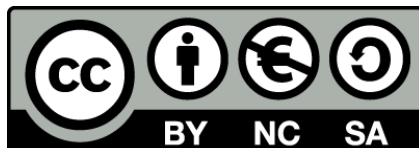




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Cellular and Molecular Insights into Schaaf-Yang Syndrome: A Multi-Model Analysis from Fibroblasts to iPSC-Derived Neural Cell Types

Mónica Centeno-Pla



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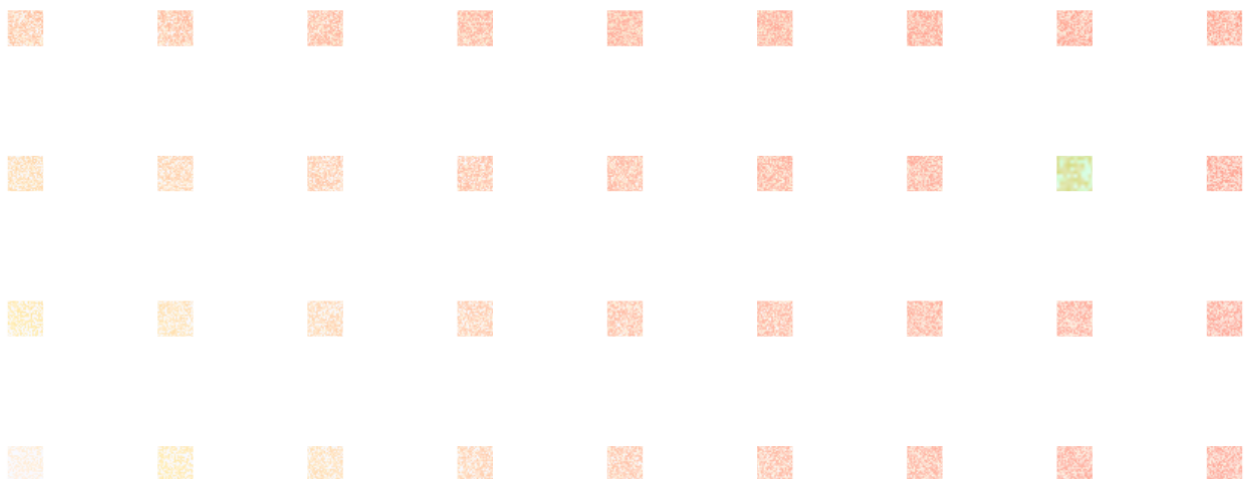
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CELLULAR AND MOLECULAR INSIGHTS INTO **SCHAAF-YANG SYNDROME**

A MULTIMODEL ANALYSIS FROM
FIBROBLASTS TO iPSC-DERIVED NEURAL
CELL TYPES

Mónica Centeno-Pla





UNIVERSITAT DE
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Facultat de Biologia

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Programa de Doctorat en Genètica

**Cellular and Molecular Insights into Schaaf-Yang Syndrome:
A Multimodel Analysis from Fibroblasts to iPSC-Derived Neural Cell Types**

**Anàlisi Cel·lular i Molecular de la Síndrome de Schaaf-Yang:
Estudi Multimodel des de Fibroblasts fins a Cèl·lules Neurals Derivades d'iPSC**

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Fa sis anys, quan la Susanna i el Dani em van explicar per primera vegada la mítica història dels orígens del grup d'Opitz, mai no hauria imaginat que acabaria embarcant-me en una tesi doctoral. En aquell moment tot just començava un camí que no sabia fins on em portaria. Ara, mirant enrere, m'adono que aquesta tesi no és només el resultat d'hores de feina i d'experiments, sinó sobretot el fruit de totes les persones que m'han acompanyat, donat suport i fet costat durant aquest trajecte.

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ABSTRACT

Schaaf-Yang syndrome (SYS) is an ultra-rare neurodevelopmental disorder caused by truncating mutations in the maternally imprinted gene *MAGEL2*. Clinically, SYS shares many features with Prader-Willi syndrome (PWS), such as neonatal hypotonia, feeding difficulties, endocrine dysfunction, neurodevelopmental delay and a consequent intellectual disability, but also presents with distinctive traits including joint contractures and a higher degree of intellectual difficulties. Due to its rarity, SYS remains poorly understood, with limited consensus on management, and molecular mechanisms. This thesis addresses these challenges through an integrated clinical and molecular approach.

In this thesis, phenotypic and genetic reviews of all reported SYS cases have been performed, enabling the development of the first clinical guidelines for patient management. A curated catalogue of *MAGEL2* variants has been established, resolving misannotations and highlighting the diagnostic importance of parental origin assessment in this imprinted gene. To elucidate disease mechanisms, we have studied the behaviour of truncated *MAGEL2* proteins using heterologous expression systems, patient-derived fibroblasts, and induced pluripotent stem cell (iPSC)-based neural models. Our findings show that truncating mutations produce stable proteins that mislocalise to the nucleus rather than being degraded. This aberrant localisation potentially reframes SYS as a dual loss- and gain-of-function disorder distinct from PWS. Multi-omic studies across different patient-derived cell models have shown both overlapping and cell type-specific dysregulations. Specifically, in CNS cell types, transcriptomic profiling has identified disruptions in synaptic and neuronal function, extracellular matrix organisation, and developmental pathways. Preliminary calcium imaging assays have potentially uncovered impaired GABAergic neuronal activity, linking transcriptional dysregulation to altered physiology. Finally, we have observed alterations in amyloid- β secretion across patient-derived cell types, establishing its potential as a robust, disease-relevant biomarker.

This work is among the first to use human iPSC-derived neuronal models to investigate SYS, providing direct access to potentially disease-relevant cell types. Using multiple patient-derived platforms, we refine the molecular definition of SYS and reveal potential pathogenic mechanisms alongside candidate biomarkers. By integrating clinical, multi-omic, and functional analyses, this thesis advances our understanding of SYS pathophysiology and establishes a foundation for future studies aimed at dissecting molecular mechanisms and identifying therapeutic targets.

KEYWORDS: rare diseases, human genetics, human cell culture, molecular biology, developmental disabilities

RESUM

La síndrome de Schaaf-Yang (SYS) és una malaltia ultrarara del neurodesenvolupament causada per mutacions truncants en el gen *MAGEL2*. Clínicament, la SYS comparteix nombroses característiques amb la síndrome de Prader-Willi (PWS), com ara la hipotonia neonatal, les dificultats d'alimentació, la disfunció endocrina, el retard del neurodesenvolupament i la consegüent discapacitat intel·lectual. No obstant això, també presenta trets distintius, com contractures articulars i un grau més elevat de dificultats intel·lectuals. A causa de la seva raresa, la SYS continua sent poc coneguda, amb un consens limitat pel que fa al maneig clínic i als mecanismes moleculars subjacents. Aquesta tesi aborda aquests reptes mitjançant un enfocament tant clínic com molecular.

En aquesta tesi, s'ha dut a terme una revisió fenotípica i genètica de tots els casos de SYS descrits fins ara, que ha permès desenvolupar les primeres pautes clíniques per al maneig dels pacients. S'ha establert un catàleg curat de variants de *MAGEL2*, resolent anotacions errònies i destacant la importància diagnòstica d'avaluar l'origen parental de les mutacions. Per tal d'elucidar els mecanismes de la malaltia, s'ha estudiat el comportament de proteïnes *MAGEL2* truncades mitjançant sistemes d'expressió heteròlegs, fibroblasts derivats de pacients i models neurals diferenciats a partir de cèl·lules pluripotents induïdes (iPSC). Els resultats mostren que les mutacions truncants produeixen proteïnes estables que es localitzen erròniament al nucli, en lloc de degradar-se. Aquesta localització aberrant podria reformular la SYS com un trastorn amb un doble mecanisme de pèrdua i guany de funció, diferent de la PWS. Els estudis multiòmics en diversos models cel·lulars derivats de pacients han revelat desregulacions tant compartides com específiques de cada tipus cel·lular. En concret, el perfil transcriptòmic de cèl·lules del sistema nerviós central, ha identificat alteracions en la funció sinàptica i neuronal, l'organització de la matriu extracel·lular i en les vies de desenvolupament. A més, assaigs preliminars d'imatges de calci suggereixen una activitat neuronal GABAèrgica alterada, establint un vincle entre la desregulació transcripcional i les disfuncions fisiològiques observades. Finalment, s'han detectat alteracions en la secreció de β -amiloide en els models cel·lulars derivats de pacients, la qual cosa apunta al seu potencial com a biomarcador robust i rellevant per a la malaltia.

Aquest treball és un dels primers a utilitzar models neuronals derivats d'iPSCs humanes per investigar la SYS, oferint accés directe a tipus cel·lulars potencialment rellevants per a la patologia. Mitjançant aquestes i altres plataformes derivades de pacients, es refina la definició molecular de la SYS i es revelen possibles mecanismes patogènics, així com biomarcadors candidats. Integrant l'anàlisi clínic, multiòmica i funcional, aquesta tesi avança en la comprensió de la fisiopatologia de la SYS i estableix les bases per a futurs

estudis orientats a dissecionar-ne els mecanismes moleculars i identificar possibles dianes terapèutiques.

PARAULES CLAU: malalties rares, genètica humana, cultius cel·lulars humans, biologia molecular, trastorns del desenvolupament

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ABBREVIATIONS

ACMG: American College of Medical Genetics

AD: Alzheimer's disease

AESYS: Asociación Española del Síndrome de Schaaf-Yang

AICD: APP intracellular domain

AMC: Arthrogryposis multiplex congenita

APP: Amyloid precursor protein

ASD: Autism spectrum disorder

ASO: Antisense oligonucleotide

A β : β -amyloid

BDNF: Brain-derived neurotrophic factor

BioID: Biotin identification

BMP: Bone morphogenetic protein

BNLC: Banco nacional de líneas celulares

CNS: Central nervous system

DEG: Differentially expressed gene

E-I: Excitatory-Inhibitory

EB: Embryoid body

ECM: Extracellular matrix

ESC: Embryonic stem cell

FGF: Fibroblast growth factor

GABA: Gamma-aminobutyric acid

GECI: Genetically encoded calcium indicator

GH: Growth hormone

GPCR: G-protein-coupled receptor

HA: Hemagglutinin

HGVS: Human Genome Variation Society

HOX: Homeobox

HPO: Human phenotype ontology

HSJD: Hospital Sant Joan de Déu

iA: Induced astrocyte

ICC: Immunocytochemistry

ID: Intellectual disability

iN: Induced neuron

IPSC: Induced pluripotent stem cell

MAGE: Melanoma antigen gene expression

MAGEL2: MAGE Family Member L2

MHD: MAGE homology domain

mRNA: Messenger RNA

MS: Mass spectrometry

mTOR: Mammalian target of rapamycin

NDD: Neurodevelopmental delay

NES: Nuclear export signal

NLS: Nuclear localization signal

NMD: Nonsense mediated decay

NPC: Neural progenitor cell

OXT: Oxytocin

POMC: Pro-opiomelanocortin

PWS: Prader-Willi syndrome

rhGH: Recombinant human growth hormone

RNAi: RNA interference

SG: Secretory granule

SHRC: WASH regulatory complex

shRNAs: Short hairpin RNA

siRNA: Small interfering RNA

SYS: Schaaf-Yang syndrome

TCA: Tricarboxylic acid

TF: Transcription factor

TGF- β : Transforming growth factor-beta

TGN: Trans-Golgi network

TPM: Transcripts per million

U7BS: USP7 binding site

UTR: Untranslated region

VUS: Variant of uncertain significance

WT: Wild type

INTRODUCTION

1. SCHAAF-YANG SYNDROME

Schaaf-Yang syndrome (SYS; MIM #615547) is an ultra-rare neurodevelopmental disease caused by truncating mutations in *MAGEL2* (MAGE Family Member L2; MIM* 605283). The syndrome was first described and named in 2013, after the identification of disease-causing truncating mutations in the paternal allele of *MAGEL2* in four patients exhibiting a syndromic neurodevelopmental condition with some features resembling the Prader-Willi syndrome (PWS; MIM #176270) phenotype but normal methylation patterns and absence of 15q11q13 deletion [1]. Since then, the literature has reported over 200 patients carrying more than 50 different truncating mutations in the paternally inherited allele of *MAGEL2*, since the maternal allele is silenced due to methylation [2–38]. Most mutations associated with SYS are nonsense or frameshift mutations that result in premature stop codons within the *MAGEL2* coding sequence (**Figure 1**).

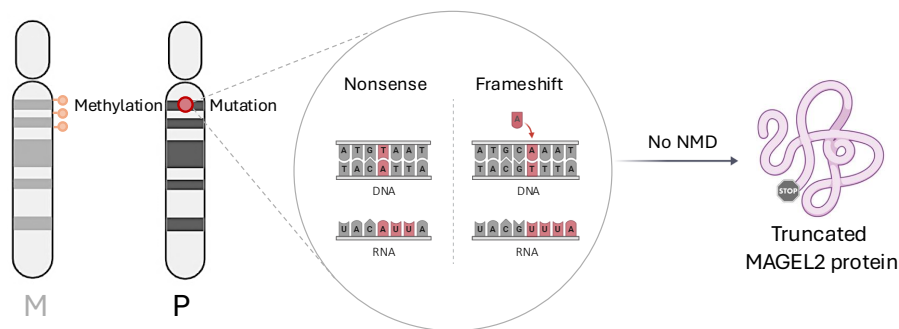


Figure 1: Schaaf-Yang syndrome mechanism of disease. Mutations in the paternally expressed *MAGEL2* gene escape nonsense mediated decay (NMD), leading to the production of a truncated *MAGEL2* protein.

SYS is diagnosed through molecular genetic testing and the identification of a heterozygous pathogenic variant in the paternal copy of *MAGEL2*. A proportion of patients inherit their mutation from a clinically unaffected father (who himself carries the variant on his maternal allele), while others are *de novo*. The syndrome shows full penetrance, although the severity and specific clinical manifestations vary between cases (reviewed in [39]). SYS is extremely rare, with an estimated prevalence below 1 per 1,000,000 individuals worldwide (Orphanet; Accessed August 2025).

SYS was initially considered a PWS-like phenotype due to patients manifesting overlapping clinical symptoms, especially noticeable during the early years of life. These include neonatal hypotonia, feeding difficulties, respiratory problems, hypogonadism, neurodevelopmental delay (NDD), and intellectual disability (ID) [4]. Despite the similarities, there are important differences that make SYS a distinct diagnosis from

PWS. SYS patients often display more severe NDD, and ID, greater limitations in functionality and are more likely to exhibit autism spectrum disorder (ASD) typical behaviours [40]. Moreover, a key feature in most individuals with SYS is the presence of joint contractures (arthrogryposis multiplex congenita; AMC), which are exceptional in PWS [4]. Conversely, standardised criteria for PWS diagnosis like hypopigmentation, a characteristic facial gestalt, obesity, and obsessive-compulsive behaviour are rarely seen in SYS (**Figure 2A**). In fact, many children with SYS tend to be underweight in infancy and early childhood due to feeding difficulties, contrasting with the classic hyperphagia and weight gain in PWS. However, over time, the phenotypic differences between both syndromes can become less pronounced, making them more challenging to differentiate clinically in adulthood [20].

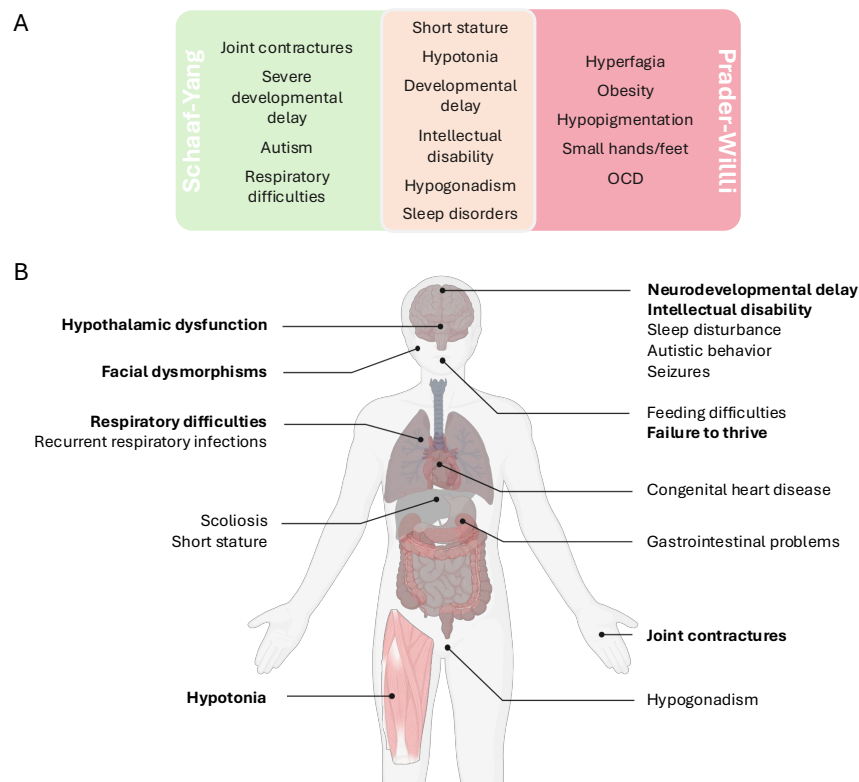


Figure 2: Phenotypic characteristics of *MAGEL2*-related syndromes. **(A)** Phenotypic overlap between *MAGEL2*-related syndromes Prader-Willi syndrome and Schaaf-Yang syndrome. Adapted from [4]. **(B)** Phenotypic spectrum of Schaaf-Yang syndrome. Most common symptoms are shown in bold.

One of the most consistent features across SYS reported cases is early-onset NDD, affecting both motor and language milestones (**Figure 2B**). Hypotonia, often profound in the neonatal period, is frequently one of the first signs and contributes to significant delays in reaching motor milestones. Many children with SYS are unable to sit

independently until after 12 months, and walking, if achieved, may not occur until 2-3 years or later. Fine motor skills and speech development are significantly impaired, with some patients remaining nonverbal. ID is also common, ranging from moderate to severe [41,42]. Additionally, as previously mentioned, ASD is highly prevalent in SYS, with studies estimating that over 75% of individuals meet criteria [20]. Feeding difficulties are another hallmark of SYS, particularly in infancy. Hypotonia impairs the coordination of sucking and swallowing, leading to dysphagia and failure to thrive, with frequent need for nasogastric or gastrostomy tube feeding [4]. As patients grow, other clinical symptoms begin to show. Joint contractures, particularly involving the elbows, wrists, and fingers, are observed in a large proportion of individuals. However, in many cases, these are also present at birth, causing AMC. Contractures can limit mobility and may require orthopaedic intervention or physical therapy [3].

An overlap between the hormonal and metabolic phenotypes of PWS and SYS has also been noted. A hallmark feature in both conditions is growth hormone (GH) deficiency, which contributes to short stature, increased fat mass, and reduced levels of IGF-1 and IGFBP-3 [12,43]. In SYS, this deficiency often comes with other pituitary hormone abnormalities, including low levels of thyroid-stimulating hormone, antidiuretic hormone, adrenocorticotrophic hormone, and gonadotropins [10,13]. These combined deficiencies likely result from hypothalamic dysfunction, highlighting the essential role of *MAGEL2* in regulating the hypothalamo-pituitary axis [44].

Elevated ghrelin levels are also observed in individuals with both SYS and PWS [12,45]. Since ghrelin is a hormone that increases appetite and is produced by the stomach during fasting, these findings support the hyperphagia observed in most PWS patients and a small number of SYS patients. The same study also noted a higher prevalence of both glucose intolerance and diabetes mellitus in SYS compared to PWS [12]. However, further research is needed to definitively establish a pathogenetic relationship between these manifestations and the disease. Low bone mineral density and scoliosis are prevalent in SYS patients, indicating significant bone health issues. Lastly, disturbances in thermoregulation, such as episodes of hypothermia or reduced response to fever, have been noted in some patients [12]. These features, though underrecognised, may have clinical relevance during infections or postoperative recovery.

As part of this thesis (Article 1), an updated and expanded review of the clinical and genetic data from all reported SYS cases was carried out in collaboration with specialists at Hospital Sant Joan de Déu (HSJD).

1.1. THERAPEUTIC STRATEGIES FOR SYS

Current treatment for SYS is mainly symptomatic, reflecting its complex, multisystemic nature. The management of clinical complications in SYS is primarily reactive rather than preventive, focusing on treating problems as they arise. Although symptoms may vary between patients, today there is enough knowledge to provide some general recommendations for the typical complications. After a review of evidence and reports in the literature, together with personal experience, recommendations for the clinical management of SYS patients, can be found in Article 1 of this thesis. The guideline encompasses a range of supportive therapies, including speech and physical therapy, psychiatric care, nutritional support, and interventions for sleep apnoea. Beyond symptomatic care, several targeted therapeutic approaches are under investigation:

- **Recombinant human GH (rhGH):** rhGH is an established therapy for PWS and has recently gained attention as a promising treatment for SYS. In PWS, rhGH is approved in many countries and has been shown to improve growth, reduce fat mass, increase lean body mass, and positively influence motor development and cognition [46–48]. Emerging studies in SYS suggest that similar benefits may be achieved. For instance, GH treatment in SYS patients led to improvements in growth, muscle tone, and motor milestones, and may have also enhanced cognitive and social skills [35,49]. However, larger studies are still needed to assess long-term outcomes and to define optimal dosing and timing for SYS.
- **Oxytocin (OXT) system modulation:** The OXT system has gained interest for its involvement in feeding, social behaviour, and neurodevelopment, key features affected in SYS. Also, given that *MAGEL2* is highly expressed in OXT-producing neurons, its loss has been linked to impaired OXT signalling pathways [50]. Preclinical studies using *Magel2*-knockout (KO) mouse models have shown that early administration of OXT can improve social interactions, cognitive performance, thermoregulation, and neuronal development [51–53]. These reports also suggest that the timing of treatment is crucial, and that combining OXT with behavioural therapy may further enhance its therapeutic effects. Based on this preclinical evidence, clinical trials in SYS patients are currently being explored.

- Brain-derived neurotrophic factor (*BDNF*) gene therapy: *BDNF* is another compelling target, acting downstream of hypothalamic feeding pathways and contributing to the regulation of both energy balance and behaviour. [54]. In *Magel2*-KO mice, AAV-mediated *Bdnf* gene therapy improved metabolic parameters such as body weight and fat mass, as well as behavioural traits like social interaction and anxiety [55,56]. Supporting its relevance to human neurons, overexpression of *BDNF* in wild type (WT) human induced pluripotent stem cell (iPSC)-derived neural cultures has been shown to enhance neuronal activity and promote axonal growth, indicating a potential to improve neural connectivity and function in disease contexts [57]. However, safely and effectively delivering gene therapies to the human brain, especially to deep hypothalamic areas, is a significant technical and regulatory challenge.

1.2. IN VIVO AND IN VITRO MODELS OF SYS

The understanding of the pathogenesis of SYS has been significantly advanced by the development of various *in vivo* and *in vitro* models targeting *MAGEL2*.

1.2.1. RODENT MODELS OF *MAGEL2* DEFICIENCY

The human *MAGEL2* gene and its murine homologue *Magel2* share similar genomic structures. In mice, *Magel2* is located in chromosome 7, in a similar region to the human Prader-Willi critical region (15q11-q13). Both genes are intronless, paternally expressed, and embedded within CpG islands containing direct tandem repeats in the 5' untranslated region (UTR), features commonly found in imprinted genes [58]. The proteins are also similar in size, measuring 1,249 and 1,284 amino acids long, respectively, and have an overall identity of 60%.

The first *Magel2*-KO mouse model was generated in 2007 by replacing the open reading frame with an in-frame LacZ knock-in expression cassette [59]. Its phenotype was extensively characterised and recapitulated fundamental aspects of PWS such as excessive weight gain, neonatal growth retardation, increased adiposity, and endocrine dysfunction [44,60–62]. Brain-specific defects included reduced overall volume, lower levels of serotonin, decreased dopamine and leptin receptors, and abnormal development of hypothalamic arcuate nucleus projections [63–65]. Electrophysiological studies showed decreased OXT neuron activity, with reduced excitatory and increased inhibitory currents, as well as a marked reduction of OXT receptor-expressing astrocytes in certain brain regions [50,66]. Impaired autophagy and increased mammalian target of

rapamycin (mTOR) activity were also noted [67]. Behaviourally, young KO mice displayed neurobehavioral dysfunction, altered exploratory activity, changed social interactions, and a lack of preference for social novelty [68]. This mouse strain is now commercially available and has been used in studies showing that *Magel2* in hypothalamic pro-opiomelanocortin (POMC) neurons modulates stress-induced anxiety-like behaviour, regulates leptin-mediated depolarisation, and influences spatial learning associated with food rewards [69–71]. Additionally, these mice also show a reduction in components of secretory granules (SGs) in the hypothalamus [72].

A second *Magel2*-KO mouse was generated in 2010 using a LoxP-Hygromycin-LoxP cassette to disrupt the promoter and most of the coding sequence [53]. Studies in these mice highlighted *Magel2*'s role in regulating feeding behaviour and social cognition, linked again to altered OXT signalling [73,74]. In these adult mice, authors also observed deficits in social recognition and interaction, reduced learning ability, and difficulty to overcome social fear [52,75]. Furthermore, *Magel2* deficiency led to abnormal neuronal maturation, irregular neurite outgrowth, fewer glutamatergic synapses, and a delayed excitatory-to-inhibitory GABAergic developmental sequence [51,75,76].

In 2020, Ieda *et al.* developed two new mouse models to specifically investigate the effects of truncated *Magel2* [77]. These approaches better reflect the molecular defect observed in SYS, in which the MAGEL2 protein is truncated. One was a transgenic line overexpressing the N-terminal (proline-rich) domain of *Magel2*, which resulted in high perinatal mortality likely due to the ubiquitous overexpression of the truncated protein. The second was a genome-edited model carrying truncating mutation (p.Glu564Serfs*130) as observed in SYS patients. However, these mice only showed mild growth retardation without the broader phenotype seen in SYS.

More recently, a rat model carrying a truncating *Magel2* mutation (p.Ser132Glyfs*728) was generated [78]. This model exhibited paternal expression of *Magel2* in the hypothalamus and displayed increased anxiety-like behaviour and altered sociability. Additionally, physiological tests revealed changes in body composition, cardiac function, and breathing patterns, supporting the conservation of *Magel2*'s functional role across rodent species.

Lastly, a mouse model carrying a deletion in genes *Ndn* and *Magel2* was generated by Barelle *et al.* [79]. The mouse showed a range of phenotypes relevant to PWS, including neonatal lethality, early sensory delays, altered social behaviour, and metabolic

dysregulation. Furthermore, these mice exhibited respiratory abnormalities, dysregulation of circadian and axonal function-related genes, and impaired maturation of OXT neurons and melanocortin circuits. As seen, several studies have extensively characterised these mouse models, with the most relevant findings summarised in **Table 1**.

Table 1: Comparative summary of murine and cellular models of *MAGEL2*-related syndromes.

Model	Growth and metabolism	Behaviour	Molecular/cellular phenotypes	References
Mouse KO (2007)	Growth retardation, obesity, endocrine dysfunction, progressive infertility	Altered exploratory activity, altered social interaction, lack of preference for social novelty, decreased locomotor activity	Increased mTOR activity, impaired autophagy, increased leptin, decreased dopamine receptors and orexin, decreased OXTR astrocytes, low OXT neuron activity, reduced SGs	[44,50,59–68,72]
Mouse KO (2010)	Feeding difficulties, growth retardation	Impaired learning and memory, social recognition deficits, social fear	Irregular neurite outgrowth, decreased glutamatergic synapses, decreased OXT, delayed GABA switch	[51–53,73–76]
Mouse p.Glu564Serfs*130	Mild neonatal growth retardation	No major behavioural symptoms observed	-	[77]
Mouse N-terminal overexpression	High fetal/neonatal lethality	-	-	[77]
Mouse <i>Ndn-Magel2</i> deletion	Low body weight, reduced fat mass, altered puberty, increase in apneas, improved glucose tolerance	Altered exploratory activity	Dysregulation of circadian genes, impaired maturation of OXT neurons, disrupted development of melanocortin circuits	[79]
Rat p.Ser132Glyfs*728	Altered body composition, cardiac/respiratory changes	Increased anxiety-like behaviour, reduced sociability	-	[78]
SYS patient-derived fibroblasts (p.Gln666Profs*47, p.Gln638*)	Lipid droplet accumulation	-	Increased mTOR activity, impaired autophagy, decreased P-AMPK levels	[67]
SYS patient-derived iNs (p.Gln666Profs*47, p.Gln638*)	-	-	Increased mTOR activity, altered dendrite morphology	[67]

1.2.2. CELLULAR MODELS OF SYS

To our knowledge, only one published study has used patient-derived cell lines carrying *MAGEL2* truncating mutations to investigate cellular dysfunction in SYS [67]. In this study, Crutcher *et al.* showed that three fibroblast lines from SYS patients exhibit significantly increased mTOR expression, which is linked to impaired autophagy. This impairment of autophagy in SYS may also lead to the accumulation of lipid droplets, as the normal clearance and recycling of lipids may be impaired in these cells. In the same article, day 30 excitatory induced neurons derived from SYS patient iPSCs exhibited similar dysregulations: increased mTOR expression and altered dendrite formation, specifically affecting dendrites originating from the soma (**Table 1**). Importantly, these changes were rescued by treatment with mTORC1 inhibitor rapamycin, suggesting potential therapeutic strategies to mitigate these dysfunctions.

2. PRADER-WILLI SYNDROME

Prader-Willi syndrome (MIM #176270) is a complex genetic disorder caused by an alteration in the so-called Prader-Willi critical region, a 6Mb region within the range 15q11-q13. This locus contains a cluster of genes subject to genomic imprinting, where most are expressed from the paternal allele, while the corresponding maternal alleles are epigenetically silenced. Key genes affected in PWS include *MAGEL2*, *NDN*, *MKRN3*, and the *SNURF-SNRPN* transcript, as well as several non-coding RNAs such as the *SNORD* cluster (**Figure 3A**). While not all genes in the region follow the same imprinting pattern, the hallmark of PWS is the absence or dysfunction of the maternally imprinted genes, resulting from paternal deletion (65%-75%), maternal uniparental disomy (20%-30%), or defects of the imprinting centre (1%-3%) (**Figure 3B**) [80]. Despite extensive research, the individual contribution of each gene within the PWS region to the syndrome's phenotype remains unclear. The estimated prevalence of PWS is 1 in 10,000 to 20,000 individuals and although consensus clinical diagnostic criteria is available and accurate, the main method for diagnosis is genetic testing.

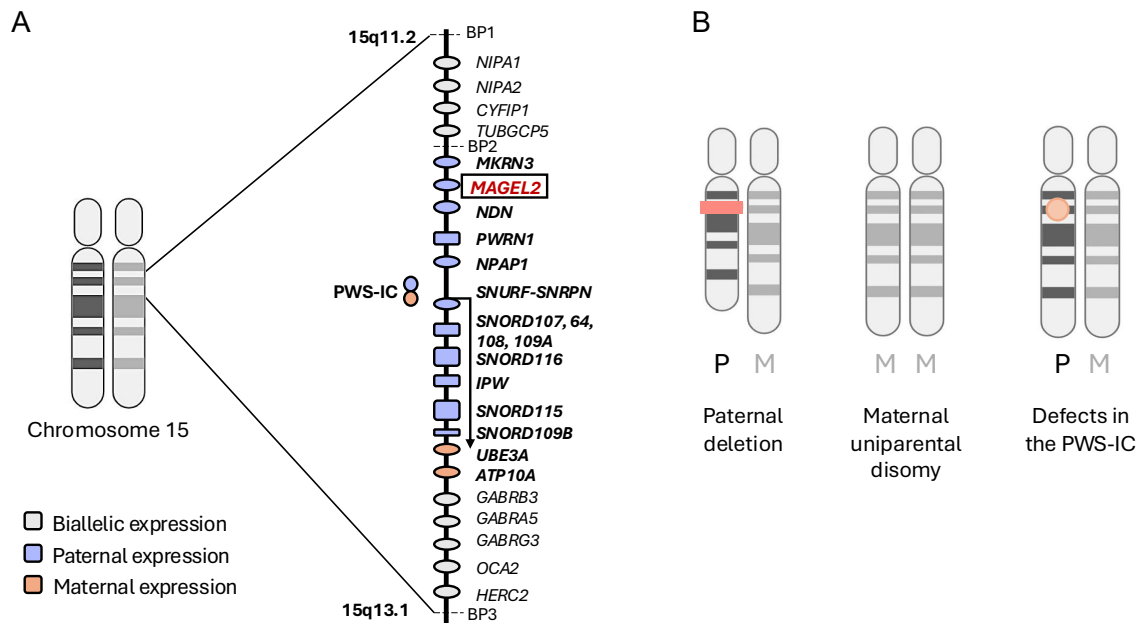


Figure 3: *MAGEL2* genomic context in Prader-Willi syndrome (PWS). **(A)** Genomic location and imprinting status of *MAGEL2* and other genes within the 15q11-q13 region. Ovals indicate protein-coding genes, while rectangles represent RNA genes. **(B)** Possible PWS mechanisms of disease.

Historically, the clinical progression of PWS is divided into two stages: the first is characterized by dysphagia and failure to thrive, followed by a later stage marked by hyperphagia and the development of obesity. However, more detailed studies have refined this model into five to seven nutritional phases, including prenatal growth restriction, early infant feeding issues, gradual weight gain with normal appetite, progressive hyperphagia, and ultimately a potential decline in appetite during late adulthood [81].

In addition to these feeding-related phenotypes, individuals with PWS often show severe hypotonia, which leads in many cases to the clinical suspicion in the neonatal units. Lethargy and hypotonia during the first weeks of life is followed by delayed motor and language milestones. It has been said that motor milestones are achieved at twice the expected age and problems with speech development are also very frequent. Later, mild to moderate ID, neuropsychiatric and behavioural issues such as stubbornness, temper tantrums, manipulative tendencies, skin-picking, and obsessive-compulsive behaviours, are common [82]. Hypogonadism is also noted, with males often presenting cryptorchidism, genital hypoplasia, and infertility; and females delayed puberty and ovarian dysfunction. Short stature, often due to GH insufficiency (or insufficient response), is typical, along with distinctive facial features (such as triangular-shaped face, almond shaped eyes, downturned mouth corners, and thin upper lip), small hands

and feet, strabismus, and scoliosis. There is also a high incidence of sleep disturbances due to upper airway obstruction during sleep, and type II diabetes, particularly in those PWS patients who become obese [83].

3. *MAGEL2*

MAGEL2 (MIM* 605283) is a protein-coding gene located within the Prader-Willi critical region (15q11-q13). Like other genes in this locus, *MAGEL2* is subject to parental imprinting and is expressed only from the paternal allele. Therefore, mutations on the maternally inherited allele have no phenotypic consequence, whereas disruptions of the paternal allele may manifest as disease. A characteristic of *MAGEL2* is its single-exon structure, spanning 4,319 base pairs. The absence of introns prevents the activation of the nonsense mediated decay (NMD) pathway in response to nonsense mutations, leading to the production of truncated proteins.

MAGEL2 is predominately expressed in the brain, particularly the hypothalamus, with weaker expression in the pituitary gland, basal ganglia, and cerebral cortex (**Figure 4**) [15,84]. The hypothalamus plays a central role in coordinating whole-body homeostasis. Through its connections with the pituitary gland, it regulates hormone release in endocrine organs such as the adrenal glands, thyroid, and gonads. These pathways are essential for controlling growth, stress responses, metabolism, and reproduction. In parallel, the hypothalamus integrates neural signals to regulate functions like body temperature, feeding behaviour, and sleep; many of which are disrupted in SYS and PWS [85].

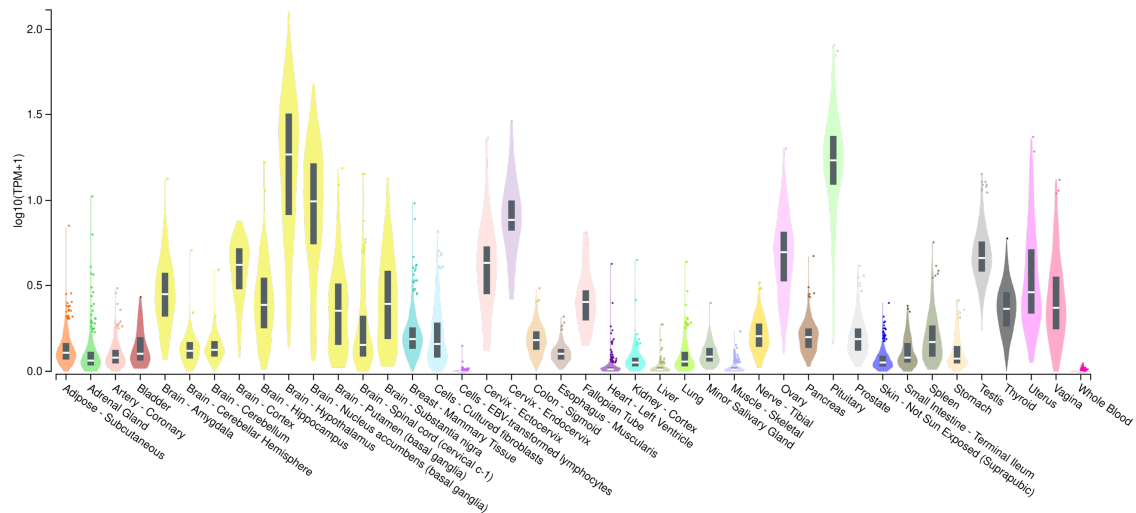


Figure 4: Bulk tissue gene expression profile of *MAGEL2* across multiple human tissues. Expression levels, measured in transcripts per million (TPM), show predominant expression in brain regions, most notably the hypothalamus and basal ganglia, as well as in the pituitary gland, indicating a central nervous system and neuroendocrine bias. Data source: GTEx Portal; Accessed September 2025 [84].

MAGEL2 is broadly expressed during embryonic development, suggesting a key role in early cellular programs and the potential to influence the onset of neurodevelopmental disorders. In mouse embryos, *Magel2* expression is detected throughout the central nervous system (CNS), including the neural tube, diencephalon, forebrain, and developing hypothalamus. It also appears in non-neural tissues such as the genital tubercle, middle intestine, placenta, tongue, and musculoskeletal structures [58,86,87]. As development progresses, *Magel2* expression becomes largely restricted to the brain, especially the hypothalamus, where it localises to nuclei involved in neuroendocrine signalling and circadian rhythm regulation [72]. Evidence from both human and animal studies suggests that *MAGEL2* expression can be modulated by genetic and environmental factors. In neuronal cells from nine patients carrying pathogenic *NF1* mutations, *MAGEL2* expression was significantly reduced [88]. In mice, transcription of hypothalamic *Magel2* is altered in response to the synthetic estrogen diethylstilbestrol [89]. These findings support the view of *MAGEL2* as a dynamically regulated gene, with functions that extend from early developmental programs to specialised neural processes.

The *MAGEL2* gene encodes for the MAGEL2 protein, which comprises 1,249 amino acids. MAGEL2 belongs to the melanoma antigen gene (MAGE) family, a group of proteins initially discovered due to their expression in melanomas and testicular tissue, but later found to play important roles in development, cell cycle control, and ubiquitin

signalling. Within this family, MAGEL2 is classified as a Type II MAGE, as it is not restricted to the X chromosome and is expressed in many tissues (reviewed in [90]). MAGEL2 has three relevant structural and functional elements: a proline-rich region, a USP7 binding site (U7BS), and a MAGE homology domain (MHD). The proline-rich region at the N-terminus is highly unstructured and has been associated with proteins that participate in cellular stress responses and mRNA metabolism [91]. The U7BS region acts as a binding site for deubiquitinating enzyme USP7 [92]; and at the C-terminus, spanning amino acids 1,027 to 1,195, is the MHD. This highly conserved sequence, present in both Type I and Type II MAGE proteins, allows for highly specific protein-protein interactions. A common feature of the MAGE family is the ability to bind E3 RING proteins through their MHD [93]. Specifically, MAGEL2 interacts with the coiled-coil (CC) domain of TRIM27 and mediates its recruitment to the endosomes [94].

3.1. CELLULAR FUNCTIONS OF MAGEL2

The role of MAGEL2 in the retrograde endosomal protein recycling is its most studied function in the cell. This pathway is essential for sorting and recycling transmembrane proteins, such as receptors and transporters, from endosomes back to the plasma membrane or to the trans-Golgi network (TGN), rather than allowing their degradation in lysosomes [95]. MAGEL2 regulates this process through its interaction with the retromer complex, and ubiquitinating and deubiquitinating enzymes.

More specifically, along E3 ubiquitin ligase TRIM27 and deubiquitinating enzyme USP7, MAGEL2 forms the MUST complex [94]. This complex is recruited to the endosomes, where it binds to the retromer through an interaction between MAGEL2 and VPS35. The retromer complex recognises sorting motifs on transmembrane cargo proteins and packages them into recycling tubules for retrograde trafficking. As well as recognising cargo proteins, the retromer also recruits the WASH regulatory complex (SHRC) to sites where actin remodelling is needed (reviewed in [96]). The SHRC features five main elements: CCDC53, FAM21, SWIP, Strumpellin, and WASH. In the endosomes, TRIM27 activates the SHRC by ubiquitinating WASH with K63-linked chains. This modification stabilises the association of SHRC with the membrane and stimulates F-actin polymerisation (**Figure 5**) [94,97]. Actin patches facilitate the segregation and trafficking of cargo proteins to their correct destinations by marking and remodelling specific regions of the endosome.

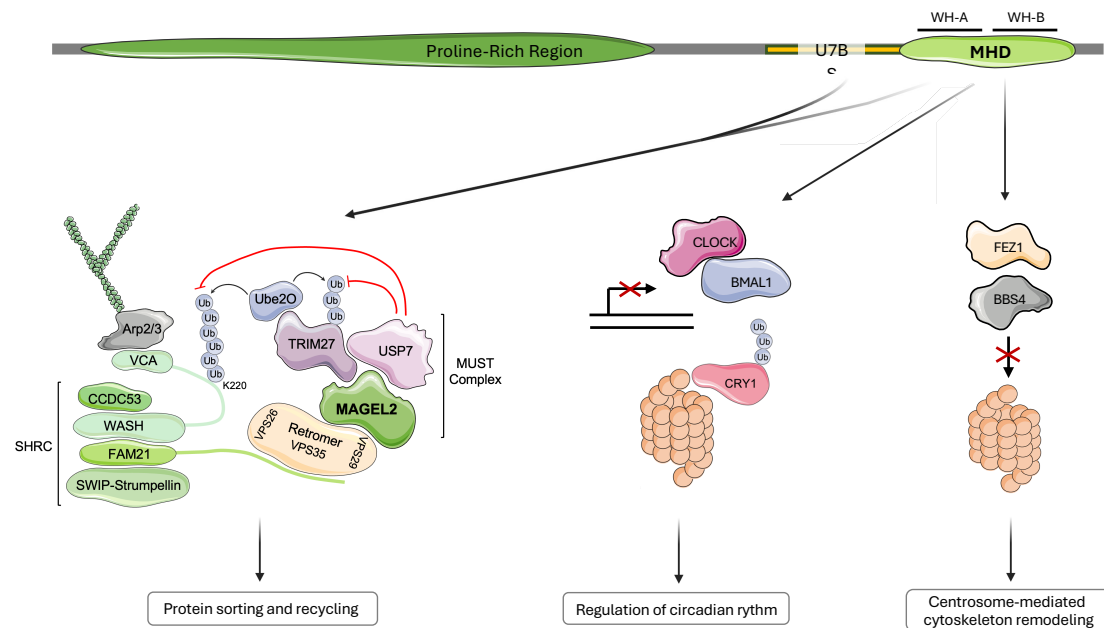


Figure 5: Described roles and functions of the MAGEL2 protein, encompassing the MAGE homology domain (MHD). Image created with Servier Medical Art.

USP7 modulates this process by reversing ubiquitination, allowing recycling to be turned off or reset, and preventing hyperactivation of WASH. Therefore, the MUST complex acts as a molecular switch controlling the efficiency and timing of protein recycling. Specifically, MAGEL2 itself serves as a platform or scaffold, recruiting TRIM27 and USP7 to the SHRC and retromer, and ensuring actin nucleation occurs only at specific membrane domains. This well-coordinated trafficking network is essential for proper cellular distribution and function of a wide variety of proteins.

The failure of retrograde transport mechanisms can disrupt many cellular processes. For example, loss of *MAGEL2* reduces the number of SGs and decreases downstream neuropeptide production and release [72]. This occurs because impaired endosome-mediated retrograde transport leads to the early lysosomal breakdown of essential proteins. SGs are crucial for the release of neuropeptides and hormones, especially in hypothalamic neurons, as many proteins rely on proper recycling and trafficking to be incorporated into maturing granules [98]. Therefore, MAGEL2 contributes to the proper sorting and recycling of these granule-resident proteins through its role in the retromer-mediated recycling pathway.

It was also proposed that Magel2 binds to and prevents proteasomal degradation of Fez proteins and BBS4, involved in centrosome-mediated cytoskeletal rearrangements during neurite growth (**Figure 5**) [99]. By stabilising these proteins, Magel2 may indirectly

support microtubule dynamics and neuronal morphology during development. However, it is important to note that the construct used in this study is based on a C-terminal 521 amino acid Magel2. This sequence corresponds to earlier genome annotations, whereas the latest GRCm39 mouse genome assembly identifies Magel2 as a 1,284-residue protein. Therefore, this study may not fully capture the function of the complete protein.

MAGEL2 is also recognised for its circadian expression in both mice and humans, functioning as one of several clock-regulated genes [100]. Circadian rhythm proteins show rhythmic variations in their subcellular localisation and abundance, a cycle that depends on tightly regulated protein ubiquitination and proteasomal degradation. In this context, MAGEL2 helps balance the ubiquitination of CRY1, a key component in the circadian rhythm feedback loop [101]. It also influences the subcellular localisation of circadian rhythm proteins Bmal1 and Clock and represses Clock:Bmal1 mediated transcription (**Figure 5**) [102]. Supporting this, and in line with *Magel2* expression in the hypothalamus (where the suprachiasmatic nucleus acts as the central circadian pacemaker in mammals), *Magel2*-KO mice show lower activity amplitude and increased activity during the daytime [59]. Furthermore, significant differences in circadian period lengths were seen between PWS neurons and controls [103].

Beyond its established role in protein trafficking, MAGEL2 appears to participate in other processes through its proline-rich domain. This intrinsically disordered region, containing Pro-X_n-Gly motifs, is predicted to facilitate liquid-liquid phase separation and the formation of RNA-regulatory condensates [91]. In fact, MAGEL2 has been shown to bind mRNA m⁶A methylation readers (YTHDF1/2/3) and RNA interference factors (TNRC6 family). This suggests that MAGEL2 may help regulate mRNA stability and localisation through m⁶A-dependent mechanisms. Further studies also highlight MAGEL2's interaction with RNA processing regulators, including SMN and FMRP, both of which are implicated in neurodevelopmental disorders [104].

4. CELL-BASED MODELS FOR STUDYING DISEASE

Cell-based models have become indispensable tools for investigating the molecular mechanisms underlying rare diseases like SYS. These *in vitro* systems enable the analysis of how mutations disrupt cellular functions such as protein trafficking, metabolic homeostasis, and stress responses, all in a human cellular context. Beyond their mechanistic value, cell models also support precision medicine by allowing the testing of potential therapies directly on disease-relevant cell types, bridging the gap between

basic research and clinical application. Recent advances in 3D cultures and organ-on-chip technologies further improve the relevance of these models, especially for disorders that affect multiple systems (reviewed in [105]). This is particularly important for SYS, as current animal models do not completely reproduce the human phenotype, making human cell-based approaches a more representative platform. Additionally, unlike animal models, *in vitro* systems avoid many ethical and logistical issues.

The complexity of SYS, characterised by neurodevelopmental abnormalities and systemic symptoms, requires complementary cellular approaches (**Figure 6**). In this thesis, we used heterologous expression systems to investigate *MAGEL2* variants in a controlled human cell background, a strategy made necessary by the lack of reliable antibodies for detecting the truncated protein forms present in SYS patients. In addition, patient-derived fibroblasts provided a physiologically relevant cellular context that preserves the genetic, epigenetic, and environmental background of the individual, and also served as a source for generating iPSCs. While both models offer unique advantages, neither replicates the neural environment where *MAGEL2* function may be most critical. Therefore, we also used iPSC differentiation into CNS cell types to investigate SYS-related neurodevelopmental mechanisms in a more relevant setting. Each model has its own benefits and limitations, and selecting the most appropriate system depends on the specific research question, whether it involves studying neurodevelopmental pathways, modelling endocrine dysfunction, or testing targeted therapies.

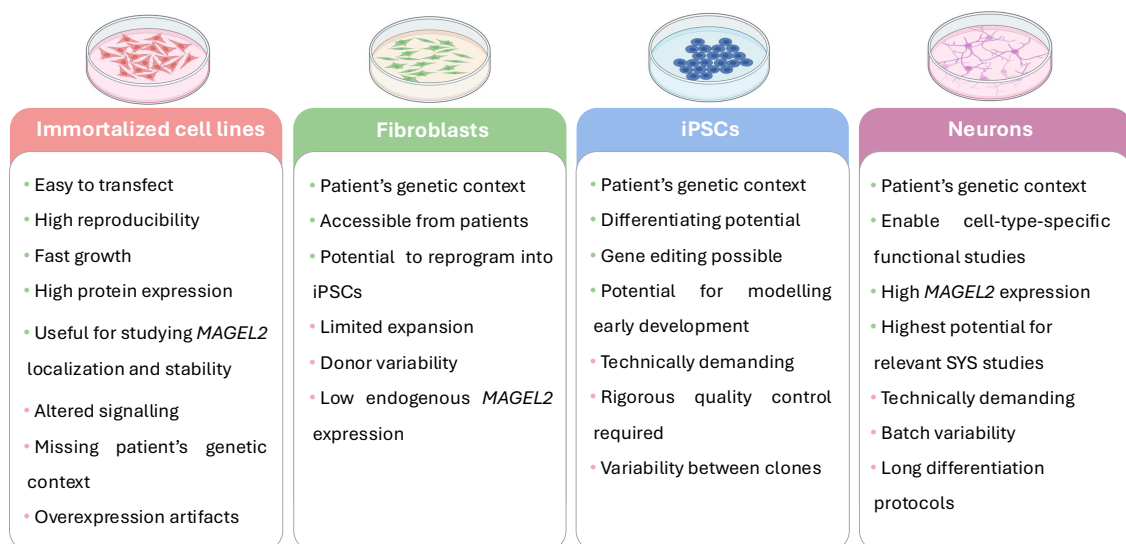


Figure 6: Comparative overview of cellular models used in this study to investigate SYS. Immortalised cell lines, fibroblasts, iPSCs, and differentiated neurons each offer distinct advantages and limitations for SYS-related research.

4.1. THE USE OF iPSCs IN DISEASE MODELLING

iPSCs are a transformative approach in the field of disease modelling, offering major advantages. A primary strength of iPSCs is the fact that they are derived from a patient's own somatic cells. This preserves the individual's unique genetic background, enabling the creation of personalised *in vitro* models. Second, they retain the ability to generate derivatives of all three germ layers. Therefore, they provide access to otherwise unobtainable CNS cell types, which are central to SYS pathophysiology. Lastly, iPSCs can be used to mirror cellular developmental processes and gene expression patterns characteristic of early embryogenesis, allowing to study and model SYS initiation and progression *in vitro*.

Despite their many advantages, iPSCs are not without challenges (**Figure 6**). Reprogramming can introduce genetic and epigenetic alterations [106], and residual “epigenetic memory” from the somatic source cell can bias differentiation outcomes [107]. Extended culture can lead to the expansion of clones with growth advantages, potentially linked to oncogenic mutations or loss of pluripotency [108]. Additionally, genetic background, reprogramming method, and passage number have all been shown to cause variability in the differentiation efficiency and potential of iPSC lines [107,109,110].

iPSCs were first generated by Takahashi and Yamanaka through the expression of four transcription factors (TFs), *OCT4*, *SOX2*, *KLF4*, and *c-MYC*, in mouse fibroblasts [111], and soon after in human cells [112]. Since these initial breakthroughs, the field has grown considerably. Reprogramming protocols have been refined and successfully applied to various human somatic cell types [113–115]. Moreover, alternative TF combinations and small molecule supplements have been developed to improve efficiency and reproducibility and reduce the use of potentially oncogenic genes such as *c-MYC* [116,117].

The methods used to deliver reprogramming factors into adult somatic cells are also evolving. They fall into two broad categories: integrative and non-integrative techniques. While integrative methods, such as those using retroviruses and lentiviruses, show great efficiency and stable transgene expression, they have limitations for clinical application because of their propensity for permanent genomic modifications. Furthermore, problems linked to the inefficient silencing or subsequent reactivation of these integrated transgenes can impact the differentiation potential and long-term stability of the derived

iPSCs [118]. Consequently, efforts have focused on refining non-integrative strategies, such as adenoviral and adeno-associated vectors, Sendai virus, episomal plasmids, direct delivery of synthetic mRNA etc. [119–121]. These approaches avoid permanent changes to the genome, lowering the risk of unwanted mutations or gene reactivation.

After the establishment of iPSC colonies, a rigorous characterisation is essential to confirm their pluripotency and genetic integrity. This validation is crucial for later applications in disease modelling, drug testing, and potential therapeutic uses. Additionally, it is also a prerequisite for banking iPSC lines in public repositories. Today, many biobanks store patient-derived and control iPSC lines for a range of diseases, including rare neurodevelopmental disorders [122,123]. These valuable resources empower the research community to accelerate translational research and facilitate cross-laboratory reproducibility. As recommended by the International Stem Cell Banking Initiative [123], this validation typically includes: (i) assessment of pluripotency markers (alkaline phosphatase or embryonic stem cell (ESC) marker expression); (ii) *in vitro* differentiation to demonstrate the capacity to form tissues representative of the three primordial germ layers; (iii) karyotype analysis to detect chromosomal abnormalities; (iv) determination of cell identity via DNA fingerprinting; and (v) microbiological screening to ensure contaminant-free cultures. A detailed description of the characterisation procedures used to validate and bank the iPSC lines generated in our laboratory and used in this study is provided in the **Annex**.

4.2. CNS CELL TYPES TO MODEL NEURODEVELOPMENTAL DISORDERS

Modelling the cellular complexity of the CNS *in vitro* is extremely useful for uncovering the mechanisms behind neurological disorders and developing effective therapies. As previously discussed, iPSCs are a valuable tool in this field, providing a patient-specific platform to generate disease-relevant cell types. Given the severe neurological phenotypes associated with SYS, and the brain-specific expression pattern of *MAGEL2*, iPSC-derived CNS cell models are a great option to understand the cellular and molecular basis of the disease in a controlled and physiologically relevant environment.

The CNS is a highly complex tissue composed of diverse, interacting cell types, broadly classified into neurons and glial cells. Neurons show extensive variation in morphology, connectivity, and molecular identity (reviewed in [124]), making their classification technically and conceptually challenging. For clarity, this introduction will broadly focus

on glutamatergic (excitatory) and GABAergic (inhibitory) neurons, which establish the excitatory-inhibitory (E-I) balance essential for normal brain function.

- Glutamatergic neurons constitute most neurons in the cerebral cortex and are usually characterised by their triangular soma, extensive apical dendrites, and long-range axonal projections [125]. Their ability to integrate and transmit information across cortical and subcortical regions makes them central to many aspects of brain function. Glutamatergic neurons release glutamate, the brain's primary excitatory neurotransmitter. When released into the synaptic cleft, glutamate binds to receptors such as AMPA and NMDA receptors, leading to membrane depolarisation. AMPA receptors open first, letting Na^+ into the postsynaptic neuron and causing depolarisation (reviewed in [126]). NMDA receptors activate only when the cell is already depolarised and glutamate is bound, letting Ca^{2+} flow into the neuron. This Ca^{2+} influx activates enzymes like CaMKII, which in turn promotes the insertion of more AMPA receptors into the synapse and triggers changes in the shape of dendritic spines. These changes make the synapse stronger and more responsive and are the cellular basis of learning and memory.
- GABAergic neurons release gamma-aminobutyric acid (GABA), the brain's principal inhibitory neurotransmitter. GABA binds to GABA type A and B receptors, causing membrane hyperpolarisation or stabilisation of its potential. GABA type A receptors are fast-acting ion channels that let Cl^- into the cells to rapidly hyperpolarise neurons. In contrast, GABA type B receptors are slow-acting G-protein-coupled receptors (GPCRs) that modulate activity more gradually over time by activating K^+ channels and inhibiting Ca^{2+} release (reviewed in [127]). This coordinated inhibitory effect is crucial for maintaining neural circuit stability and preventing excessive, uncontrolled neuronal firing. GABAergic neurons come in many shapes and sizes, reflecting the diversity of roles they play in the brain. Unlike glutamatergic pyramidal neurons, which tend to have long axons reaching distant targets, GABAergic neurons are more locally focused. Their axons branch extensively but stay within the same brain region, and their dendrites are generally shorter and spread out in multiple directions (reviewed in [128]). Inhibitory neurons are essential for temporal synchronisation and network oscillations, and their dysfunction has been implicated in a wide range of disorders, including epilepsy, ASD, and schizophrenia [129–131].

Glial cells are the most abundant and diverse cell population in the human brain. Traditionally seen as passive structural elements providing metabolic and physical

support to neurons, research has now established their active role in many CNS processes, including myelination, synaptic modulation, homeostasis, and responses to injury (reviewed in [132]). The CNS contains four main types of glial cells: astrocytes, oligodendrocytes, microglia, and ependymal cells. While all glial cell types contribute to CNS function through diverse mechanisms, this section will concentrate on astrocytes and their critical interactions with neurons, with the others mentioned briefly for context.

- Astrocytes are star-shaped glial cells that act as regulators of neural function through their roles in neurotransmitter recycling, ionic homeostasis, lipid metabolism, and intercellular signalling. One of the most well-characterised functions of astrocytes is their role in synaptogenesis, as they secrete molecules like hevin and SPARC to control the formation, maturation, and plasticity of synapses [133]. In fact, in the mature CNS, astrocytes form the "tripartite synapse" closely surrounding pre and postsynaptic neurons. When sensing glutamate, astrocytes exhibit intracellular Ca^{2+} signalling, which can lead to the release of gliotransmitters such as glutamate, ATP or D-serine (reviewed in [134]). These astrocyte signals can modulate both presynaptic and postsynaptic neuronal activity, affecting neurotransmitter release probability, receptor sensitivity, and even synaptic plasticity mechanisms [135]. Beyond synaptic regulation, astrocytes provide essential metabolic support to neurons. They are responsible for buffering extracellular K^+ , recycling neurotransmitters like glutamate and GABA, and supplying neurons with lactate through the astrocyte-neuron lactate shuttle [136]. This metabolic coupling is critical for maintaining the excitability and function of neuronal networks.
- Oligodendrocytes are responsible for producing myelin, a lipid-rich sheath that wraps around axons and increases the speed and efficiency of electrical signal propagation (reviewed in [137]). In addition, oligodendrocytes may also provide metabolic support to neurons by shuttling lactate and pyruvate directly to axons for ATP production [138]. Oligodendrocyte development follows a well-defined lineage progression, from oligodendrocyte precursor cells to pre-myelinating and finally mature myelinating oligodendrocytes, and this maturation is tightly regulated and can be influenced by neuronal activity [139].
- Microglia are the resident immune cells of the CNS. In their resting state, microglia contribute to synaptic pruning during development, phagocytosis of apoptotic cells, and secretion of factors that support neuronal survival. When exposed to injury, infection, or stress, microglia become activated and can adopt a range of

phenotypes, ranging from pro-inflammatory to anti-inflammatory. This plasticity allows them to participate in diverse processes, including neuroinflammation, axon regeneration, and remyelination [140,141].

- Ependymal cells are a relatively uniform population of ciliated glial cells that form a thin, continuous layer along the walls of the brain's ventricles and the central canal of the spinal cord. Their main function is to facilitate the circulation of cerebrospinal fluid, contributing to the distribution of nutrients, removal of metabolic waste, and maintenance of intracranial pressure (reviewed in [142]).

The complex relationships and organisation of these diverse cell types are essential for normal CNS development and function. Understanding their individual roles and collective interactions is key for modelling and understanding neurological diseases *in vitro*.

4.2.1. IPSC DIFFERENTIATION INTO CNS CELL TYPES

Robust and efficient differentiation into CNS lineages is central to the application of iPSCs in neurodevelopmental disorder research. This process must mirror the complex molecular cascades that control neurogenesis *in vivo*, producing cells that have the morphological, electrophysiological, and molecular characteristics of their natural counterparts. Two main strategies to differentiate iPSCs into CNS cell types have been used in this work: small molecule-based methods and TF-based methods, each presenting its own advantages and considerations for generating physiologically relevant disease models. A schematic overview of the cellular reprogramming and differentiation strategies used in this study to model SYS can be found in **Figure 7**.

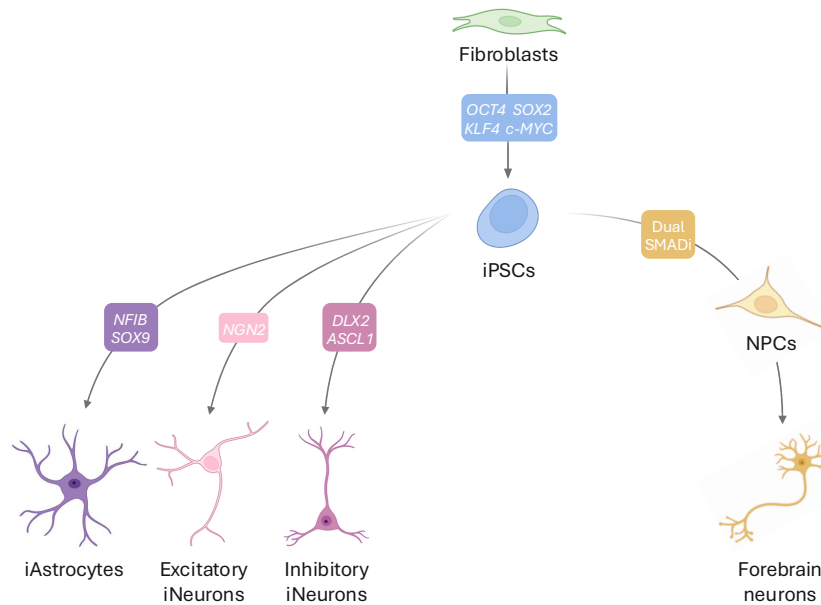


Figure 7: Schematic overview of the cellular reprogramming and differentiation strategies used in this study to model SYS. Fibroblasts were reprogrammed into iPSCs using Yamanaka factors. iPSCs were subsequently differentiated into neural progenitor cells (NPCs) through dual SMAD inhibition, followed by lineage-specific differentiation into forebrain neurons. iAstrocytes, and excitatory and inhibitory iNeurons were obtained using TF-based protocols.

4.2.1.1. Small molecule-based methods

Small molecule-based differentiation protocols guide iPSCs through diverse differentiation stages by mimicking the developmental signals and morphogen gradients that take place during embryonic development. Through temporal control of these signalling factors, cellular differentiation is directed to specific neuronal lineages and can be organised into key phases:

- **Neural induction:** In this initial phase, iPSCs are directed to form neural progenitor cells (NPCs). This is mainly achieved through the inhibition of signalling pathways that maintain pluripotency. A widely adopted example of this strategy is dual SMAD inhibition, which concurrently blocks both the transforming growth factor-beta (TGF- β) and bone morphogenetic protein (BMP) pathways using inhibitors like SB431542 (targeting TGF- β receptor type I kinase) and LDN-193189 (targeting BMP receptor type I) [143]. By inhibiting these pathways, which typically promote non-ectodermal fates, iPSCs are efficiently directed towards neuroectodermal lineage specification.

- **Patterning and specification:** Once NPCs have been generated through neural induction, the next step is to guide them toward specific identities. This is essential because the CNS is highly organised into different regions such as the forebrain, midbrain, and hindbrain, each giving rise to unique types of neurons and glial cells. Therefore, by introducing specific factors, NPCs can be guided to differentiate into region-specific neural progenitors. For instance, the anterior-posterior identity of neural progenitors is largely determined by the activity of the Wnt and fibroblast growth factor (FGF) pathways. Low Wnt/FGF activity typically promotes anterior identities, while higher levels favour posterior fates [144]. Similarly, dorsoventral patterning is often guided by factors like Sonic Hedgehog for ventral identities and BMPs for dorsal identities [145]. In addition, timing and lineage commitment are fine-tuned by modulating other developmental pathways. For example, as Notch signalling maintains NPCs in an undifferentiated state, inhibitors such as DAPT are often used to promote neuronal differentiation [146].
- **Terminal differentiation:** In this final stage, progenitor cells transition into mature neurons or glial cells with functional and morphological characteristics. For neurons, this process involves the development of mature electrophysiological properties, including the ability to fire action potentials and form functional synaptic connections. At the molecular level, terminal differentiation is characterised by the expression of lineage-specific markers. Mature neurons typically express NeuN, while astrocytes express GFAP. The maturation process can be enhanced by co-culture systems, which have been shown to promote synapse formation, network activity, and overall neuronal health [147]. Additionally, the supplementation with neurotrophic factors can accelerate and support the maturation of both neurons and glia [148].

Given the complexity of CNS development, it is difficult to define a universal protocol. Nevertheless, many highly referenced protocols fall into two main categories: monolayer protocols and embryoid body (EB) protocols. In monolayer-based protocols, iPSC colonies are maintained as a monolayer during neural induction and develop directly into characteristic neural rosettes without aggregation, typically due to the consistent action of SMAD signalling inhibitors [143,149]. In contrast, a typical EB differentiation protocol involves the initial formation of 3D cellular aggregates in suspension culture. These EBs are then adhered to a substrate and exposed to specific differentiation factors, allowing them to spread out and form neural rosettes [150]. NPCs emerge from these rosettes, which can then be passaged to establish relatively stable cells that will ultimately give

rise to mature neurons. While some differentiation protocols described aim to obtain any general neural lineage, others have developed more precise methods to yield specific neuronal types, such as dopaminergic neurons [151] or GABAergic neurons [152].

When optimised, small-molecule differentiation protocols can produce consistent and relatively pure neural populations. From a practical point of view, small molecules are typically cost-effective and can be applied to large-scale cultures, making them suitable for applications like drug screening. Despite these benefits, several challenges exist. Many small molecule-based differentiation protocols are lengthy, often requiring weeks to months to generate neural populations. Also, differentiated neurons may sometimes exhibit immature phenotypes, lacking the full electrophysiological complexity and synaptic integration of adult neurons *in vivo* [153]. Additionally, despite efforts to establish defined conditions, batch-to-batch variations in small molecule activity or iPSC line characteristics can influence differentiation outcomes.

4.2.1.2. Transcription factor-based methods

TF-based differentiation, also known as forced or direct programming, is a strategy that uses the overexpression of lineage-specific TFs to rapidly drive iPSCs, or even somatic cells, into a desired cell type. These TFs are commonly delivered using gene transfer systems like lentiviral or adenoviral vectors, or plasmid transfection. To ensure robust expression and improve purity, antibiotic resistance genes are often included for selecting successfully modified cells. Furthermore, researchers can also control how long the TFs stay active, using either constitutively active promoters or inducible systems like Tet-On.

A defining strength of TF-based reprogramming lies in its rapid induction of neural identity. Unlike traditional small-molecule differentiation, these methods can generate functional neurons in as little as two to three weeks. Another key advantage of direct differentiation lies in its speed and consistency, producing a high percentage of differentiated cells, with some reports indicating yields close to 100% [154]. Despite these benefits, the success of direct reprogramming largely depends on how effectively the genetic material is introduced. Low transfection or transduction efficiency can limit the number of cells that respond to the TFs, reducing the overall yield and consistency.

A variety of TF-based methods have been developed to generate specific neuronal subtypes from iPSCs, each using different lineage-defining factors to drive cell fate specification. For example, overexpressing *NGN2* using lentiviral constructs converts human iPSCs into excitatory neurons in just two weeks [154]. Similarly, specific combinations of TFs have been used to generate other neuronal populations: a combination of *ASCL1* and *DLX2* yields GABAergic neurons [155], while dopaminergic neurons can be derived using *ASCL1*, *NURR1*, and *LMX1A* [156]. For motor neurons, *NGN2* combined with *ISL1*, and *LHX3* have proven effective [157]. Not limited to neurons, TF-driven methods have also successfully generated functional astrocytes. The overexpression of *NFIB* and *SOX9* has reprogrammed human iPSCs into induced astrocyte populations that closely resemble adult human astrocytes after only two weeks [158].

OBJECTIVES

The main aim of this thesis is to advance the understanding of the molecular and cellular pathophysiology of Schaaf-Yang syndrome (SYS), with a focus on elucidating the effects of *MAGEL2* truncating mutations and identifying potential therapeutic targets.

To address this general aim, the following specific objectives were defined:

1) Characterisation of SYS and functional insights.

- To conduct a revision and curation of the phenotypic and genetic landscapes of previously reported SYS cases, aiming to establish a unified clinical picture and genetic spectrum of the disease.
- To assess the molecular behaviour of truncated *MAGEL2* proteins using heterologous systems by comparing their stability, degradation dynamics, and subcellular localisation relative to the WT protein.
- To perform a characterisation and functional analysis of fibroblasts derived from SYS patients, in order to identify cellular dysregulations associated with *MAGEL2* truncation in patient-derived cells.

2) Generation and molecular profiling of disease-relevant cell types.

- To characterise patient-derived induced pluripotent stem cell (iPSC) lines as a foundational step to generate *in vitro* neural models for SYS.
- To establish transcription factor and small molecule-based differentiation strategies to generate central nervous system (CNS) cell types relevant to SYS pathophysiology.
- To investigate molecular signatures of *MAGEL2* dysfunction on CNS cell types differentiated from SYS patient-derived iPSCs through multi-omic and functional analyses.
- To identify conserved molecular signatures and candidate biomarkers, for potential use in diagnosis, patient stratification, or therapeutic monitoring.

RESULTS

REPORT OF THE SUPERVISORS ON THE CONTRIBUTION OF THE PhD CANDIDATE TO THE ARTICLES INCLUDED IN THIS THESIS

Title of the thesis: “Cellular and Molecular Insights into Schaaf-Yang Syndrome: A Multimodel Analysis from Fibroblasts to iPSC-Derived Neural Cell Types”

Author: Mónica Centeno-Pla

Supervisors: Dr. Raquel Rabionet, Dr. Roser Urreizti, and Dr. Mercedes Serrano

Dr. Raquel Rabionet, Dr. Roser Urreizti, and Dr. Mercedes Serrano, acting as co-directors of the thesis entitled “Cellular and Molecular Insights into Schaaf-Yang Syndrome: A Multimodel Analysis from Fibroblasts to iPSC-Derived Neural Cell Types” presented by Mónica Centeno-Pla as a compendium of 3 research articles, detail the precise contribution of the PhD candidate to each publication below.

ARTICLE 1

Title: Advancing in Schaaf-Yang syndrome pathophysiology: from bedside to subcellular analyses of truncated MAGEL2

Authors: Laura Castilla-Vallmanya*, Mónica Centeno-Pla*, Mercedes Serrano*, Héctor Franco-Valls, Raúl Martínez-Cabrera, Aina Prat-Planas, Elena Rojano, Juan A. G. Ranea, Pedro Seoane, Clara Oliva, Abraham J. Paredes-Fuentes, Gemma Marfany, Rafael Artuch, Daniel Grinberg, Raquel Rabionet*, Susanna Balcells*, Roser Urreizti*

Journal: Journal of Medical Genetics **Number:** 60(4)

Pages: 406-415

Impact Factor (2023): 3.6

Quartile: Q2

Category: Genetics & Heredity

Contribution of the PhD candidate: The candidate contributed to the collection and review of previously published genetic and clinical data. She took part in experiments studying the heterologous expression of the mutated MAGEL2 protein and in quantifying Aβ1-40 secretion levels. She played an active role in analysing metabolomic data and performing immunocytochemistry quantification. She also collaborated in the preparation of the tables and figures presented in the manuscript.

A preliminary unpublished draft of this scientific article was included in Dr. Castilla-Vallmanya's PhD thesis “Combining exome sequencing and functional studies to identify causal genes of ultra-rare neurodevelopmental disorders” as Article 9.

ARTICLE 2

Title: Subcellular localisation of truncated MAGEL2 proteins: insight into the molecular pathology of Schaaf-Yang syndrome

Authors: Mónica Centeno-Pla, Estefanía Alcaide-Consuegra, Sophie Gibson, Aina Prat-Planas, Juan Diego Gutiérrez-Ávila, Daniel Grinberg, Susanna Balcells*, Roser Urreizti*, Raquel Rabionet*

Journal: Journal of Medical Genetics **Number:** 61(8)

Pages: 780-782

Impact Factor (2024): 3.7

Quartile: Q2

Category: Genetics & Heredity

Contribution of the PhD candidate: Mónica Centeno-Pla participated in the design and cloning of the plasmids used in the study. She performed the analysis and interpretation of the experimental results, and prepared the tables and figures included in the manuscript. She drafted the first version of the manuscript and was also involved in the revision and modifications of the final version following feedback from co-authors and journal reviewers.

ARTICLE 3

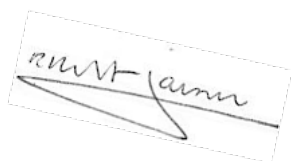
Title: A Multi-Omic and Functional Perspective on Neural Cell Dysfunction in Schaaf-Yang Syndrome

Authors: Mónica Centeno-Pla, Aina Prat-Planas, Juan Diego Gutiérrez-Ávila, Clàudia Puigsasllas-Pastor, Elena Rojano, Pedro Seoane, Daniel Grinberg, Susanna Balcells, Daniel Tornero, Isaac Canals, Roser Urreizti, Raquel Rabionet

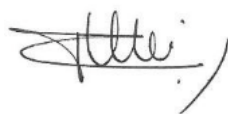
Journal: To be submitted to the European Journal of Human Genetics.

Contribution of the PhD candidate: The candidate performed the differentiation of iPSCs into neural cultures and carried out their immunochemical characterization. She prepared RNA and cellular extract samples for transcriptomic and proteomic analyses and conducted quantification experiments of extracellular A β 1-40. She also participated in the analysis of all multi-omic and functional experiments. In addition, she prepared the tables and figures included in the manuscript, drafted the first version of the article, and was responsible for implementing the revisions based on feedback from the co-authors.

Barcelona,



Raquel Rabionet



Roser Urreizti



Mercedes Serrano

CHAPTER 1: CHARACTERISATION OF SCHAAF-YANG SYNDROME AND FUNCTIONAL INSIGHTS

ARTICLE 1: ADVANCING IN SCHAAF-YANG SYNDROME PATHOPHYSIOLOGY: FROM BEDSIDE TO SUBCELLULAR ANALYSES OF TRUNCATED MAGEL2

Summary:

Background: Schaaf-Yang syndrome (SYS) is caused by truncating mutations in *MAGEL2*, mapping to the Prader-Willi region (15q11-q13), with an observed phenotype partially overlapping that of Prader-Willi syndrome. *MAGEL2* plays a role in retrograde transport and protein recycling regulation. Our aim is to contribute to the characterisation of SYS pathophysiology at clinical, genetic and molecular levels.

Methods: We performed an extensive phenotypic and mutational revision of previously reported patients with SYS. We analysed the secretion levels of amyloid- β 1-40 peptide (A β 1-40) and performed targeted metabolomic and transcriptomic profiles in fibroblasts of patients with SYS (n=7) compared with controls (n=11). We also transfected cell lines with vectors encoding wild-type (WT) or mutated *MAGEL2* to assess stability and subcellular localisation of the truncated protein.

Results: Functional studies show significantly decreased levels of secreted A β 1-40 and intracellular glutamine in SYS fibroblasts compared with WT. We also identified 132 differentially expressed genes, including non-coding RNAs (ncRNAs) such as *HOTAIR*, and many of them related to developmental processes and mitotic mechanisms. The truncated form of *MAGEL2* displayed a stability similar to the WT but it was significantly switched to the nucleus, compared with a mainly cytoplasmic distribution of the WT *MAGEL2*. Based on the updated knowledge, we offer guidelines for the clinical management of patients with SYS.

Conclusion: A truncated *MAGEL2* protein is stable and localises mainly in the nucleus, where it might exert a pathogenic neomorphic effect. A β 1-40 secretion levels and *HOTAIR* mRNA levels might be promising biomarkers for SYS. Our findings may improve SYS understanding and clinical management.

Reference:

Castilla-Vallmanya L, Centeno-Pla M, Serrano M, *et al.* Advancing in Schaaf-Yang syndrome pathophysiology: from bedside to subcellular analyses of truncated *MAGEL2*. *J Med Genet* 2023;60:406–415 doi:10.1136/jmedgenet-2022-108690

Functional genomics



Original research

Advancing in Schaaf-Yang syndrome pathophysiology: from bedside to subcellular analyses of truncated MAGEL2

Laura Castilla-Vallmanya ,^{1,2,3} Mónica Centeno-Pla ,^{1,2,4} Mercedes Serrano ,^{2,3,5} Héctor Franco-Valls,¹ Raúl Martínez-Cabrera,¹ Aina Prat-Planas ,^{1,2,3} Elena Rojano,⁶ Juan A G Ranea,^{3,6} Pedro Seoane,^{3,6} Clara Oliva,^{2,4} Abraham J Paredes-Fuentes ,^{2,4} Gemma Marfany ,^{1,2,3} Rafael Artuch ,^{2,3,4} Daniel Grinberg ,^{1,2,3} Raquel Rabionet ,^{1,2,3} Susanna Balcells ,^{1,2,3} Roser Urreizti ,^{2,3,4}

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ABSTRACT

Background Schaaf-Yang syndrome (SYS) is caused by truncating mutations in *MAGEL2*, mapping to the Prader-Willi region (15q11-q13), with an observed phenotype partially overlapping that of Prader-Willi syndrome. *MAGEL2* plays a role in retrograde transport and protein recycling regulation. Our aim is to contribute to the characterisation of SYS pathophysiology at clinical, genetic and molecular levels.

Methods We performed an extensive phenotypic and mutational revision of previously reported patients with SYS. We analysed the secretion levels of amyloid- β 1–40 peptide ($A\beta_{1-40}$) and performed targeted metabolomic and transcriptomic profiles in fibroblasts of patients with SYS (n=7) compared with controls (n=11). We also transfected cell lines with vectors encoding wild-type (WT) or mutated *MAGEL2* to assess stability and subcellular localisation of the truncated protein.

Results Functional studies show significantly decreased levels of secreted $A\beta_{1-40}$ and intracellular glutamine in SYS fibroblasts compared with WT. We also identified 132 differentially expressed genes, including non-coding RNAs (ncRNAs) such as *HOTAIR*, and many of them related to developmental processes and mitotic mechanisms. The truncated form of *MAGEL2* displayed a stability similar to the WT but it was significantly switched to the nucleus, compared with a mainly cytoplasmic distribution of the WT *MAGEL2*. Based on the updated knowledge, we offer guidelines for the clinical management of patients with SYS.

Conclusion A truncated *MAGEL2* protein is stable and localises mainly in the nucleus, where it might exert a pathogenic neomorphic effect. $A\beta_{1-40}$ secretion levels and *HOTAIR* mRNA levels might be promising biomarkers for SYS. Our findings may improve SYS understanding and clinical management.

INTRODUCTION

In 2013, truncating mutations in *MAGEL2* (OMIM 605283) were associated with a new clinical entity,¹ first described as a Prader-Willi-like syndrome and currently named as Schaaf-Yang syndrome (SYS, OMIM 615547). *MAGEL2* is one of the

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ *MAGEL2*-truncating mutations cause Schaaf-Yang syndrome (SYS), but the functional effects of the truncated *MAGEL2* protein have been poorly defined.

WHAT THIS STUDY ADDS

⇒ By expressing truncated *MAGEL2* in cell lines, we now know that a truncated version of the protein is retained in the nucleus, thus exerting a novel behaviour in addition to the loss of some of its main functions. Patients' fibroblasts show reduced levels of excreted amyloid- β 1–40 and intracellular glutamine as well as an altered transcriptomic profile, including overexpression of the major regulator *HOTAIR*.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Based on a comprehensive review of genetic and clinical aspects of all reported cases, families and physicians will benefit from the Clinical Management Recommendations that we provide here.

five maternally imprinted protein-coding genes contained in the Prader-Willi region (15q11-q13). Lack of expression of the paternal alleles in this region causes Prader-Willi syndrome (PWS; OMIM 176270). In contrast, non-sense or frameshift mutations in the paternal allele of *MAGEL2* are predicted to encode a truncated protein lacking the MAGE Homology Domain (MHD) and have been associated with SYS.¹ Since then, over a hundred patients with SYS have been reported and phenotypically described.^{1–28} Patients with SYS and PWS show overlapping clinical phenotypes, including neonatal hypotonia, intellectual disability (ID), developmental delay (DD), early feeding difficulties, endocrinological disturbances (hypogonadism and other hormonal imbalances) and sleep disorders. However, some of the clinical criteria for PWS diagnosis, such as hypopigmentation, characteristic

facial dysmorphisms, small hand and feet, hyperphagia, obesity and obsessive-compulsive behaviours, are frequently absent in patients with SYS, who, on the other hand, present more frequently with severe ID, autism spectrum disorder (ASD) behaviours and joint contractures.^{29–31} Some truncating variants in *MAGEL2* have also been associated with Chitayat-Hall syndrome (CHS).³² However, a systematic review of all patients with SYS and CHS showed that there is no discernible genetic or clinical difference between both syndromes, and the latter has been renamed as SYS in OMIM.¹⁷ In contrast, two particular *MAGEL2* truncating mutations have been recurrently identified in patients affected by lethal arthrogryposis multiplex congenita (AMC), a much more severe phenotype, distinct from SYS.^{4 10 27 33 34} All in all, there is no specific constellation of symptoms pathognomonic or specific for any of these clinical syndromes; furthermore, they probably conform to a clinical continuum, therefore denoting the need to address clinical denomination according to molecular findings.

MAGEL2 shows a wider expression in human fetal tissues than in adult tissues, where it is predominantly present in brain (according to GTEx³⁵). In adult mice, it also becomes mostly restricted to the central nervous system, specifically to the amygdala and the hypothalamus, and predominantly in the suprachiasmatic, the paraventricular and the supraoptic nuclei.^{36–38} It is a single-exon gene that encodes one of the largest proteins of the type II MAGE protein family consisting of 1249 amino acids. At a structural level, the N-terminal region of *MAGEL2* contains a proline-rich domain, whose function remains unclear.³⁹ At the C-terminus, from amino acids 1027 to 1195, there is the MHD, a highly conserved 170-amino acid sequence present in both type I and type II MAGEs, crucial for protein–protein interaction.⁴⁰ Through it, *MAGEL2* recognises and binds the coiled-coil domain of the E3 ubiquitin ligase TRIM27. The MHD is also crucial for binding VPS35, a subunit of the retromer cargo-selective complex. *MAGEL2*, TRIM27 and USP7 form the MUST complex, which is recruited to endosomes through direct binding of *MAGEL2* to VPS35 and plays a role in retrograde endosomal transport.^{41 42} These specialised endosomes participate in endosomal export pathways that deliver membrane protein cargoes either to the trans-Golgi network through retrograde pathways or to the plasma membrane through recycling pathways.⁴³

A dysfunction of the retrograde transport could be disturbing for many cellular processes. Loss of *MAGEL2* expression causes a reduction in secretory granules protein levels due to impaired endosomal protein trafficking and subsequent lysosomal degradation, resulting in a reduction of circulating bioactive hypothalamic hormones.³⁶ A well-coordinated trafficking network is also key for the correct regulation of amyloid precursor protein (APP) cleavage.⁴⁴ APP family members are relevant for neuronal differentiation and migration during cortical development^{45 46} and proper neuromuscular junction formation and neurotransmission.⁴⁷ Many studies support a model where retromer deficiency leads to increased APP cleavage, A β peptide production and exocytosis.^{48–50} In addition, protein levels of the glucose transporter GLUT1 in the cell membrane are reduced after VPS35 and SNX27 inhibition, showing that they are also tightly regulated by the retromer.⁵¹

Here, we have performed an extensive literature revision and based on it, we have expanded the clinical and genetic delineation of SYS and developed a standardised set of guidelines for its clinical management. We also contribute to the knowledge of the cellular phenotype by assessing the effect of a recurrent truncating variant on *MAGEL2* protein stability and subcellular

localisation using heterologous expression vectors. Finally, we have performed a transcriptomic and metabolomic characterisation of fibroblasts derived from patients with SYS and interpreted the results in the context of molecular and clinical findings.

RESULTS

Clinical management of patients with SYS requires a coordinated multidisciplinary approach

We performed a systematic revision of all the published patients with SYS to date, who were all carriers of *MAGEL2* mutations (table 1 and online supplemental table 1), carefully inspecting both the molecular and phenotypic data, with the aim to propose a set of guidelines for SYS clinical management.

The literature-based recommendations have been divided into two life periods: the perinatal period (first 28 days of life) and infancy/adolescence. They include the most relevant medical problems associated with each period and the specific concerns or interventions advisable for each area. A schematic version of the different clinical areas and tests included in the guidelines are represented in figure 1, and a detailed, printable version is supplied as online supplemental table 2 (both in Spanish as online supplemental figure 1 and online supplemental table 3).

In most instances, pregnancy was uneventful (only polyhydramnios has been reported in some cases), although there is a high rate of caesarean sections (over 50% of the patients where delivery information is available). Clinical symptoms appear early in life showing a complex phenotype that includes neuromuscular symptoms, respiratory and endocrinological problems, feeding difficulties and dysmorphic traits. Other clinical issues can appear later and may affect almost any organ or system, requiring a coordinated multidisciplinary approach.

SYS variants are mostly truncating and located in the C-terminal domain

To date, 61 different variants have been associated with SYS or AMC (figure 2). Mutation p.(Gln666Profs*47), present in 80 individuals, is considered a recurrent mutational hotspot. These variants are mostly truncating and predominantly located in the C-terminal region of the protein, leading to a partial or a total lack of the MHD domain and compromising the functions that *MAGEL2* carries out through this domain. Three different missense variants and a small in-frame duplication have also been associated with SYS phenotypes (in grey in figure 2). These atypical variants are not predicted to encode a truncated form of the protein, and there are no functional studies to support their pathogenicity. Thus, their clinical implication remains to be proven.

Fibroblasts from patients with SYS show altered gene expression patterns

To better understand the effect of *MAGEL2* truncating mutations on gene expression patterns, we performed an mRNA whole transcriptome analysis (mRNASeq) on fibroblasts from six healthy donors and three SYS subjects: a girl carrying p.(Gln638*) and a boy and an unrelated girl, both carrying p.(Gln666Profs*47). Using the ExpHunter Suite, we identified 132 differentially expressed genes, 76 upregulated and 56 downregulated (online supplemental table 4 and online supplemental figure 2A,B). The top 10 upregulated and downregulated genes, showing the most significant changes in the expression fold, are listed in table 2. Four genes were tested by quantitative PCR (qPCR) in fibroblasts from five patients with SYS and four control individuals,



Functional genomics

Table 1 Clinical overview of patients with MAGEL2-related disorders

		Total	
	Diagnostic	SYS	AMC
	Individuals	122	13
	Gender	43 F; 42 M; 38 UnK	7 F; 5 M; 1 UnK
	Protein change	59 different mutations	p.Gln666Serfs*36; p.Leu708Trpfs*7
Phenotype (HPO term)			
Pregnancy complications			
Decreased fetal movement	HP:0001558	12/37 (32.4%)	4/4
Polyhydramnios	HP:0001561	7/16 (43.75%)	5/5
Neurology and neurodevelopment			
Neurodevelopmental delay	HP:0012758	100/100 (100%)	
Intellectual disability	HP:0001249	92/97 (94.8%)	
Autistic behaviour	HP:0000729	55/76 (72.4%)	
Abnormality of brain morphology	HP:0012443	19/32 (59.4%)	1/1
Infantile lethargy/weak cry	HP:0001254/HP:0001612	15/24 (62.5%)	
Epilepsy	HP:0001250	24/79 (30.4%)	
Respiratory			
Respiratory difficulties	HP:0002098	60/74 (81.8%)	1/1
Apnoea	HP:0002104	11/18 (61.6%)	
Sleep apnoea	HP:0010535	53/75 (70.7%)	
Respiratory failure requiring mechanical ventilation	HP:0004887	44/73 (60.3%)	
Respiratory distress requiring endotracheal intubation	HP:0004887	16/50 (32.0%)	
Recurrent respiratory infections	HP:0002205	5/6 (83.3%)	
Feeding and growth			
Feeding difficulties	HP:0011968	92/106 (86.8%)	1/1
Poor suck	HP:0002033	72/81 (88.9%)	
Dysphagia	HP:0002015	35/50 (70.0%)	
Nasogastric tube feeding in infancy	HP:0011470	41/63 (65.1%)	
Gastrostomy tube feeding in infancy	HP:0011471	26/54 (48.5%)	
Hyperphagia	HP:0002591	17/63 (27.0%)	
Increased body weight/obesity	HP:0004324/HP:0001513	24/79 (30.4%)	
Gastro-oesophageal reflux	HP:0002020	38/76 (50.0%)	
Chronic constipation	HP:0012450	46/76 (60.5%)	
Physical characteristics			
Neonatal hypotonia	HP:0001319	54/79 (68.4%)	2/2
Congenital contractures	HP:0002803	93/106 (87.7%)	
AMC	HP:0002804	15/31 (48.4%)	11/11
Abnormality of the eye	HP:0000478	41/52 (78.8%)	
Scoliosis	HP:0002650	38/66 (57.6%)	
Short stature	HP:0004322	34/49 (69.4%)	
Small hands	HP:0200055	30/49 (61.2%)	
Campodactyly of finger	HP:0100490	27/54 (50.0%)	4/4
Tapered fingers	HP:0001182	15/44 (34.1%)	
Small feet	HP:0001773	14/38 (36.8%)	
Bilateral clubfoot	HP:0001776	8/27 (29.6%)	4/4
Facial dysmorphism	HP:0001999	64/70 (91.4%)	5/5
Endocrinology			
Hypopituitarism	HP:0040075	7/13 (53.8%)	1/1
Growth hormone deficiency	HP:0000824	16/22 (72.7%)	
Hypothyroidism	HP:0000821	8/27 (29.6%)	
Hypoglycaemia	HP:0001943	14/22 (63.6%)	
Temperature instability	HP:0005968	39/62 (62.9%)	
Diabetes insipidus	HP:0000873	5/17 (29.4%)	
Hypogonadism	HP:0000135	40/80 (50%)	2/2
Cryptorchidism	HP:0000028	8/12 (66.7%)	
Other alterations			
Congenital heart defect	HP:0001627	7/20 (35.0%)	
Bradycardia	HP:0001662	3/4 (75.0%)	
Sleep disturbance	HP:0002360	13/13 (100%)	
Death in infancy/childhood	HP:0001522/HP:0003819	10 cases	13 cases

AMC, arthrogryposis multiplex congenita; F, female; HPO, Human Phenotype Ontology; M, male; SYS, Schaaf-Yang syndrome; UnK, unknown.

Schaaf-Yang Syndrome Clinical Management

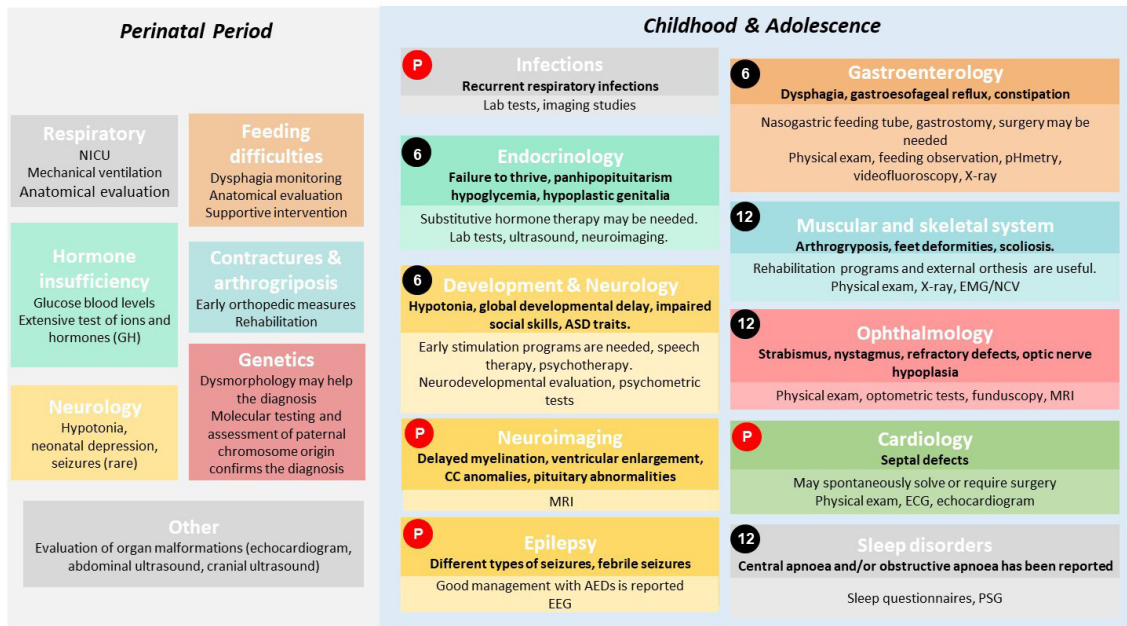


Figure 1 Schematic guidelines for Schaaf-Yang syndrome clinical management. AEDs, anti epileptic drugs; ASD, autism spectrum disorder; CC, corpus callosum; EEG, electroencephalogram; EMG/NCV, electromyogram/nerve conduction velocity; GH, growth hormone; NICU, neonatal intensive care unit; PSG, polysomnography. Perinatal period: Boxes include the medical area of disease. Medical problems and management requirements are detailed below. Childhood and adolescence: Boxes include medical area of disease. Medical problems and diagnosis are detailed below in bold. Management recommendations and complementary examinations are below, not in bold letters with lighter colours at the bottom. Numbers in black dots state the recommended periodicity for clinical evaluation in months for every specialty for all the individuals. In contrast, a P in a red dot means that those complementary examinations and management tools need to be personalised, and applied only if patient's personal characteristics require them, always following the medical team criteria. For instance, MRI and cardiology are recommended at diagnosis/birth for all patients, but follow-up depends on comorbidities. Please see online supplemental table 2 for more details. A Spanish version of this figure is included as online supplemental figure 1.

which confirmed significant upregulation of *HOTAIR* and *PITX1* and downregulation of *TBX5* (online supplemental figure 2C).

Enrichment analysis on the 132 identified DEGs highlighted a group of five genes related to 'collagen formation' and various mitosis-related REACTOME categories, such as 'Resolution of Sister Chromatid Cohesion' and 'Mitotic Spindle Checkpoint' (online supplemental figure 2D). Consistently, most of those genes were also present in the 'Kinetochore' and 'Chromosome, centromeric region' categories according to Gene Ontology cellular component enrichment (online supplemental table 5).

SYS fibroblasts show decreased $A\beta_{1-40}$ peptide secretion levels

Given MAGEL2 function in the retromer, the truncation of MAGEL2 could affect APP cleavage and $A\beta_{1-40}$ peptide production rates. Thus, levels of $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides were measured by ELISA in the extracellular medium from SYS, PWS and control fibroblasts in the search for a potential biomarker of truncated MAGEL2 function. SYS fibroblasts showed significantly decreased extracellular levels of the $A\beta_{1-40}$ processed peptide, both compared with PWS and control fibroblasts. No differences were observed in the PWS group compared with the control (figure 3A). Levels of $A\beta_{1-42}$ peptide were extremely low

and no differences were observed between conditions (data not shown).

SYS fibroblasts show altered levels of organic acids and amino acids (metabolic profiling in fibroblasts)

Mass spectrometry analysis of intracellular metabolites from extracts of SYS ($n=4$), PWS ($n=9$) and control ($n=5$) fibroblasts showed a robust and significant decrease in glutamine levels for SYS fibroblasts (figure 3B), as well as a significant increase of suberic, sebatic, adipic and malic organic acids levels (online supplemental figure 3).

We investigated if any of the genes involved in these metabolites' pathways were differentially expressed in the transcriptomic analysis (online supplemental table 4). The only differentially expressed gene involved in glutamine (GO:0006541) or organic acid (GO:0006082) metabolic processes was *ME1* (ENSG00000065833), encoding the NADP-dependent malic enzyme protein, which was upregulated in SYS fibroblasts.

Truncated form of MAGEL2 remains stable in the cell

To evaluate the stability and recycling of a truncated form of MAGEL2, HEK293T cells were transfected with haemagglutinin

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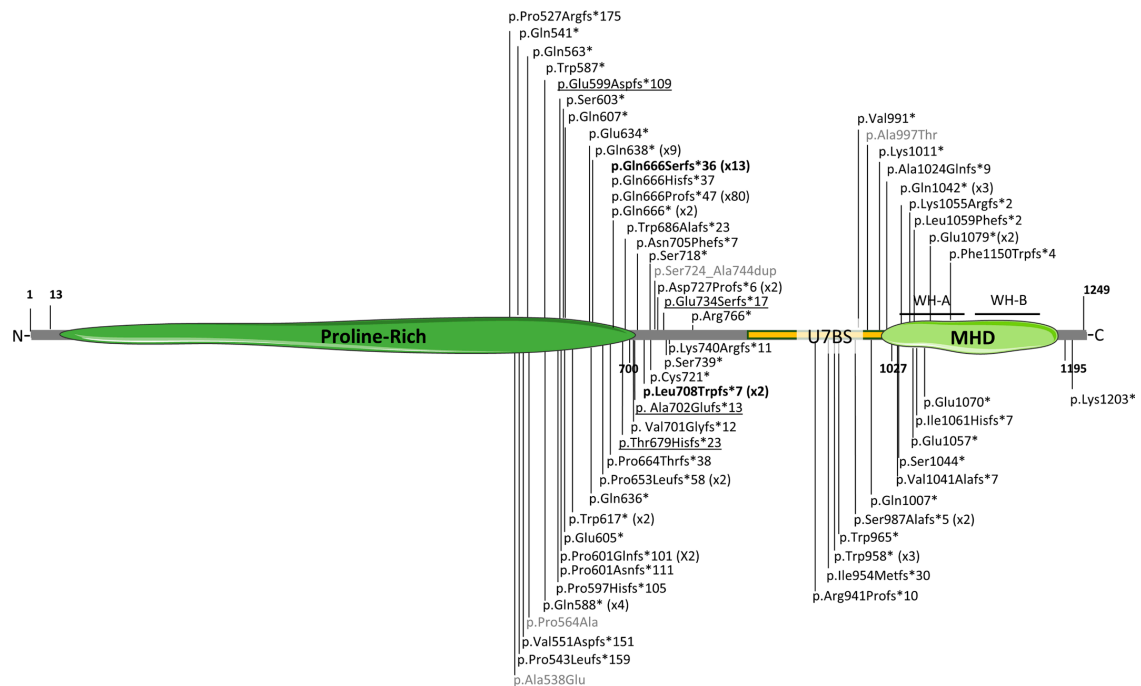


Figure 2 Schematic representation of *MAGEL2* disease-associated variants. MHD, MAGE Homology Domain. The number in brackets indicates the number of individuals carrying recurrent mutations. In bold, mutations p.Gln666Serfs*36 (x13) and p.Leu708Trpfs*7 (x2) associated with arthrogryposis multiplex congenita. In grey, atypical, non-truncating variants. Previously misreported variants c.1797_1820del (p.Glu599Aspfs*109 (c.1797_1810del)), c.224delC (p.Thr679Hisfs*23 (c.2034delC)), c.2015delC (p.Ala702Glufs*13 (c.2105delC)) and c.390delA (p.Glu734Serfs*17 (c.2199delA))¹⁰ are underlined. All variants are referenced to the *MAGEL2* hg38 transcript NM_019066.5. The complete list of variants, their cDNA annotation and reference are collected in online supplemental table 4. Image created with Servier Medical Art (smart.servier.com).

(HA)-tagged expression vectors containing either the wild-type (WT) cDNA sequence of *MAGEL2* (*MAGEL2*-WT) or the c.1912C>T; p.Gln638* (*MAGEL2*-Gln638*) mutation, which encodes a protein 611 amino acids shorter than the WT form and has been reported in nine patients (online supplemental table 1). Blocking of the proteasomal degradation pathway with MG132 (online supplemental figure 4A) or the lysosomal pathway with bafilomycin (online supplemental figure 4B) showed that *MAGEL2* is mostly degraded via proteasome with no differences between WT and truncated *MAGEL2* (online supplemental figure 4A,B). A time-course of cycloheximide treatment (2, 4, 6, 12 hours) showed also a similar stability and half-life for both *MAGEL2* forms (online supplemental figure 4C).

Increased nuclear localisation of the truncated form of *MAGEL2*

To determine the subcellular localisation of the p.Gln638* truncated form of *MAGEL2*, HEK293T, SAOS-2 and HeLa cells were transfected with the expression vectors *MAGEL2*-WT or *MAGEL2*-Gln638*. Immunocytochemistry assays detecting HA showed that in SAOS-2 cells transfected with the *MAGEL2*-Gln638* construct and quantified by Mander's coefficient there was a shift of the protein towards the nucleus, while the *MAGEL2*-WT form was mainly located in the cytoplasm (figure 3C,D). A similar localisation pattern was observed in HEK293T and HeLa cells (data not shown), supporting the idea that presence of variant p.Gln638*, which leads to the lack of

part of the C-terminal sequence, affects protein subcellular localisation independently of the cell type. This result is consistent with predictions obtained following the Scandinavian Protocol, which combines different online tools to predict protein subcellular localisation.^{52,53}

DISCUSSION

Since its first description in 2013,¹ more than 150 SYS individuals carrying *MAGEL2*-truncating mutations have been published. Most publications include one or a reduced group of patients, often with scarce clinical descriptions, hampering the definition of the syndrome's characteristics. Families, already burdened by the solitude of having an ultrarare condition, lack clear and established clinical guidelines for their treatment and follow-up. Once available, such evidence-based recommendations will help reduce inequity in healthcare and empower both families and clinicians facing such a rare disease. To develop the recommendations, we performed an extensive revision of all the SYS subjects published so far at both the phenotypic and genetic level and elaborated a comprehensive and detailed follow-up programme. Despite the comprehensive revision, to date, no clear underlying phenotype-genotype correlation was observed, with the exception of two particular variants [p.(Gln666Serfs*36) and p.(Leu708Trpfs*7)] which are associated with the much more severe phenotype of AMC leading to perinatal death.

Genetically, SYS is caused by non-sense or frameshift mutations. Only one *MAGEL2* missense variant (c.1613C>A;

Table 2 Top 10 upregulated and downregulated DEGs identified after mRNASeq analysis of skin fibroblasts in patients with SYS and controls

	Gene	ID	Log ₂ FC	FDR
Upregulated	HOTAIR	ENSG00000228630	7.470	8.02E-30
	MTRNR2L1	ENSG00000256618	5.523	1.14E-05
	PTPRD	ENSG00000153707	4.625	1.23E-06
	SLITRK4	ENSG00000179542	4.524	8.81E-03
	GATA2-AS1	ENSG00000244300	4.304	1.41E-18
	PITX1	ENSG00000069011	4.234	8.16E-03
	CLDN1	ENSG00000163347	4.145	1.12E-06
	ISL2	ENSG00000159556	4.088	1.93E-03
	RARB	ENSG00000077092	4.000	1.49E-02
	VANGL2	ENSG00000162738	3.975	8.09E-07
Downregulated	ANGPTL1	ENSG00000116194	-7.084	3.66E-06
	TBX5	ENSG00000089225	-6.982	9.18E-39
	PI16	ENSG00000164530	-6.385	1.29E-03
	TBX5-AS1	ENSG00000255399	-5.457	5.44E-12
	INMT	ENSG00000241644	-5.041	2.56E-03
	CRLF1	ENSG00000006016	-5.029	6.80E-04
	ASPA	ENSG00000108381	-4.813	1.02E-03
	WISP2	ENSG00000064205	-4.420	3.87E-08
	KY	ENSG00000174611	-4.248	1.30E-11
	APOD	ENSG00000189058	-4.213	6.00E-05

Genes analysed by quantitative PCR are given in bold.
DEGs, differentially expressed genes; FC, fold change; FDR, false discovery rate ; mRNASeq, mRNA sequencing; SYS, Schaaf-Yang syndrome .

p.Ala538Glu) has been reliably described as potentially disease-associated.¹⁷ While at the moment of its publication, this change was classified as ‘disease causing’ based on available in silico and frequency data, currently, updated information, including a gnomAD V3.1 Amish MAF of 0.03, supports its reclassification as ‘likely benign’ according to the American College of Medical Genetics (ACMG) guidelines.⁵⁴ Clinically, the patient carrying this variant presented with DD, ASD and dysmorphic traits. While her presentation shares some traits with SYS, the high frequency of this variant in the Amish population, together with the non-specific character of these traits, would suggest that it is not the main cause of the disease. Two other missense mutations have been identified in two different patients: p.Pro564Ala and p.Ala997Thr (with two and four carriers in gnomAD v3.1.1, respectively). However, their potential causality was not further discussed and there are no clinical descriptions of the patients.¹⁰ An in-frame duplication in the paternal chromosome (p.Ser724_Ala744dup, two carriers in gnomAD v3.1) has also been identified in a patient with a clinical presentation sharing some resemblances with SYS, but the scarcity of available information makes it difficult to fully understand the clinical role of this variant.²¹ We have also noticed some missannnotations, probably due to the fact that, originally, *MAGEL2* was predicted to encode a 529-amino acid protein (instead of the current 1249 amino acids) lacking the N-terminal domain (hg38). This is the case for reported variants p.(Thr76Hisfs*23) and p.(Glu131Serfs*17),¹⁰ which we have relabelled as p.(Thr679Hisfs*23) and p.(Glu734Serfs*17). To sum up, while the pathogenicity of *MAGEL2*-truncating mutations has been clearly established and documented, it remains to be clarified whether any *MAGEL2* missense or in-frame mutation is really a causal variant for SYS.

The phenotypic overlap between SYS and PWS suggests that the alteration of *MAGEL2* may contribute to some aspects of

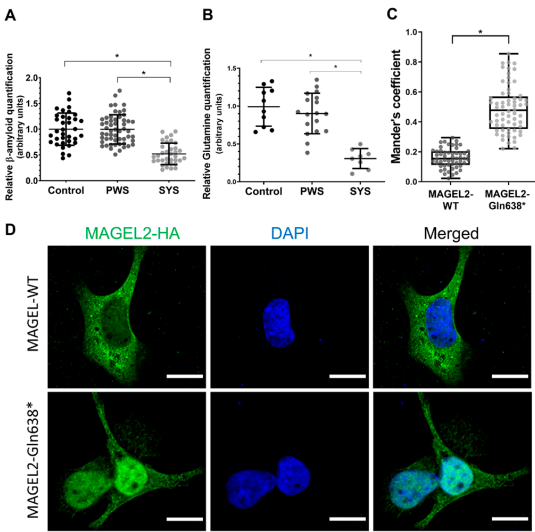


Figure 3 Molecular and cellular biomarkers for Schaaf-Yang syndrome (SYS). (A) Amyloid- β 1–40 ($A\beta_{1-40}$) peptide levels in control, Prader-Willi syndrome (PWS) and SYS fibroblasts’ extracellular medium. Data obtained from at least three independent experiments (SYS: n=5; PWS: n=9; control: n=5). (B) Glutamine levels in control, PWS and SYS fibroblasts (SYS: n=6; PWS: n=9; control: n=6). Values from two independent experiments have been normalised to the mean of the control group. Horizontal lines represent mean values and error bars represent the SD. Statistical analyses were performed using one-way analysis of variance and Tukey’s multiple comparisons test in GraphPad Prism. * $p < 0.001$. (C) Colocalisation quantification using Mander’s coefficient between the haemagglutinin (HA) fluorescence signal and DAPI (4’,6-diamidino-2-phenylindole) in *MAGEL2*-wild-type (WT) and *MAGEL2*-Gln638* transfected cells normalised by the total HA intensity. n=118 from six independent experiments. * $p < 0.001$. (D) Representative immunofluorescence images of SAOS-2 cells transfected with *MAGEL2*-WT and *MAGEL2*-Gln638* plasmids, stained with anti-HA (green, tagging *MAGEL2*) or DAPI (blue, cell nuclei). Scale bar represents 15 μ m.

the PWS phenotype, but the extent of this contribution is still an open question. Two patients carrying atypical deletions involving only *MAGEL2*, *NDN* and *MKRN3* did not show a full PWS phenotype: one patient showed only mild delayed motor skills and the other displayed obesity, DD and high pain threshold.^{55,56} At the same time, neither does the deletion lead to SYS, further supporting that it is the presence of the truncated form of the *MAGEL2* protein that leads to some of the particular aspects of SYS. As our results show, the truncated protein is synthesised, it is stable and it is not degraded any faster than the WT form in the first 12 hours. We propose that this truncated form could be exerting toxic effects in addition to the negative effects caused by the lack of WT *MAGEL2* protein.

The role of *MAGEL2* in the regulation of endosomal protein trafficking and recycling has been widely studied.^{41,42,57} Consistent with this, loss of paternal *MAGEL2* expression in *Magel2*^{P^{+/}} mice and neuronal cell models derived from patient with PWS leads to decreased levels of secretory granule proteins, which lead to reduced levels of circulating bioactive hormones and of mature secretory granules in neurons.³⁶ Also, dental pulp stem cell-derived neurons from several patients with PWS and

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one patient with SYS showed impaired trafficking of M6PR (cation-dependent mannose-6-phosphate receptor), indicating impaired endosome-mediated retrograde transport. We hypothesised that this impaired trafficking could also be reflected in an aberrant A β peptide production and exocytosis.^{48–50} Indeed, A β _{1–40} peptide extracellular levels of SYS fibroblasts were significantly decreased compared with those of PWS and control groups, which showed no difference between them. This result supports the idea that the truncated form of the protein may have a different effect on protein trafficking than the complete loss of the protein itself and establishes A β _{1–40} levels as a potential biomarker for the disease in patient-derived cells.

Despite being its most deeply studied function, dysregulation of protein trafficking and recycling is not the only consequence of MAGEL2 malfunction. The subcellular localisation of the heterologously expressed full MAGEL2 protein^{41,42} and its C-terminal part alone³⁹ has been previously studied, both presenting a mainly cytoplasmic localisation. Our immunocytochemistry assays in different transfected cell lines showed that, in contrast, the heterologously expressed MAGEL2-Gln638* (N-terminal part of the protein) was predominantly located inside the nucleus. While the lack of a properly functioning MAGEL2 antibody hampers the validations of these experiments in the endogenous MAGEL2, they point to new functions of the truncated protein. The increased nuclear localisation of the protein suggests that, in addition to the loss of the MAGEL2 normal functions, including the translocation of YTHDF2 to the nucleus,³⁹ SYS-associated mutations could involve new functions of unknown consequences.

To explore if the truncated form of MAGEL2 could be affecting the expression of other genes, we applied an unbiased transcriptomic approach in fibroblasts. We found an enrichment in genes related to mitosis, nuclear function and localisation, as well as in developmental and neurological processes.

Previous studies on the full-length and C-terminal parts of the MAGEL2 protein have shown that this protein is involved in RNA metabolic processes.³⁹ In our transcriptomic analysis, *HOTAIR* expression was clearly increased in SYS fibroblasts. *HOTAIR* is a well-known long non-coding RNA that mediates transcriptional silencing in trans. This gene's promoter contains binding sites for numerous transcription factors, including AP1 (activator protein 1), Sp1 (specificity protein 1) and NF- κ B (Nuclear Factor Kappa B Subunit 1).⁵⁸ It is able to exert epigenetic functions through H3K27 trimethylation and H3K4 demethylation, among other regulatory functions, and has a widely studied role in cancer (reviewed in Ref. 59). *HOTAIR* promotes transcriptional silencing of the *HOXD* locus, which plays an essential role in determining body axes and orchestrating organ formation during vertebrate development.⁶⁰ *HOTAIR* is also a regulator of mTOR, increasing its phosphorylation and mTOR-mediated exosome release,⁶¹ and its regulation of GLUT1 levels (figure 4).⁶² While our mRNAseq data do not show an increase in mTOR levels, SYS-derived fibroblasts have been reported to show an increase in mTOR expression and activity.⁶³

Endocrine and metabolic alterations in PWS have been widely studied.⁶⁴ However, little is known regarding metabolic impairment in SYS. A study including five female and four male patients with SYS showed that they may present some but not all the endocrine alterations also observed in PWS: patients benefit from growth hormone (GH) therapy, show increased ghrelin levels and present a high risk of developing diabetes mellitus.¹⁰ A review on the literature on endocrine abnormalities in MAGEL2-related syndromes²⁶ showed that the most common hormonal alterations involve GH, thyroid-stimulating hormone,

adrenocorticotrophic hormone, antidiuretic hormone and gonadotropins, probably caused by hypothalamic impairment.

The targeted metabolomic analysis of SYS-derived and PWS-derived fibroblasts was aimed at finding biomarkers that assess the lysosomal, peroxisomal, mitochondrial and cytosolic metabolic pathways. The most relevant finding was a significant reduction in glutamine levels in SYS fibroblasts compared with the other two groups, whereas glutamate levels remained unchanged. Glutamine supplies nitrogen and carbon for biosynthetic reactions in rapidly proliferating cells,⁶⁵ but in many contexts its key role relies on providing glutamate (figure 4) which has a wider range of metabolic functions than glutamine itself. An example of the relevant functions of glutamine and glutamate is the glutamate/GABA-glutamine cycle in neurons.⁶⁶ In fact, it has been stated that alterations of the GABAergic system may play an important role in aspects of the pathophysiology of PWS.⁶⁷ A hypothesis to explain the decrease in glutamine levels in SYS fibroblasts could be a dysregulation in the retrieval and recycling of a glutamine transporter, such as SLC1A5, whose retrieval and recycling is promoted by the retromer and whose degradation is enhanced on retromer knockout.⁶⁸ Another explanation could be related to the hyperactivation of the mTOR pathway, as mTOR plays a role in the glutamine metabolism by increasing glutamate dehydrogenase activity through the inhibition of SIRT4 (figure 4).⁶⁹ The hyperactivation of mTOR could drive a glutamate depletion which would be compensated by an increase in glutamine deamidation.

In conclusion, our results support the hypothesis that the SYS-specific phenotype might be explained by a neomorphic effect of the truncated protein, rather than by a dose-reduction situation. This is supported by the subtle changes in gene expression patterns and metabolite levels observed in fibroblasts, which suggest novel effects for the truncated protein, which warrant further exploration. In addition, we provide a comprehensive phenotypical delineation of the syndrome and a standardised set of guidelines aimed at the improvement of clinical management of patients with SYS. Finally, we propose to improve SYS

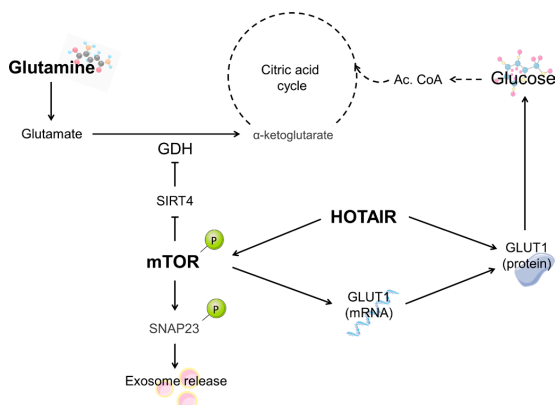


Figure 4 Schematisation of the relationship between the different biomarkers identified in the Schaaf-Yang syndrome (SYS) fibroblasts. The increased levels of *HOTAIR* could lead to the upregulation of mTOR (mammalian target of rapamycin) previously seen in the literature as well as the deregulation of glucose uptake through GLUT1 (glucose transporter 1). Ac.CoA, Acetyl-coenzyme A; GDH, glutamate dehydrogenase; SIRT4, siirtuin 4; SNAP23, Synaptosome Associated Protein 23.

diagnosis by testing the pathogenicity of doubtful missense mutations through the in vitro overexpression system.

An extended version of the methodology is included as online supplemental material. Briefly, we performed a systematic review of the literature indexed in PubMed from the date of the first clinical description of pathology associated with variants in *MAGEL2* until February 2022 using the terms: 'MAGEL2', 'SYS' and 'Schaaf-Yang syndrome'. All mutations have been referenced to the *MAGEL2* hg38 main transcript NM_019066.5. Fibroblasts were obtained from skin biopsies of 7 SYS individuals, 9 patients with PWS and 11 controls (online supplemental table 6). All cell cultures (fibroblasts, HEK293T, HeLa and SAOS-2) were cultured in standard cell culture conditions. The cDNA sequences of interest were cloned in the mammalian expression vector pcDNATM3.1⁽⁺⁾ (Invitrogen, ThermoFisher Scientific) including a HA tag and transfected into the different cell lines. Cells were treated with MG132, bafilomycin or cycloheximide. Whole RNA was extracted from fibroblasts and RNA-Seq was performed by LEXOGEN and analysed with the ExpHunter Suite.⁷⁰ Expression levels of four selected genes were analysed by qPCR. Protein was extracted using RIPA buffer, resolved by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and immunoblotted following standard biochemical techniques. For immunocytochemistry, cells were fixed in 4% paraformaldehyde (PFA), permeabilised and blocked. Coverslips were incubated with anti-HA primary antibody and DAPI. For ELISA quantification, the supernatant of the collected media (without FBS (fetal bovine serum)) was obtained and analysed by the A β_{1-40} high-sensitive ELISA or the A β_{1-42} high-sensitive ELISA (IBL International). For metabolomics, amino acids were analysed using ultrahigh-performance liquid chromatography–tandem mass spectrometry and organic acids with gas chromatography–mass spectrometry. Statistical analysis was performed using R-Studio. Differences were considered significant if $p < 0.05$.

All skin donors or their legal representative gave their written informed consent. Their samples and data were obtained in accordance with the Declaration of Helsinki 1964, as revised in October 2013 (Fortaleza, Brazil).

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Contributors MC-P, AP-P, RR, DG and RU gathered and reviewed all the clinical and mutation data previously published and elaborated the corresponding figures and tables. HF-V, LC-V, MC-P, RMC, SB and GM performed and analysed the heterologous expression experiments, WB and ICC. HF-V, RM-C, LC-V, MC-P and RU performed amilod Beta experiments. RA, CO, AJP-F, MC-P and RU performed and analyzed the metabolomics studies. LC-V, ER, JAGR, DG, SB and PS performed and analysed the mRNAseq experiments and further analysis. LCV, MS, RR, SB and RU drafted the manuscript. RU acts as a guarantor for this manuscript. All authors have critically reviewed and approved the manuscript.

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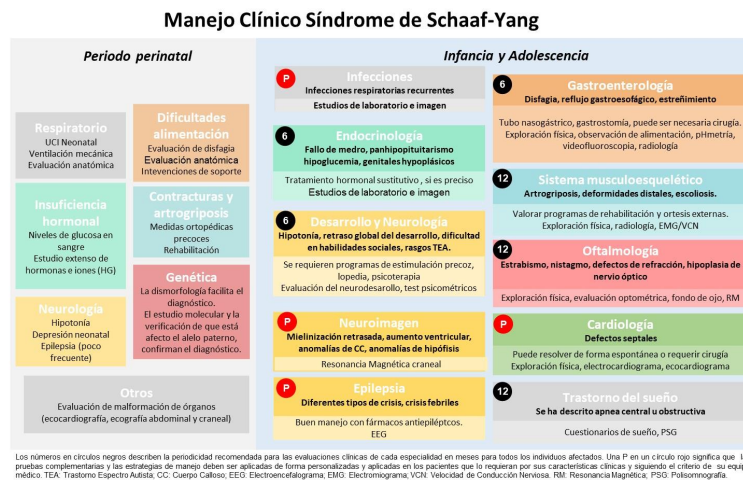
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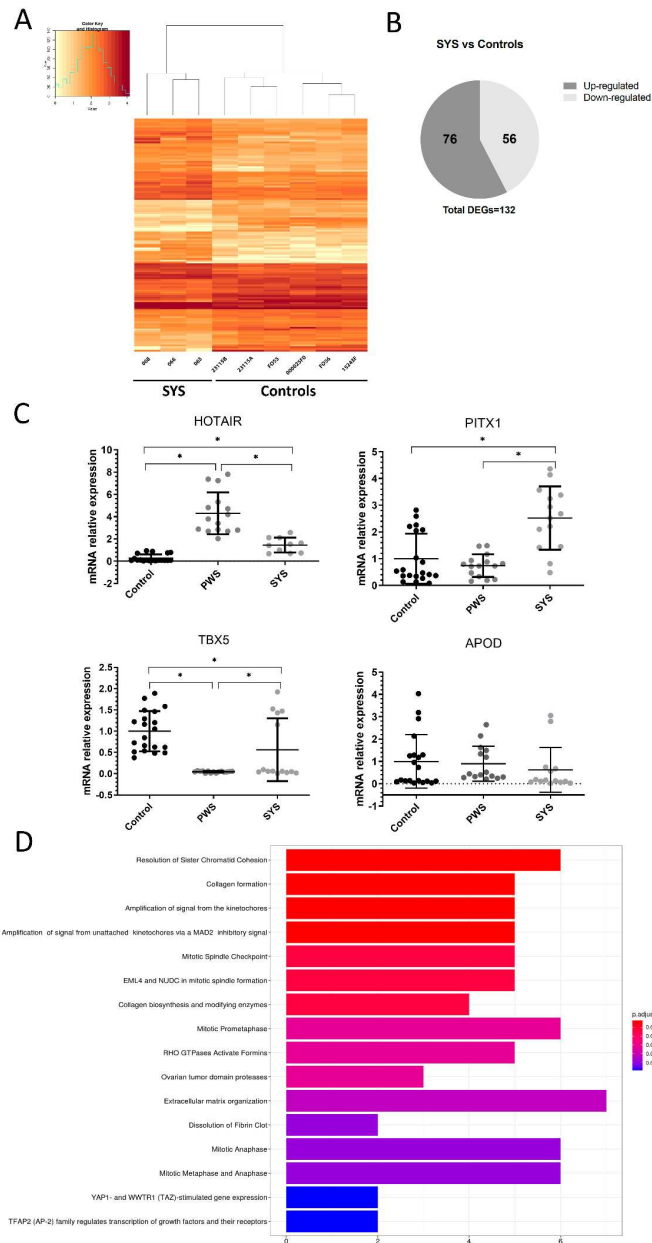
Laura Castilla-Vallmanya^{1,2,3#}, Mónica Centeno-Pla^{1,3,4#}, Mercedes Serrano^{2,3,5#}, Héctor Franco-Valls¹, Raúl Martínez-Cabrera¹, Aina Prat-Planas^{1,2,3}, Elena Rojano⁶, Juan A. G. Ranea^{2,6}, Pedro Seoane^{2,6}, Clara Oliva^{3,4}, Abraham J. Paredes-Fuentes^{3,4}, Gemma Marfany^{1,2,3}, Rafael Artuch^{2,3,4}, Daniel Grinberg^{1,2,3}, Raquel Rabionet^{1,2,3#}, Susanna Balcells^{1,2,3#}, Roser Urreiziti^{2,3,4#}

SUPPLEMENTARY FIGURES:



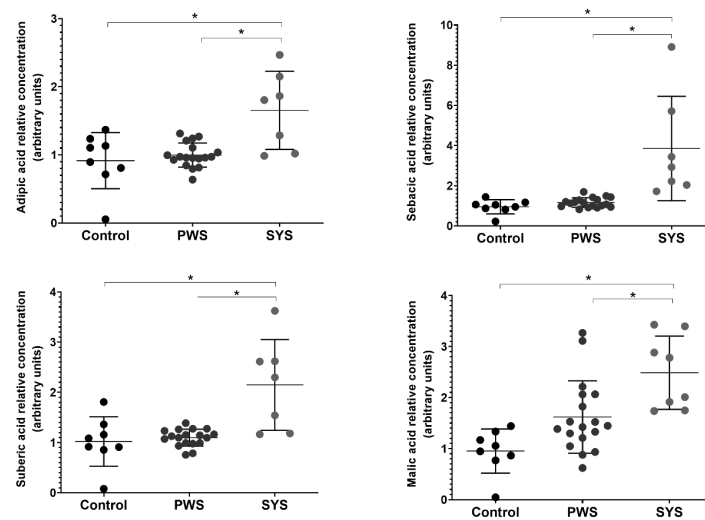
Supplementary Figure 1: Spanish version of Figure 1.

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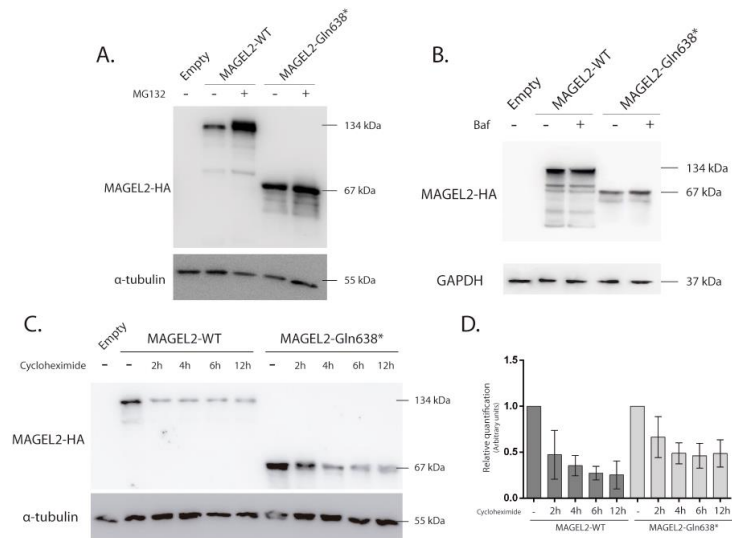
Suppl. 2

Supplementary Figure 2: A) Heatmap of the DEGs identified in SYS and control fibroblasts. B) Proportion of up- and down-regulated transcripts in SYS and control fibroblasts. C) qPCR analysis of *HOTAIR*, *PITX1*, *TBX5* and *APOD* in 5 SYS patients, 5 PWS patients and 7 control individuals. Values from 3 replicates have been normalised to the mean of the control group. Horizontal lines represent mean values and error bars represent the standard deviation. Statistical analyses were performed using One-Way ANOVA and Tukey's multiple comparisons test in GraphPad Prism.* $p < 0.001$. D) REACTOME enrichment analysis of DEGs based on over-representation analysis (ORA) obtained with Cluster Profiler. The length of the bar represents the number of genes observed in each category. Colour indicates adjusted p-value of each category.



Supplementary Figure 3: Targeted metabolomics in fibroblasts. Adipic, Suberic, Sebatic and Malic acid are significantly increased in SYS derived fibroblasts compared to controls and PWS derived fibroblasts. (SYS: n=6; PWS: n=9; control: n=6). Values from 2 replicates have been normalised to the mean of the control group. Horizontal lines represent mean values and error bars represent the standard deviation. Statistical analyses were performed using One-Way ANOVA and Tukey's multiple comparisons test in GraphPad Prism.* $p < 0.001$.

Suppl. 3



Supplementary Figure 4: Degradation rate and stability of MAGEL2-WT and MAGEL2-Gln638* proteins. A) Representative Western Blot of heterologously expressed MAGEL2 proteins with or without MG132 treatment (10μM for 16 hours) (n=3). B) Representative Western Blot of heterologously expressed MAGEL2 proteins with or without Baf treatment (1μM for 16 hours) (n=3). C) Representative Western Blot of heterologously expressed MAGEL2 proteins with or without Cx treatment (150μM) during 2, 4, 6 or 12 hours (n=3). D) Quantification of three independent experiments normalised to the expression levels of MAGEL2-WT or MAGEL2-Gln638* without Cx treatment.

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SUPPLEMENTARY MATERIAL AND METHODS:

Literature review: A Cochrane Library search and a PubMed database search was performed from the date of the first clinical description of pathology associated with variants in *MAGEL2* till May 31, 2022, using the following terms: 'MAGEL2', 'SYS', 'Schaaf-Yang syndrome', and different combinations of these terms. Concerning the data extraction, it included first author, publication year, molecular data (identified mutation and residue changes, *de novo* or inherited condition), pregnancy and perinatal information, multiorgan clinical data, complementary exams information, neuroimaging, and age and cause of death. Unfortunately, in some clinical series, much clinical information is not detailed.

RNA-sequencing and data analysis: Whole RNA was extracted from fibroblasts using the High Pure RNA Isolation Kit (Roche). RNA-Seq was performed by LEXOGEN, Inc. using the QuantSeq 3' messenger RNA (mRNA)-Seq FWD kit for library preparation. ExpHunter Suite [1] was used to analyse the expression data and perform functional enrichment analyses. The differential expressed genes (DEGs) are obtained using an adjusted p-value of 0.05, a minimum log fold change of 1 and Deseq2 and EdgeR packages give as DEG the analyzed gene. These DEGs are used as input for a functional enrichment analyses using REACTOME database and an adjusted p-value of 0.05.

qPCR analysis of selected genes: Whole RNA was extracted from fibroblasts using the High Pure RNA Isolation Kit (Roche). The extracted RNA was reverse transcribed using the High Capacity cDNA reverse transcription kit (Applied Biosystems) following the manufacturer's instructions. qPCR was performed using LightCycler® 480 SYBR Green I Master (04887352001, Roche). The human *GAPDH* gene served as a housekeeping gene and the efficiency of each reaction was calculated according to the standard curve. Melting-curves were conducted at the end of amplification to ensure data quality. Each sample was performed in triplicate. Primer sequences are available on demand.

Cell culture: Fibroblasts were obtained from skin biopsies of seven SYS patients, nine PWS patients and eleven controls (**Supplementary Table 7**). Corresponding informed consent and institutional ethics approval were obtained (Ethics Committee of the Universitat de Barcelona, IRB00003099). Fibroblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, Merck) supplemented with 10% Foetal Bovine Serum (FBS) (Gibco, LifeTechnologies), 1% Penicillin–Streptomycin (Gibco, LifeTechnologies) and 1% GlutaMAX (Gibco, LifeTechnologies). Conditioned medium

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for ELISA was only supplemented with 1% Penicillin–Streptomycin (Gibco, LifeTechnologies) and was collected after 72 h. HEK293T, HeLa and SAOS-2 cells were cultured in DMEM (Sigma-Aldrich, Merck) supplemented with 10% FBS (Gibco, LifeTechnologies) and 1% Penicillin–Streptomycin (Gibco, LifeTechnologies). The cDNA sequences of interest (ENST00000650528.1) were cloned in the mammalian expression vector pcDNATM3.1⁽⁺⁾ (Invitrogen, ThermoFisher Scientific) including a hemagglutinin (HA) tag at the C-terminal end (courtesy of the CRG). The vectors were transfected into 60% confluent HEK293T, HeLa and SAOS-2 cells using LipofectamineTM 3000 (Invitrogen, ThermoFisher Scientific) and Opti-MEMTM (Gibco, LifeTechnologies). Cells were treated with 10 µmol/L MG132 (Calbiochem) or 1 µmol/L bafilomycin (Sigma-Aldrich, Merck) for 16 h or 150 µmol/L cycloheximide (Sigma-Aldrich, Merck) for 2, 4, 8 or 12 h prior to total protein extraction.

Protein extraction and western blotting: Total protein extraction was performed using RIPA buffer supplemented with protease inhibitors (04693159001, Roche, Merck) and N-ethylmaleimide (Sigma-Aldrich, Merck) and quantified using the PierceTM BCA Protein Assay kit (ThermoFisher Scientific). Twenty µg of total protein per lane were run in 10% acrylamide/bis-acrylamide gels, transferred to a PVDF membrane (Millipore, Merck) and blocked with 5% skimmed milk in TBS-Tween 1X. Different antibodies were used: Anti-HA tag (ab18181, Abcam), anti-α-Tubulin (T5168, Sigma-Aldrich), anti-GAPDH (sc-47724, Santa Cruz Biotechnology) and anti-Mouse IgG (Fc specific)–Peroxidase (A0168, Sigma-Aldrich). Results were visualised using the LAS-4000 Luminescent Image Analyzer (Fujifilm) and the LuminataTM Forte Western HRP Substrate (WBLUF0100, Millipore). The detected bands were quantified using ImageJ.[2]

Immunocytochemistry: Cells were fixed in 4% Paraformaldehyde (PFA), permeabilized with 0,1 mol/L glycine and 0,1% Triton X-100 in PBS and blocked with 0,3 mol/L glycine, 0,05% Triton X-100 and 10% Normal Donkey Serum (#S30-100M, Merck Millipore) in PBS. Coverslips were incubated with anti-HA primary antibody (ab18181, Abcam), Donkey anti-Mouse Cy2 antibody (#715-225-150, Jackson ImmunoResearch) and DAPI (#D1306, Invitrogen) and mounted with MOWIOL (#475904, Millipore). Images were acquired using a Zeiss confocal microscope LSM 880 and analysed with ImageJ. [2]

ELISA analysis: The supernatant of the conditioned medium was obtained after short centrifugation. Total protein concentration was quantified using the PierceTM BCA Protein Assay kit (ThermoFisher Scientific) and Aβ₁₋₄₀ and Aβ₁₋₄₂ amyloid peptide using the Amyloid-beta (1-40) High Sensitive ELISA and Amyloid-beta (1-42) High Sensitive

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ELISA (IBL International GmbH). Statistical analyses were performed using One-Way ANOVA and Tukey's multiple comparisons test in GraphPad Prism.

Metabolomics analyses: Amino acid analyses were performed using a ultra-high-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) method, as previously reported.^[3] Gas chromatography-mass spectrometry (GC-MS) analyses were performed to identify different organic acids, as described in [4, 5]. Statistical analyses were performed using One-Way ANOVA and Tukey's multiple comparisons test in RStudio (<https://www.rstudio.com/>).

Ethical issues: For the patients whose fibroblasts have been studied, parents gave their written informed consent. Their samples and data were obtained in accordance with the Helsinki Declaration of 1964, as revised in October 2013 (Fortaleza, Brazil). The study was approved by the Institutional Review Board (IRB00003099) of the Bioethical Commission of the University of Barcelona (October 5, 2020) and Hospital Sant Joan de Déu (PIC-111-19).

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Suppl. 7

Supplementary Table 1: Complete list of patients and HPOs.

	TOTAL	Schaaf et al., 2013	Soden et al., 2014	Mejlachowicz et al., 2015	Fountain et al., 2016		Palomares-Bralo et al., 2017	Urreiziti et al., 2017	Bayat et al., 2018	Enya et al., 2018	Hidalgo-Santos et al., 2018	Kleinendorst et al., 2018	Matuszewska et al., 2018
Diagnosis		SYS	SYS	Lethal AMC	SYS/AMC		SYS	SYS	SYS	SYS	SYS	SYS	SYS
New described cases	135	4	2	4	16	Patients 5 and 6	1	1	1	3	1	1	2
Gender	46F; 40M; 49Unk	4M	2F	3F; 1M	2F; 3M; 11Unk	1M; 1F	M	F	M	2F; 1M	F	F	2F
Protein change		p.Val551Aspfs*151; p.Pro601Glnfs*101; p.Ile1061Hisfs*7; p.Gln1042*	p.Gln666Profs*47	p.Gln666Serfs*36 (x3); p.Leu708Trpfs*7	p.Gln666Profs*47 among others	p.Gln666Serfs*36 (x2)	p.Gln666Profs*47	p.Gln638*	p.Gln666Profs*47	p.Ser1044*; p.Gln638* (x2)	p.Gln1007*	p.Trp617*	p.Gln666Hisfs*37; p.Trp965*
Inheritance		de novo (x3), ? (x1)	gonadal mosaicism	Inherited (x3); de novo		Inherited (x2)	inherited	de novo	?	Inherited (x2); de novo	de novo	Inherited	de novo
Cognitive/Neurologic/Behavioural													
Developmental delay	101/101	4/4	2/2		14/14		1/1	1/1	1/1	3/3	1/1	1/1	2/2
Intellectual disability	87/87	4/4	2/2		14/14		1/1	1/1	1/1	3/3	1/1	1/1	2/2
ASD Diagnosis	55/76	4/4	2/2		4/7					1/3	1/1 (signs)		2/2 (signs)
Full Scale IQ (avg.)													
Seizures	24/40	1/4	1/2		2/13		1/1			2/3			
Sleep apnea	53/66	2/4			9/13			1/1				1/1	2/2
Sleep disturbance	5/5							1/1					1/1
Brain MRI findings	20/32	1/1						1/1	1/1	2/3	0/1		0/1
Self-injurious behavior/Skin picking	16/21	2/4			7/12								
Aggressive behavior	4/14												
Behavioral abnormalities (impulsivity, compusitivity...)	13/13	2/4			9/11								
Temper tantrums, agressivity	7/17	2/4											
Infantile lethargy, weak cry	14/22	3/4	1/2							2/3			
Neurological deterioration following Febrile illness	5/8												
Speech articulation defects	6/8	3/4											
Pregnancy complications													
Decreased fetal movement	18/33			4/4	6/15	2/2				0/3	0/1		
Prenatal or perinatal death	7/7			4/4		2/2						1/1	
Polyhydramnios	12/20			4/4									1/2
Respiratory distress													
Apneic episodes	11/18		2/2	1/1				1/1	1/1	3/3	1/1	1/1	2/2
Intubation	22/47												2/2
Mechanical Ventilator	44/72								1/1	3/3		1/1	2/2
Tracheostomy	16/20									2/2			2/2
Dyspnea	2/2												
Neonatal pneumonia	7/8										1/1		
Recurrent respiratory infections	5/6							1/1		2/3	1/1		
Feeding issues													
Poor suck in infancy	78/88	3/4	2/2		15/16		1/1	1/1	1/1	3/3	1/1	1/1	2/2
Dysphagia	35/49		2/2		14/15		1/1				1/1		2/2
Hyperphagia	13/14	2/4			4/13			1/1		1/3	1/1		1/2
Use of ng tube	39/63	2/4							1/1			0/1	
Use of g tube	27/54								1/1			1/1	2/2
Excessive weight gain or obesity	23/35	3/4			5/13				0/1			1/1	
Gastroesophageal Reflux	38/65				8/14						1/1		
Chronic intestinal pseudo-obstruction	1/1								1/1				
Chronic constipation	46/65				4/11		1/1		1/1				
Physical characteristics													
Hypotonia (at time of exam)	61/64				13/13						1/1	1/1	
Neonatal hypotonia	63/72	3/4	2/2	1/1	14/15		1/1	1/1	1/1	3/3	1/1	1/1	2/2
Decreased muscle mass	2/3		1/2								1/1		
Increased fatty tissue	6/8		1/2									1/1	
Congenital distal contractures	96/109	2/4	2/2		15/18	2/2	1/1	1/1	1/1		1/1		2/2

CHARACTERISATION OF SCHAAF-YANG SYNDROME AND FUNCTIONAL INSIGHTS



	TOTAL	Schaaf et al., 2013	Soden et al., 2014	Mejlachowicz et al., 2015	Fountain et al., 2016		Palomares-Bralo et al., 2017	Urreizti et al., 2017	Bayat et al., 2018	Enya et al., 2018	Hidalgo-Santos et al., 2018	Kleinendorst et al., 2018	Matuszewska et al., 2018
Arthrogyposis	21/30			4/4	2/18	2/2				3/3		1/1	2/2
Flexion of elbows ans knees	4/7			3/3									
Scoliosis	35/57	0/2			7/14	0/2			1/3				
Kyphosis	6/19					0/2							
Short neck	8/18		1/5	4/4								1/1	
Short stature	35/52	2/4			10/16	0/2	1/1	1/1			1/1		
Hypogonadism	41/44	3/4			8/11 (M); 1/1 (F)	0/2	1/1	1/1		1/2	0/1		
Cryptorchidism	8/13	2/4					1/1		1/1	1/2			
Eye abnormalities	36/44	3/4	1/1		11/14			1/1	1/1 (strabismus)		1/1		
Microcephaly	3/5												
Macrocephaly	4/13												
Short limbs	2/3												
Small Hands	30/49	1/4			12/14			1/1					
Cranofacial disproportion	1/1												
Camptodactyly	27/41	2/4		4/4	1		1/1	1/1	1/1	3/3			
Polydactyly	1/5												
Bilateral ulnar deviation	4/10	1/4											
Tapering fingers	14/26	1/4			1		1/1				1/1		
Lateral deviation of wrists	1/1												
Small Feet	15/31	1/4			8/15	0/2							
Bilateral club foot	13/28			4/4				1/1		1/3			1/2
Equinovarus feet	2/5										1/1		
Abnormality of the skin	1/2												
Hypopigmentation	4/6												
Hirsutism	2/5												
Facial dysmorphisms	70/76	4/4	2/2	4/4	16/18	2/2	1/1	1/1	1/1	3/3	1/1	1/1	2/2
Wide mouth	4/7	2/4						1/1					
Small mouth	3/8								1/1				1/2
Down-turned mouth corners	2/5												
Thick vermillion upper and lower lip	2/5												
Limited opening of the mouth	1/1											1/1	
High arched palate	15/33		2/2						1/1	1/3			1/2
Cleft palate	3/8									1/3			
Thick palatal ridges	1/1							1/1					
Glossoptosis	2/5												
Widely spaced teeth	3/11						1/1						
Teeth malposition	1/1							1/1					
Toothed gum	2/7												
Shallow nasolabial fold	4/6												
Broad nasal root	10/17						1/1		1/1 (prominent)	1/3			
Depressed nasal root	3/8									1/3			
High nasal bridge	3/7												2/2
Wide nasal bridge	2/10												
Flat nasal bridge													
Depressed nasal bridge	1/5												
Short nose	2/6												
Anteverted nares	3/6							1/1					
Large and broad nose	1/4	1/4						1/1					
Smooth philtrum	4/6		2/2							1/3			
Deep philtrum	2/6						1/1						
Short philtrum	1/5												

	TOTAL	Schaaf et al., 2013	Soden et al., 2014	Mejlachowicz et al., 2015	Fountain et al., 2016	Palomares-Bralo et al., 2017	Urreizti et al., 2017	Bayat et al., 2018	Enya et al., 2018	Hidalgo-Santos et al., 2018	Kleinendorst et al., 2018	Matuszewska et al., 2018
Long philtrum	2/7											
Ptosis	2/2		2/2									
Microphthalmos												
Hyperthelormism	8/17			3/4					1/3			
Deep set eyes	5/10							1/1				
Prominent epicanthic folds	3/7											1/2
Telecanthus	2/5											
Upslanting palpebral fissures	2/3										1/1	1/2
Downslanting palpebral fissures	2/5											
Narrow palebral fissures	5/11			3/4								1/2
Almond-shaped palpebral fissures	4/10	1/4							1/3			
Low set ears	15/24					1/1			1/3			2/2
Large ears	9/18							1/1				
Bitemporal narrowing	8/20	1/4										2/2
Frontal bossing	10/25											
Microretrognathia	6/10			4/4								
Retrognathia	7/7							1/1				
Micrognathia	16/26								3/3	1/1		1/2
Mandibular malformations	1/1											
Bulbar palsy	1/4											
Scaphocephaly	3/5							1/1				
Dolicocephaly	2/6					1/1						
Brachycephaly	1/5											
Trigonocephaly	2/6						1/1					
Ridge over the metopic suture	6/7											
Broad forehead	1/1									1/1		
High forehead	4/12											
Prominent forehead	4/14											
Flat forehead	2/5											
Square-shaped face	2/6										1/1	
Long face	4/4											
Pierre Robin sequence	1/5											
Horizontal chin crease	1/1										1/1	
Endocrinological issues	0/1										0/1	
Hypopituitarism	6/12								1/3	1/1		
Growth Hormone deficiency	18/24								1/3	1/1		
Hypothyroidism	8/28							1/1	1/3	1/1		
Hypoglycemia	14/22		2/2			1/1						
Diabetes insipidus	4/16								1/3			
Other endocrinological issues	9/9								2/3	1/1		
Other alterations												
Heart defects	7/19											
Bradycardia	3/4		1/2				1/1					
Tracheomalacia	1/5											
Jaundice	3/11											
Miopathy	1/1											
Skeletal abnormalities	1/5											
Normal pathological examination of brain	4/4			4/4								
Temperature instability	37/45				6/13		1/1	0/1				
Excessive salivation	1/1						1/1					
Premature death	23			4/4	2/2						1/1	



	McCarthy et al., 2018	McCarthy et al., 2018	Poliak et al., 2018	Tong et al., 2018	Jobling et al., 2018	Gregory et al., 2019	Negishi et al., 2019	Patak et al., 2019		Xiao et al., 2019	Guo et al., 2019	de Andrade et al., 2020
Diagnosis	SYS	SYS	SYS	SYS	CHS->SYS	SYS	SYS	SYS	SYS like (mild)	SYS	AMC	SYS
New described cases	2	35	1	2	6	4	6	4	1 (Missense)	5	1	1
Gender	2F	NS	F	1F; 1M	6F	3F; 1M	1F; 5M	2F; 2M	F	2F; 3M	M	M
Protein change	p.Gln666Profs*47 (x2)	NS	p.Gln638*	p.Pro543Leufs*159; p.Gln666Profs*47	p.Gln666Profs*47; p.Asp727Profs*6(x2); p.Gln588*(x2); p.Ala966fs	p.Gln666Profs*47	p.Gln638*(x2); p.Trp587*; p.Gln666Profs*47; p.Gln588*(x2)	p.Val1041Alafs*7; p.Gln666Profs*47; p.Glu1057*; p.Gln666Hisfs*37	p.Ala538Glu	p.Gln666Profs*47	p.Gln666Serfs*36	p.Val701Glyfs*12
Inheritance	de novo	?	inherited	inherited; de novo	inherited (x1); ? (x3); de novo (x2)	de novo (x2); ? (x2) (non-identical twins)	?; de novo (x3); inherited (x2)	de novo(x2); inherited (x1); 4% paternal mosaicism	inherited	inherited	inherited	Inherited
Cognitive/Neurologic/Behavioural												
Developmental delay	2/2	35/35	1/1	2/2	6/6	4/4	6/6	4/4	1/1			
Intellectual disability	2/2	34/34			6/6		5/5	4/4	1/1			
ASD Diagnosis	2/2	25/32			1/1	1/4	1/1	3/4	1/1			
Full Scale IQ (avg.)	8.00	59.60										
Seizures	1/2	7/34	1/1	2/2			2/6	2/4				
Sleep apnea	2/2	22/28	1/1	1/2		4/4	1/5	2/4				
Sleep disturbance			1/1		2/2							
Brain MRI findings			1/1	0/1	4/4	2/4	2/2	2/4	1/1		1/1?	
Self-injurious behavior/Skin picking						1/4	4/6	1/4	1/1			
Aggressive behavior					1/1			0/4	1/1			
Behavioral abnormalities (impulsivity, compulsivity...)												
Temper tantrums, aggressivity							1/5					
Infantile lethargy, weak cry			1/1				5/6	1/4				
Neurological deterioration following Febrile illness				1/2			4/6					
Speech articulation defects				1/2	1/1							
Pregnancy complications												
Decreased fetal movement				1/2	1/5			1/4	0/1			
Prenatal or perinatal death											1/1	
Polyhydramnios						2/4		2/4			1/1	
Respiratory distress												
Apneic episodes	2/2	22/35	1/1		4/6	4/4		4/4		5/5		1/1
Intubation	2/2	15/34	1/1		1/6					5/5		
Mechanical Ventilator	2/2	15/35	1/1	1/2	1/6			3/4				1/1
Tracheostomy	0/1	5/34		1/2		3/4		3/4		5/5		1/1
Dyspnea				2/2								
Neonatal pneumonia										4/5		1/1
Recurrent respiratory infections			1/1									
Feeding issues												
Poor suck in infancy	1/1	33/35	1/1	2/2	4/6	1/4	6/6	2/4		5/5	1/1	1/1
Dysphagia	1/1	33/35	1/1	1/1			6/6	2/4				
Hyperphagia	0/1	27/35	1/1					1/4				
Use of ng tube	0/1	7/34										
Use of g tube	1/1	22/34	1/1	1/2			4/6 (not specified)					
Excessive weight gain or obesity	2/2	14/33	1/1		2/6			2/4				1/1
Gastroesophageal Reflux	0/1	6/35					3/6	1/4				
Chronic intestinal pseudo-obstruction	2/2	15/34	1/1		4/6	2/4		2/4				1/1
Chronic constipation	1/2	25/33			2/6	3/4		1/4				
Physical characteristics												
Hypotonia (at time of exam)	2/2	32/32	1/1		6/6	1/4		4/4				
Neonatal hypotonia			1/1	2/2		1/3	6/6	4/4	0/1	4/5	1/1	1/1
Decreased muscle mass												
Increased fatty tissue					3/4							1/1
Congenital distal contractures	2/2	31/35	1/1	2/2	6/6	4/4	6/6	3/4	1/1 (knee)		1/1	

	McCarthy et al., 2018	McCarthy et al., 2018	Poliak et al., 2018	Tong et al., 2018	Jobling et al., 2018	Gregory et al., 2019	Negishi et al., 2019	Patak et al., 2019	Xiao et al., 2019	Guo et al., 2019	de Andrade et al., 2020
Arthrogryposis					1/1	4/4					
Flexion of elbows and knees						1/4					
Scoliosis	2/2	10/19			6/6	3/4		1/4	1/1		
Kyphosis	1/1	4/19							1/5		
Short neck					1/6						
Short stature					6/6	4/4	5/6	1/4	0/1		
Hypogonadism	0/1	9/35	1/1	1/1			4/6	2/2	0/1	3/3	1/1
Cryptorchidism				1/1		1/1		1/2	0/1		
Eye abnormalities			1/1		3/6	4/4	5/6	4/4	1/1		
Microcephaly			1/1								
Macrocephaly						2/4		0/4	1/1		
Short limbs											1/1
Small Hands			1/1			1/4	6/6	1/4	0/1	2/5	
Cranofacial disproportion											1/1
Camptodactyly					3/6	1/4		2/4	0/1	5/5	
Polydactyly										1/5	
Bilateral ulnar deviation					3/6						
Tapering fingers					1/6		4/6	3/4	0/1		
Lateral deviation of wrists											1/1
Small Feet			1/1			1/4		1/4	0/1	1/5	
Bilateral club foot					2/6			1/4	0/1		
Equinovarus feet											
Abnormality of the skin				1/2							
Hypopigmentation							4/6				
Hirsutism								1/4	1/1		
Facial dysmorphisms			1/1	1/2	6/6	4/4	4/6	4/4	1/1	4/5	1/1
Wide mouth											
Small mouth					1/5						
Down-turned mouth corners					2/5						
Thick vermillion upper and lower lip								1/4	1/1		
Limited opening of the mouth											
High arched palate					1/5	3/4		1/4	0/1	3/5	
Cleft palate								1/4	1/1		
Thick palatal ridges											
Glossoptosis						1/4					1/1
Widely spaced teeth					1/5			1/4	0/1		
Teeth malposition											
Toothed gum										1/5	
Shallow nasolabial fold							4/6				
Broad nasal root					4/5			1/4	0/1		
Depressed nasal root											
High nasal bridge								0/4	1/1		
Wide nasal bridge					1/5			1/4	0/1		
Flat nasal bridge											
Depressed nasal bridge					1/5						
Short nose					1/5						
Anteverted nares					2/5						
Large and broad nose											
Smooth philtrum											1/1
Deep philtrum								1/4	0/1		
Short philtrum								0/4	1/1		

	McCarthy et al., 2018	McCarthy et al., 2018	Poliak et al., 2018	Tong et al., 2018	Jobling et al., 2018	Gregory et al., 2019	Negishi et al., 2019	Patak et al., 2019	Xiao et al., 2019	Guo et al., 2019	de Andrade et al., 2020
Long philtrum					1/5						
Ptosis											
Microphthalmos											
Hyperthelorm					1/5			2/4	1/1		
Deep set eyes					3/5			1/4			
Prominent epicanthic folds								1/4	1/1		
Telecanthus								1/4	1/1		
Upslanting palpebral fissures											
Downslanting palpebral fissures								1/4	1/1		
Narrow palpebral fissures					1/5						
Almond-shaped palpebral fissures											
Low set ears			3/5	1/2	3/5			2/4	1/1		
Large ears					1/5		4/6				
Bitemporal narrowing					1/5	3/4		1/4	0/1		
Frontal bossing					2/5	2/4		1/4	0/1	2/5	
Microretrognathia									1/5		
Retrognathia					5/5						1/1
Micrognathia			1/1	1/2		4/4		2/4	0/1	1/5	1/1
Mandibular malformations										1/1	
Bulbar palsy						1/4					
Scaphocephaly						2/4					
Dolicocephaly								1/4	0/1		
Brachycephaly					1/5						
Trigonocephaly					1/5						
Ridge over the metopic suture					5/5						
Broad forehead											
High forehead					1/5			1/4	0/1		
Prominent forehead					1/5	2/4		1/4	0/1		
Flat forehead					2/5						
Square-shaped face					1/5						
Long face						4/4					
Pierre Robin sequence									1/5		
Horizontal chin crease											
Endocrinological issues											
Hypopituitarism			1/1					1/4	0/1		
Growth Hormone deficiency					6/6	4/4	3/3	1/4	0/1		
Hypothyroidism					1/5			1/4	0/1		
Hypoglycemia			1/1	1/2	5/6	1/1		2/4	0/1		
Diabetes insipidus						2/4					
Other endocrinological issues						3/4		3/4	0/1		
Other alterations											
Heart defects			1/1		2/6	1/4		0/4	1/1		
Bradycardia											1/1
Tracheomalacia								0/4	1/1		
Jaundice					2/6			0/4	1/1		
Miopathy					1/1						
Skeletal abnormalities								1/4	0/1		
Normal pathological examination of brain											
Temperature instability	1/2	27/35	1/1								
Excessive salivation											
Premature death				2/2					3/5 (apneic seizures)	Prenatal death	

	Chen et al., 2020	Ahn et al., 2020	Marbach et al., 2020	Llamos-Paneque et al., 2020	Halloun et al., 2021	Duan et al., 2021	Nunes et al., 2021	Bertoli-Avella et al., 2021	Laquerriere et al., 2021
Diagnosis	SYS	SYS	SYS	SYS	SYS	SYS	SYS	SYS	AMC/SYS
New described cases	2	4	8	1	1	1	1	2	9
Gender	2F	3M; 1F	4F; 4M	M	F	M	M	NA	4F; 4M; 1UnK
Protein change	p.Gln666Profs*47	p.Gln666Profs*47(x2); p.Lys740Argfs*11; p.Phe1150Trpfs*4	p.Pro597Hisfs*105; p.Pro601Glnfs*101; p.Gln607*; p.Gln666Profs*47; p.Ser724_Ala744dup; p.Arg941Profs*10; p.Trp958*; p.Ser987Alafs*5	p.Leu1059Phefs*2	p.Gln638*	p.Gln563*	p.Gln666Profs*47	p.Ser603*	p.Gln666Serfs*36(x5); p.Gln666Profs*47(x2); p.Leu708Trpfs*7; p.Val991*
Inheritance	de novo (x2)	de novo (x2); Inherited (x2)	de novo (x3); Inherited (x2); unknown (x3)	inherited	de novo	?	?	?	Inherited (x5); de novo (x4)
Cognitive/Neurologic/Behavioural									
Developmental delay	2/2	4/4		1/1			1/1	2/2	
Intellectual disability		4/4				1/1			
ASD Diagnosis		2/4	4/7	1/1					
Full Scale IQ (avg.)									
Seizures	1/2	1/4		0/1		0/1			
Sleep apnea			5/8	0/1					
Sleep disturbance									
Brain MRI findings	0/2	2/4							
Self-injurious behavior/Skin picking									
Aggressive behavior			2/8						
Behavioral abnormalities (impulsivity, compulsivity...)			2/8						
Temper tantrums, aggressivity			4/8						
Infantile lethargy, weak cry	1/2								
Neurological deterioration following Febrile illness									
Speech articulation defects				1/1					
Pregnancy complications									
Decreased fetal movement	0/2		2/8				0/1		
Prenatal or perinatal death									
Polyhydramnios		1/4					1/1		
Respiratory distress									
Apneic episodes	2/2	2/4							
Intubation									
Mechanical Ventilator	2/2	3/4	4/8	0/1					
Tracheostomy				0/1					
Dyspnea									
Neonatal pneumonia					1/1				
Recurrent respiratory infections									
Feeding issues									
Poor suck in infancy	2/2	3/4				0/1			
Dysphagia	2/2	2/4	6/8	1/1					
Hyperphagia				1/1					
Use of ng tube		2/4	4/8	0/1					
Use of g tube	1/2			0/1	1/1				
Excessive weight gain or obesity			3/8	0/1		1/1			
Gastroesophageal Reflux	1/2	1/4		0/1		0/1			
Chronic intestinal pseudo-obstruction									
Chronic constipation		1/4	6/8	1/1					
Physical characteristics									
Hypotonia (at time of exam)									
Neonatal hypotonia	2/2	3/4	6/8	1/1			1/1		
Decreased muscle mass									
Increased fatty tissue									
Congenital distal contractures	2/2	3/4	6/8	1/1					



	Chen et al., 2020	Ahn et al., 2020	Marbach et al., 2020	Llamos-Paneque et al., 2020	Halloun et al., 2021	Duan et al., 2021	Nunes et al., 2021	Bertoli-Avella et al., 2021	Laquerriere et al., 2021
Arthrogryposis		1/4					1/1		9/9
Flexion of elbows and knees									
Scoliosis			4/8	0/1		0/1			
Kyphosis				0/1		0/1			
Short neck	1/2								
Short stature		2/4			1/1	1/1			
Hypogonadism			3/8	1/1		1/1			1/1
Cryptorchidism									
Eye abnormalities									
Microcephaly		2/4							
Macrocephaly		1/4							
Short limbs	1/2								
Small Hands	1/2	1/4				1/1		2/2	
Cranofacial disproportion									
Camptodactyly	1/2	2/4							
Polydactyly									
Bilateral ulnar deviation									
Tapering fingers	2/2								
Lateral deviation of wrists									
Small Feet		1/4				1/1			
Bilateral club foot	1/2	1/4					1/1		
Equinovarus feet		1/4							
Abnormality of the skin									
Hypopigmentation									
Hirsutism									
Facial dysmorphisms	2/2	4/4							
Wide mouth	1/2								
Small mouth									
Down-turned mouth corners									
Thick vermillion upper and lower lip									
Limited opening of the mouth									
High arched palate	1/2	1/4							
Cleft palate									
Thick palatal ridges									
Glossoptosis									
Widely spaced teeth									
Teeth malposition									
Toothed gum	1/2								
Shallow nasolabial fold									
Broad nasal root	2/2								
Depressed nasal root		1/4			1/1				
High nasal bridge									
Wide nasal bridge									
Flat nasal bridge									
Depressed nasal bridge									
Short nose									
Anteverted nares									
Large and broad nose									
Smooth philtrum									
Deep philtrum									
Short philtrum									

	Chen et al., 2020	Ahn et al., 2020	Marbach et al., 2020	Llamos-Paneque et al., 2020	Halloun et al., 2021	Duan et al., 2021	Nunes et al., 2021	Bertoli-Avella et al., 2021	Laquerriere et al., 2021
Long philtrum	1/2								
Ptosis									
Microphthalmos									
Hyperthelormism									
Deep set eyes									
Prominent epicanthic folds									
Telecanthus									
Upslanting palpebral fissures									
Downslanting palpebral fissures									
Narrow palpebral fissures									
Almond-shaped palpebral fissures	1/2				1/1				
Low set ears					1/1				
Large ears	1/2	2/4							
Bitemporal narrowing									
Frontal bossing		1/4						2/2	
Microretrognathia					1/1				
Retrognathia									
Micrognathia	1/2								
Mandibular malformations									
Bulbar palsy									
Scaphocephaly									
Dolicocephaly									
Brachycephaly									
Trigonocephaly									
Ridge over the metopic suture	1/2								
Broad forehead									
High forehead	2/2								
Prominent forehead									
Flat forehead									
Square-shaped face									
Long face									
Pierre Robin sequence									
Horizontal chin crease									
Endocrinological issues									
Hypopituitarism								2/2	1/1
Growth Hormone deficiency				1/1	1/1				
Hypothyroidism		0/4	3/8		0/1				
Hypoglycemia		0/2		0/1	1/1				
Diabetes insipidus			0/8		1/1				
Other endocrinological issues									
Other alterations									
Heart defects	2/2				0/1				
Bradycardia									
Tracheomalacia									
Jaundice									
Miopathy									
Skeletal abnormalities									
Normal pathological examination of brain									
Temperature instability				1/1		0/1			
Excessive salivation									
Premature death	2/2								6

Supplementary Table 2: Management and surveillance proposed protocol for Schaaf-Yang Syndrome (English version).

Supplemental material

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J Med Genet

Perinatal period		
	Description	Special problems and their management
Respiratory difficulties	They frequently require Neonatal ICU, mainly due to the need for respiratory assistance during the perinatal period that varies from oxygen therapy to invasive mechanical ventilation, ranging from a few hours of support to a few months. Laryngeal stridor, glossoptosis, tracheomalacia, and pulmonary hypoplasia have also been reported.	NICU. Mechanic ventilation. Evaluation by otorhinolaryngologists and pulmonologists to rule out anatomical abnormalities is essential. Respiratory support from oxygen therapy to invasive mechanical ventilation and need of tracheostomy.
	Feeding difficulties with frequent episodes of choking or ineffective sucking are very common. The combination of hypotonia, lethargy, dysphagia, and a high palate can make oral feeding very difficult to achieve.	Nutritionists and dysphagia specialists are needed. An anatomical evaluation to rule out malformations is necessary. Supportive nutritional intervention may be necessary, ranging from a nasogastric tube to a permanent gastrostomy or parenteral nutrition.

Supplementary Table 2: Management and surveillance proposed protocol for Schaaf-Yang Syndrome

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Supplemental material

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J Med Genet

Perinatal period		
	Description	Special problems and their management
Contractures	Clubfoot, arthrogryposis, contractures, and other joint movement restrictions are frequently present at birth. Fetal hypokinesia can lead to severe arthrogryposis.	Early intervention through orthopedics may be essential to ameliorate the prognosis. It is important to refer to specialists on rehabilitation and children's orthopedics .
	Several endocrine abnormalities have been detected during the perinatal period, including diabetes insipidus, hyponatremia, growth hormone (GH) deficiency, hypoglycemia, and hypocalcemia (see Endocrinology section).	Blood glucose levels need to be controlled during the perinatal period. Laboratory tests at this age should include a comprehensive analysis of ions, glucose, and hormones to rule out frequent abnormalities. Refer to the pediatric endocrinologist .
Hormonal problems	In the perinatal period, an evaluation by a child neurologist and a geneticist/dysmorphologist is recommended. A detailed physical examination and a standardized protocol are recommended to rule out congenital malformations that include echocardiogram, abdominal ultrasound, cranial ultrasound...	

Supplementary Table 2: Management and surveillance proposed protocol for Schaaf-Yang Syndrome

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Childhood and Adolescence		
SPECIALTY	STUDIES AND FOLLOW-UP	SPECIFIC CARE AND RECOMMENDED STUDIES
Endocrinology (E)	Every 6 months/ Physical exam	E1 Failure to thrive during infancy and childhood, but also excessive increase in weight, trend to obesity, and hyperphagia beginning after infancy. Dietary intervention may be necessary. E2 Short stature (-1.5 SD to -5 SD), sometimes GH may be needed (consider the increased risk of obstructive apnea when prescribing GH). E3 Diabetes insipidus has been described presenting with polyuria, low urine density, hyposthenuria, and hypernatremia. Hormone replacement therapy may be required. E4 Panhypopituitarism caused by pituitary gland hypoplasia has been described, but also with normal MRI. It is necessary to monitor thyroid function, somatomedin C, GH, adrenal hormones, testosterone, LH and FSH. Hyperprolactinemia has been detected. Hormone treatment, including GH, levothyroxine, and hydrocortisone, may be necessary. Hormone replacement therapy. Adolescence → estrogens.
	Assessments: Laboratory Ultrasound Neuroimaging (MRI)	E5 Hypoglycemia . Guarantee the intake and maintenance of glucose. Rule out hyperinsulinemia. E6 Temperature instability. Support measures. E7 Hypoplastic genitalia, micropenis and cryptorchidism. Assess testosterone, surgery.

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Childhood and Adolescence		
SPECIALTY	STUDIES AND FOLLOW-UP	SPECIFIC CARE AND RECOMMENDED STUDIES
Gastroenterology (G)	Every 6 months/ Physical exam Feeding Observation Assessment: pHmetry Video fluoroscopy Image (Rx) Laboratory	G1 Feeding problems are almost constant. Initial feeding difficulties are due to dysphagia, recurrent respiratory aspiration, and sometimes a nasogastric tube and gastrostomy are required. G2 Early-onset chronic constipation and gastroesophageal reflux are also common. G3 Infrequently reported complications: Intestinal pseudo-obstruction, velopharyngeal insufficiency, eosinophilic esophagitis, and food allergies.
	Every 12 months/ Physical exam Assessment: Image (Rx) Electromyogram and nerve conduction	MS1 Abnormal muscle tone is very frequent, especially hypotonia. MS2 Arthrogryposis is very common: contractures, shortening of limbs, elbows, knees, hips. Camptodactyly, clinodactyly, brachydactyly of the fingers, adducted thumbs. MS3 Club feet and equinovarus feet have been repeatedly described. MS4 Scoliosis , kyphosis, lordosis and asymmetric chest. Early rehabilitation programs and external bracing may be needed. Periodic X-ray monitoring of the hip in children who cannot walk and images of the spine X-ray as a whole is recommended. Consider surgery if necessary. MS5 Less frequently: mesomelic and rhizomelic shortening of the limbs, hip dysplasia, and distal muscular atrophy of the limbs.

Supplementary Table 2: Management and surveillance proposed protocol for Schaaf-Yang Syndrome

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Childhood and Adolescence		
SPECIALTY	STUDIES AND FOLLOW-UP	SPECIFIC CARE AND RECOMMENDED STUDIES
Neurodevelopmental problems and intellectual development (ND)	Every 6-12 months Physical exam Assessment: Scales and developmental assessments either on the patient or completed by the parents	ND1 Abnormal muscle tone and arthrogryposis are very common. Hypotonia is the most prevalent. ND2 Gross motor development may be severely impaired by delayed acquisition of head control, sitting position, and gait (not achieved by many patients). ND3 Fine motor development is also atypical due to motor abnormalities, arthrogryposis, and camptodactyly. The purposeful use of the hands, which is based on cognition and social skills, may be impaired. Rehabilitation programs, early stimulation and, later, occupational therapy programs are essential. ND4 Social skills and communication are often severely affected. Only some of the patients can develop, speech and language (generally poor). ND5 ASD traits and behavioral abnormalities are very frequent and can interfere with communication skills. Evaluation by speech therapists is recommended. Alternatives to oral communication, such as pictographs or electronic devices, can be of help. Psychotherapy may be necessary for patients with ASD, to treat not only the communicative difficulties but also rigidity, repetitive behaviors, hypersensoriality,...

Supplementary Table 2: Management and surveillance proposed protocol for Schaaf-Yang Syndrome

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Childhood and Adolescence		
SPECIALTY	STUDIES AND FOLLOW-UP	SPECIFIC CARE AND RECOMMENDED STUDIES
Epilepsy (E)	Every 12 months Clinical interview Assessment: EEG	E1 Febrile seizures have been reported. E2 Epilepsy was found in less than 50% of patients, including different types of seizures (partial, generalized). Different AEDs were used successfully.
Neuroimaging (NI)	At the time of diagnosis. Afterwards, neuroimaging studies if new symptoms are present: MRI	NI1 Delayed myelination, ventricular enlargement, abnormalities of the corpus callosum (thinning, dysplasia or agenesis). NI2 Normal or hypoplastic pituitary gland is frequently reported. NI3 Rarely described: global cerebral atrophy, increased T2 signal in the caudate nucleus or putamen, globus pallidus, hypoplastic vermis, localized cerebellar hemorrhages.
Sleep disorders (SD)	Every 12 months Assessment: Sleep questionnaires Polysomnography (PSG)	SD1 Central and/or obstructive apnea have been reported and can worsen the cognitive and behavioral phenotype, as well as can be the cause of premature death. Periodic PSG is recommended.

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Childhood and Adolescence		
SPECIALTY	STUDIES AND FOLLOW-UP	SPECIFIC CARE AND RECOMMENDED STUDIES
Infections (I)	Every 12 months	I1 Recurrent respiratory infections have been reported in patients with respiratory assistance but also without respiratory support. Chronic lung disease has also been reported due to repeated bronchopneumonia or recurrent aspirations. Imaging studies may be required to evaluate the lung parenchyma and rule out malformations. Invasive studies are rarely necessary. I2 No predisposition to infections in other organs or systems is reported.
	Assessment: Laboratory Lung imaging studies	
Cardiology (C)	At the time of diagnosis and if new symptoms develop	C1 Septal defects (CSA) have been reported as the most common structural cardiac defects, sometimes solving spontaneously. C2 Bradycardia has been described in a 1-month-old baby.
	Physical exam Assessment: ECG Echocardiography	
Ophthalmology (O)	Every 12 months	O1 Strabismus and nystagmus have been described as oculomotor disorders. O2 Refractive errors have been reported. O3 Hypoplasia or atrophy of the optic nerve can be present. O4 Vision is usually difficult to assess in patients with SYS. Pediatric ophthalmologists are required. Lack of eye tracking has been described, however probably features of ASD underlie the abnormal ocular pursuit. O5 Rare: Xerophthalmia, caused by sleep with opened eyes.
	Physical exploration Assess: Acuity assessment Fundus Imaging techniques	

Supplementary Table 2: Management and surveillance proposed protocol for Schaaf-Yang Syndrome

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Supplementary Table 3: Propuesta de protocolo de estudio y seguimiento para el Síndrome de Schaaf-Yang (Spanish version).

Periodo perinatal		
	Descripción	Problemas especiales y su manejo
Dificultades respiratorias	Con frecuencia precisan UCI Neonatal, principalmente debido a la falta de reactividad neonatal. La necesidad de asistencia ventilatoria durante el periodo perinatal varía desde la oxigenoterapia hasta la ventilación mecánica invasiva, desde unas horas de apoyo hasta algunos meses. También se han notificado estridor laríngeo, glosptosis, traqueomalacia e hipoplasia pulmonar.	UCIN. Ventilación mecánica. Evaluación por otorrinolaringólogos y neumólogos para descartar anomalías anatómicas. Soporte respiratorio desde oxigenoterapia hasta ventilación mecánica invasiva y necesidad de traqueostomía.
	Son muy frecuentes las dificultades para alimentarse con frecuentes episodios de atragantamiento o succión ineficaz. La hipotonía, el letargo, la disfagia y un paladar alto, todos juntos pueden dificultar la nutrición por boca.	Se necesitan nutricionistas y especialistas en disfagia . Es importante realizar una evaluación anatómica para descartar malformaciones . Puede ser necesaria una intervención nutricional de apoyo, desde una sonda nasogástrica hasta una gastrostomía permanente o nutrición parenteral.

Supplementary Table 3: Propuesta de protocolo de estudio y seguimiento para el Síndrome de Schaaf-Yang

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Periodo perinatal		
	Descripción	Problemas especiales y su manejo
Contracturas	El pie zambo, la artrogriposis, las contracturas y otras restricciones de movimiento articular están presentes con frecuencia al nacer. La hipocinesia fetal puede provocar una artrogriposis grave.	La intervención temprana con medidas ortopédicas puede resultar esencial para obtener un mejor pronóstico. Es importante remitir a especialistas en rehabilitación y ortopedia infantil .
	Se han detectado varias alteraciones endocrinológicas durante el período perinatal, como la diabetes insípida, hiponatremia, deficiencia de hormona del crecimiento (GH), hipoglucemia e hipocalcemia (ver sección de Endocrinología).	Controlar los niveles de glucosa en sangre durante el período perinatal. Las pruebas de laboratorio a esta edad deben incluir un análisis exhaustivo de iones, glucosa y hormonas para descartar alteraciones prevalentes. Consultar con el endocrinólogo infantil.
Problemas hormonales	En la época perinatal se recomienda la evaluación por un neurólogo infantil y un genetista/ dismorfólogo. Se recomienda un examen físico detallado y un protocolo estandarizado para descartar malformaciones orgánicas que incluyen ecocardiograma, ecografía abdominal, ecografía craneal....	

Supplementary Table 3: Propuesta de protocolo de estudio y seguimiento para el Síndrome de Schaaf-Yang

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Infancia y Adolescencia		
ESPECIALIDAD	SEGUIMIENTO Y ESTUDIOS	CUIDADOS ESPECÍFICOS Y ESTUDIOS RECOMENDADOS
Endocrinología (E)	Cada 6 meses/	E1 Fallo de medro durante la infancia y la niñez, pero también sobrepeso temprano, obesidad e hiperfagia que comienzan después de la infancia. Puede ser necesaria una intervención dietética. E2 Estatura baja (de -1,5 DE a -5 DE), a veces necesita GH (considerar la apnea obstructiva cuando se prescribe GH) E3 Se ha descrito diabetes insípida que cursa con poliuria, densidad urinaria baja, hipostenuria e hipernatremia. Terapia hormonal sustitutiva.
	Exploración física Valorar: Laboratorio Ecografía Neuroimagen (RMc)	E4 Se ha descrito panhipopituitarismo causado por hipoplasia de la glándula pituitaria, pero también con resonancia magnética normal. Es necesario controlar la función tiroidea, somatomedina C, GH, hormonas suprarrenales, testosterona, LH y FSH. Se ha detectado hiperprolactinemia. Puede ser necesario un tratamiento con hormonas, que incluyen GH, levotiroxina e hidrocortisona. Terapia hormonal sustitutiva. Adolescencia → estrógenos. E5 Hipoglicemia . Garantizar la ingesta y mantenimiento de glucosa. Descartar hiperinsulinemia. E6 Inestabilidad de temperatura . Medidas de apoyo. E7 Genitales hipoplásicos, micropene y criptorquidia. Valorar testosterona, cirugía.

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Infancia y Adolescencia		
ESPECIALIDAD	SEGUIMIENTO Y ESTUDIOS	CUIDADOS ESPECÍFICOS Y ESTUDIOS RECOMENDADOS
Gastroenterología (G)	Cada 6 meses/ Exploración física Observación de la alimentación Valorar: pHmetría Videofluoroscopia Imagen (Rx) Laboratorio	G1 Los problemas de alimentación son casi constantes. Las dificultades iniciales de alimentación se deben a disfagia, aspiración respiratoria recurrente y, en ocasiones, se requiere sonda nasogástrica y gastrostomía. G2 También son frecuentes el estreñimiento crónico de aparición temprana y el reflujo gastroesofágico . G3 Complicaciones informadas con poca frecuencia: pseudoobstrucción intestinal, insuficiencia velofaríngea, esofagitis eosinofílica y alergias alimentarias.
	Cada 12 meses/ Exploración física Valorar: Imagen (Rx) Electromiograma y Conducción nerviosa	MS1 El tono muscular anormal es muy frecuente, sobre todo la hipotonía. MS2 La artrogriposis es muy frecuente: contracturas, acortamiento de extremidades, codos, rodillas, caderas. Camptodactilia, clinodactilia, braquidactilia de los dedos, pulgares en aducción. MS3 Se han descrito repetidamente los pies zambos y equinovaros . MS4 Escoliosis , cifosis, lordosis y tórax asimétrico. Es posible que se necesiten programas de rehabilitación temprana y ortesis externa. Se recomienda el control periódico de rayos X de la cadera en los niños que no pueden caminar y de la columna en total. Cirugía cuando sea necesario. MS5 Con menor frecuencia: acortamiento mesomélico y rizomélico de las extremidades, displasia de cadera y atrofia muscular distal de las extremidades.

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Infancia y Adolescencia		
ESPECIALIDAD	SEGUIMIENTO Y ESTUDIOS	CUIDADOS ESPECÍFICOS Y ESTUDIOS RECOMENDADOS
Problemas de neurodesarrollo y desarrollo intelectual (ND)	Cada 6-12 meses Exploración física Valorar: Escalas y evaluaciones del desarrollo sobre el paciente o completadas por los padres	ND1 El tono muscular anormal y la artrogriposis son muy frecuentes. La hipotonía es la más prevalente. ND2 El desarrollo de la motricidad gruesa puede verse gravemente afectado por el retraso en la adquisición del control de la cabeza, la sedestación y la marcha (no logrado por muchos pacientes). ND3 El desarrollo de la motricidad fina también es anormal debido a anomalías motoras, artrogriposis y camptodactilia. ND4 El uso propositivo de las manos, que se basa en la cognición y las habilidades sociales, puede verse alterado. Los programas de rehabilitación, estimulación temprana y, posteriormente, los programas de terapia ocupacional son fundamentales. ND4 Las habilidades sociales y la comunicación suelen verse gravemente afectadas. Solo algunos pacientes pueden desarrollar un lenguaje escaso. ND5 Los rasgos de TEA y las alteraciones del comportamiento son muy frecuentes. Esto puede interferir con las habilidades de comunicación. Se recomienda la evaluación de los terapeutas del habla. Las alternativas a la comunicación oral, como pictogramas o dispositivos electrónicos, pueden ayudar. La psicoterapia puede ser necesaria para los pacientes con TEA, para tratar no solo la esfera comunicativa sino también la rigidez, las conductas reiterativas, la hipersensibilidad...

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Infancia y Adolescencia		
ESPECIALIDAD	SEGUIMIENTO Y ESTUDIOS	CUIDADOS ESPECÍFICOS Y ESTUDIOS RECOMENDADOS
Epilepsia (E)	Cada 12 meses	E1 Se han notificado convulsiones febriles.
	Entrevista clínica Valorar: EEG	E2 Epilepsia en menos del 50% de los pacientes, diferente tipología (parcial, generalizada), diferentes FAEs utilizados con éxito.
Neuroimagen (NI)	En el momento diagnóstico.	NI1 Mielinización retrasada, agrandamiento ventricular, anomalías del cuerpo calloso (adelgazamiento, displasia o agenesis).
	Posteriormente, valorar si aparecen nuevos síntomas: MRI	NI2 Con frecuencia se informa de glándula pituitaria normal o hipoplásica. NI3 Raramente descrito: atrofia cerebral global, aumento de la señal T2 en el núcleo caudado o en el putamen, globo pálido, vermis hipoplásico, hemorragias cerebelosas puntiformes.
Problemas de sueño (SD)	Cada 12 meses	SD1 Se ha informado de apnea central y / o apnea obstructiva que pueden empeorar los problemas cognitivos y de comportamiento, así como ser la causa de muerte prematura.
	Valorar: Cuestionarios de sueño Polisomnografía (PSG)	Se recomienda PSG periódica.

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Infancia y Adolescencia		
ESPECIALIDAD	SEGUIMIENTO Y ESTUDIOS	CUIDADOS ESPECÍFICOS Y ESTUDIOS RECOMENDADOS
Infecciones (I)	Cada 12 meses	I1 Se han notificado infecciones respiratorias recurrentes en los pacientes que necesitan asistencia respiratoria y en los que no la tienen, así como enfermedad pulmonar crónica debida a bronconeumonía repetida o aspiraciones recurrentes. Es posible que se requieran estudios de imágenes para verificar el parénquima pulmonar y descartar malformaciones. Rara vez son necesarios estudios invasivos.
	Valorar: Laboratorio Estudios de imagen pulmonares	I2 No se informa de una predisposición a infecciones en otros órganos o sistemas.
Cardiología (C)	Al diagnóstico y si se desarrollan nuevos síntomas	C1 Los defectos septales (CSA) se han informado como las anomalías cardíacas estructurales más frecuentes, que en ocasiones se resuelven espontáneamente.
	Exploración física Valorar: ECG Ecocardiografía	C2 Se ha descrito bradicardia en un bebé de 1 mes.
Oftalmología (O)	Cada 12 meses	O1 Se han descrito estrabismo y nistagmo , como trastornos oculomotores.
	Exploración física Valorar: Valoración de la agudeza Fondo de ojo Técnicas de imagen	O2 Se han descrito defectos de refracción. O3 Hipoplasia o atrofia del nervio óptico. O4 La vista es normalmente difícil de evaluar en pacientes con SYS. Se requieren oftalmólogos pediátricos. Se ha descrito falta de seguimiento con los ojos, probablemente los rasgos de TEA subyacen a este síntoma. O5 Raras: Xeroftalmia, causada por dormir con los ojos abiertos.

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Supplementary Table 4: Full list of mutations depicted in Figure 2.

cDNA change	Protein Change	Reported phenotype	Number of individuals	Publication	Comments
NM_019066.5:c.1580delC	p.Pro527Argfs*175	SYS	1	Fountain et al., 2016	
NM_019066.5:c.1613C>A	p.Ala538Glu	mild SYS	1	Patak et al., 2019	It is already described as missense in the paper.
NM_019066.5:c.1621C>T	p.Gln541*	SYS	1	Fountain et al., 2016	
NM_019066.5:c.1628delC	p.Pro543Leufs*159	SYS	1	Tong et al., 2018	
NM_019066.5:c.1652delT	p.Val551Aspfs*151	SYS	1	Schaaf et al., 2013	
NM_019066.5:c.1687C>T	p.Gln563*	SYS	1	Duan et al., 2021	
NM_019066.5:c.1690C>G	p.Pro564Ala	SYS	1	McCarthy et al., 2018	
NM_019066.5:c.1761G>A	p.Trp587*	SYS	1	Negishi et al., 2019	
NM_019066.5:c.1762C>T	p.Gln588*	Chitayat-Hall (SYS)	4	Jobling et al., 2018; Negishi et al., 2019	
NM_019066.5:c.1790delC	p.Pro597Hisfs*105	SYS	1	Marbach et al., 2020	ESHG Poster P08.100.A - The adult phenotype of Schaaf-Yang syndrome
NM_019066.5:c.1797_1810del	p.Glu599Aspfs*109	SYS	1	McCarthy et al., 2018	In the paper it is reported as c.1797_1820del (p.Ile600_Gln607del), but in HGMD is shown as c.1797_1810del (p.Glu599Aspfs*109) which correlates more with the SYS phenotype.
NM_019066.5:c.1800delT	p.Pro601Glnfs*101	SYS	1	Marbach et al., 2020	ESHG Poster P08.100.A - The adult phenotype of Schaaf-Yang syndrome
NM_019066.5:c.1801_1802del	p.Pro601Asnfs*111	SYS	1	Fountain et al., 2016	
NM_019066.5:c.1802delC	p.Pro601Glnfs*101	SYS	1	Schaaf et al., 2013	
NM_019066.5:c.1808C>G	p.Ser603*	SYS	1	Bertoli-Avella et al., 2021	
NM_019066.5:c.1813G>T	p.Glu605*	SYS	1	McCarthy et al., 2018	
NM_019066.5:c.1819C>T	p.Gln607*	SYS	1	Marbach et al., 2020	ESHG Poster P08.100.A - The adult phenotype of Schaaf-Yang syndrome
NM_019066.5:c.1850G>A	p.Trp617*	SYS	2	Kleinendorst et al., 2018	One of the patients is ours.
NM_019066.5:c.1900G>T	p.Glu634*	SYS	1	McCarthy et al., 2018	
NM_019066.5:c.1906C>T	p.Gln636*	SYS	1	McCarthy et al., 2018	
NM_019066.5:c.1912C>T	p.Gln638*	Opitz C/ MAGEL2 syndrome/ SYS	9	Urreizti et al., 2017; Fountain et al., 2016; Enya et al., 2018; Poliak et al., 2018; Negishi et al., 2019; Halloun et al., 2021; Stranneheim et al., 2021	
NM_019066.5:c.1958_1962del	p.Pro653Leufs*58	SYS	2	McCarthy et al., 2018	
NM_019066.5:c.1990_1991delinsA	p.Pro664Thrfs*38	SYS	1	McCarthy et al., 2018	
NM_019066.5:c.1996delC	p.Gln666Serfs*36	AMC	13	Mejlachowicz et al., 2015; Fountain et al., 2016; McCarthy et al., 2018; Laquerriere et al., 2021; Guo et al., 2019	
NM_019066.5:c.1996dupC	p.Gln666Profs*47	SYS; Chitayat-Hall	80	Soden et al., 2014; Fountain et al., 2016; Jobling et al., 2018; Palomares-Bralo et al., 2017; Matuszewska et al., 2018; McCarthy et al., 2018; Negishi et al., 2019; Patak et al., 2019; Gregory et al., 2019; Marbach et al., 2020; Chen et al., 2020; Ahn et al., 2020; Laquerriere et al., 2021; Nunes et al., 2021	
NM_019066.5:c.1996_1997dup	p.Gln666Hisfs*37	SYS	1	Patak et al., 2019	
NM_019066.5:c.1996C>T	p.Gln666*	SYS	2	McCarthy et al., 2018	
NM_019066.5:c.2034delC	p.Thr679Hisfs*23	SYS	1	McCarthy et al., 2018	In the paper it is reported as c.224delC. Our hypothesis is that it was annotated in GRCh37.
NM_019066.5:c.2056_2066del	p.Trp686Alafs*23	SYS	1	McCarthy et al., 2018	
NM_019066.5:c.2101dupG	p.Val701Glyfs*12	SYS	1	de Andrade et al., 2020	



NM_019066.5:c.2105delC	p.Ala702Glufs*13	SYS	1	McCarthy et al., 2018	In the paper it is reported as c.2015delC, but in that position there is no C. In HGMD it is reported as c.2105delC (p.Ala702Glufs*13) which correlates with the SYS phenotype
NM_019066.5:c.2118delT	p.Leu708Trpfs*7	AMC/SYS	2	Mejlachowicz et al., 2015; Laquerriere et al., 2021	
NM_019066.5:c.2113_2114del	p.Asn705Phefs*7	SYS	1	McCarthy et al., 2018	
NM_019066.5:c.2153C>A	p.Ser718*	SYS	1	McCarthy et al., 2018	
NM_019066.5:c.2163C>A	p.Cys721*	SYS	1	McCarthy et al., 2018	
NM_019066.5:c.2170_2232dup	p.Ser724_Ala744dup	SYS	1	Marbach et al., 2020	ESHG Poster P08.100.A - The adult phenotype of Schaaf-Yang syndrome. Features suggestive of SYS.
NM_019066.5:c.2179_2180del	p.Asp727Profs*6	Chitayat-Hall (SYS)	2	Jobling et al., 2018	
NM_019066.5:c.2199delA	p.Glu734Serfs*17	SYS	1	McCarthy et al., 2018	In the paper it is reported as c.390delA. It does not seem to be correctly annotated, in this position there is a G. Our hypothesis is that it could be annotated in GRCh37.
NM_019066.5:c.2216delC	p.Ser739*	SYS	1	Ahn et al., 2020	in the paper it is reported as c.2217delC, but we have corrected it as c.2216delC
NM_019066.5:c.2296C>T	p.Arg766*	SYS	1	McCarthy et al., 2018	
NM_019066.5:c.2821dupC	p.Arg941Profs*10	SYS	1	Marbach et al., 2020	ESHG Poster P08.100.A - The adult phenotype of Schaaf-Yang syndrome
NM_019066.5:c.2861_2862insG	p.Ile954Metfs*30	SYS	1	McCarthy et al., 2018	
NM_019066.5:c.2873G>A	p.Trp958*	SYS	3	Fountain et al., 2016; McCarthy et al., 2018; Marbach et al., 2020	Marbach: ESHG Poster P08.100.A - The adult phenotype of Schaaf-Yang syndrome
NM_019066.5:c.2894G>A	p.Trp965*	SYS	1	Matuszewska et al., 2018	
NM_019066.5:c.2958delG	p.Ser987Alafs*5	SYS	2	McCarthy et al., 2018; Marbach et al., 2020	
NM_019066.5:c.2970delC	p.Val991*	AMC	1	Laquerriere et al., 2021	
NM_019066.5:c.2989G>A	p.Ala997Thr	SYS	1	McCarthy et al., 2018	
NM_019066.5:c.3019C>T	p.Gln1007*	SYS	1	Hidalgo-Santos et al., 2018	
NM_019066.5:c.3031A>T	p.Lys1011*	SYS	1	McCarthy et al., 2018	
NM_019066.5:c.3070delG	p.Ala1024Glnfs*9	SYS	1	McCarthy et al., 2018	
NM_019066.5:c.3122delT	p.Val1041Alafs*7	SYS	1	Patak et al., 2019	
NM_019066.5:c.3124C>T	p.Gln1042*	SYS	3	Schaaf et al., 2013; McCarthy et al., 2018	
NM_019066.5:c.3131C>A	p.Ser1044*	SYS	1	Enya et al., 2018	
NM_019066.5:c.3164_3165del	p.Lys1055Argfs*2	SYS	1	Stranneheim et al., 2021	
NM_019066.5:c.3169G>T	p.Glu1057*	SYS	1	Patak et al., 2019	
NM_019066.5:c.3175_3176dup	p.Leu1059Phefs*2	SYS	1	Llamos-Paneque et al., 2020	
NM_019066.5:c.3181_3182del	p.Ile1061Hisfs*7	SYS	1	Schaaf et al., 2013	
NM_019066.5:c.3208G>T	p.Glu1070*	SYS	1	Bowling et al., 2017; McCarthy et al., 2018	
NM_019066.5:c.3235G>T	p.Glu1079*	SYS	2	Fountain et al., 2016; McCarthy et al., 2018	
NM_019066.5:c.3449_3450delTT	p.Phe1150Trpfs*4	SYS	1	Ahn et al., 2020	
NM_019066.5:c.3607A>T	p.Lys1203*	SYS	1	McCarthy et al., 2018	

Supplementary Table 5: Complete list of DEGs.

Gene ID	Gene symbol	Combined FDR	Mean LogFCs
UPregulated			
ENSG00000228630	<i>HOTAIR</i>	0.00000000	74.697
ENSG00000256618	<i>MTRNR2L1</i>	0.00001135	55.233
ENSG00000153707	<i>PTPRD</i>	0.00000123	46.245
ENSG00000179542	<i>SLITRK4</i>	0.00880923	45.239
ENSG00000244300	<i>GATA2-AS1</i>	0.00000000	43.041
ENSG00000069011	<i>PITX1</i>	0.00816110	42.342
ENSG00000163347	<i>CLDN1</i>	0.00000112	41.450
ENSG00000159556	<i>ISL2</i>	0.00193059	40.878
ENSG00000077092	<i>RARB</i>	0.01493986	40.001
ENSG00000162738	<i>VANGL2</i>	0.00000081	39.748
ENSG00000250133	<i>HOXC-AS2</i>	0.00000000	37.501
ENSG00000114115	<i>RBP1</i>	0.00010963	36.565
ENSG00000251151	<i>HOXC-AS3</i>	0.00002460	35.799
ENSG00000170412	<i>GPRC5C</i>	0.00318874	35.355
ENSG00000158258	<i>CLSTN2</i>	0.00001884	32.582
ENSG00000205403	<i>CFI</i>	0.00481319	32.156
ENSG00000176887	<i>SOX11</i>	0.00617501	31.216
ENSG00000050344	<i>NFE2L3</i>	0.00406723	30.615
ENSG00000118785	<i>SPP1</i>	0.00001245	29.987
ENSG00000179348	<i>GATA2</i>	0.00002887	28.457
ENSG00000165194	<i>PCDH19</i>	0.00016182	28.371
ENSG00000189120	<i>SP6</i>	0.00048115	28.068
ENSG00000110328	<i>GALNT18</i>	0.01328474	27.797
ENSG00000145794	<i>MEGF10</i>	0.00006520	27.100
ENSG00000145569	<i>FAM105A</i>	0.00486515	26.858
ENSG00000158164	<i>TMSB15A</i>	0.00000078	26.185
ENSG00000213190	<i>MLLT11</i>	0.00000008	25.159
ENSG00000116741	<i>RGS2</i>	0.00007873	25.044
ENSG00000144355	<i>DLX1</i>	0.00483280	25.016
ENSG00000139364	<i>TMEM132B</i>	0.00011399	24.618
ENSG00000122378	<i>FAM213A</i>	0.00287714	24.111
ENSG00000146250	<i>PRSS35</i>	0.00561639	23.849
ENSG00000260549	<i>MT1L</i>	0.00000992	21.904
ENSG00000273038	<i>AL365203.3</i>	0.00000000	19.057
ENSG00000151725	<i>CENPU</i>	0.00000002	18.965
ENSG00000090530	<i>P3H2</i>	0.00086613	18.937
ENSG00000149948	<i>HMGA2</i>	0.00670181	18.504
ENSG00000131203	<i>IDO1</i>	0.00013629	18.327
ENSG00000138135	<i>CH25H</i>	0.00008401	17.432
ENSG00000156970	<i>BUB1B</i>	0.00373261	17.240
ENSG00000104368	<i>PLAT</i>	0.00860139	17.015
ENSG00000143228	<i>NUF2</i>	0.00632092	16.711
ENSG00000114268	<i>PFKFB4</i>	0.00014643	16.596
ENSG00000121621	<i>KIF18A</i>	0.00034690	16.581
ENSG00000170312	<i>CDK1</i>	0.00213696	15.856

ENSG00000115163	<i>CENPA</i>	0.00279515	15.844
ENSG00000164687	<i>FABP5</i>	0.00080168	15.778
ENSG00000111110	<i>PPM1H</i>	0.00645193	15.669
ENSG00000197632	<i>SERPINB2</i>	0.01326318	14.831
ENSG00000150995	<i>ITPR1</i>	0.00769409	14.761
ENSG00000124813	<i>RUNX2</i>	0.00001473	14.757
ENSG00000176971	<i>FIBIN</i>	0.01272529	14.381
ENSG00000254726	<i>MEX3A</i>	0.00017300	14.358
ENSG00000102096	<i>PIM2</i>	0.00093743	14.035
ENSG00000197646	<i>PDCD1LG2</i>	0.01170811	13.984
ENSG00000259207	<i>ITGB3</i>	0.00648175	13.957
ENSG00000118193	<i>KIF14</i>	0.00636966	13.816
ENSG00000152952	<i>PLOD2</i>	0.00006319	13.715
ENSG00000050730	<i>TNIP3</i>	0.00404054	13.498
ENSG00000168679	<i>SLC16A4</i>	0.00212062	13.118
ENSG00000150051	<i>MKX</i>	0.00001061	12.801
ENSG00000171812	<i>COL8A2</i>	0.01530689	12.742
ENSG00000135048	<i>TMEM2</i>	0.00436512	12.558
ENSG00000171502	<i>COL24A1</i>	0.00000125	12.508
ENSG00000140263	<i>SORD</i>	0.01645580	12.443
ENSG00000188342	<i>GTF2F2</i>	0.00000000	12.364
ENSG00000137269	<i>LRRC1</i>	0.00543204	12.269
ENSG00000135916	<i>ITM2C</i>	0.00288275	12.159
ENSG00000057019	<i>DCBLD2</i>	0.00000000	12.142
ENSG00000140945	<i>CDH13</i>	0.00007483	11.570
ENSG00000176697	<i>BDNF</i>	0.00008341	11.214
ENSG00000067177	<i>PHKA1</i>	0.00188460	11.207
ENSG00000154188	<i>ANGPT1</i>	0.00002689	11.025
ENSG00000130066	<i>SAT1</i>	0.00760640	10.727
ENSG00000103187	<i>COTL1</i>	0.00983754	10.350
ENSG00000132429	<i>POPDC3</i>	0.00001490	10.274
DOWNregulated			
ENSG00000178695	<i>KCTD12</i>	0.00106130	-10.194
ENSG00000188112	<i>C6orf132</i>	0.00000368	-10.303
ENSG00000099377	<i>HSD3B7</i>	0.00134145	-10.580
ENSG00000176658	<i>MYO1D</i>	0.00404705	-11.198
ENSG00000173517	<i>PEAK1</i>	0.00000101	-11.545
ENSG00000182179	<i>UBA7</i>	0.00009340	-11.594
ENSG00000189320	<i>FAM180A</i>	0.00180989	-12.136
ENSG00000138131	<i>LOXL4</i>	0.00469257	-12.375
ENSG00000138744	<i>NAAA</i>	0.00357893	-12.792
ENSG00000239653	<i>PSMD6-AS2</i>	0.00185430	-13.232
ENSG00000138356	<i>AOX1</i>	0.00195770	-13.434
ENSG00000157404	<i>KIT</i>	0.00002769	-13.879
ENSG00000176928	<i>GCNT4</i>	0.00415318	-14.183
ENSG00000229847	<i>EMX2OS</i>	0.00241163	-14.284
ENSG00000266094	<i>RASSF5</i>	0.00012660	-14.698

ENSG00000116667	<i>C1orf21</i>	0.00005050	-15.643
ENSG00000139132	<i>FGD4</i>	0.00322221	-17.180
ENSG00000152117	<i>AC073869.1</i>	0.00144781	-17.284
ENSG00000228784	<i>LINC00954</i>	0.00041045	-17.334
ENSG00000153993	<i>SEMA3D</i>	0.00004346	-17.822
ENSG00000206538	<i>VGLL3</i>	0.00000000	-18.117
ENSG00000196935	<i>SRGAP1</i>	0.00000972	-19.512
ENSG00000204682	<i>CASC10</i>	0.00032805	-20.733
ENSG00000035664	<i>DAPK2</i>	0.00001064	-20.752
ENSG00000149292	<i>TTC12</i>	0.00930174	-21.262
ENSG00000172348	<i>RCAN2</i>	0.00062630	-21.702
ENSG00000287733	novel transcript (lncRNA)	0.00002544	-21.759
ENSG00000235505	<i>AP002004.1</i>	0.00073579	-21.960
ENSG00000107738	<i>VSIR</i>	0.00000000	-22.341
ENSG00000215386	<i>MIR99AHG</i>	0.00000168	-22.998
ENSG00000130038	<i>CRACR2A</i>	0.00001310	-23.839
ENSG00000110852	<i>CLEC2B</i>	0.00000106	-24.671
ENSG00000258479	<i>LINC00640</i>	0.00278439	-26.137
ENSG00000091831	<i>ESR1</i>	0.00000033	-26.370
ENSG00000176771	<i>NCKAP5</i>	0.00041109	-28.014
ENSG00000133083	<i>DCLK1</i>	0.00000099	-28.172
ENSG00000287516	novel transcript (lncRNA)	0.00005411	-28.253
ENSG00000171840	<i>NINJ2</i>	0.00000002	-28.859
ENSG00000003400	<i>CASP10</i>	0.00032486	-30.075
ENSG00000198483	<i>ANKRD35</i>	0.00000000	-31.637
ENSG00000101265	<i>RASSF2</i>	0.00000000	-32.207
ENSG00000150594	<i>ADRA2A</i>	0.00013274	-33.274
ENSG00000136869	<i>TLR4</i>	0.00058424	-35.545
ENSG00000102802	<i>MEDAG</i>	0.00000326	-37.459
ENSG00000185742	<i>C11orf87</i>	0.00000298	-40.083
ENSG00000155011	<i>DKK2</i>	0.00014551	-40.656
ENSG00000189058	<i>APOD</i>	0.00006002	-42.130
ENSG00000174611	<i>KY</i>	0.00000000	-42.483
ENSG00000064205	<i>WISP2</i>	0.00000004	-44.203
ENSG00000108381	<i>ASPA</i>	0.00101640	-48.129
ENSG00000006016	<i>CRLF1</i>	0.00067991	-50.286
ENSG00000241644	<i>INMT</i>	0.00256196	-50.410
ENSG00000255399	<i>TBX5-AS1</i>	0.00000000	-54.574
ENSG00000164530	<i>PI16</i>	0.00129264	-63.849
ENSG00000089225	<i>TBX5</i>	0.00000000	-69.819
ENSG00000116194	<i>ANGPTL1</i>	0.00000366	-70.837

Supplementary Table 6: Significantly enriched Reactome categories.

ID	Description	pvalue	p.adjust	qvalue	Gene symbol	Gene Count
REACTOME						
R-HSA-2500257	Resolution of Sister Chromatid Cohesion	0.0000927	0.0223995	0.0212078	<i>CENPA,KIF18A,NUF2,CENPU,BUB1B,CDK1</i>	6
R-HSA-141424	Amplification of signal from the kinetochores	0.0002435	0.0223995	0.0212078	<i>CENPA,KIF18A,NUF2,CENPU,BUB1B</i>	5
R-HSA-69618	Mitotic Spindle Checkpoint	0.0005179	0.0332646	0.0314948	<i>CENPA,KIF18A,NUF2,CENPU,BUB1B</i>	5
R-HSA-1474290	Collagen formation	0.0001799	0.0223995	0.0212078	<i>P3H2, LOXL4, PLOD2, COL24A1, COL8A2</i>	5
GO Cellular Components						
GO:0000776	Kinetochores	0.0001378	0.0130901	0.0123286	<i>RASSF2,CENPA,KIF18A,NUF2,CENPU, BUB1B</i>	6
GO:0000775	Chromosome, centromeric region	0.0009309	0.0294791	0.0277642	<i>RASSF2,CENPA,KIF18A,NUF2,CENPU, BUB1B</i>	6

Supplementary Table 7: List of fibroblast lines.

Group	MAGEL2 mutation	Age (years)	Sex
SYS	(p.Gln638*)	16-20	Female
SYS	(p.Trp617*)	0-5	Male
SYS	(p.Gln666Profs*47)	6-10	Male
SYS	(p.Gln666Profs*47)	6-10	Female
SYS	(p.Gln1007*)	11-15	Female
SYS	(p.Trp686Alafs*23)	6-10	Male
SYS	(p.Ala703Glyfs*9)	0-5	Male
SYS	(p.Ser671*)	Adult	Female
PWS	Paternal deletion	0-5	Male
PWS	disomy	16-20	Male
PWS	disomy	11-15	Male
PWS	Imprinting defect	6-10	Female
PWS	Paternal deletion	6-10	Female
PWS	Paternal deletion	0-5	Male
PWS	disomy	6-10	Female
PWS	Paternal deletion	6-10	Female
PWS	Paternal deletion	11-15	Female
Control	-	Adult	Male
Control	-	Adult	Male
Control	-	Adult	Female
Control	-	Adult	Male
Control	-	Adult	Female
Control	-	16-20	Male
Control	-	Adult	Male
Control	-	11-15	Male
Control	-	Adult	Female
Control	-	11-15	Female
Control	-	Adult	Male

Adult: >21 years old

ARTICLE 2: SUBCELLULAR LOCALISATION OF TRUNCATED MAGEL2 PROTEINS:
INSIGHT INTO THE MOLECULAR PATHOLOGY OF SCHAAF-YANG SYNDROME**Summary:**

Schaaf-Yang syndrome (SYS) is an ultra-rare neurodevelopmental disorder caused by truncating mutations in *MAGEL2*. Heterologous expression of wild-type (WT) or a truncated (p.Gln638*) C-terminal HA-tagged MAGEL2 revealed a shift from a primarily cytoplasmic to a more nuclear localisation for the truncated protein variant. We now extend this analysis to six additional SYS mutations on a N-terminal FLAG-tagged MAGEL2. Our results replicate and extend our previous findings, showing that all the truncated MAGEL2 proteins consistently display a predominant nuclear localisation, irrespective of the C-terminal or N-terminal position and the chemistry of the tag. The variants associated with arthrogryposis multiplex congenita display a more pronounced nuclear retention phenotype, suggesting a correlation between clinical severity and the degree of nuclear mislocalisation. These results point to a neomorphic effect of truncated MAGEL2, which might contribute to the pathogenesis of SYS.

Reference:

Centeno-Pla M, *et al.* Subcellular localisation of truncated MAGEL2 proteins: insight into the molecular pathology of Schaaf-Yang syndrome. *J Med Genet* 2024;61:780–782. doi:10.1136/jmg-2024-109898

Genotype-phenotype correlations

Short report

Subcellular localisation of truncated MAGEL2 proteins: insight into the molecular pathology of Schaaf-Yang syndrome

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ABSTRACT

Schaaf-Yang syndrome (SYS) is an ultra-rare neurodevelopmental disorder caused by truncating mutations in *MAGEL2*. Heterologous expression of wild-type (WT) or a truncated (p.Gln638*) C-terminal HA-tagged *MAGEL2* revealed a shift from a primarily cytoplasmic to a more nuclear localisation for the truncated protein variant. We now extend this analysis to six additional SYS mutations on a N-terminal FLAG-tagged *MAGEL2*. Our results replicate and extend our previous findings, showing that all the truncated *MAGEL2* proteins consistently display a predominant nuclear localisation, irrespective of the C-terminal or N-terminal position and the chemistry of the tag. The variants associated with arthrogryposis multiplex congenita display a more pronounced nuclear retention phenotype, suggesting a correlation between clinical severity and the degree of nuclear mislocalisation. These results point to a neomorphic effect of truncated *MAGEL2*, which might contribute to the pathogenesis of SYS.

INTRODUCTION

MAGEL2 (OMIM #605283) is one of the maternally imprinted protein-coding genes of the Prader-Willi/Angelman syndrome critical region (15q11-q13). It is a single-exon gene, encoding one of the largest proteins of the type II MAGE protein family, comprising 1249 amino acids. Within the N-terminal region of *MAGEL2*, there is a proline-rich domain that has been associated with RNA metabolism,¹ while in the C-terminal end, spanning amino acids 1027 to 1195, lies a MAGE Homology Domain (MHD). MHD is a highly conserved sequence common to both type I and type II MAGEs that plays a crucial role in facilitating protein-protein interactions in retrograde endosomal transport or circadian rhythm processes.^{2,3} Since the identification of *MAGEL2* truncating mutations as the cause of Schaaf-Yang syndrome (SYS, OMIM #615547) in 2013,⁴ over 80 different mutations, all resulting in the production of truncated proteins lacking the MHD, have been reported.

SYS is an ultra-rare neurodevelopmental syndrome which shares some clinical features (neonatal hypotonia, developmental delay or sleep disorders) with the more common Prader-Willi syndrome (OMIM

#176270), caused by the lack of expression of paternal genes in 15q11-q13. However, individuals with SYS more frequently exhibit severe intellectual disability (94.8%), autism spectrum disorder behaviours (72.4%) and joint contractures (87.7%).^{5,6} In addition, three particular *MAGEL2* truncating variants, p.Gln666Serfs*36, p.Leu708Trpfs*7 and p.Val991*, are associated with a significantly more severe phenotype characterised by arthrogryposis multiplex congenita (AMC), which ultimately leads to perinatal death.^{7–10}

In a previous study, to characterise the functional consequences of *MAGEL2* truncation, we transfected vectors encoding haemagglutinin (HA)-tagged wild-type (WT) or one particular truncated *MAGEL2* (*MAGEL2*-Gln638*) and assessed their subcellular localisation.⁶ Immunocytochemistry (ICC) assays showed that in cells transfected with the WT construct, *MAGEL2* was mainly located in the cytoplasm, while there was a shift towards the nucleus when transfecting the truncated protein. Importantly, this phenomenon was seen consistently across different recipient cell lines. However, it could be argued that the phenomenon was mutation-specific and/or that the C-terminal HA tag might be mediating this effect.

To establish the generalisability of the results, we have now investigated a broader range of *MAGEL2* truncating variants. We generated additional plasmids expressing a N-terminal FLAG-tagged *MAGEL2* protein, in which we introduced six truncating *MAGEL2* mutations as illustrated in online supplemental figure 1. Four of them (p.Trp617* (c.1850G>A), p.Gln638* (c.1912C>T), p.Gln666Profs*47 (c.1996dupC), and p.Trp686Alafs*23 (c.2056_2066del)) were identified in SYS patients (in 2, 15, 99 and 1 cases, respectively); while the other two (p.Gln666Serfs*36 (c.1996delC) and p.Leu708Trpfs*7 (c.2118delT)) have been associated with severe AMC and perinatal death in all diagnosed cases (11 and 2, respectively) (online supplemental table 1).^{7–10}

MATERIALS AND METHODS

ICC assays were conducted in transfected HeLa cells to detect the presence of FLAG and 4',6'-diamidino-2-phenylindole (DAPI). Cloning, cell culture and ICC are described in supplementary materials and methods. Images were acquired

Genotype-phenotype correlations

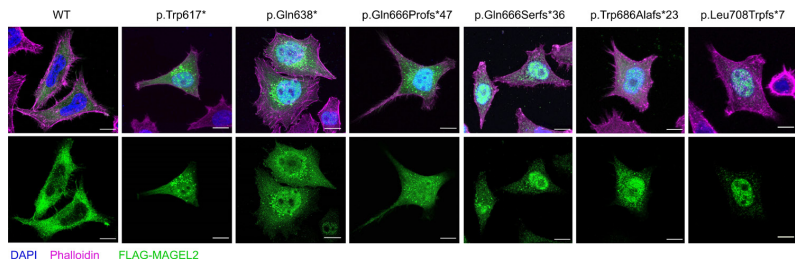


Figure 1 Representative immunofluorescence images of HeLa cells transfected with wild-type (WT) or truncated *MAGEL2* vectors carrying mutations p.Trp617*, p.Gln638*, p.Gln666Profs*47, p.Gln666Serfs*36, p.Trp686Alafs*23 and p.Leu708Trpfs*7, respectively, stained with 4',6-diamidino-2-phenylindole (DAPI) (blue), Phalloidin (magenta) and anti-FLAG (green). Scale bar represents 10 µm.

using a Zeiss confocal microscope LSM 880 and analysed with ImageJ.¹¹ To quantitatively assess the nuclear localisation, we used Mander's co-localisation coefficient between the FLAG fluorescent signal and DAPI. Statistical significance was determined using Welch analysis of variance (ANOVA), and values of $p < 0.01$ were considered statistically significant.

RESULTS

Our ICC assays revealed that all six heterologously expressed truncated proteins are predominantly found inside the nucleus, in contrast with the cytoplasmic localisation of WT *MAGEL2* (figure 1). These findings were consistently observed across multiple experimental replicates, suggesting a genuine shift in the localisation pattern induced by *MAGEL2* truncating mutations. No discernible changes in cell shape or viability were observed in the transfected cells, neither with the wild-type or with any of the truncated proteins.

A quantitative analysis of co-localisation of *MAGEL2* and DAPI revealed significant differences between all six truncated *MAGEL2* variants and the full-length wild-type form and hinted at a connection between the degree of nuclear localisation and the clinical severity of certain mutations (figure 2A). Notably,

AMC mutations (p.Gln666Serfs*36 and p.Leu708Trpfs*7) exhibit a more pronounced nuclear localisation (figure 2B). This finding adds a layer of complexity to our understanding of *MAGEL2* in cellular processes, linking the subcellular behaviour of its truncating variants to their phenotypical manifestation.

DISCUSSION

In our study, we made concerted efforts to address potential subcellular localisation effects attributable to the tagging method. Our data consistently demonstrated a notable nuclear localisation pattern of all exogenously expressed truncated *MAGEL2* proteins. This strengthens the validity and specificity of our findings, suggesting that the observed localisation reflects the inherent properties of truncated *MAGEL2*.

While the lack of a properly functioning antibody against the N-terminus of *MAGEL2* hinders the validation of these findings with endogenous *MAGEL2*, the consistent and significant shift in localisation points to a pathogenic neomorphic effect of the truncated protein in the nucleus. Furthermore, our findings hint at the intriguing possibility that the extent of nuclear localisation of truncated *MAGEL2* might play a role in the manifestation and severity of joint contractures in patients with SYS.

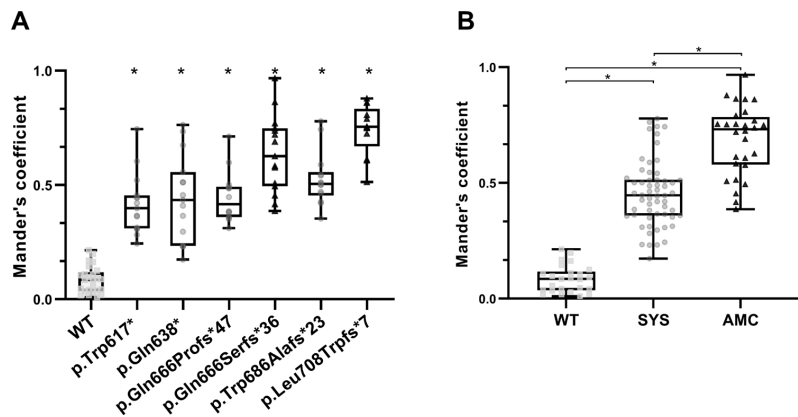


Figure 2 A) Mander's coefficient quantification of the co-localisation between the FLAG fluorescence signal and 4',6-diamidino-2-phenylindole (DAPI) in cells transfected with wild-type (WT) FLAG-MAGEL2 or truncated FLAG-MAGEL2. The symbol '*' denotes significant differences of truncating mutations when compared with the WT group * $p < 0.01$. (B) Co-localisation quantification using Mander's coefficient between the FLAG fluorescence signal and DAPI in *MAGEL2*-WT and *MAGEL2*-truncated transfected cells carrying Schaaf-Yang syndrome (SYS) or arthrogryposis multiplex congenita (AMC) mutations, respectively. The symbol '*' denotes significant differences between groups * $p < 0.01$. Statistical analyses were performed using Welch analysis of variance (ANOVA) in GraphPad Prism. Data are presented as mean $\pm$ SD from at least two independent experiments ($n \geq 2$). WT, SYS and AMC variants are differentiated by shape (square, circle and triangle, respectively).

Genotype-phenotype correlations

This correlation could be invaluable for predicting the potential impact of newly identified *MAGEL2* mutations based on their subcellular localisation patterns, offering a novel angle for prognosis.

Subcellular localisation significantly dictates the functional dynamics of proteins, establishing protein-protein interactions, post-translational modifications and integration into cellular processes. Consequently, aberrant subcellular localisation of proteins due to mutations has been acknowledged to trigger disease.¹² As *MAGEL2* is involved in multiple cellular signalling pathways including those related to retrograde endosomal transport,² circadian rhythm,³ dendrite formation¹³ or RNA metabolism,¹ the nuclear localisation of truncated *MAGEL2* might interfere with these signalling cascades and lead to altered cellular responses. In addition to the disruption of normal cellular processes, the truncated protein might also acquire new functions in the nucleus, engaging in interactions with a different set of nuclear proteins compared with the WT. Truncation could yield abnormal protein products susceptible to aggregation, which may result in the aberrant regulation of gene expression and nuclear processes.

In conclusion, our study consistently shows a nuclear shift in truncated *MAGEL2* variants, potentially disrupting cell functions and causing harmful effects associated with the SYS phenotype. Besides, the suggestion that an increased nuclear localisation might correlate with the severity of AMC is open to additional investigation. Collectively, our results shed new light on the complex molecular mechanisms underlying the pathogenicity of SYS-associated mutations and lay the groundwork for more research on the role of truncated *MAGEL2* in the nucleus. Further studies could explore the mechanisms driving the nuclear export of *MAGEL2*, and therapeutic targeting to correct mislocalisation of truncated *MAGEL2*. Additionally, being aware that our study focused on exogenously expressed *MAGEL2*, we recognise that, to establish their clinical relevance, it would be essential to validate our results in the context of endogenous protein expression, preferably in neuronal cells given the neurological features of SYS. Alternative approaches, such as CRISPR/Cas9 (Clustered regularly interspaced palindromic repeats / CRISPR-associated protein 9)-mediated tagging of endogenous *MAGEL2*, may enable more accurate assessment of its subcellular localisation and potential interactions with other nuclear components.

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Contributors RU, EA-C, AP-P and MC-P designed the cloning strategy. EA-C, MC-P and SG participated in the cloning of the plasmids. MC-P and SG conducted the cell culture, transfection and immunofluorescence staining experiments. MC-P and JDG-A analysed the ICC assays. MC-P and EA-C elaborated the corresponding figures and tables. SB, DG, RR and RU supervised the overall research process. MC-P, SB, RR and RU drafted the manuscript. All authors have critically reviewed and approved the manuscript.

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with an overhang to incorporate Kozak, FLAG and *ScaI* target sequences at N-terminal of *MAGEL2*. PCR primers are available in Supplementary Table 1. PCR product was cloned in a pGEM-T vector (A137A, Promega). Ligation was carried out using the Rapid DNA Dephos & Ligation Kit (Roche) following the manufacturer's instructions. Transformation of the ligation product was done in XL1-Blue (Agilent) competent cells with SOC media. Plasmids were purified (PureLink™ Quick Plasmid Miniprep Kit, Invitrogen) and Sanger sequenced. Then, this FLAG-MAGEL2 N-terminal region was incorporated into the full pcDNA3.1(+) *MAGEL2* plasmid using restriction enzymes *ScaI* (FD0434, ThermoFisher Scientific) and *HindIII* (FD0504, ThermoFisher Scientific), and the GFX PCR DNA and Gel Band Purification Kit (28903470, Cytiva).

To incorporate the six SYS patient mutations in the truncated FLAG-MAGEL2 plasmids, and a stop codon sequence in the WT plasmid, the last 1947 bps at C-terminal of *MAGEL2* were cloned in a pUC19 vector (SD1172, GenScript) using enzymes *EcoRI* (FD0274, ThermoFisher Scientific) and *XbaI* (FD0684, ThermoFisher Scientific). Mutations c.1850G>A, c.1912C>T, c.1996dupC, c.1996delC, c.2056_2066del, c.2118delT and a TAA stop codon sequence were introduced respectively by site-directed mutagenesis using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent). Mutagenesis primers can be found in Supplementary Table 2.

Cell culture: HeLa cells were cultured in DMEM (Sigma-Aldrich, Merck) supplemented with 10% FBS (Gibco, LifeTechnologies) and 1% Penicillin–Streptomycin (Gibco, LifeTechnologies). The vectors were transfected into 60% confluent HeLa cells using Lipofectamine™ 3000 (Invitrogen, ThermoFisher Scientific) and Opti-MEM (Gibco, LifeTechnologies).

Immunocytochemistry: HeLa cells were fixed in 4% Paraformaldehyde (PFA), permeabilized with 0,1 mol/L glycine and 0,1% Triton X-100 in PBS and blocked with 0,3 mol/L glycine, 0,05% Triton X-100 and 10% Normal Donkey Serum (#S30-100M, Merck Millipore) in PBS. Coverslips were incubated with anti-FLAG primary antibody (F3165, Sigma), Donkey anti-Mouse Cy2 antibody (#715-225-150, Jackson Immunoresearch), Phalloidin (#A30107, Invitrogen), and DAPI (#D1306, Invitrogen) and mounted with MOWIOL (#475904, Millipore). Images were acquired using a Zeiss confocal microscope LSM 880 and analysed with ImageJ[2].

Suppl. 2

SUPPLEMENTARY TABLES

Supplementary Table 1. Clinical overview of patients carrying mutations p.Trp617*, p.Gln638*, p.Gln666Profs*47, p.Trp686Alafs*23, p.Gln666Serfs*36, and p.Leu708Trpfs*7 in MAGEL2, respectively.

		c.1850G>A	c.1912C>T	c.1996dupC	c.2056_2066del	c.1996delC	c.2118delT
	Protein change	p.Trp617*	p.Gln638*	p.Gln666Profs*47	p.686Alafs*23	p.Gln666Serfs*36	p.Leu708Trpfs*7
	Diagnostic Cases	SYS 2	SYS 11	SYS 45	SYS 1	AMC 8	AMC 2
	Reference	[3], Not published	[4-10]	[4], [10], [11-26]	Not published	[4], [23], [27], [28]	[23], [27]
Phenotype (HPO term)							
Pregnancy complications							
Decreased fetal movement	HP:0001558	1/1	1/1	3/6	1/1	5/5	1/1
Polyhydramnios	HP:0001561			2/2	1/1	4/4	1/1
Development & Intellectual disability							
Intellectual disability	HP:0001249	2/2	2/2	23/23	1/1		
Autistic behavior	HP:0000729		2/2	13/17	0/1		
Abnormality of brain morphology	HP:0012443		2/3	6/10			
Neurodevelopmental delay	HP:0012758	2/2	5/5	26/26	1/1		
Respiratory							
Respiratory difficulties	HP:0002098	1/1	6/6	20/20			1/1
Sleep apnea	HP:0010535	1/1	3/4	16/22	1/1		
Respiratory failure requiring mechanical ventilation	HP:0004887	1/1	6/6	13/13			
Recurrent respiratory infections	HP:0002205		6/6	3/3			
Feeding and growth							
Feeding difficulties	HP:0011968	1/1	10/10	31/32	1/1	1/1	
Poor suck	HP:0002033		6/6	13/13	1/1		
Dysphagia	HP:0002015		2/2	1/1			
Nasogastric tube feeding in infancy	HP:0011470	1/1	1/1	4/4			
Gastrostomy tube feeding in infancy	HP:0011471		2/2	6/7			
Hyperphagia	HP:0002591	0/1		1/6			
Increased body weight/Obesity	HP:0004324/ HP:0001513	1/1	1/1	2/12			
Gastroesophageal Reflux	HP:0002020		2/2	9/14			
Chronic constipation	HP:0012450		1/1	11/15	1/1		
Physical characteristics							
Neonatal hypotonia	HP:0001319	2/2	8/10	33/34		1/1	1/1
Congenital contractures	HP:0002803		7/8	30/30	1/1	1/1	
Arthrogryposis multiplex congenita	HP:0002804	0/1		6/7		5/5	1/1
Abnormality of the eye	HP:0000478	1/2	2/2	17/19			
Scoliosis	HP:0002650			8/11		0/2	
Short stature	HP:0004322		3/3	9/12	1/1	0/2	
Small hands	HP:0200055	1/1	4/6	12/20			
Camptodactyly of finger	HP:0100490		3/3	10/14	1/1	3/3	1/1
Tapered fingers	HP:0001182	1/1		5/8			
Small feet	HP:0001773		2/2	5/13		0/2	
Bilateral club foot	HP:0001776		1/1			3/3	1/1
Facial dysmorphism	HP:0001999	2/2	5/6	25/29	1/1	5/5	1/1
Endocrinology							

Suppl. 3



Growth hormone deficiency	HP:0000824	0/1	2/2	7/7	1/1		
Hypothyroidism	HP:0000821	0/1	1/2	0/2			
Hypoglycemia	HP:0001943	0/1	2/2	6/6			
Temperature instability	HP:0005968	0/1	3/4	2/4			
Diabetes insipidus	HP:0000873	0/1	2/2	3/6			
Hypogonadism	HP:0000135	1/1	7/9	15/21	1/1	1/3	
Cryptorchidism	HP:0000028	1/2	1/1	3/6			
Other alterations							
Seizures	HP:0001250		3/5	8/13			
Congenital heart defect	HP:0001627		4/5	4/7			1/1
Bradycardia	HP:0001662		1/1	1/2			
Sleep disturbance	HP:0002360		2/2	1/1			
Death in infancy/childhood	HP:0001522/ HP:0003819	1	1	11	-	8	2

Supplementary Table 2: Primer sequences used in the cloning process of the FLAG-MAGEL2 plasmids.

c.1850G>A	Primer F	5' -CTGGGCCTGCTAGGCCAGCGCCT-3'
	Primer R	5' -AGGCGCTGGCCTAGCAGGCCAG-3'
c.1912C>T	Primer F	5' -GGGGGGAGCCTACCTCTGGGCCT-3'
	Primer R	5' -AGGCCAGAGGTAGGCTCCCCC-3'
c.1996dupC	Primer F	5' -CCTGGGCCTGCTGGGGGGGTAGCT-3'
	Primer R	5' -AGCTACCCCCCAGCAGGCCAGG-3'
c.1996delC	Primer F	5' -CCTGGGCCTGCTGGGGGGTAGTG-3'
	Primer R	5' -CAGCTACCCCCCAGCAGGCCAGG-3'
c.2056_2066del	Primer F	5' -CGCTCCAGCCTTCCGCCTGCAGTCTTGC-3'
	Primer R	5' -GCAAGACTGCAGCGGAAGGCTGGAGCG-3'
c.2118delT	Primer F	5' -CGGTAGCAGCGGAAATTTCCCTGGGCT-3'
	Primer R	5' -AGCCCAGGGGAAATTGCCGCTGCTACCG-3'
TAA stop codon	Primer F	5' -CCCCCTCCCGCTAACTCGAGTCTAGAG-3'
	Primer R	5' -CTCTAGACTCGAGTTAGCGGGGAGGGGG-3'
Kozak+FLAG+ScaI	Primer F	5' -AAGCTTGCCACCATGGACTACAAAGACGATG ACGACAAGAGTACTTCGCAGCTAAGTAAGAAT-3'
	Primer R	5' -CCTAGGGCAGGAGGCTGGGT-3'

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Suppl. 6

CHAPTER 2: GENERATION AND MOLECULAR PROFILING OF DISEASE-RELEVANT CELL TYPES

ARTICLE 3: A MULTI-OMIC AND FUNCTIONAL PERSPECTIVE ON NEURAL CELL DYSFUNCTION IN SCHAAF-YANG SYNDROME

Summary:

Schaaf-Yang syndrome (SYS) is a rare neurodevelopmental disorder characterised by features like developmental delay, intellectual disability, neonatal hypotonia, joint contractures, and autistic features. It is caused by truncating mutations in *MAGEL2*, a gene within the Prader-Willi critical region (chr15q11-q13). In this study, we use iPSC-derived neural cell types from SYS patients to investigate disease mechanisms through integrative transcriptomic, proteomic, and targeted functional analyses. A consistent reduction in secreted A β 1-40 was observed in SYS patient-derived cells, which reinforces A β 1-40 as a possible biomarker for SYS. This functional impairment was accompanied by widespread transcriptional alterations in iNeurons derived from SYS patients, including downregulation of synaptic programs and upregulation of extracellular matrix and developmental genes. Similarly, SYS patient-derived iAstrocytes demonstrated increased expression of extracellular matrix components alongside less capacity for extracellular remodelling. Calcium imaging showed that, although baseline neuronal network activity was comparable between SYS and control lines, GABA A receptor blockade induced higher hypersynchronous bursting in SYS networks, consistent with impaired maturation and function of inhibitory signalling. Together, these findings reveal shared molecular changes across both cell types, highlighting common pathways disrupted by *MAGEL2* truncation, and providing a foundation for future therapeutic research.

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A Multi-omic and Functional Perspective on Neural Cell Dysfunction in Schaaf-Yang Syndrome

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Abstract

Schaaf-Yang syndrome (SYS) is a rare neurodevelopmental disorder characterised by features like neurodevelopmental delay, intellectual disability, neonatal hypotonia, joint contractures, and autistic features. It is caused by truncating mutations in *MAGEL2*, a gene within the Prader-Willi critical region (chr15q11-q13). In this study, we use iPSC-derived neural cell types from SYS patients to investigate disease mechanisms through integrative transcriptomic, proteomic, and targeted functional analyses. A consistent reduction in secreted A β 1-40 was observed in SYS patient-derived cells, which reinforces A β 1-40 as a possible biomarker for SYS. This functional impairment was accompanied by widespread transcriptional alterations in iNeurons derived from SYS patients, including downregulation of synaptic programs and upregulation of extracellular matrix and developmental genes. Similarly, SYS patient-derived iAstrocytes demonstrated increased expression of extracellular matrix components alongside less capacity for extracellular remodelling. Calcium imaging showed that, although baseline neuronal network activity was comparable between SYS and control lines, GABA A receptor blockade induced higher hypersynchronous bursting in SYS networks, consistent with impaired maturation and function of inhibitory signalling. Together, these findings reveal shared molecular changes across both cell types, highlighting common pathways disrupted by *MAGEL2* truncation, and providing a foundation for future therapeutic research.

Introduction

Schaaf-Yang syndrome (SYS; MIM #615547), first described in 2013 [1], is a rare neurodevelopmental disorder caused by truncating mutations in the paternal copy of *MAGEL2* (MAGE Family Member L2; MIM *605283). This gene is located within the Prader-Willi critical region (chr15q11–q13) and is one of five maternally imprinted protein-coding genes in this locus. *MAGEL2* consists of a single exon, which prevents the activation of nonsense-mediated mRNA decay (NMD). As a result, truncating mutations produce stable proteins that are thought to contribute to disease pathogenesis [2].

Clinically, SYS is characterised by a combination of developmental and endocrine features. Common early symptoms include neonatal hypotonia, feeding difficulties, joint contractures and respiratory complications. As children grow, developmental delay, intellectual disability, and speech impairments become more evident [3]. Growth hormone and other pituitary hormone deficiencies have been reported [4,5], and other metabolic issues such as hypoglycaemia, altered glucose tolerance, and low bone mineral density are also frequently described [4,6].

During embryonic development, *MAGEL2* is expressed throughout the central nervous system (CNS), including the neural tube, diencephalon, forebrain, and developing hypothalamus, as well as in non-neural tissues such as the genital tubercle, middle intestine, placenta, tongue, and musculoskeletal structures [7–9]. As development progresses, *MAGEL2* expression becomes more restricted, with the highest levels in the adult brain, particularly within the hypothalamus, and weaker expression in the spinal cord, pituitary gland, and cerebellum (GTEx Portal; Accessed August 2025) [5,10]. This spatial and temporal expression pattern suggests an important role for *MAGEL2* in brain development and maturation.

MAGEL2 encodes a protein that belongs to the type II Melanoma Antigen Gene (MAGE)

protein family. Its N-terminal region features a proline-rich domain predicted to have functions related to mRNA metabolism [11,12]. Additionally, *MAGEL2* contains a USP7-binding site that facilitates its interaction with the deubiquitinating enzyme USP7 [13]. The MAGE homology domain (MHD) is located at the C-terminal region, encompassing residues 1,027–1,195. This conserved stretch of approximately 170 amino acids is found in both type I and type II MAGE proteins and plays a key role in mediating protein-protein interactions [14].

The best-characterised function of *MAGEL2* is its role in retromer-mediated endosomal protein trafficking. Through its MHD, *MAGEL2* recognises and binds the E3 ubiquitin ligase TRIM27 and deubiquitinating enzyme USP7 [13,15]. This multi-protein assembly forms the MUST complex. Upon recruitment to endosomes via *MAGEL2* interaction with VPS35, a critical component of the retromer, the MUST complex coordinates with both retromer and WASH regulatory complexes to control retrograde trafficking dynamics. This machinery determines if membrane protein cargoes are directed to the trans-Golgi network via retrograde transport or returned to the plasma membrane through recycling pathways, maintaining cellular protein homeostasis and membrane composition [16]. Disruption of this pathway, as seen in *Magel2*-deficient models, impairs secretory granule formation and neuropeptide release [14,17].

MAGEL2 also contributes to neuronal development by stabilizing proteins such as Fez1, which is involved in cytoskeletal remodelling during neurite outgrowth [18]. Recent studies using human excitatory induced neurons (iNs) derived from SYS patient induced pluripotent stem cells (iPSCs) have reported increased mTOR signalling and altered dendritic development, suggesting broader disruptions in neurodevelopmental signalling pathways [19]. Additionally, *MAGEL2* is a clock-controlled gene and plays a role in maintaining circadian rhythm by regulating the ubiquitination and localisation of

core clock proteins, such as CRY1, BMAL1, and CLOCK [20].

In this study, we used iPSC-derived neural models to investigate the molecular consequences of *MAGEL2* truncating mutations associated with SYS. Given the enriched expression of *MAGEL2* in hypothalamic and GABAergic neuronal populations, we focused our analysis on inhibitory iNs, performing bulk mRNA sequencing and quantitative proteomics. To complement this, we also studied induced astrocytes (iAs); glial cells essential for neurotransmitter recycling, synaptogenesis, and intercellular communication [21,22]. In addition, we evaluated functional outcomes related to disease pathology by assessing secreted β -amyloid (A β) 1-40 levels using ELISA [2] and performed calcium imaging studies to probe neuronal network activity in neuronal cultures. This is among the first studies to characterise SYS molecular phenotypes in human iPSC-derived neural models, providing mechanistic insights into disease pathogenesis and potential targets for future therapeutic investigation.

Experimental procedures

Human iPSCs culture: Seven iPSC lines were used in this study: four healthy controls and three SYS patient-derived lines (Suppl. Table 1). Lines generated in our laboratory have been banked and are available at the Banco Nacional de Líneas Celulares (BNLC). iPSCs were maintained using mTeSR™ Plus medium (Stemcell™ Technologies, 5825) with 0.5% Penicillin Streptomycin (P/S, ThermoFisher Scientific, 15070063) on Matrigel® (Corning®, 356234)-treated plates and maintained at 37°C in humidified air with 5% CO₂, with medium changed every 2-3 days. Cells were passaged with TrypLE™ Express (1X) (Gibco™, 12605010), when reaching approximately 80% confluency. To improve survival rate, 10 μ M Rock Inhibitor (Sigma-Aldrich, Y0503) was added to the medium for 24 h after plating.

Lentiviral production: Lentiviruses were produced in HEK293T cells as previously described [23]. Briefly, cells were cotransfected with the lentivectors M2-rtTA (Addgene, 20342), TetO-Ascl1-Puro (Addgene, 97329), TetO-Dlx2-Hygro (Addgene, 97330), TetO-Sox9-Puro (Addgene, 117269), and TetO-Nfib-Hygro (Addgene, 117271); and the packaging plasmids pMD2.G (Addgene, 12259), pRSV-Rev (Addgene, 12253), and pMDLg/pRRE (Addgene, 12251) using Polyethyleneimine (Polysciences, PN 23966). For two T175 flasks, 5,5 μ g of pMD2.G, 3,9 μ g of pRSV-Rev, 7,5 μ g of pMDLg/pRRE, and 23 μ g of the desired lentivector plasmids were transfected. After 48 hours, viral particles were collected and concentrated by centrifugation at 19,500 xg for 2 hours at 4 °C. The supernatant was carefully removed, and 100 μ L of Dulbecco's Modified Eagle Medium (DMEM; Gibco™, 11965092) was added directly onto the pellet without mixing. On the following day, the pellet was resuspended, divided into aliquots, and stored at -80 °C.

Generation of iNeurons and iAstrocytes from iPSCs: Both neuronal induction and astrocyte induction protocols were carried out as previously described with minor modifications [24,25]. Protocol schemes are shown in Suppl. Figure 1. When human iPSCs reached roughly 80% confluence, they were dissociated using Accutase (Gibco™, A11105), and 3 $\times$ 10⁵ cells were seeded into Matrigel-coated six-well plates in mTeSR™ Plus medium supplemented with 10 μ M ROCK inhibitor. After 24 hours, the medium was refreshed with mTeSR™ Plus, and 1.5 μ L of each viral vector was added per well (rtTA, Dlx2, and Ascl1 for iNs; rtTA, Nfib, and Sox9 for iAs). On day 0, the medium was exchanged for fresh mTeSR™ Plus containing 2.5 μ g/mL doxycycline (Sigma, D9891), which was maintained for the duration of the experiment.

For neuronal induction, from day 1, medium was changed daily using BrainPhys™ Neuronal Medium (Stemcell™ Technologies, 05790), 2% (50x) B-27™ supplement (Gibco™, 17504044), 1% (100x) N-2

supplement (Gibco™, 17502001), and 0,5% P/S. Selection of transduced cells was carried out on days 1-3 adding 1.25 µg/mL puromycin (Gibco™, A1113830) and on days 1-5 adding hygromycin (200 µg/mL) (Sigma, 10687010). On day 7, cells were dissociated with Accutase and passed through 40 µm strainers (VWR, 732-2757) to eliminate cell aggregates. Then, 5·10⁵ cells were replated in Matrigel-treated 0.4 µm Falcon® Permeable Supports (Corning®, 353090) for proteomics and transcriptomics experiments, and 1,25·10⁵ cells were replated in 24 Well µ-Plates (IBIDI, 82426) for immunocytochemistry experiments.

For astrocyte induction, cells were cultured in Expansion medium (DMEM/F-12, 10% FBS (Gibco™, 17914671), 1% (100x) N-2 supplement, 1% GlutaMAX and 0,5% P/S). Selection of transduced cells was carried out on days 1-3 adding 1.25 µg/mL puromycin (Gibco™, A1113830) and on days 1-5 adding hygromycin (200 µg/mL) (Sigma, 10687010). After day 3 Expansion medium was gradually replaced by FGF medium: Neurobasal, 2% (50x) B-27™ supplement, 1% NEAA (Gibco™, 11350912), 1% GlutaMAX, and 1% FBS, 8 ng/mL FGF2 (R&D Systems, 233-FB), 5 ng/mL CNTF (Peprotech, 450-13), and 10 ng/mL BMP4, (Peprotech, 120-05ET). On day 7, cells were dissociated with Accutase until a single cell suspension was achieved. Then, 3·10⁵ cells were replated in Matrigel-treated six-well plates for transcriptomics experiments using FGF medium alone. For immunocytochemistry, 5·10⁴ cells were replated in 24 well IBIDI plates.

Generation of forebrain neurons from iPSCs: Neural progenitor cells were generated using the embryoid body protocol of the STEMdiff™ SMADi Neural Induction Kit (StemCell™ Technologies, 08581) in 6 well plates coated with Matrigel according to the manufacturer's instructions. Sequentially, forebrain neurons were differentiated from NPCs in 24 Well ibidi µ-Plates coated with Poly-L-Ornithine (PLO, Sigma, P4957) and Laminin (Gibco™, A29249) following the instructions of the STEMdiff™ Forebrain Neuron Differentiation Kit (StemCell™

Technologies, 08600). Differentiated cells were maintained for up to 50 days with media changes after 2-3 days using BrainPhys™ Neuronal Medium and STEMdiff™ Forebrain Neuron Maturation Supplement (StemCell™ Technologies, 08606). For each cell line, three co-culture replicates were generated in each of two independent differentiations.

Immunocytochemistry: Cells were fixed in 4% Paraformaldehyde, permeabilised with 0,025% Triton X-100 in PBS and blocked with 0,025% Triton X-100 and 5% Normal Donkey Serum (Merck Millipore, #S30-100M) in PBS. Cells were incubated with primary antibodies overnight at 4°C, and with secondary antibodies and DAPI (Invitrogen, #D1306) for 2 hours at RT. Used antibodies are listed in Suppl. Table 2. Preparations were mounted with MOWIOL (Merck Millipore, #475904). Images were acquired using a Zeiss confocal microscope LSM 880 and analysed with ImageJ [26].

ELISA analysis: The supernatant of 48 h conditioned medium of iN and iA cocultures differentiation day (dd) 28 was obtained after short centrifugation. Total protein concentration was quantified using the Pierce BCA Protein Assay kit (ThermoFisher Scientific, 23225) and Aβ1-40 peptide using the Amyloid-beta (1-40) High Sensitive ELISA (IBL International GmbH, JP27718). Each sample was measured in duplicate, and replicates from two independent differentiations were included in the experiment.

mRNA-sequencing analyses: Whole RNA was extracted from iNs and iAs at dd28 using RNeasy Mini Kit (Qiagen, 74104). mRNASeq was performed at the Spanish Centre Nacional d'Anàlisi Genòmica (CNAG). RNA-Seq libraries were generated using the KAPA Stranded mRNA-Seq Kit for Illumina platforms (Roche, KR0960), according to the supplier's instructions. Sequencing was performed on an Illumina NovaSeq 6000 system with paired-end reads of 2 × 51 bp, employing the dual-indexing protocol provided by the manufacturer. RNA-Seq samples were first

processed with SeqtrimBB using the standard transcriptomic template to eliminate sequencing artifacts, adapter sequences, and low-quality bases. The preprocessed reads were then aligned to the human reference genome (GRCh38.p13) and quantified using STAR (v2.5.3). Alignment produced count tables, indicating the number of reads mapped to each gene per sample. These raw counts were normalised to Counts Per Million (CPM), correcting for sequencing depth across libraries and enabling comparison of expression levels between samples. Expression analyses were subsequently carried out using ExpHunterSuite [27]. The suite currently includes edgeR, limma, NOISeq and DESeq2 (using default parameters). Genes are considered prevalent DEGs if they are detected by at least as many packages as the user-defined threshold, while possible DEGs are those identified by at least one method but fewer than the threshold. The differential expressed genes (DEGs) are obtained using an adjusted p-value (FDR) <0.05, and a minimum $|\log_2 \text{FC}|$ of 1. For functional enrichment using Gene Ontology, the EdgeR package was employed, using a p-value < 0.05 and $|\log_2 \text{FC}|$ of 1.5.

Proteomics analyses: dd28 iN pellets were sent to the Mass Spectrometry and Proteomics Core Facility (MSPCF) of the Institute for Research in Biomedicine (IRB Barcelona). Samples were lysed and proteins extracted using the Beatbox from PreOmics. The total protein amount was quantified using Pierce™ 660 nm Protein Assay Reagent. A total of 75 µg of sample were digested with trypsin/LysC and cleaned up using the iST PreOmics Kit. The trypsinised samples were analysed by nanoLC-MS/MS using a data independent acquisition, library-free, label-free strategy. Differential abundance analysis in Patients vs Controls was performed using a label free quantification approach. MaxLFQ [28] protein group level quantification values from DIA-NN [29] were used and standard cutoffs for the fold change ($|\text{FC}| > 1.5$) and the p-value ($p < 0.05$) were applied to define significant protein groups. For integrated transcriptomic and proteomic analysis in iNeurons, we combined

datasets by plotting transcript $\log_2 \text{FCs}$ against matched protein $\log_2 \text{FCs}$, classifying each gene-protein pair as RNA-only, protein-only, or dysregulated in both assays.

qPCR analysis of selected genes: Whole RNA was extracted from iNs and iAs at dd28 using RNeasy Mini Kit. Complementary DNA (cDNA) was synthesised from the extracted RNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4374967), following the supplier's protocol. Quantitative PCR was then carried out using the LightCycler® 480 SYBR Green I Master Mix (Roche, 04887352001). *GAPDH* was used as the internal control gene, and reaction efficiencies were determined using standard curves. To verify specificity, melting curve analysis was performed after amplification. All reactions were run in triplicate. Primer sequences are available upon request.

Calcium fluorescence imaging: Lentivirus (0.5 µL/mL from a titer of $2 \cdot 10^8$ TU/mL) containing the plasmid for the expression of the fluorescent genetically encoded calcium indicator (GECI) GCaMP6s were used. The expression of this vector is driven by the Synapsin I promoter that is only expressed in mature neurons [30]. Dynamic changes in neuronal activity were monitored using GCaMP6s-based intracellular calcium imaging. Recordings were conducted on an Opera-Phenix high content system (Revvity) in the non-confocal mode. Images were acquired at 20 frames per second (fps), in 8-bit greyscale format. Cells were visualised with an $\times 10$ objective lens, providing a field of view of $1.1 \times 1.1 \text{ mm}^2$ and a spatial resolution of 1.08 µm/pixel . Spontaneous neuronal activity was recorded for 8.5 minutes approximately every 3-4 days, and cultures were tracked from around d22 for approximately 4 weeks. At the final timepoint (dd55), recordings were performed before and after the addition of Bicuculline (Sigma-Aldrich, 5.05875) 40µM to explore the role of the GABAergic component.

Calcium imaging analysis: Calcium fluorescence activity was analysed with the NETCAL 7.4.1 software

(<http://www.itsnetcal.com/>) [31,32]. For each experiment, a high-contrast fluorescence image was produced from the averaged recordings. Neuronal somas were first detected automatically and subsequently adjusted manually. Each soma was designated as a region of interest (ROI), and mean fluorescence values were extracted from these ROIs to obtain raw traces. Fluorescence signals were normalised to each neuron's baseline intensity. To reduce noise, the traces were smoothed using a moving average filter, followed by the application of a high-pass filter preserving frequencies above 0.01 Hz. Neuronal activity events were then identified using the Oasis inference algorithm [33]. The inferred spikes were displayed in raster plots. From these data, network activity was quantified as the proportion of neurons exhibiting at least two detected events during the recording. Mean activity was quantified as the number of spikes per neuron per minute. Population-level activity was extracted from the raster plots. This measure was used to assess network synchrony, calculated as the percentage of collective events involving at least 40% of neurons, relative to the total number of events during the recording. Functional connectivity was estimated from the inferred spike trains using generalised transfer entropy (GTE), following established methods [32], and implemented in MATLAB 2021a. This approach generated adjacency matrices that reflected significant information flow between neurons, which were subsequently visualised as spatial connectivity networks using Gephi 0.10.1. The resulting matrices were then applied to characterise network-level properties under different culture conditions. Topological features of these networks were further quantified with the Brain Connectivity Toolbox [34]. In this context, $\langle k \rangle$ represents the mean degree, calculated as the total number of links per neuron averaged across the population.

Statistical analyses: Statistical analyses were performed using GraphPad Prism 10. For comparisons between two independent groups, unpaired two-tailed Student's *t*-tests were applied. The assumption of normality was

assessed using the Shapiro-Wilk test, and equality of variances was evaluated using an F-test. For ELISA and qPCR experiments, values were first normalised to the mean of the control group before analysis. For calcium imaging analysis, Tukey's multiple comparisons test was used to compare control and SYS groups across different timepoints. Data were averaged from a minimum of three values per condition. Data are presented as the mean $\pm$ standard error of the mean (SEM). Statistical significance was defined as $p < 0.05$.

Ethical statements: This research was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants (four SYS patients and two healthy controls), including approval for skin fibroblast collection and subsequent reprogramming into iPSC lines. Two additional control iPSC lines were obtained from BNLC and Coriell Institute. The project received ethical approval from the Bioethics Committee of the Universitat de Barcelona (IRB00003099) and from the Health Department of the Catalan Government, authorising the use of iPSC lines.

Results

Successful generation of iNs and iAs to model SYS

To establish relevant *in vitro* models for investigating SYS pathophysiology, iPSCs from three SYS patients and four healthy controls were subjected to transcription factor (TF) forward programming for 28 days. This protocol enabled the efficient and reproducible generation of two key central nervous system (CNS) cell types: inhibitory iNs and iAs. Successful neuronal differentiation was confirmed by immunocytochemistry. As shown in Figure 1, all iN populations exhibited robust expression of MAP2, confirming neuronal lineage. Additionally, the presence of GAD67 indicated that these iNs predominantly adopted a GABAergic identity, consistent with the specific forward programming strategy employed [24]. Astrocytic differentiation was assessed by the expression of Vimentin (VIM).

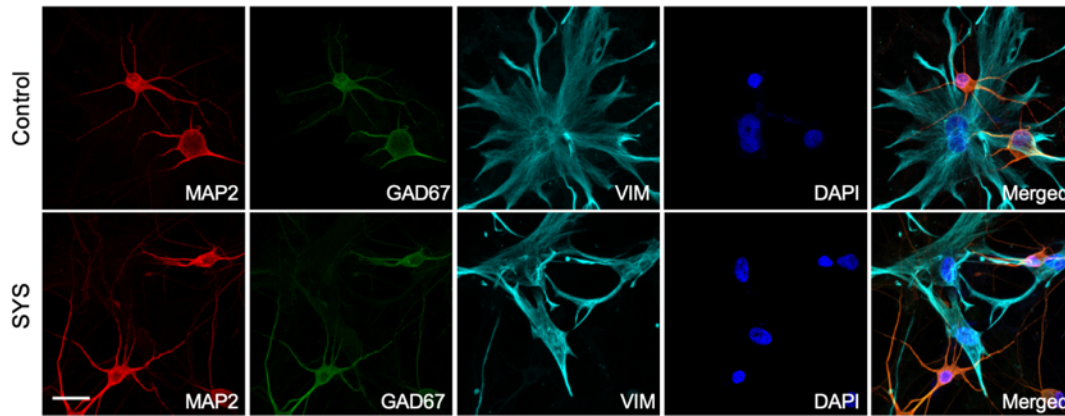


Figure 1: Representative images of cocultures of iNeurons (iNs) and iAstrocytes (iAs) differentiated from control and SYS patient-derived iPSCs. iNs stained positive for the neuronal marker MAP2 (red) and the GABAergic marker GAD67 (green). iAs stained positive for VIM (cyan). Nuclei were counterstained with DAPI (blue). Scale bar, 25 μ m.

To further validate cell type identity, we examined gene expression profiles from bulk RNA sequencing data. Transcriptomic data confirmed the expression of additional markers in the respective populations, including *TUBB3* and *NEUROD1* in iNs, and *GFAP*, *S100B*, and *ALDH1L1* in iAs (data not shown), reinforcing the neuronal and astrocytic nature of the differentiated cells. Importantly, marker expression and cellular morphology were comparable between control and SYS lines for both iNs and iAs, supporting the consistency of the differentiation protocol independently of the genotype.

SYS patient-derived neural cultures show decreased β -amyloid 1-40 peptide secretion levels

Building on our previous findings [2], which reported reduced secretion of A β 1-40 in SYS patient-derived fibroblasts, we investigated whether a similar alteration occurs in human neural cells. Given that *MAGEL2* participates in retromer-mediated trafficking [15], we hypothesised that truncating *MAGEL2* mutations could impair APP processing and A β peptide release. To test this, we measured extracellular levels of A β 1-40 in conditioned medium from co-cultures of iNs and iAs derived from four SYS and four control iPSCs (Suppl. Table 1). ELISA quantification showed a significant reduction in A β 1-40 secretion in SYS patient-derived cultures compared to controls (Figure 2A). This alteration was

observed in the absence of significant changes in expression levels of *APP* or its primary processing enzymes, including the β -secretase *BACE1* and γ -secretase complex component *PSEN1* (Figure 2B). In contrast, we detected a marked increase in *LRP1* mRNA expression in iNs ($\text{ILog}_2\text{FCI} > 1$, $p < 0.05$), a receptor known to mediate endocytic clearance of soluble A β , suggesting an additional mechanism contributing to the reduced extracellular peptide levels (Figure 2B).

SYS iNeurons exhibit suppressed synaptic programs and upregulation of ECM-related transcripts

To investigate the impact of *MAGEL2* truncating mutations on gene expression, we performed bulk mRNA sequencing on inhibitory iNs differentiated for 28 days from iPSCs of three SYS patients and four unaffected controls. Our analysis identified 14 differentially expressed genes (DEGs) (defined by $\text{ILog}_2\text{FCI} > 0.5$ and $\text{FDR} < 0.05$), with 6 genes significantly upregulated and 8 significantly downregulated in SYS iNs relative to controls (Table 1).

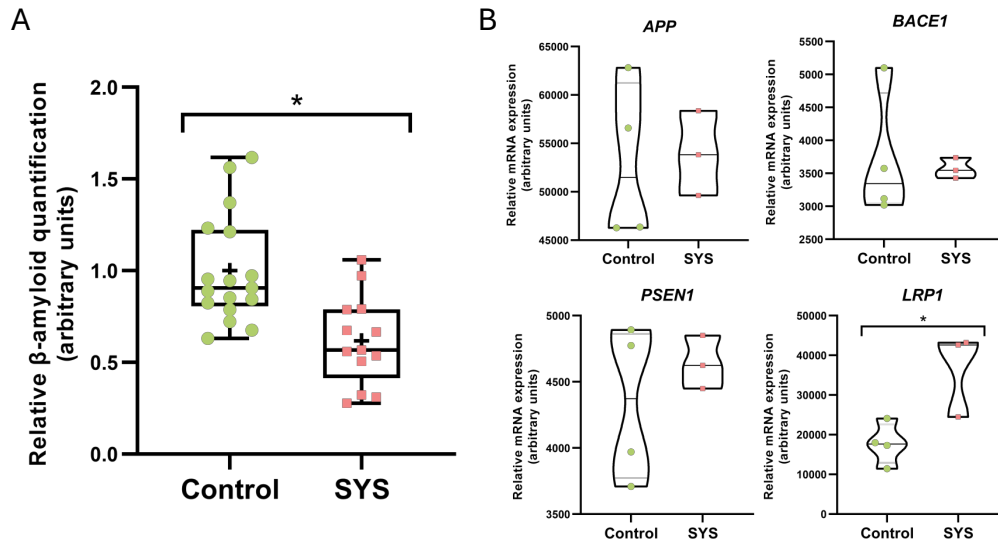


Figure 2: Altered A β 1-40 secretion in SYS-derived neural cultures. A) Quantification of secreted β -amyloid 1-40 in neural cultures derived from SYS patients and controls. Values were normalised to the mean of the control group. B) mRNA expression of *APP*, its primary processing β and γ -secretase complex component enzymes, and *LRP1* in iNeurons. Plots show median, interquartile range, and individual data points. Statistical analyses were performed using t test in GraphPad Prism. * $p < 0.05$.

Table 1: Differentially expressed genes identified after mRNASeq analysis of inhibitory iNeurons derived from patients with Schaaf-Yang syndrome and controls (defined by $|\text{Log}_2\text{FC}| > 0.5$ and $\text{FDR} < 0.05$).

	Gene	ID	FDR	Log2 FC
Upregulated	<i>SHISA3</i>	ENSG00000178343	0.02220	2.068
	<i>GATA4</i>	ENSG00000136574	0.01660	1.654
	<i>MGP</i>	ENSG00000111341	0.02254	1.295
	<i>CYP27C1</i>	ENSG00000186684	0.00258	1.283
	<i>FLRT2</i>	ENSG00000185070	0.00001	1.091
	<i>COL12A1</i>	ENSG00000111799	0.00130	0.982
Downregulated	<i>SIK1</i>	ENSG00000142178	0.00901	-7.207
	<i>ESRG</i>	ENSG00000265992	0.04917	-3.901
	<i>HLA-DRB1</i>	ENSG00000196126	0.00035	-1.964
	<i>MST1L</i>	ENSG00000186715	0.00001	-1.937
	<i>PI4KAP1</i>	ENSG00000274602	0.00000	-1.783
	<i>NNAT</i>	ENSG00000053438	0.00178	-1.752
	<i>KCNH8</i>	ENSG00000183960	0.00185	-1.223
	<i>CRNDE</i>	ENSG00000245694	0.00649	-0.594

To gain functional insights into these observed transcriptional changes in inhibitory iNs, we expanded the set of genes for Gene Ontology (GO) enrichment analysis using a more permissive threshold ($|\text{Log}_2\text{FC}| > 1.5$, unadjusted $p < 0.05$), consistent with common practices for exploratory enrichment analysis when gene numbers are low [35]. Based on these criteria, we identified 176 differentially expressed genes, with 47 upregulated and 129 downregulated transcripts (Figure 3A, Suppl. Table 3). Analysis of upregulated transcripts revealed significant enrichment for gene ontology (GO) terms associated with tissue morphogenesis and structural remodelling, including "ECM organisation", "proximal/distal pattern formation", "striated muscle development", and "collagen fibril organisation" (Figure 3D). Notably, closer inspection of genes ranked by nominal significant p-value revealed upregulation of several *HOX* cluster genes (including *HOXD10*, *HOXB13*, *HOXA10*, *HOXA7*, *HOXA11*, *HOXA9*, *HOXC9*, *HOXA13*, and *HOXB9*), although these did not meet previous FDR significance. By contrast, downregulated genes were significantly enriched in GO categories central to neuronal identity and synaptic function. Top enriched GO categories

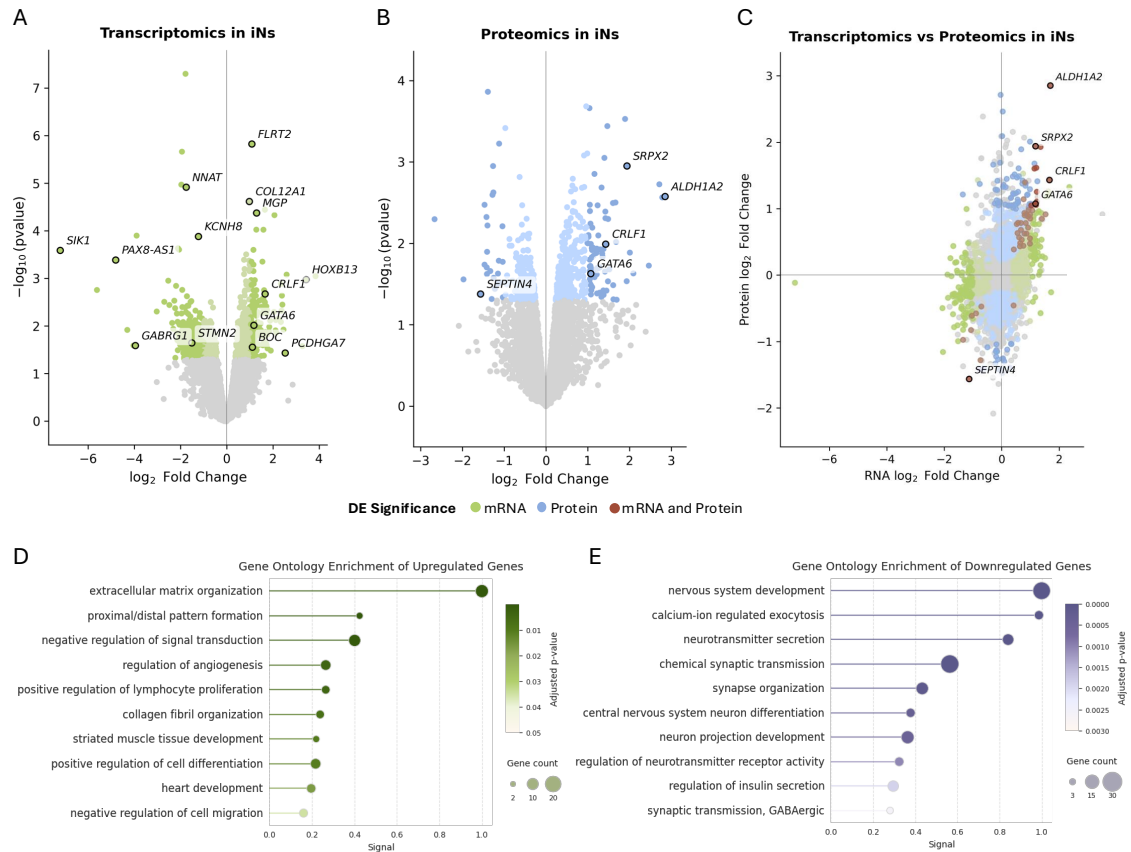


Figure 3: Multi-omic analyses of SYS inhibitory iNeurons (iNs). A) Volcano plot showing differential gene expression in iNs transcriptomic analysis, with significantly upregulated and downregulated genes highlighted in green. B) Volcano plot displaying differential protein expression in iNs proteomic analysis, with significantly altered proteins shown in blue. C) Correlation plot comparing mRNA and protein fold changes of iNs. Colours indicate significance in RNA (green), protein (blue), or both (brown). (D) Gene Ontology enrichment analysis of upregulated genes in SYS iNs mRNA sequencing. (E) Gene Ontology enrichment analysis of downregulated genes in SYS iNs mRNA sequencing. Circle size represents gene count and colour intensity indicates adjusted p-value significance.

included "nervous system development," "neurotransmitter secretion," and "chemical synaptic transmission" (Figure 3E). Together, these results point to a broad functional shift in SYS-derived inhibitory iNs, characterised by the repression of neuronal programs and a gain of expression in genes associated with embryonic patterning, ECM remodelling, and developmental organisation.

Proteomic profiling of SYS iNeurons reveals subtle protein expression changes

Quantitative mass spectrometry-based proteomics was also performed on samples matching the ones used for transcriptomic analysis, enabling integration of gene and protein expression data across layers.

Proteomic analysis identified a total of 9,083 proteins present in all samples. Following quality control and filtering, a final set of 8,247 proteins was retained for differential analysis. While presence of MAGEL2 was confirmed in both SYS and control samples, it was identified by a single unique peptide, so it was excluded from further analyses. Differential expression analysis using adjusted p-values (FDR<0.05) did not yield any significantly dysregulated proteins (DEPs). To increase sensitivity, we focused subsequent analyses on proteins reaching nominal significance ($p < 0.05$). Based on this criteria, differential expression analysis revealed 235 proteins significantly dysregulated, with 159 upregulated and 76 downregulated in SYS iNs relative to controls (Figure 3B, Suppl. Table 4).

GO enrichment analysis of this dataset ($|\text{Log}_2\text{FC}| > 1$ and $p < 0.05$) revealed dysregulation of different processes (Suppl. Table 5). The most enriched term was "regulation of phosphatidylinositol 3-kinase (PI3K) activity". Other enriched categories included "regulation of collagen fibril organisation", "muscle tissue morphogenesis", and "positive regulation of cell motility", suggesting that truncation of MAGEL2 may broadly affect pathways linked to cell signalling, migration and ECM dynamics. There was little overlap among the top DEGs identified in transcriptomic and proteomic analyses. However, the most enriched pathways in proteomics data supported the transcriptomic results, as detailed in the following integrative analysis.

Integrated transcriptomic and proteomic analysis in iNeurons

Integrative analysis of the transcriptomic and proteomic datasets identified 12 common genes that exhibited nominally significant changes at both the transcriptomic and proteomic levels ($|\text{Log}_2\text{FC}| > 1$ and $p < 0.05$) (Figure 3C, Table 2). Strikingly, 11 of these genes were upregulated, with only one consistently downregulated target. This asymmetry likely reflects a technical limitation of mass spectrometry-based proteomics, which tends to under-detect low-abundance or rapidly degraded proteins.

Table 2: Commonly dysregulated genes in mRNA Sequencing and proteomic studies of SYS patient-derived iNeurons ($|\text{Log}_2\text{FC}| > 1$ and $p < 0.05$).

Gene	ID	mRNA		Protein	
		p value	Log ₂ FC	p value	Log ₂ FC
ALDH1A2	ENSG00000128918	0.0228	1.6949	0.0026	2.8520
CRLF1	ENSG00000006016	0.0021	1.6641	0.0102	1.4304
REN	ENSG00000143839	0.0248	1.3472	0.0292	1.9323
RFLNB	ENSG00000183688	0.0167	1.2212	0.0345	1.1068
BST2	ENSG00000130303	0.0096	1.2156	0.0202	1.2541
QNG1	ENSG00000165118	0.0136	1.2011	0.0488	1.6148
IL33	ENSG00000137033	0.0176	1.1894	0.0217	1.0554
SRPX2	ENSG00000102359	0.0257	1.1789	0.0011	1.9391
GATA6	ENSG00000141448	0.0097	1.1777	0.0236	1.0737
GFPT2	ENSG00000131459	0.0272	1.1332	0.0032	1.6042
NFATC1	ENSG00000131196	0.0182	1.0276	0.0033	1.0505
SEPTIN4	ENSG00000108387	0.0154	-1.132	0.0420	-1.5645

Transcriptomic profiling of SYS iAstrocytes and shared molecular signatures across neural lineages

Bulk mRNA sequencing on iAs at dd28 revealed 32 genes with significant dysregulation, with 19 upregulated and 13 downregulated relative to controls ($|\text{Log}_2\text{FC}| > 0.5$ and $\text{FDR} < 0.05$) (Suppl. Table 6) providing insights into astrocytic dysfunction in SYS. The 10 most significantly upregulated and downregulated genes, ranked by fold change, are presented in Table 3.

Table 3: Top 10 upregulated and downregulated differentially expressed genes identified after mRNASeq analysis of iAstrocytes of patients with Schaaf-Yang syndrome and controls.

	Gene	ID	FDR	Log2 FC
Upregulated	BCAN	ENSG00000132692	0.0000081	3.8099
	ADCYAP1R1	ENSG00000078549	0.0341335	3.3656
	PAX3	ENSG00000135903	0.0001951	2.9529
	CTSF	ENSG00000174080	0.0020318	2.5459
	CLDN10	ENSG00000134873	0.0089490	1.6255
	LHX9	ENSG00000143355	0.0000233	1.6042
	SLC6A14	ENSG00000268104	0.0000194	1.5873
	GEM	ENSG00000164949	0.0159133	1.5822
	NPY1R	ENSG00000164128	0.0472595	1.4950
	FMO1	ENSG00000010932	0.0000081	1.4248
Downregulated	SIK1	ENSG00000142178	0.0068098	-8.6916
	TFAP2B	ENSG00000008196	0.0163130	-6.5483
	PKP3	ENSG00000184363	0.0073237	-5.3676
	PAX8-AS1	ENSG00000189223	0.0019225	-5.1268
	PRR15	ENSG00000176532	0.0424940	-5.1194
	IGF2	ENSG00000167244	0.0231839	-4.7889
	XKR7	ENSG00000260903	0.0473962	-4.1817
	CXCL14	ENSG00000145824	0.0308858	-3.2774
	BCAR1	ENSG00000050820	0.0376793	-1.0137
	CAB39L	ENSG00000102547	0.0385603	-1.0022

To determine whether these astrocytic changes reflect broader, lineage-independent consequences of *MAGEL2* dysfunction, we compared gene expression profiles across iNs and iAs. Given that SYS is caused by truncating mutations in *MAGEL2*, we first determined whether *MAGEL2* mRNA levels were altered in patient-derived iNs and iAs. Transcripts were detected in both cell types, with higher expression in inhibitory iNs (average counts: 746 in iNs vs. 423 in iAs). No significant differences between SYS and control lines were observed in either cell type, indicating that the alleles encoding truncated *MAGEL2* remain transcriptionally active in SYS-derived neural cultures.

We then compared differentially expressed genes in SYS iNs and iAs, applying a threshold of $|\text{Log}_2\text{FC}| > 1$ and $p < 0.05$, using nominal p-values rather than adjusted FDR to increase sensitivity. This analysis identified 718 genes significantly dysregulated in SYS iAs (Suppl. Figure 2A, Suppl. Table 7), while the

corresponding iN dataset included 521 genes (Figure 3A). Comparison of both datasets revealed 48 genes commonly dysregulated in SYS iNs and iAs (Suppl. Figure 2B&C, Suppl. Table 8), suggesting shared downstream consequences of *MAGEL2* truncation across neural lineages. Four genes, *NNAT*, *MGP*, *AHNAK*, and *SIK1*, were further validated by qPCR in both cell types (Suppl. Figure 3).

Monitoring of network activity reveals distinct responses after disinhibition in SYS patient-derived neuronal cultures

For calcium imaging experiments, we generated mature neural cultures from human iPSC-derived NPCs. Cells were transduced with lentiviruses carrying the GECI GCaMP6s driven by the human Synapsin I promoter. Transduction efficiency and GCaMP6s expression were comparable across lines, with approximately 80% of MAP2-positive neurons expressing GCaMP6s (Figure 4). At the final timepoint (dd55), ICC analysis showed that around 70% of cells were positive for the neuronal marker MAP2, while roughly 30% were astrocytes expressing GFAP (Suppl. Figure 4). One control line (Ctrl135) showed weaker astrocytic representation, which seemed to coincide with poorer network maturation phenotypes described below.

From dd25 to dd55, both SYS and control groups showed a progressive increase in the number of active neurons, indicating that functional excitatory networks were established (Suppl. Figure 5A). At the late stage (from dd45 to dd55), no differences were observed between SYS and control lines in either the number of connections per neuron ($\langle k \rangle$) or the number of connected neurons (Suppl. Figure 5B&C). However, controls displayed pronounced inter-line variability in temporal coordination. Some control cultures developed robust synchronous bursting by late time points, whereas others remained dominated by sparse, weakly correlated events as shown by respective raster plots at dd55 (Suppl. Figure 5C). Among controls, line

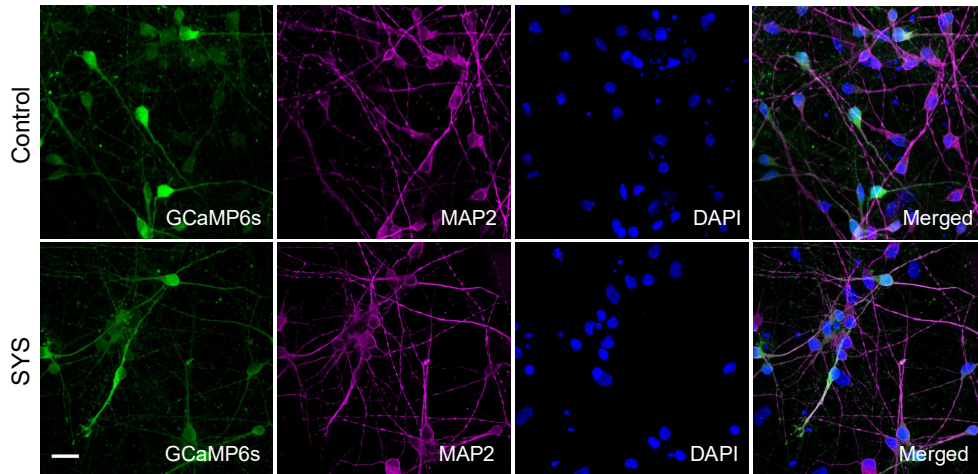


Figure 4: Representative images of immunofluorescence staining of neurons differentiated from SYS patients and controls. At dd55 cells show GCaMP6s (green), and MAP2 (magenta), confirming expression of the genetically encoded calcium indicator and neuronal identity. Nuclei are counterstained with DAPI (blue). Scale bar represents 25 μm .

GM28907 most closely matched the expected maturation profile [32], steadily rising event rates with the emergence of periodic synchronous network events by ~dd45-dd55. For this reason, it was selected as the primary reference line for comparisons with SYS samples. To assess inhibitory tone in SYS patient-derived neuronal networks, we applied the GABA A receptor antagonist bicuculline during calcium imaging, and visualised network responses using raster plots (Figure 5A). Bicuculline drove all lines into a hypersynchronous state, indicating successful network disinhibition. At baseline, average population activity did not differ significantly between control and SYS lines (Figure 5B). Upon bicuculline addition, both groups showed a marked increase in the proportion of active neurons. However, analysis of mean firing rates (spikes/min) revealed divergent responses. In the control line, bicuculline produced an increase in per-neuron mean activity (Figure 5B). In contrast, among SYS lines, 63.1 manifested high synchrony with higher mean activity, whereas 66.1 and 68.3 reorganised into bursts that yielded lower mean spikes/min per neuron despite greater event participation. This pattern suggests that while bicuculline unmasks latent excitability in SYS networks, individual neurons in some patients may be unable to sustain enhanced activity.

Discussion

This study used iPSC-derived inhibitory iNs and iAs from SYS patients to examine the molecular effects of *MAGEL2* truncating mutations. By combining multi-omics profiling with functional assays, we aimed to uncover disease-associated alterations in neuronal and glial biology, identify candidate biomarkers, and provide mechanistic insights into truncated *MAGEL2* dysfunction.

We observe a reduction of secreted A β 1-40 in SYS patient-derived iN and iA co-cultures. In concordance with our previous results in fibroblasts [2], this indicates a potentially systemic phenotype affecting A β metabolism across cell types and unveils extracellular A β 1-40 as a candidate biomarker for SYS. The reduction in A β 1-40 occurred despite similar mRNA levels of *APP*. This rules out transcriptional downregulation as the mechanism for reduced A β 1-40 levels, suggesting that the effect arises from post-transcriptional regulation or alterations in protein trafficking.

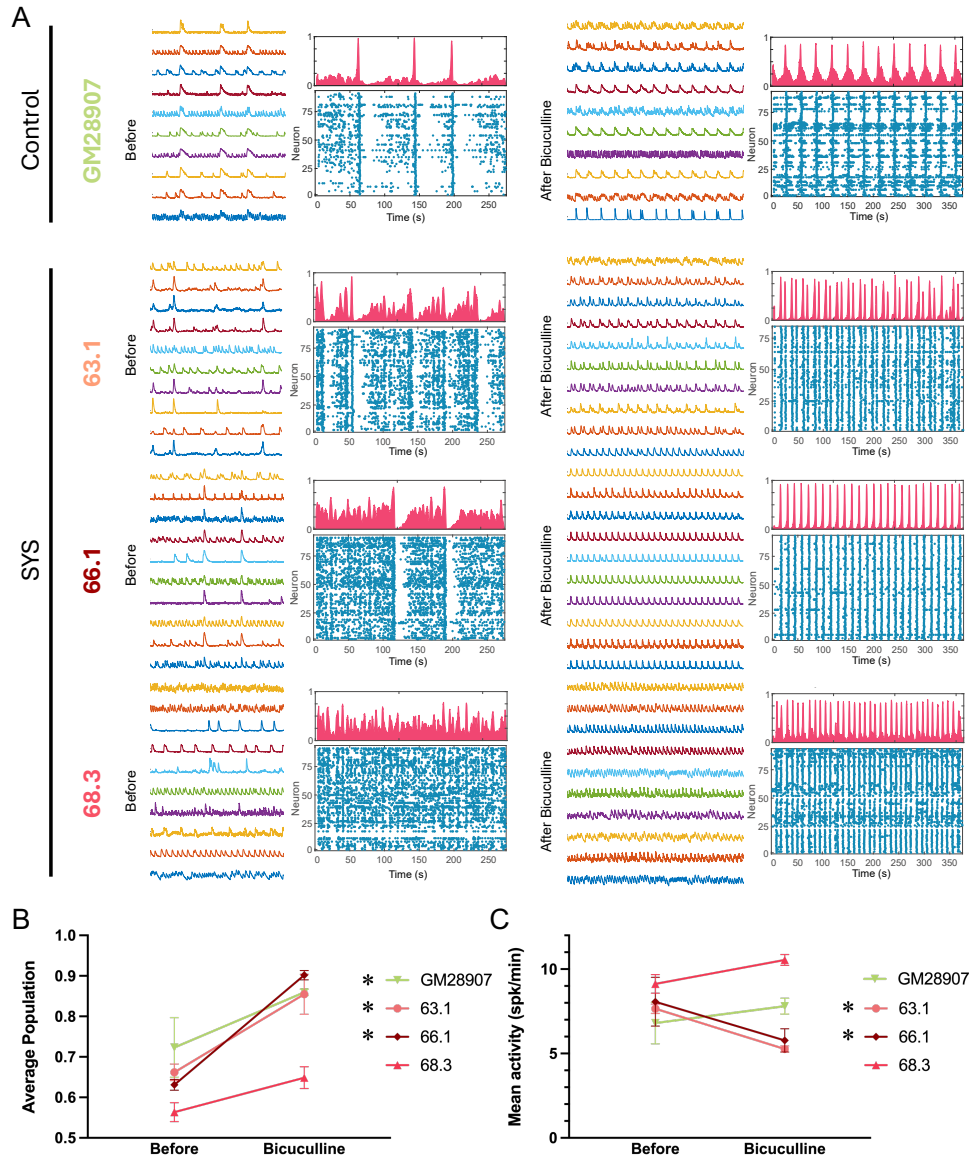


Figure 5: Network activity before and after bicuculline treatment. A) Representative calcium traces (left) and raster plots (right) of neuronal activity in control (GM28907) and SYS lines (63.1, 66.1, 68.3) before and after bicuculline treatment. B) Quantification of average population activity, showing significant increases after bicuculline in all lines. C) Mean activity rate (spikes/min) before and after bicuculline. Error bars represent SEM. Asterisks indicate significance. Statistical analyses were performed using Tukey's multiple comparison test in GraphPad Prism. * $p < 0.05$.

A compelling candidate for disruptions in APP trafficking is the retromer complex, a key component of endosomal protein sorting (Figure 6A). Composed of VPS35, VPS26 and VPS29, the retromer functions at the endosomes to retrieve APP and return it to the trans-Golgi network or the plasma membrane [16]. Studies in cellular and animal models show that retromer dysfunction disrupts normal APP trafficking, often altering A β peptide secretion [36]. Interestingly, retromer

deficiency does not always change total APP levels but can reduce A β 40 while increasing the A β 42:A β 40 ratio [37,38]. These findings suggest that the reduced production of A β 40 peptides in SYS patient-derived cells may result from impaired retromer-mediated retrieval.

Such retromer dysfunction could be explained by the contribution of MAGEL2 to the endosomal recycling pathway. The retromer is

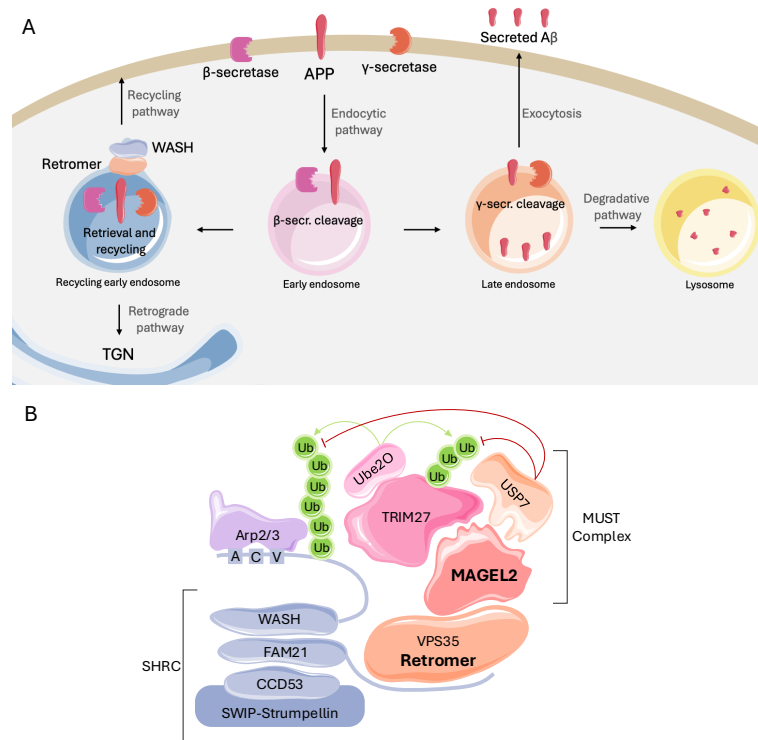


Figure 6: APP trafficking, processing, and the role of MAGEL2 in the MUST complex. A) Diagram of APP processing and trafficking. APP undergoes endocytosis and is cleaved by β -secretase in early endosomes. Some APP, BACE1, and γ -secretases are retrieved by the retromer-WASH complex from the recycling endosome and trafficked to the trans-Golgi network (TGN) or plasma membrane. Non-retrieved cargo proceeds to late endosomes for γ -secretase cleavage and $A\beta$ generation, followed by degradation or secretion. B) Schematic representation of the MUST complex, highlighting the role of MAGEL2 in association with TRIM27, USP7, and the retromer core component VPS35.

a key regulator of endosomal sorting, shuttling APP away from amyloidogenic compartments and back to the TGN or plasma membrane [38]. It is composed of subunits VPS26, VPS29, and VPS35; the latter being where MAGEL2 acts as a scaffold by linking it with TRIM27 and USP7 (Figure 6B) [15]. Most truncating mutations in *MAGEL2* eliminate the MHD and U7BS domains at the C-terminus, which are essential for functional cooperation with TRIM27 and USP7. This disruption could impair the recruitment of MAGEL2 to endosomes or alter ubiquitin editing of retromer cargo, ultimately affecting APP recycling. Beyond APP localisation, the subcellular distribution of BACE1 and γ -secretase components is also tightly regulated by endosomal trafficking. Although BACE1 was not detected in our proteomic analysis, likely due to low abundance or membrane association, studies have shown that its redistribution within endosomal compartments affects β -cleavage of APP [39]. Therefore,

MAGEL2 truncation could affect APP processing by disrupting not only APP routing but also secretase localisation.

In addition to processing alterations, clearance deficits could also contribute to lowered extracellular $A\beta$ 1-40. *LRP1* transcripts were upregulated (at the nominal level) in SYS patient-derived iNs with a similar trend in iAs. Consistently, proteomic analysis of iNs indicated a modest increase in LRP1, although it did not reach statistical significance. LRP1 is one of the main endocytic receptors for soluble $A\beta$, and its knockout impairs $A\beta$ clearance [40,41]. Observations in AD models show that increased LRP1 activity can facilitate extracellular $A\beta$ clearance, leading to reduced detectable levels in the interstitial space even when production is unchanged [42]. However, the fact that only nominally significant changes in *LRP1* expression accompany a clear reduction in extracellular $A\beta$ 1-40 suggests that other factors must be contributing.

In SYS patient-derived iNs, transcriptomic profiling revealed a suppression of canonical neuronal programs alongside an upregulation of genes associated with development, tissue patterning, and ECM organisation. Genes essential for synaptic function and neurotransmission such as *NNAT*, *KCNH8*, and *STMN2* were downregulated, suggesting a disruption of key molecular signatures linked to neuronal development, vesicle trafficking or synapse formation [43–45]. These coordinated gene expression changes suggest that truncated *MAGEL2* may interfere with late-stage neuronal differentiation and the establishment or maintenance of functional synapses. Complementing these findings, a subset of genes involved in ECM composition and cell adhesion, including *PCDHGA7*, *MGP*, *FLRT2*, and *COL12A1*, were markedly upregulated. These transcriptional changes may reflect compensatory remodelling or the adoption of alternative cellular states that impair network assembly [46–48]. The functional importance of the ECM is demonstrated by numerous genetic disorders resulting from mutations in ECM components [49]. Interestingly, we also observed upregulation of *HOX* genes governing developmental patterning and regional identity, as well as morphogen modulators such as *SHISA3*, *BOC*, and *GATA4*, regulating Wnt, FGF, and Sonic hedgehog signalling [50–53]. This shift suggests that truncated *MAGEL2* may reactivate developmental programs normally silent in mature inhibitory neurons, potentially disturbing neuronal specification and positional identity. GO analysis supported this dichotomy: SYS patient-derived iNs showed enrichment of downregulated genes in “nervous system development” and “synaptic transmission” terms, whereas upregulated genes clustered in “ECM organisation” and morphogenetic processes. Overall, these transcriptomic patterns suggest that truncating *MAGEL2* mutations tilt inhibitory neurons away from a mature synaptic phenotype and toward a more embryonic, ECM-driven state.

At the proteomic level, while no DEPs reached FDR-corrected thresholds, exploratory GO enrichment from nominal hits identified shared pathways, including dysregulation of PI3K signalling, cytoskeletal regulation, and collagen-related processes. This partial overlap in pathways critical for neurogenesis and synapse stabilisation strengthens the hypothesis that *MAGEL2* truncation affects developmental and structural programs within the neuron. In fact, several nominal hits stand out for their relevance to neuronal development and function. *ALDH1A2*, which regulates retinoic acid synthesis, critical for brain development, showed strong upregulation at both the transcript and protein level [54]. *SRPX2*, which protects synapses from complement-mediated elimination [55], and *CRLF1*, associated with neuronal survival and neuroprotection [56], both also showed increased expression. Notably, *SEPTIN4* was the only gene that was consistently downregulated, suggesting possible impacts on cytoskeletal organisation and neuronal migration [57]. The convergence of several dysregulated targets across transcript and protein levels strengthens their relevance to SYS pathophysiology and highlights potential candidates for future functional investigation.

The potential neuronal misprogramming aligns with the dual functional roles of *MAGEL2* in both cytoskeletal remodelling and endosomal protein trafficking. On one hand, *MAGEL2* contributes to neurite outgrowth by stabilizing cytoskeletal regulators involved in actin and microtubule dynamics [18]. These processes are essential for shaping neuronal morphology and axon guidance. On the other hand, *MAGEL2* also participates in retromer-dependent endosomal trafficking [13], which is critical for the recycling of membrane proteins, including receptor subunits, ion channels, and synaptic vesicle components. Truncating mutations in *MAGEL2* may therefore impair both structural and functional aspects of neuronal maturation, by disrupting cytoskeletal remodelling and hindering the surface delivery of proteins essential for synaptic connectivity.

Although *MAGEL2* expression is lower in iAs than in inhibitory iNs, SYS iAs also exhibited a transcriptional signature, suggesting cell type-specific dysregulation. Among the upregulated genes were key extracellular matrix proteoglycans linked to synaptic stability and plasticity [58,59]. Astrocyte-derived ECM is known to modulate neuronal synaptogenesis and plasticity and has been linked to epilepsy or autism [59–61]. Therefore, excessive or aberrant ECM as seen in upregulation of genes like *NCAN* and *BCAN*, could affect synapse formation and contribute to the synaptic deficits indicated by the neuronal transcriptome. Conversely, several genes involved in transcriptional regulation and development were significantly downregulated. These included *TFAP2B*, a TF essential for neuronal differentiation and neural crest development, and *IGF2*, a growth factor with key roles in neurogenesis and neuroprotection [62,63]. The observed dysregulation may contribute to altered astrocyte-neuron communication and extracellular environment remodelling in SYS-derived neural circuits. To date, research on astrocytes in SYS is limited to a single study conducted in a mouse model [64]; which reported reduced GFAP expression in the hypothalamus, whereas in our human iPSC-derived models we did not observe a reduction in GFAP expression.

We next examined genes whose expression was altered in both SYS patient-derived iNs and iAs to identify shared molecular pathways affected by *MAGEL2* truncation. The parallel downregulation of *SIK1* in both iNs and iAs may point to a disruption of circadian regulation, consistent with sleep disturbances frequently reported in SYS patients. *SIK1* is a key kinase in the *CRTC1*-*CREB* signalling axis that resets the molecular clock in response to environmental cues [65]. Notably, *MAGEL2* itself is a clock-controlled gene, with evidence showing its protein product regulates the stability and localisation of core circadian machinery [20,66]. The combination of *MAGEL2* truncation and decreased *SIK1* expression indicates that SYS may involve a

complex, multi-level disruption of circadian signalling across the CNS.

One consistent finding across both SYS patient-derived iNs and iAs in our model was the downregulation of *OXTR*, a gene encoding the oxytocin receptor. This aligns with observations in *Magel2*-KO mouse models, which also show reductions in *Oxtr* expression across brain regions [64,67]. Both SYS and Prader-Willi syndrome (MIM #176270) are associated with reduced oxytocin production and signalling [68,69], making oxytocin pathway deficiency a shared hallmark of *MAGEL2*-related disorders. Given the central role of oxytocin signalling in social behaviour, stress regulation, and hypothalamic function, *OXTR* downregulation in SYS may contribute directly to the neurodevelopmental and behavioural symptoms observed in SYS patients.

To our knowledge, only one published study has used patient-derived neuronal cell lines carrying *MAGEL2* truncating mutations to investigate cellular dysfunction in SYS [19]. In this work, Crutcher *et al.* differentiated iPSCs from SYS patients into excitatory neurons and reported increased *mTOR* expression. However, in our own datasets, we did not detect significant dysregulation of *mTOR* at the transcriptomic or proteomic levels, indicating possible model- or context-specific differences.

Our longitudinal calcium imaging experiments revealed that neuronal cultures derived from SYS patients followed a broadly similar trajectory of maturation. However, we observed variability among control lines, particularly in terms of network synchrony. Differences in astrocyte content, donor-specific genetic background, residual epigenetic memory from reprogramming, and subtle variation in differentiation efficiency may all also affect the pace and extent of neuronal network maturation [21,70,71]. No significant differences were detected in either population activity or mean firing rates in SYS cells relative to controls; and our proteomic data, although obtained from iNs, showed no

reduction in synaptic markers such as PSD95 or Synapsin-1. This confirms that SYS patient neurons are able to form active networks under standard differentiation protocols. However, our transcriptomic analysis revealed that canonical neuronal programs, including key synaptic genes, were downregulated in SYS cells. This suggests that while baseline network activity appears normal, underlying transcriptional deficits may compromise network stability and fine-tuning, particularly under stress conditions.

These underlying differences between SYS and control cultures became apparent after bicuculline treatment. The rhythmic bursting in SYS neuronal networks after GABA A receptor inhibition was visually more hyperactive and synchronous compared to the control. The exaggerated bicuculline response in SYS neuronal networks may reflect a specific deficit in the maturation and effectiveness of GABAergic signalling. Studies in *Magel2*-KO hippocampal neurons have shown that the developmental switch of GABA from depolarizing to inhibitory is delayed, leaving networks in a state of reduced inhibitory control during critical stages of maturation [72]. Our transcriptomic data showing nominally significantly reduced expression of *GABRG1*, *GABRG2*, and *GABRB2*, key γ and β -subunits of the GABA A receptor complex, suggests compromised receptor assembly and function, further weakening inhibition. Normally, different interneuron subtypes act cooperatively to fine-tune E-I balance and stabilise network activity [73]. In SYS networks, the combination of delayed GABAergic maturation and reduced receptor function may impair this cooperative action. To maintain apparent baseline activity, SYS networks might rely disproportionately on the available inhibitory tone, but when this fragile inhibition is blocked, the compensatory system may be collapsing, unmasking latent hyperexcitability.

Under resting conditions, network stability may be maintained by compensatory mechanisms, including homeostatic scaling of excitatory inputs or reliance on tonic inhibition [74].

Research shows that neurons sense changes in their own firing rates via intracellular calcium dynamics and then adjust postsynaptic receptor trafficking accordingly [75]. Consistent with this model, our transcriptomic data revealed a nominal downregulation of AMPA and NMDA receptor subunits (*GRIA1*, *GRIA2*, *GRIN1*) which could reflect an adaptive response aimed at dampening excitatory drive to counterbalance weakened GABAergic inhibition. Nevertheless, once bicuculline removes the inhibitory tone, the system may become unstable, leading to either uncontrolled synchrony or irregular bursting. Together, these observations may highlight disinhibition as a disease mechanism in SYS and call for replication in additional patient lines, with detailed analysis of inhibitory synapse density and function.

This study provides the first integrative multi-omic and functional characterisation of SYS patient-derived neural cells, revealing consistent disruptions in genes and pathways central to neurodevelopment, synaptic function, ECM remodelling, and neuronal-glia communication. These findings not only deepen our understanding of SYS pathogenesis but also highlight molecular targets that may inform future therapeutic strategies. While our multi-omic study focused primarily on GABAergic neurons, the convergence of transcriptomic and proteomic dysregulation suggests robust and disease-relevant signatures. At the functional level, calcium imaging uncovered a disinhibition phenotype across SYS cultures, consistent with impaired GABAergic signalling. However, these conclusions are based on a limited number of SYS and control lines, and patient-specific variability remains a confounding factor. Future work should incorporate additional patient diversity, different neuronal subtypes, single-cell resolution, and *in vivo* validation to fully elucidate the cellular complexity of SYS and evaluate the translational potential of these findings.

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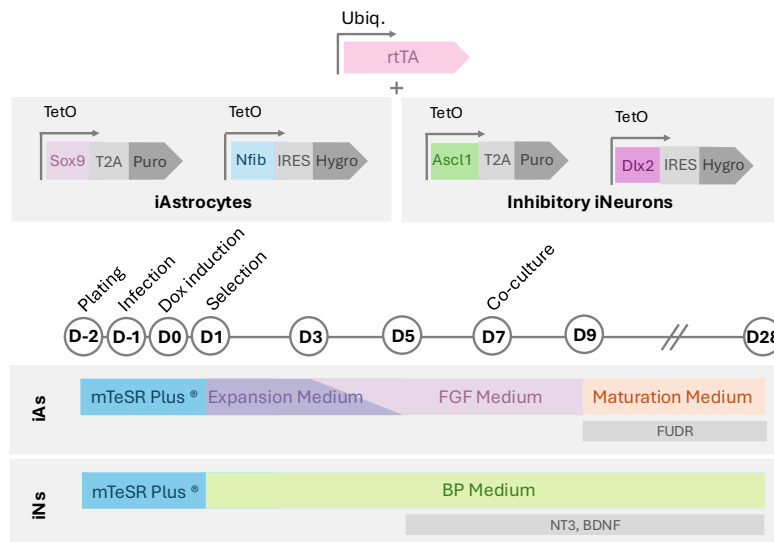
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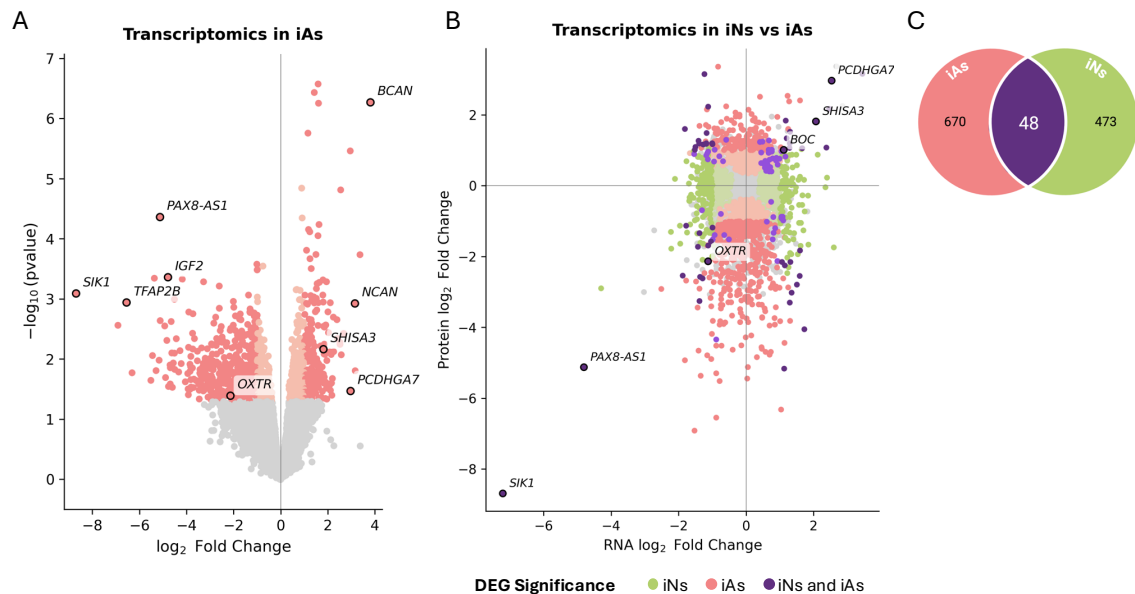
Supplementary information for:

A Multi-omic and Functional Perspective on Neural Cell Dysfunction in Schaaf-Yang Syndrome

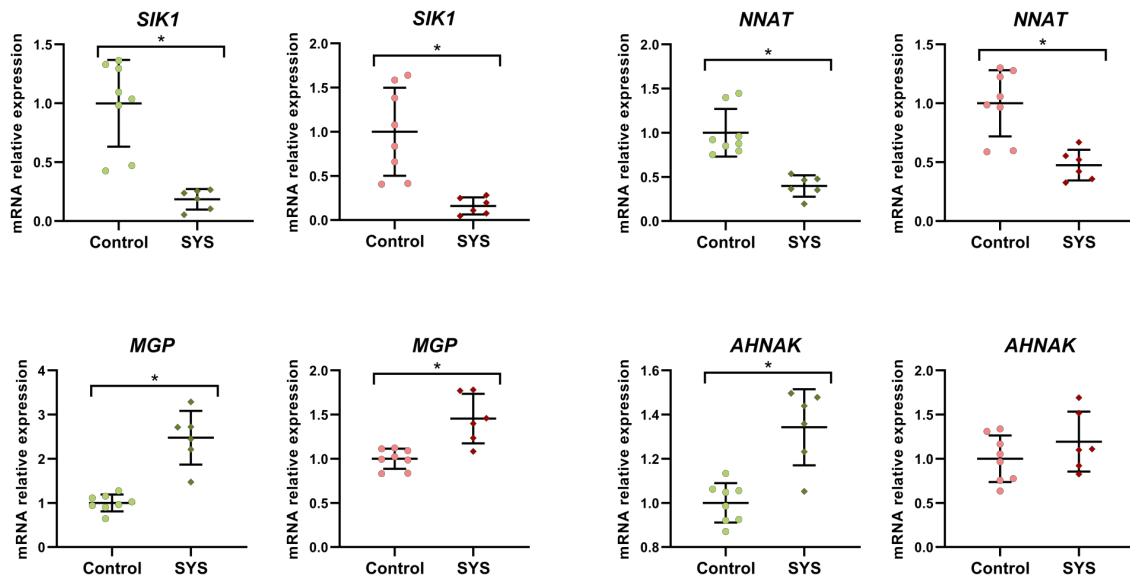
Mónica Centeno-Pla^{1,2,3}, Aina Prat-Planas^{1,2}, Juan Diego Gutiérrez-Ávila^{1,2}, Clàudia Puigasllas-Pastor⁴, Elena Rojano^{2,5,6}, Pedro Seoane^{2,5,6}, Daniel Grinberg^{1,2}, Susanna Balcells^{1,2}, Daniel Tornero⁴, Isaac Canals⁷, Roser Urreizti^{2,3}, Raquel Rabionet^{1,2}



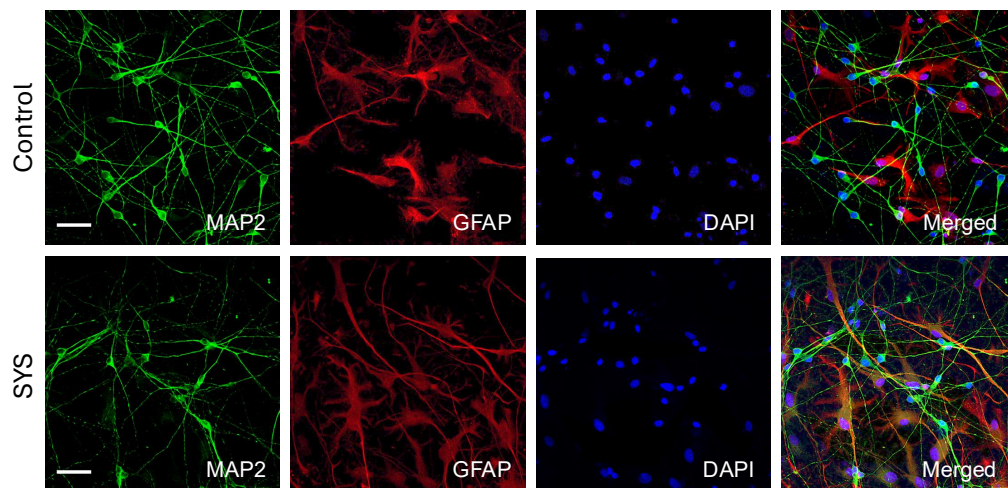
Suppl. Figure 1: Schemes describing the protocols for induction of inhibitory iNeurons and iAstrocytes using TF-mediated programming. Adapted from [24,25].



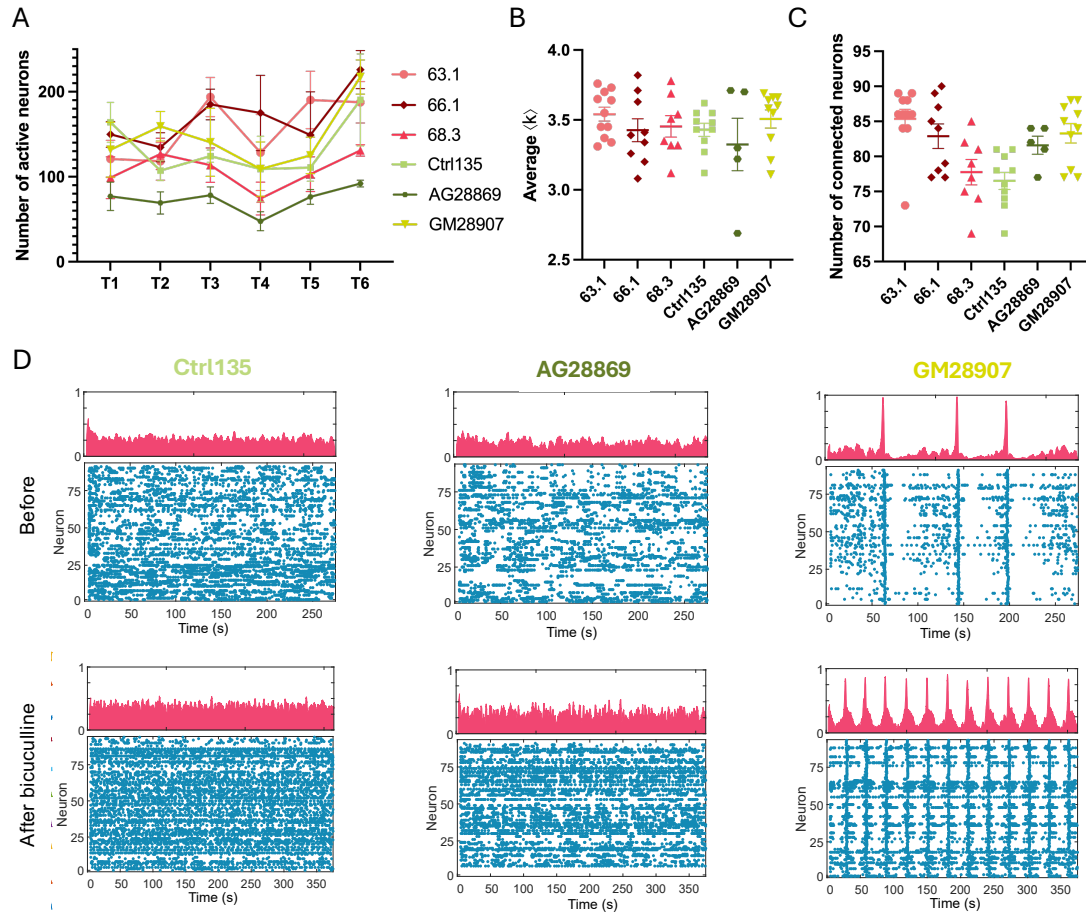
Suppl. Figure 2: Transcriptomic and alterations in SYS iAs and iNs. (A) Volcano plot showing differential gene expression in iAs transcriptomics analysis, with significantly upregulated and downregulated genes highlighted in pink. (B) Correlation plot comparing mRNA fold changes of iNs and iAs. Colours indicate significance in iNs (green), iAs (red), or both (purple). (C) Venn diagram showing overlap of DEGs between SYS iNs and iAs. $|\text{Log}_2 \text{FC}| > 1$ and $p < 0.05$.



Suppl. Figure 3: qPCR validation of transcriptomic alterations in SYS iNeurons (green) and iAstrocytes (pink). RNA levels of genes *SIK1*, *NNAT*, *MGP*, and *AHNK*, were measured in 3 SYS patients and 3 controls. Values from 2 replicates have been normalised to the mean of the control group. Horizontal lines represent mean values and error bars represent the standard deviation. Statistical analyses were performed using unpaired two-tailed Student's t-test in GraphPad Prism *p< 0.05.



Suppl. Figure 4: Representative immunofluorescence stainings showing the expression of neural markers in iPSC-derived cultures from SYS patients and controls. Neurons were identified by MAP2 staining (green), while astrocytes were labelled with GFAP (red). Nuclei were counterstained with DAPI (blue). Merged images show the coexistence of neurons and astrocytes in both control and SYS cultures. Scale bar: 50 μm.



Suppl. Figure 5: Neuronal activity analysis during differentiation. A) Number of active neurons over the differentiation period for SYS patient lines 63.1, 66.1, 68.3, and controls Ctrl135, AG28869, and GM28907. B) Graphs showing the average number of connections per neuron ($\langle k \rangle$) measured at the late stage (from dd45 to dd55) in SYS and control lines. C) Number of active neurons at the late stage in SYS patients and controls, with error bars showing SEM. D) Raster plots of neuronal activity in controls at dd55 before and after bicuculline.

Suppl. Table 1: Induced pluripotent stem cell lines used in this study; Banco Nacional de Líneas Celulares (BNLC).

Cell line code	Group	MAGEL2 mutation	Age (years)	Sex	Origin
AG28869	Control	-	21-25	F	Coriell
GM28907	Control	-	16-20	F	Coriell
Ctrl2	Control	-	0-5	M	BNLC
Ctrl135	Control	-	11-15	F	Our lab
63.1	Patient	p.Q638*	16-20	F	Our lab
66.1	Patient	p.Q666Pfs*47	6-10	M	Our lab
68.3	Patient	p.Q666Pfs*47	6-10	F	Our lab

Suppl. Table 2: List of primary and secondary antibodies employed for immunocytochemistry, including dilution, and supplier information.

Antibody	Supplier	Cat. N°	Dilution
Rabbit IgG anti-GFAP	Dako	z0334	1:1000
Mouse IgG anti-Vimentin	Dako	M0725	1:500
Chicken IgG anti-MAP2	Abcam	ab5392	1:1000
Mouse IgG anti-GAD67	Millipore	MAB5406	1:1000
Donkey anti-mouse IgG A488	Abcam	ab150105	1:400
Donkey anti-rabbit IgG A555	Abcam	ab150074	1:400
Donkey anti-chicken IgG A647	Abcam	ab150171	1:500
Donkey anti-mouse IgG A555	Abcam	ab150106	1:400

Suppl. Table 3: List of differentially expressed genes (DEGs) in SYS iNeurons compared to controls. ($|\text{Log}_2\text{FC}| > 1.5$, unadjusted $p < 0.05$).

Gene	p value	Log2FC
DOWNREGULATED		
<i>SIK1</i>	0.000259671	-7.207224436
<i>USP6</i>	0.001760428	-5.617862436
<i>PAX8-AS1</i>	0.000413527	-4.805009098
<i>HLA-DRB5</i>	0.012146452	-4.304717314
<i>FAM135B</i>	0.025886392	-4.034000347
<i>GABRG1</i>	0.025920284	-3.952187117
<i>ESRG</i>	0.000126824	-3.901129677
<i>VWC2L</i>	0.004985662	-3.029487562
<i>FZD10-AS1</i>	0.003121143	-2.702774568
<i>ENSG00000243004</i>	0.001123472	-2.530015330
<i>TTC9B</i>	0.003639466	-2.481534948
<i>C22orf42</i>	0.008660904	-2.361555566
<i>ISL1-DT</i>	0.011524332	-2.314803090
<i>PNMA6F</i>	0.002954933	-2.268021675
<i>RIMBP2</i>	0.004243118	-2.232977410
<i>NPIP2</i>	0.046842520	-2.222891537
<i>MIR124-1HG</i>	0.001442152	-2.191993001
<i>POU2F2</i>	0.005513314	-2.155112869
<i>FOXA2</i>	0.014093845	-2.144499666
<i>ENSG00000235934</i>	0.020005852	-2.131680259
<i>STXBP5-AS1</i>	0.008215344	-2.126536945
<i>ADGRG4</i>	0.032430619	-2.116764072
<i>KLHL1</i>	0.000233958	-2.110169288
<i>GRIP2</i>	0.017593284	-2.067203221
<i>GHRH</i>	0.000250049	-2.063330673
<i>TH</i>	0.021827601	-2.050203421
<i>GABRG2</i>	0.009397460	-2.046998054
<i>GPR22</i>	0.016699859	-2.044054193
<i>ONECUT3</i>	0.008760646	-2.033946300
<i>SCRT1</i>	0.024958413	-2.014915399
<i>KCNJ9</i>	0.015438480	-1.987837621
<i>HLA-DRB1</i>	0.000010770	-1.964187846

<i>ABCA12</i>	0.005489933	-1.956728267
<i>CNTNAP5-DT</i>	0.018183933	-1.946606746
<i>MNX1</i>	0.017955441	-1.946296837
<i>ENSG00000287900</i>	0.019568394	-1.945774822
<i>MST1L</i>	0.000002185	-1.936846530
<i>CALY</i>	0.012370319	-1.926814357
<i>SLC4A10</i>	0.001751824	-1.924708426
<i>ASIC4</i>	0.004018296	-1.920515324
<i>PROC</i>	0.010219198	-1.904987037
<i>SKOR1</i>	0.023578119	-1.896059938
<i>SYNDIG1L</i>	0.029497697	-1.894372236
<i>PPP1R17</i>	0.025397339	-1.893643179
<i>CALCR</i>	0.008298611	-1.884755259
<i>THCAT155</i>	0.037133500	-1.879109201
<i>HPCAL4</i>	0.013675232	-1.868947549
<i>HLA-DPB2</i>	0.035248034	-1.858236977
<i>POU2F2-AS2</i>	0.038060320	-1.849881951
<i>NECAB1</i>	0.014662892	-1.848317136
<i>FREM3</i>	0.048819188	-1.844893930
<i>GFY</i>	0.045579475	-1.844830064
<i>KCNA1</i>	0.017930178	-1.819636278
<i>GLT1D1</i>	0.040828747	-1.811772054
<i>MIR124-2HG</i>	0.010391954	-1.808425211
<i>ZDHHC11B</i>	0.004083244	-1.799043696
<i>PPFIA2</i>	0.020419327	-1.793686980
<i>ENSG00000260918</i>	0.017676171	-1.790541739
<i>OPRM1</i>	0.028718665	-1.789113424
<i>ENSG00000274602</i>	0.000000051	-1.783315894
<i>TMEM196</i>	0.024757948	-1.776481177
<i>STK32A</i>	0.019767572	-1.774294243
<i>CNGB1</i>	0.021619525	-1.772867027
<i>CACNG3</i>	0.021158695	-1.765924131
<i>FMNL1-DT</i>	0.013592775	-1.765146008
<i>KCNC2</i>	0.005135626	-1.762563045



<i>CCDC177</i>	0.016515742	-1.754795832
<i>NNAT</i>	0.000012207	-1.752281257
<i>MYT1L</i>	0.035719645	-1.735051020
<i>ENSG00000186369</i>	0.021506775	-1.730889622
<i>PHOX2B</i>	0.042963888	-1.729529314
<i>ENSG00000242686</i>	0.002382846	-1.722277358
<i>SYT5</i>	0.026522436	-1.714642827
<i>RUNDC3B</i>	0.017355048	-1.713970420
<i>PHF24</i>	0.027015909	-1.700828523
<i>PDIA2</i>	0.011182354	-1.699071353
<i>DDC</i>	0.009513821	-1.696419389
<i>NAP1L2</i>	0.003761313	-1.695725989
<i>APC2</i>	0.024083218	-1.689425602
<i>ATP1A3</i>	0.031707164	-1.685550759
<i>ST18</i>	0.018908684	-1.682577611
<i>SCN2A</i>	0.008853365	-1.679605439
<i>ELAVL3</i>	0.015664686	-1.674142397
<i>PAK5</i>	0.020858622	-1.671925260
<i>ENSG00000226486</i>	0.005609871	-1.664654033
<i>NDRG4</i>	0.003773067	-1.660578324
<i>TUNAR</i>	0.019994000	-1.650883884
<i>CELF4</i>	0.017681177	-1.645152007
<i>NWD2</i>	0.024754277	-1.644226730
<i>PTPRN2</i>	0.008379280	-1.642682655
<i>DGKK</i>	0.039007961	-1.637957177
<i>FGF17</i>	0.034109414	-1.635706091
<i>TMEM151B</i>	0.017041884	-1.635639350
<i>RGS11</i>	0.011012025	-1.632453885
<i>SNCB</i>	0.035364520	-1.621219665
<i>GLRA3</i>	0.007645949	-1.616499722
<i>MAP6</i>	0.010563538	-1.611306913
<i>GNG3</i>	0.015477364	-1.606408604
<i>CELF3</i>	0.018644327	-1.595278997
<i>ABCC8</i>	0.025417197	-1.594517798
<i>GAD1</i>	0.026100951	-1.591073605
<i>CPNE7</i>	0.024977893	-1.586269619
<i>ACVR1C</i>	0.014480502	-1.584571618
<i>ENSG00000249460</i>	0.024990262	-1.581466326
<i>CAMKV</i>	0.040682351	-1.574096176
<i>OVOS2</i>	0.035332744	-1.574056601
<i>GRIN1</i>	0.025335445	-1.571755087
<i>HEPACAM2</i>	0.036485662	-1.571480186
<i>FUT9</i>	0.006338339	-1.571394468
<i>INSM1</i>	0.021671265	-1.571032225
<i>B4GALNT1</i>	0.021184785	-1.569721978
<i>ENSG00000269001</i>	0.003127894	-1.564244549
<i>VWA5B2</i>	0.044680496	-1.562175060
<i>NKAIN2</i>	0.013179394	-1.551284163
<i>RMST</i>	0.011886006	-1.549390147
<i>GNG13</i>	0.023373421	-1.549329050
<i>SYP</i>	0.011958608	-1.545140201
<i>CPNE4</i>	0.016786980	-1.539484188
<i>ENSG00000261770</i>	0.003009806	-1.530833987
<i>CAMK2N2</i>	0.024035914	-1.522178425

<i>KIF5A</i>	0.017063206	-1.521479145
<i>GPM6A</i>	0.014614221	-1.520234794
<i>SHISA7</i>	0.016882453	-1.514858520
<i>CKMT1B</i>	0.033872114	-1.514093230
<i>KRTAP5-AS1</i>	0.022444645	-1.513317848
<i>STMN2</i>	0.022777332	-1.509741742
<i>RGS7</i>	0.020807782	-1.502707308
<i>GPR68</i>	0.027182962	-1.502283205
<i>TMEM145</i>	0.024099124	-1.500469203
UPREGULATED		
<i>HOXD10</i>	0.000904021	3.847799327
<i>HOXB13</i>	0.001069590	3.437948693
<i>ZNF208</i>	0.026627315	3.279522119
<i>DKK1</i>	0.000836803	2.595138873
<i>PCDHGA7</i>	0.037141435	2.534404772
<i>ENSG00000225178</i>	0.011965527	2.459486097
<i>CYSLTR1</i>	0.003573879	2.411743608
<i>HOXA10</i>	0.002310535	2.391630835
<i>SLC22A3</i>	0.004792516	2.375529209
<i>KDR</i>	0.001314070	2.347955779
<i>HOXA7</i>	0.008254066	2.347302216
<i>CD1D</i>	0.007059507	2.236565323
<i>HOXA11</i>	0.013789548	2.150313055
<i>HOXA9</i>	0.007528842	2.127842837
<i>ENSG00000244649</i>	0.012427913	2.114539889
<i>SHISA3</i>	0.000047100	2.068061412
<i>ENSG00000176320</i>	0.005105646	2.051021086
<i>RPS10P8</i>	0.033063260	1.908607634
<i>HOXC9</i>	0.007752364	1.880840467
<i>FDCSP</i>	0.010464914	1.843732724
<i>RGCC</i>	0.012976890	1.835004030
<i>SLC39A2</i>	0.029748641	1.769935389
<i>HAGLR</i>	0.004651303	1.747932887
<i>HOXA13</i>	0.015234361	1.747761548
<i>G0S2</i>	0.005519731	1.725306460
<i>RFX6</i>	0.025092645	1.723517945
<i>P2RY1</i>	0.003874646	1.715370957
<i>ALDH1A2</i>	0.022863788	1.694909116
<i>SCAT8</i>	0.013501141	1.675739042
<i>CRLF1</i>	0.002136245	1.664085684
<i>S1PR1</i>	0.003195485	1.653933033
<i>GATA4</i>	0.000036200	1.653880594
<i>PCDHGA11</i>	0.003110518	1.639664533
<i>C3orf80</i>	0.017058292	1.623214653
<i>GABRQ</i>	0.001777375	1.619546977
<i>HOXB9</i>	0.042052106	1.611072162
<i>GATA5</i>	0.022914067	1.595036801
<i>SVEP1</i>	0.000095300	1.590633864
<i>IGF1</i>	0.004210151	1.589920301
<i>ENSG00000146001</i>	0.002644552	1.587859980
<i>KCNE4</i>	0.013792016	1.586679729
<i>AGTR2</i>	0.002680969	1.566589754
<i>P4HA3</i>	0.025504109	1.565634574
<i>HMCN1</i>	0.000446603	1.546987930

<i>TFPI2</i>	0.005392988	1.527880348
<i>CMKLR1</i>	0.042565109	1.502409519
<i>PTGS1</i>	0.003974771	1.501429714

Suppl. Table 4: List of significantly dysregulated proteins in SYS iNeurons compared to controls. ($p < 0.05$).

Gene	p value	Log2FC
DOWNREGULATED		
<i>MAGEA3</i>	0.005057	-2.66572
<i>ECHDC3</i>	0.027767	-1.97334
<i>SEPTIN4</i>	0.042014	-1.56453
<i>LCP1</i>	0.003367	-1.46946
<i>S100P</i>	0.018306	-1.46931
<i>TGM2</i>	0.004757	-1.4233
<i>OSBPL3</i>	0.019779	-1.41747
<i>DKK3</i>	0.02385	-1.41149
<i>KIFC3</i>	0.012724	-1.40877
<i>ABCG2</i>	0.006042	-1.39968
<i>MYLK</i>	0.000138	-1.3899
<i>S100A16</i>	0.021933	-1.33193
<i>UBASH3B</i>	0.048355	-1.29818
<i>NEBL</i>	0.002542	-1.27941
<i>SLC1A3</i>	0.001125	-1.26581
<i>OLFML2A</i>	0.002374	-1.25807
<i>PTGES</i>	0.026539	-1.25197
<i>LRATD2</i>	0.036292	-1.22205
<i>HNMT</i>	0.023573	-1.14834
<i>PTK2B</i>	0.031253	-1.14746
<i>C1orf198</i>	0.016365	-1.12839
<i>FZD1</i>	0.000597	-1.12075
<i>DOCK9</i>	0.030365	-1.08619
<i>GRAMD2B</i>	0.006176	-1.04082
<i>CAVIN1</i>	0.016891	-1.03525
<i>CAV1</i>	0.046077	-1.03129
<i>DISP2</i>	0.000384	-0.97284
<i>CAVIN3</i>	0.042775	-0.96496
<i>RANBP17</i>	0.032039	-0.93958
<i>PLEKHA6</i>	0.02963	-0.93876
<i>SORT1</i>	0.024833	-0.93015
<i>CDKN2A</i>	0.029922	-0.90872
<i>RHOB</i>	0.008866	-0.87388
<i>FZD7</i>	0.010699	-0.86895
<i>BCL11A</i>	0.045161	-0.83122
<i>PHYHIPL</i>	0.048562	-0.82528
<i>MIIP</i>	0.010709	-0.82278
<i>PRKAG2</i>	0.028671	-0.80259
<i>ZNF521</i>	0.048531	-0.7709
<i>TPM1</i>	0.038935	-0.74109
<i>ANXA2</i>	0.030698	-0.73987
<i>FAM107B</i>	0.046959	-0.72706
<i>MARVELD2</i>	0.046766	-0.7262
<i>SYNE2</i>	0.037564	-0.72142

<i>EFNA5</i>	0.014879	-0.71835
<i>GSTT1</i>	0.01325	-0.71121
<i>GALC</i>	0.014671	-0.70435
<i>LRRN1</i>	0.033137	-0.7001
<i>DDR1</i>	0.039601	-0.6949
<i>AADAT</i>	0.044441	-0.68717
<i>IGFBP7</i>	0.021822	-0.67996
<i>CHML</i>	0.019073	-0.67146
<i>GPR173</i>	0.009185	-0.66642
<i>SLC29A2</i>	0.028944	-0.65922
<i>JMJD7</i>	0.049501	-0.65024
<i>HEPH</i>	0.021157	-0.64914
<i>AP5S1</i>	0.047093	-0.64387
<i>RASSF2</i>	0.03299	-0.64355
<i>ARHGDIB</i>	0.016351	-0.64036
<i>PIK3C2B</i>	0.029544	-0.63452
<i>CUTA</i>	0.001532	-0.63034
<i>BBC3</i>	0.021054	-0.62869
<i>VWA1</i>	0.046364	-0.62853
<i>CD59</i>	0.042111	-0.62814
<i>SLC38A1</i>	0.016616	-0.62499
<i>PCDHGB6</i>	0.048258	-0.6144
<i>CPVL</i>	0.048217	-0.612
<i>CTNND2</i>	0.046545	-0.61025
<i>SHANK2</i>	0.023668	-0.60661
<i>CABLES2</i>	0.039933	-0.60551
<i>HSPG2</i>	0.027168	-0.60413
<i>DYSF</i>	0.032471	-0.59776
<i>DGKA</i>	0.027588	-0.59718
<i>DAG1</i>	0.014769	-0.59685
<i>EPPK1</i>	0.016977	-0.59636
<i>DIP2A</i>	0.008682	-0.5867
UPREGULATED		
<i>ALDH1A2</i>	0.002665	2.851989
<i>GGT1</i>	0.002732	2.783397
<i>DGLUCY</i>	0.001885	2.713636
<i>TNFAIP8L3</i>	0.018802	2.462047
<i>ZFPL1</i>	0.023435	2.092116
<i>HSPB6</i>	0.013018	2.008206
<i>SRPX2</i>	0.001123	1.939131
<i>REN</i>	0.029242	1.93228
<i>KYNU</i>	0.000297	1.893537
<i>MAPK12</i>	0.049364	1.692177
<i>CRISPLD2</i>	0.009628	1.678116
<i>DYNLT3</i>	0.003155	1.664046
<i>QNG1</i>	0.048811	1.614841
<i>HSPA2</i>	0.032696	1.609201
<i>GFPT2</i>	0.003202	1.604185
<i>EGFLAM</i>	0.020918	1.516426
<i>TENT5A</i>	0.005028	1.487904
<i>SRPX</i>	0.029337	1.483842
<i>GALNT14</i>	0.000363	1.469559
<i>LIFR</i>	0.038143	1.448007
<i>CRLF1</i>	0.010249	1.430439

PITX2	0.004918	1.4238
PCOLCE2	0.004078	1.421659
LAMC3	0.022267	1.412012
TRIM38	0.000892	1.398753
PDE1C	0.043038	1.379957
MAMDC2	0.011748	1.377888
ASCL1	0.037953	1.37308
CACNB2	0.014654	1.356805
FLT1	0.013403	1.347054
CAT	0.002064	1.330849
RTL1	0.045526	1.327929
ADAMTS1	0.011885	1.294349
BACE2	0.019736	1.286344
FKBP5	0.040488	1.283653
BST2	0.020235	1.254137
AEBP1	0.00801	1.25316
DLK1	0.003417	1.236375
STOM	0.020074	1.231486
ANGPTL4	0.041277	1.22287
GBP4	0.011636	1.21623
NMB	0.02403	1.214922
HAND2	0.013144	1.207159
VTN	0.026031	1.163588
RIGI	0.001406	1.160948
CPXM1	0.021112	1.143367
FKBP11	0.014236	1.138724
TYMP	0.011337	1.127453
CLEC11A	0.006422	1.109151
RFLNB	0.034594	1.106842
SPOCK1	0.012099	1.102929
LIMS3;LIMS4	0.014881	1.097167
TGFBR3	0.011467	1.096636
SNX21	0.03759	1.086372
LINGO2	0.0328	1.078836
NPR3	0.042513	1.076563
RPP25	0.010193	1.074116
GATA6	0.023628	1.073676
COL6A2	0.019658	1.06952
COL6A1	0.022895	1.066775
EFEMP2	0.005739	1.06598
PRSS56	0.018977	1.064158
IL33	0.021753	1.055401
HVCN1	0.00402	1.053334
STAT5A	0.031026	1.053288
BNIP3	0.027431	1.052709
NFATC1	0.003362	1.050489
PDK3	0.019211	1.049684
PDGFRA	0.015897	1.048676
LRRC8C	0.000219	1.041954
HGF	0.008303	1.019399
HAAO	0.007939	1.016941
NCOA7	0.009995	1.000871
IFI16	0.000788	0.988092
JPH3	0.038156	0.987417

PHACTR2	0.009853	0.983359
IDNK	0.032272	0.976432
DCN	0.008802	0.974791
RADIL	0.006337	0.967171
PCDH10	0.000208	0.963573
TMEM164	0.040447	0.947774
PARP12	0.008712	0.94479
CRYBG1	0.008206	0.939266
IL6ST	0.00655	0.938073
MICOS10	0.015353	0.932392
VASH1	0.049143	0.926837
TGFB1	0.012364	0.921019
TFPI2	0.040974	0.916916
FCGRT	0.002032	0.916356
SLIT2	0.0293	0.915696
PAPSS2	0.011917	0.911341
PKIG	0.000833	0.910967
TNFRSF10B	0.015604	0.910011
CD248	0.032865	0.907634
JUNB	0.021803	0.903472
RAB27A	0.032741	0.900601
TMTC1	0.025586	0.894196
ATP5F1E	0.020996	0.88991
GTF2IRD1	0.040031	0.888139
CHSY1	0.005404	0.88428
IGDCC4	0.016808	0.884056
LDHA	0.005458	0.882511
SHFL	0.037107	0.878354
PYGM	0.043414	0.863479
NMI	0.002081	0.861933
ADAMTS17	0.039117	0.86118
SLC25A26	0.032935	0.856347
IFI35	0.008225	0.855833
ARHGAP42	0.042854	0.853482
EMILIN1	0.012772	0.849747
UGT8	0.009319	0.838974
RBPM52	0.006319	0.822713
PLCL2	0.004839	0.818991
PRKD1	0.022412	0.804812
FKBP7	0.0063	0.788589
GJA1	0.025516	0.786853
AMMECR1	0.015133	0.783531
CYP26A1	0.046172	0.7705
PLIN2	0.013007	0.769465
RAP1B	0.004567	0.769212
RBMS1	0.026709	0.767935
PCOLCE	0.02372	0.765474
NAMPT	0.009146	0.751095
RHOU	0.014942	0.724528
ITGA5	0.043395	0.718724
TMEM143	0.015835	0.716225
OPLAH	0.009367	0.711912
BCL3	0.020716	0.707019
RASAL1	0.005796	0.701411

<i>ISG20</i>	0.002165	0.699781
<i>PPIF</i>	0.004108	0.697373
<i>ADSS1</i>	0.010296	0.684394
<i>NHS</i>	0.011873	0.682903
<i>GALNT12</i>	0.037563	0.681984
<i>ALDOC</i>	0.017009	0.670442
<i>MAP4K2</i>	0.025225	0.667349
<i>HELZ2</i>	0.00313	0.664969
<i>PLOD2</i>	0.013547	0.660881
<i>PLCB1</i>	0.004126	0.643725
<i>CACNA1D</i>	0.00161	0.642182
<i>NR1H2</i>	0.004013	0.64132
<i>COL6A3</i>	0.044795	0.638969
<i>TSPAN15</i>	0.020989	0.637681
<i>TSPAN9</i>	0.001953	0.632119
<i>P4HA2</i>	0.042742	0.625992

<i>PRDX4</i>	0.020106	0.624616
<i>PVR</i>	0.035977	0.615881
<i>SOD2</i>	0.027069	0.612994
<i>GNB3</i>	0.034524	0.612484
<i>DHPS</i>	0.011195	0.608827
<i>C15orf39</i>	0.033601	0.604016
<i>PRPS1</i>	0.040102	0.600369
<i>DNLZ</i>	0.002308	0.600234
<i>PPP2R1B</i>	0.004897	0.592968
<i>RNF150</i>	0.03847	0.587821
<i>TUBG2</i>	0.048423	0.587749
<i>IFT88</i>	0.013073	0.587404
<i>PHYH</i>	0.014219	0.586944
<i>OGDHL</i>	0.046955	0.586415

Suppl. Table 5: Gene Ontology (GO) enrichment analysis of significantly dysregulated proteins in SYS inhibitory iNeurons ($\log_2 \text{FCI} > 1$, $p < 0.05$).

	Name	Adjusted P-value	Odds Ratio	Genes
1	regulation of phosphatidylinositol 3-kinase activity (GO:0043551)	1.0191E-02	26.706	<i>PDGFRA;FLT1;PTK2B;TNFAIP8L3</i>
2	muscle tissue morphogenesis (GO:0060415)	2.1907E-02	43.931	<i>TGFBR3;EFEMP2;MYLK</i>
3	positive regulation of cell motility (GO:2000147)	2.7418E-02	69.240	<i>PDGFRA;FLT1;CAVIN1;HGF;CAV1;PTK2B;MYLK</i>
4	negative regulation of apoptotic process (GO:0043066)	2.9415E-02	45.439	<i>HGF;CAV1;BNIP3;CAT;GATA6;PTK2B;ANGPTL4;ASCL1;MAGEA3;CRLF1</i>
5	regulation of collagen fibril organization (GO:1904026)	3.7846E-02	101.510	<i>EFEMP2;AEBP1</i>
6	positive regulation of cell migration (GO:0030335)	4.3258E-02	5.642	<i>PDGFRA;VTN;FLT1;HGF;CAV1;PTK2B;MYLK</i>
7	gland development (GO:0048732)	4.2316E-02	12.334	<i>TGFBR3;HAND2;CAV1;DKK3</i>
8	regulation of response to external stimulus (GO:0032101)	4.7639E-02	8.326	<i>PDGFRA;CAV1;GATA6;GGT1;PTGES</i>

Suppl. Table 6: List of differentially expressed genes in SYS iAstrocytes compared to controls. (|Log₂FC|>0.5 and FDR<0.05).

Gene	Log2FC	FDR
DOWNREGULATED		
<i>SIK1</i>	-8.6916	0.0068099
<i>TFAP2B</i>	-6.5483	0.0163131
<i>PKP3</i>	-5.3676	0.0073237
<i>PAX8-AS1</i>	-5.1268	0.0019226
<i>PRR15</i>	-5.1194	0.0424941
<i>IGF2</i>	-4.7889	0.0231840
<i>XKR7</i>	-4.1817	0.0473963
<i>CXCL14</i>	-3.2774	0.0308858
<i>BCAR1</i>	-1.0137	0.0376794
<i>CAB39L</i>	-1.0022	0.0385603
<i>RRS1</i>	-0.9839	0.0021102
<i>SBNO2</i>	-0.9639	0.0358231
<i>IRF2BPL</i>	-0.7548	0.0069723
UPREGULATED		
<i>BCAN</i>	3.8099	0.0000081
<i>ADCYAP1R1</i>	3.3656	0.0341336
<i>PAX3</i>	2.9529	0.0001951
<i>CTSF</i>	2.5459	0.0020319
<i>CLDN10</i>	1.6255	0.0089491
<i>LHX9</i>	1.6042	0.0000233
<i>SLC6A14</i>	1.5873	0.0000194
<i>GEM</i>	1.5822	0.0159133
<i>NPY1R</i>	1.4950	0.0472595
<i>FMO1</i>	1.4248	0.0000081
<i>PRRX1</i>	1.2338	0.0159978
<i>GAL3ST4</i>	1.1798	0.0015961
<i>SEZ6L</i>	1.1554	0.0000457
<i>STMN2</i>	1.0959	0.0236013
<i>RAB7B</i>	0.9742	0.0226493
<i>DCLK1</i>	0.9099	0.0035063
<i>CST3</i>	0.8888	0.0002197
<i>TIAM2</i>	0.6833	0.0323150
<i>RAB29</i>	0.6545	0.0159684

Suppl. Table 7: List of dysregulated genes in SYS iAstrocytes compared to controls (|Log₂FC|>1.5 and p<0.05). Log₂ fold changes and p values are provided for each protein.

Gene	p value	Log2FC
DOWNREGULATED		
<i>SIK1</i>	0.000813759	-8.691612163
<i>MMP9</i>	0.002751452	-6.913351691
<i>TFAP2B</i>	0.001148823	-6.548309056
<i>HOXC8</i>	0.016944398	-6.314246630
<i>ERVH48-1</i>	0.022652976	-5.514607149
<i>FGB</i>	0.008760264	-5.446345663
<i>PKP3</i>	0.000453767	-5.367640133
<i>GJD2</i>	0.015600416	-5.295219709

<i>LINC02381</i>	0.010473299	-5.165292519
<i>PAX8-AS1</i>	0.000043637	-5.126842704
<i>PRR15</i>	0.002302803	-5.119380996
<i>WNT6</i>	0.002819525	-4.926822037
<i>PLSCR5</i>	0.020343129	-4.892282475
<i>GABRP</i>	0.004907041	-4.837097303
<i>IGF2</i>	0.000436870	-4.788896371
<i>MUC16</i>	0.028592378	-4.767075330
<i>ANXA8</i>	0.012576212	-4.701363060
<i>PROK1</i>	0.026004890	-4.692536343
<i>HOXB6</i>	0.013969300	-4.688736649
<i>GPR26</i>	0.002381754	-4.652208702
<i>SLC1A6</i>	0.007938741	-4.627692885
<i>ITGB6</i>	0.029566187	-4.601619119
<i>EGFLAM</i>	0.011648861	-4.562764003
<i>PIK3C2G</i>	0.001015907	-4.515747994
<i>SPNS2</i>	0.007995523	-4.515007651
<i>ODAPH</i>	0.014897369	-4.502005275
<i>HOXC6</i>	0.004596628	-4.435552534
<i>WNT7A</i>	0.016430375	-4.335542061
<i>FLJ16779</i>	0.007269041	-4.278444717
<i>OXGR1</i>	0.011907982	-4.227357521
<i>XKR7</i>	0.000470809	-4.181658824
<i>RFX6</i>	0.023928794	-4.052798283
<i>PRG4</i>	0.017418196	-3.948699338
<i>CSF1R</i>	0.031453613	-3.933336313
<i>WNT10A</i>	0.039317799	-3.923726557
<i>SERTM2</i>	0.006392536	-3.915560963
<i>IGFN1</i>	0.001655485	-3.905499580
<i>CRYBG2</i>	0.012225595	-3.854460923
<i>GPC1-AS1</i>	0.016024927	-3.849783558
<i>HAND1</i>	0.031760191	-3.849283019
<i>ENSG00000254202</i>	0.013465295	-3.824095316
<i>CCKBR</i>	0.022029341	-3.818870006
<i>SYTL1</i>	0.009042596	-3.817140285
<i>WNT2</i>	0.029321107	-3.811554235
<i>ENSG00000280241</i>	0.014083764	-3.750986264
<i>ABCG2</i>	0.003732050	-3.744935728
<i>PITX1</i>	0.006815222	-3.736124021
<i>H19</i>	0.001767780	-3.729547068
<i>CYSLTR2</i>	0.029804921	-3.712982960
<i>VTCN1</i>	0.021598418	-3.688687359
<i>MTUS2</i>	0.004574430	-3.665228560
<i>CLDN4</i>	0.022805833	-3.588555384
<i>TRIML2</i>	0.010710734	-3.540771514
<i>SFRP2</i>	0.007432045	-3.539700012
<i>NECTIN4</i>	0.026011532	-3.484384248
<i>VGLL1</i>	0.033030922	-3.463474245
<i>CSF3R</i>	0.046235518	-3.460100963
<i>KRT8P3</i>	0.017185155	-3.437640642
<i>EGFL6</i>	0.014929592	-3.430540509
<i>CTSV</i>	0.018239997	-3.403236672
<i>EPN3</i>	0.020995445	-3.364150584
<i>P2RY6</i>	0.006536514	-3.362720968

CYP24A1	0.022028979	-3.362619766
HTR1E	0.026964537	-3.353173798
ENPEP	0.020880932	-3.348501293
ZBED2	0.013674648	-3.322356974
NPTX1	0.019986563	-3.301917495
COL17A1	0.033881684	-3.287734607
SHANK2	0.011855849	-3.286982025
CXCL14	0.000520033	-3.277430705
ISL1	0.033004712	-3.256669395
CSF2RA	0.013597315	-3.250718345
NKAIN4	0.015649937	-3.235106951
GRHL2	0.015884864	-3.214400830
ACKR3	0.009706227	-3.206349012
TINAGL1	0.010435615	-3.204328135
BLNK	0.029659129	-3.190376871
IGFBPL1	0.012891435	-3.162956596
ADGRF4	0.018465291	-3.143876619
L1TD1	0.027820793	-3.127140816
INHA	0.010882025	-3.122597912
SLC2A3	0.006756641	-3.118457705
EPCAM	0.023884345	-3.100128426
EPHA1	0.042978777	-3.089851519
SH2D3A	0.010452649	-3.069186859
VIT	0.022457740	-3.055358835
PTGS2	0.022785877	-3.021547479
SLC6A17	0.009752850	-2.995125891
ASB4	0.014426841	-2.993813318
GPR87	0.033945134	-2.977600900
IGF2-AS	0.024759929	-2.975511381
JPH2	0.020644403	-2.972467810
TMPRSS13	0.047136516	-2.963039446
KRT19	0.030123929	-2.951870849
ADAMTS17	0.011825633	-2.948107010
PTGES	0.018953089	-2.946342297
SOSTDC1	0.005609458	-2.939862764
ADAMTS2	0.027522019	-2.911690510
ENSG00000253660	0.005839581	-2.909788331
NPIP15	0.042545505	-2.908588567
TMEM125	0.014540456	-2.907571831
LMNTD2-AS1	0.020731559	-2.903976425
ACKR2	0.008272265	-2.903814435
NPPB	0.010726866	-2.881591764
HTRA3	0.022811278	-2.877075800
RAB25	0.038582387	-2.873346356
LAMC3	0.015034259	-2.868364444
ACSM3	0.002538688	-2.842035832
ADGRE2	0.039985416	-2.839179000
PCSK9	0.025136991	-2.812713743
TCIM	0.027525213	-2.798920520
CMKLR1	0.037160818	-2.793651159
KCNMA1	0.002015356	-2.763274402
KDF1	0.012327703	-2.762577401
SLC2A14	0.046341376	-2.758239241
S100A4	0.005947578	-2.739238468

ELMOD1	0.012091129	-2.727975209
ADGRG1	0.008273214	-2.722962762
WNT9B	0.004560387	-2.722778716
VEGFD	0.011198770	-2.722393343
CRHBP	0.027187549	-2.715432775
TAC1	0.005475202	-2.715058565
SFN	0.048278772	-2.705701844
ERP27	0.033760198	-2.672939569
GRIA4	0.011883837	-2.641592035
HSD17B2	0.036731520	-2.626084021
FERMT1	0.023283392	-2.623192398
HOXB7	0.026406246	-2.616064659
COL15A1	0.030376747	-2.614576329
MCHR1	0.017479241	-2.611887419
MIAT	0.000611836	-2.611381395
PKD1P3	0.001432051	-2.608495867
STYK1	0.044256728	-2.585176499
EPS8L1	0.013893920	-2.570459663
ANO9	0.003367867	-2.567745580
RAB17	0.020745660	-2.558687642
CLDN6	0.020055339	-2.556911815
KCNE4	0.017269205	-2.546445721
KCNJ2	0.015430983	-2.539546714
CALCR	0.007391020	-2.537572124
LPCAT2	0.002884157	-2.525579933
NUAK2	0.005268326	-2.496979374
CACNG4	0.004304734	-2.488671116
HAND2-AS1	0.042244119	-2.481362777
IGDCC3	0.047515150	-2.477101128
ADRA1B	0.016621431	-2.476110380
MARVELD3	0.004857778	-2.466541640
ACTC1	0.034113322	-2.466050236
TNFSF15	0.043566210	-2.450950555
RAMP1	0.015492196	-2.444987165
SOX15	0.023991483	-2.441178445
TJP3	0.036287184	-2.435785043
KRT18	0.017427678	-2.431471431
ENSG00000262160	0.025258415	-2.424968769
WNT4	0.025060522	-2.402607813
ANKS1A	0.009444286	-2.400703128
EXPH5	0.013313626	-2.399756695
CGN	0.018555866	-2.385653248
TGM2	0.015573617	-2.374373200
SEMA5A	0.011549559	-2.353767462
CDC42BPG	0.019719965	-2.349023488
SULT1B1	0.040638638	-2.328528610
SLITRK6	0.025732620	-2.318188413
SMCO3	0.023725722	-2.313874162
SLC2A1	0.006144489	-2.310146117
COL5A1	0.016067738	-2.310091206
TLR3	0.013747937	-2.308364615
IL11	0.037941612	-2.306325658
FRK	0.016384287	-2.303694497
GDF6	0.005353410	-2.289125147

PLEKHG6	0.039851752	-2.285542547
ESRP2	0.037716396	-2.283406777
AIM2	0.021097049	-2.255043124
LUM	0.013382496	-2.247098076
KRT8	0.020148235	-2.236955904
PRSS56	0.033445400	-2.231387186
TCIRG1	0.013964447	-2.227288011
SERPINE1	0.022587032	-2.225460819
PLEKHN1	0.035055952	-2.219537279
SBK1	0.022842138	-2.207350469
INHBA	0.035661597	-2.182797142
NOX4	0.008955671	-2.167795054
CYP2S1	0.031432226	-2.163909493
PLEKHG4B	0.026787813	-2.154853768
DCN	0.037121330	-2.153373577
NR2F2	0.017234704	-2.152160893
FAP	0.003337946	-2.147638490
OXTR	0.040801822	-2.135827946
GPR143	0.024794639	-2.133035949
CCDC80	0.012151087	-2.128808731
ADAMTS18	0.041827717	-2.126756911
STAP2	0.003095067	-2.123425115
SLC27A2	0.012729000	-2.119938388
FAAH2	0.034268277	-2.107783411
GREM2	0.016009040	-2.096606798
PMEL	0.047562199	-2.082955381
FMOD	0.023829965	-2.070469917
ESRP1	0.006517981	-2.070443144
FUT1	0.017480587	-2.065390318
PTHLH	0.032414735	-2.043682452
PTCHD1	0.039095539	-2.043179483
TPM1	0.011172918	-2.023636723
PWWP2B	0.002738945	-2.015459190
PPDPF	0.001101746	-2.012023965
FOXA1	0.047748313	-2.002584234
PAPPA2	0.001378374	-1.993422575
IGFBP3	0.034170689	-1.990173096
C1QTNF6	0.024681427	-1.975829120
KCNH1	0.010578935	-1.964694330
SCG2	0.009764244	-1.964018818
RYS2	0.049031009	-1.963461335
ARHGEF16	0.006132252	-1.950609052
ADAMTS6	0.010901365	-1.949259097
GREB1L	0.016750525	-1.946352147
PKP2	0.021267936	-1.946111347
IRF6	0.025556361	-1.942748456
PANX2	0.004968922	-1.940531819
EFNB2	0.024678129	-1.935375438
KIF26A	0.024079871	-1.935074855
MYOZ1	0.022611743	-1.933909933
PKD1P5	0.026498920	-1.929738791
ENSG00000133519	0.011582479	-1.929095103
ZFP42	0.049641605	-1.925032128
CILP2	0.001918879	-1.924650697

TXNDC12	0.003365658	-1.922673170
SLC7A5	0.021449871	-1.913102367
MFAP5	0.022132063	-1.910446359
CMKLR2	0.016688793	-1.902155199
GALNT6	0.008483017	-1.892157211
ELMO3	0.017241061	-1.885468339
SEMA5B	0.020824788	-1.879421110
NDST3	0.031218627	-1.868592870
LYPD6B	0.047292930	-1.863857038
STEAP4	0.025192170	-1.861612023
SYNPO2	0.016833301	-1.858465773
GUCY1A1	0.042186914	-1.855687435
PAEP	0.036247092	-1.855373623
MID1	0.020795227	-1.853270299
LNK1	0.047122073	-1.849958059
LRRC32	0.036157016	-1.849855786
CBR3-AS1	0.041522693	-1.844063579
HOXB2	0.032225068	-1.840512466
PRAG1	0.003983480	-1.838019829
IGF1	0.013930992	-1.828699257
HEYL	0.004391828	-1.827605590
OCLN	0.034804353	-1.825391797
ARID3A	0.021601658	-1.825202896
LRATD2	0.012459880	-1.824687045
KLF4	0.009805362	-1.819886073
LOC122539214	0.033172247	-1.816702128
TMEM200A	0.023851191	-1.814561362
TMEM154	0.012399613	-1.811596051
TBC1D2	0.019296626	-1.804294437
EYA2	0.017204061	-1.794078901
DSP	0.029814334	-1.773333102
VGF	0.032214511	-1.762424180
CPXM2	0.007168423	-1.752203303
CNTNAP2	0.037028885	-1.752062686
EPAS1	0.036245892	-1.751637104
SNHG32	0.002168902	-1.748876883
ENSG00000260412	0.030200972	-1.740850606
COL1A1	0.039443559	-1.733812333
SLC27A6	0.031897324	-1.728163923
HSBP1L1	0.004080288	-1.725249804
CACNA1E	0.046484451	-1.723454626
GPR83	0.010831108	-1.719146105
MZT2B	0.001782144	-1.718928498
LCP1	0.005853137	-1.715038068
GPR17	0.029433850	-1.713181461
C6orf132	0.046674697	-1.709942788
ANXA3	0.027396921	-1.702645327
SPON2	0.014075457	-1.699295974
ALDH16A1	0.002227987	-1.689170881
KLF8	0.012819346	-1.683345476
RNFT2	0.011508695	-1.677961103
RGL3	0.013813349	-1.674210064
RASGRF2	0.045846731	-1.670362656
SYTL5	0.029588711	-1.670213930

<i>RBM47</i>	0.019626929	-1.666457619
<i>CDS1</i>	0.027675606	-1.662575340
<i>NOTCH3</i>	0.018404106	-1.660841120
<i>CLMN</i>	0.018898800	-1.660243355
<i>KCNC3</i>	0.003427381	-1.658045479
<i>ARHGAP27</i>	0.022031126	-1.657879684
<i>PPP1R13L</i>	0.004342622	-1.646688822
<i>LSR</i>	0.031224809	-1.640673674
<i>AMOT</i>	0.045860148	-1.635807170
<i>BBC3</i>	0.005072611	-1.627256658
<i>KCNK12</i>	0.040657742	-1.626355745
<i>GATA3</i>	0.022473292	-1.619939522
<i>PRRG4</i>	0.016946507	-1.614531837
<i>PCDH7</i>	0.044627393	-1.611077627
<i>MYO3B</i>	0.012294404	-1.606608869
<i>RIMS2</i>	0.036830983	-1.586633236
<i>FMNL1</i>	0.028858680	-1.581998220
<i>FAM167A</i>	0.038008062	-1.577079320
<i>MTMR7</i>	0.003864442	-1.577048909
<i>PGBD5</i>	0.047392799	-1.559834780
<i>CAMSAP3</i>	0.014526622	-1.556434602
<i>SOWAHA</i>	0.042814428	-1.555660088
<i>PLD5</i>	0.043883365	-1.552989643
<i>HSPB8</i>	0.020131000	-1.542559134
<i>CELSR1</i>	0.029222036	-1.538874655
<i>GNG5</i>	0.008832280	-1.535768236
<i>PLEKHA7</i>	0.041209869	-1.527556151
<i>ZNF219</i>	0.004067730	-1.526055864
<i>CNKSR1</i>	0.014279197	-1.524268624
<i>PHLDB2</i>	0.026695186	-1.515693798
<i>ANK3</i>	0.015748032	-1.511185543
<i>ENSG00000204789</i>	0.005947766	-1.507686250
<i>SH3BGR12</i>	0.021035622	-1.503530452
<i>LUCAT1</i>	0.020815597	-1.501267481
UPREGULATED		
<i>BCAN</i>	0.000000540	3.809870684
<i>ADCYAP1R1</i>	0.000184261	3.365567237
<i>HOXB13</i>	0.015668003	3.162677028
<i>NCAN</i>	0.001192600	3.151368576
<i>PCDHGA7</i>	0.034085602	2.966038461
<i>PAX3</i>	0.000003470	2.952906597
<i>FEZF1-AS1</i>	0.003760417	2.670312111
<i>LINC01139</i>	0.008497727	2.571765549
<i>CTSF</i>	0.000015400	2.545935866
<i>AGA-DT</i>	0.005699842	2.517283028
<i>TACR1</i>	0.005261899	2.513351463
<i>PCDHGA6</i>	0.004576326	2.409425359
<i>ENSG00000180229</i>	0.002218511	2.387836302
<i>ADCY8</i>	0.007544353	2.246723997
<i>PPP1R1A</i>	0.002014327	2.235993973
<i>TBX1</i>	0.021095974	2.193925773
<i>ENSG00000225178</i>	0.040028277	2.172161742
<i>DRAXIN</i>	0.025071509	2.156398075
<i>ZBTB16</i>	0.008404792	2.143860493

<i>LHX2</i>	0.033259974	2.110463628
<i>EN2</i>	0.001159633	2.104257530
<i>ENSG00000273291</i>	0.007611258	2.087475218
<i>DNAH11</i>	0.027252271	2.073088823
<i>DIO3OS</i>	0.003641727	2.027737419
<i>CLU</i>	0.001645974	2.004679118
<i>TMEM132E</i>	0.010608239	1.957748225
<i>ZIC3</i>	0.046358851	1.956899090
<i>MYBPH</i>	0.030575421	1.943071976
<i>LPAR3</i>	0.000519298	1.942420327
<i>ENSG00000278530</i>	0.024401566	1.913406722
<i>ZIC4</i>	0.002716631	1.912458096
<i>ZIC5</i>	0.045288307	1.895403561
<i>POU3F3</i>	0.008380931	1.876927620
<i>CTXND1</i>	0.002947439	1.853410262
<i>NTRK3</i>	0.005670274	1.850998695
<i>IL7</i>	0.019915608	1.849669305
<i>PLA2G2A</i>	0.025277509	1.841100078
<i>MAP3K2-DT</i>	0.008249203	1.839995742
<i>ENSG00000287839</i>	0.002858818	1.821737452
<i>SHISA3</i>	0.006877266	1.813833032
<i>HTR4</i>	0.017801529	1.812523382
<i>GRASLND</i>	0.001130823	1.803815735
<i>SHISAL1</i>	0.000484228	1.794179591
<i>CHST9</i>	0.000863935	1.787638643
<i>LMX1B</i>	0.021738336	1.781969147
<i>FRMPD3</i>	0.006039033	1.772356235
<i>C1QTNF3</i>	0.034453216	1.770303987
<i>CA12</i>	0.013491125	1.761311634
<i>SNX22</i>	0.043246899	1.718864674
<i>ENSG00000285744</i>	0.046006771	1.713083595
<i>ALDH1L1</i>	0.024158267	1.699680476
<i>PROKR1</i>	0.006056563	1.694808460
<i>CA10</i>	0.016185222	1.669157406
<i>AIF1L</i>	0.000532390	1.636545059
<i>CLDN10</i>	0.000058100	1.625468466
<i>WSCD2</i>	0.012108526	1.615686753
<i>ZIC1</i>	0.012161256	1.612481206
<i>PPP1R1B</i>	0.044059765	1.610916077
<i>KCNA1</i>	0.029718825	1.606756131
<i>HP</i>	0.017697761	1.605829067
<i>LHX9</i>	0.000000559	1.604179912
<i>NRN1</i>	0.001156716	1.597190982
<i>ADAMTS16-DT</i>	0.013400839	1.592909636
<i>SOX2</i>	0.013625814	1.590373407
<i>GCOM1</i>	0.021510546	1.589230352
<i>SLC6A14</i>	0.000000267	1.587261494
<i>MNS1</i>	0.002770723	1.584705380
<i>STAC2</i>	0.020848809	1.583317886
<i>GEM</i>	0.000089200	1.582230761
<i>ENSG00000262580</i>	0.020352711	1.580998279
<i>CRTAC1</i>	0.015963276	1.567971682
<i>DPF3</i>	0.011339464	1.566303721
<i>VAX1</i>	0.000558625	1.545704324

<i>CXCR4</i>	0.001531329	1.529920018
<i>SLCO2B1</i>	0.009916060	1.527304443
<i>MGP</i>	0.011966689	1.524743023
<i>SSTR2</i>	0.025736667	1.524002069
<i>ADAMTSL3</i>	0.000675593	1.514825099
<i>GASK1A</i>	0.023713631	1.514278322
<i>RAP1GAP</i>	0.000978857	1.509857228

<i>IGF1</i>	0.00421	1.590	0.01393	-1.829
<i>KCNE4</i>	0.01379	1.587	0.01727	-2.546
<i>CMKLR1</i>	0.04260	1.502	0.03716	-2.794
<i>PTGS2</i>	0.01061	1.347	0.02279	-3.022
<i>FAP</i>	0.00368	1.304	0.00334	-2.148
<i>NPTX1</i>	0.03340	1.284	0.01999	-3.302
<i>LUM</i>	0.01087	1.139	0.01338	-2.247
<i>LINC02381</i>	0.04908	1.122	0.01047	-5.165
<i>DCN</i>	0.02515	1.062	0.03712	-2.153

Suppl. Table 8: Commonly dysregulated genes in SYS iNeurons and iAstrocytes ($|\text{Log}_2\text{FC}| > 1$ and $p < 0.05$).

	Gene	iNs		iAs	
		p value	log2 FC	p value	log2 FC
Downregulated	<i>SIK1</i>	0.00026	-7.207	0.00081	-8.692
	<i>PAX8-AS1</i>	0.00041	-4.805	0.00004	-5.127
	<i>CALCR</i>	0.00830	-1.885	0.00739	-2.538
	<i>ENSG00000274602</i>	0.00000	-1.783	0.00335	-1.125
	<i>GPR83</i>	0.02995	-1.433	0.01083	-1.719
	<i>ISL1</i>	0.04424	-1.388	0.03300	-3.257
	<i>PTPRO</i>	0.02643	-1.383	0.02153	-1.341
	<i>ANO9</i>	0.01663	-1.368	0.00337	-2.568
	<i>MIAT</i>	0.01265	-1.299	0.00061	-2.611
	<i>OXTR</i>	0.02262	-1.127	0.04080	-2.136
	<i>KLF8</i>	0.03684	-1.126	0.01282	-1.683
	<i>MTMR7</i>	0.04375	-1.042	0.00386	-1.577
	<i>SAMD12</i>	0.04718	-1.004	0.02857	-1.180
Upregulated	<i>HOXB13</i>	0.00107	3.438	0.01567	3.163
	<i>PCDHGA7</i>	0.03714	2.534	0.03409	2.966
	<i>ENSG00000225178</i>	0.01197	2.459	0.04003	2.172
	<i>SLC22A3</i>	0.00479	2.376	0.01820	1.076
	<i>SHISA3</i>	0.00005	2.068	0.00688	1.814
	<i>S1PR1</i>	0.00320	1.654	0.01841	1.062
	<i>MGP</i>	0.00004	1.295	0.01197	1.525
	<i>CCDC163</i>	0.00177	1.286	0.00327	1.207
	<i>CYP27C1</i>	0.00010	1.283	0.00458	1.072
	<i>QNG1</i>	0.01364	1.201	0.02669	1.345
	<i>MAP3K2-DT</i>	0.01500	1.169	0.00825	1.840
	<i>BOC</i>	0.02806	1.114	0.00550	1.011
	<i>KCNA1</i>	0.01793	-1.820	0.02972	1.607
Down iNs / Up iAs	<i>GPM6A</i>	0.01461	-1.520	0.03194	1.021
	<i>STMN2</i>	0.02278	-1.510	0.00016	1.096
	<i>DNER</i>	0.03526	-1.418	0.02258	1.272
	<i>AMER2</i>	0.02526	-1.391	0.01040	1.190
	<i>PTPRZ1</i>	0.01896	-1.274	0.01869	1.170
	<i>NCAN</i>	0.02896	-1.243	0.00119	3.151
	<i>CACNA2D3</i>	0.02969	-1.189	0.01778	1.184
	<i>JMJD7-PLA2G4B</i>	0.04317	-1.182	0.01667	1.201
	<i>PRRT2</i>	0.01584	-1.176	0.03700	1.490
	<i>PPP1R1A</i>	0.04240	-1.133	0.00201	2.236
	<i>RALGDS</i>	0.03840	-1.013	0.04719	1.203
	<i>MAPK8IP1</i>	0.04646	-1.003	0.02859	1.179
	<i>RFX6</i>	0.02509	1.724	0.02393	-4.053

DISCUSSION

This thesis started as a collaborative project with HSJD, with the goal of addressing the clinical and molecular challenges associated with SYS. One of our earliest tasks was to perform a genetic and phenotypic review of all previously reported SYS cases. In rare diseases like SYS, information is often scattered throughout individual case reports, making it difficult to form a clear picture of the disorder. This lack of well-established management protocols can lead to clinicians having to rely on personal experience or to extrapolate from related conditions, increasing the risk of poor management. Therefore, such reviews help bring together information, reduce inconsistencies, and create a reliable basis for diagnosis and treatment.

Regarding the molecular scenario, our examination brought together that, to date, 61 different variants have been linked to SYS, with most being truncating mutations including nonsense and frameshift changes. These typically cluster in the C-terminal region of *MAGEL2* and eliminate the MHD, which is essential for the interaction with proteins involved in retrograde endosomal transport and other cellular processes [92,159]. Such truncating mutations are widely accepted as pathogenic. In contrast, a small number of previously reported missense variants and in-frame duplications remain of uncertain significance. Unlike truncating alleles, their effect cannot be assumed to be a simple loss of function. In *MAGEL2*, a missense or small in-frame change could have different consequences: from benign polymorphisms to subtle conformational shifts that impair protein-protein interactions, or even dominant-negative effects. Without functional studies, predicting their impact remains difficult. In this context, functional biomarkers may be particularly useful. This work has shown that nuclear mislocalisation of *MAGEL2* and disruptions in amyloid- β (A β) secretion could be used as informative readouts, potentially helping classify variants and better understand their pathogenic consequences.

Given the imprinting pattern of *MAGEL2*, parental testing and phase determination are also fundamental for confirming a diagnosis. Because *MAGEL2* is paternally expressed, only inherited or *de novo* variants on the paternal allele are pathogenic. However, confirming the parental origin often needs extra testing (such as methylation-specific assays, microsatellite analysis, or SNP-based phasing) that is not always available in standard diagnostic laboratories due to cost, expertise, or infrastructure limitations. As a result, in many cases, the parental origin of the variant is not determined. This can lead to misclassification, where a maternal variant might be wrongly labelled as causative. This limitation also affects variant databases, which often lack parental origin information for *MAGEL2* variants. The absence of phasing data complicates the grouping of truly

pathogenic variants and may inflate the number of variants of uncertain significance (VUS).

Refining diagnostic criteria, incorporating both phenotypic characterisation and molecular analysis validation, is crucial to minimise diagnostic errors. It must not be forgotten that a precise genetic diagnosis not only confirms the root cause of a patient's symptoms but also enables better predictions about the disease's course, provides genetic counselling for families, and opens the door to specific clinical management protocols. For instance, GH therapy, sometimes considered for SYS patients [49], could be inappropriately prescribed to a child whose symptoms are actually due to another condition, potentially worsening their health. Moving forward, integrating parental origin data for imprinted genes could improve the reliability of pathogenicity assignments.

Throughout our genetic review of SYS-associated mutations, we also realised that correct variant annotation is still a critical step in the diagnosis of SYS. We found that some variants were initially mapped against a shorter *MAGEL2* reference transcript (GRCh37.p13) lacking the N-terminal domain. Such errors further contributed to the idea that SYS is a PWS-like syndrome caused by a complete loss of *MAGEL2*, when in reality a putatively mislocalised N-terminal portion of the protein remains. In addition, these misannotations hinder accurate meta-analyses and can even propagate into clinical databases if not corrected. Adhering to current Human Genome Variation Society (HGVS) nomenclature [160] is essential to avoid these errors. This includes specifying the correct reference sequence (in the case of *MAGEL2*, NM_019066.5 on hg38), clearly stating the genomic, cDNA, and protein change, and ensuring that all three correspond.

Our review revealed no clear link between phenotype and genotype, except for mutations p.Gln666Serfs*36 and p.Leu708Trpfs*7, which are associated with a severe phenotype of AMC leading to perinatal death. Consistent with this, results from our immunocytochemistry (ICC) experiments showed that these same variants cause a more pronounced mislocalisation of *MAGEL2* compared to other SYS mutations. Aside from these two exceptions, the lack of genotype-phenotype correlations suggests that additional factors may contribute to phenotypic variability. One factor may be epigenetic regulation of *MAGEL2*. In a study of individuals with autism, differences in *MAGEL2* promoter methylation were observed in blood, with autistic males showing hypomethylation near the transcription start site compared to controls [161]. Environmental influences such as diet, infections, or sex can also modulate the expression or impact of genetic variants (reviewed in [162]). Stochastic processes in

gene regulation, like random fluctuations in transcription or translation, can introduce variability in the phenotype outcome [163]. Additionally, each individual's unique genetic background creates a distinct genomic context that may influence how pathogenic mutations manifest.

In parallel with our genetic review, we also collected clinical data from all reported SYS cases using Human Phenotype Ontology (HPO) terms [164]. The use of HPOs allowed us to unify the description of clinical features, reduce variability between reports, and make comparisons across patients. Clinicians at HSJD then used this phenotypic dataset as the foundation for the first clinical guidelines for the management of SYS. These guidelines aim to provide a standardised approach to patient care. They offer recommendations for the early neonatal period, as well as ongoing monitoring and interventions during childhood and adolescence. This structure helps to anticipate complications before they become critical and ensures timely referrals to specialists. Another crucial aspect is that the guidelines promote a multidisciplinary model of care. SYS affects many organ systems, and effective management requires coordinated input from many health professionals. Studies have shown that multidisciplinary clinics improve health outcomes, reduce hospitalisations, and enhance quality of life for patients and families [165]. Moreover, the use of a follow-up protocol is essential to better describe the natural history of diseases, which is especially important when introducing new therapies. Only by understanding the natural progression of a condition can physicians accurately evaluate the real improvements resulting from a treatment or an intervention. Currently, there are significant advances in the field of genetic therapies, but the objective evaluation and assessment of clinically meaningful changes in neurodevelopmental conditions remains a major challenge. This work aims to contribute by providing a first step toward addressing this need.

The impact of the guidelines extends beyond clinical management. They are educational tools for newly diagnosed families, helping them understand the course of the condition, the rationale behind recommended investigations, and the need for surveillance. However, the guidelines' adoption needs to be accompanied by dissemination strategies, so that they are not only published but also actively implemented. Other experiences show that dissemination is rarely enough [166]; thus, active engagement and feedback loops are necessary to maintain clinical relevance. In our case, this process has already taken place: the SYS clinical guidelines have been developed and are available at HSJD, have been published open access to maximise accessibility, and

have been shared with the Asociación Española del Síndrome de Schaaf-Yang (AESYS) to disseminate them among families and caregivers.

Following the clinical and genetic characterisation of SYS, functional analyses of *MAGEL2* are essential to understand disease mechanisms. Our results provide the first direct evidence that *MAGEL2* truncating mutations produce a stable truncated protein, rather than one that is rapidly degraded. This shows that SYS pathophysiology may not be explained only by loss of *MAGEL2*. Instead, the presence of a truncated, yet stable, protein suggests that altered protein function contributes to the disease phenotype. Beyond stability, we also showed that truncated *MAGEL2* proteins exhibit altered subcellular localisation. While WT *MAGEL2* is primarily cytoplasmic, truncated proteins tend to accumulate in the nucleus. This pattern is consistent across multiple truncating variants and cell lines, indicating that nuclear mislocalisation is a general feature of truncated *MAGEL2*. In fact, other studies have later backed our results, showing that the truncated *MAGEL2* protein (p.Gln666Profs*47) mainly accumulates in the nucleus, while WT *MAGEL2* shuttles between the cytoplasm and the nucleus [104]. Understanding the full life cycle and intracellular trafficking of both WT and truncated *MAGEL2* could yield important insights into its many cellular roles. Our findings also raise the question of whether similar mislocalisation occurs in endogenous *Magel2* in animal models. Some of these models carrying truncating *Magel2* mutations exhibit only partial features of the SYS phenotype [77,78]. Investigating whether nuclear accumulation is conserved in rodents could clarify if differences in subcellular localisation contribute to the milder phenotypes.

By stating that truncated *MAGEL2* is not only stable but also mislocalised to the nucleus, these works reframe our understanding of the molecular consequences of *MAGEL2* truncating mutations. For years after its discovery, SYS was described in the literature as a “PWS-like” syndrome [1]. The absence of functional *MAGEL2* in SYS was considered analogous to the loss of expression affecting multiple genes (including *MAGEL2*) in PWS. Under these circumstances, truncating mutations in *MAGEL2* were viewed as null alleles. However, our results directly challenge this view. Indeed, quantification of extracellular A β 1-40 levels and metabolomic studies also revealed different profiles for SYS and PWS samples. This highlights a distinct molecular phenotype in SYS that is not shared with classical PWS, despite both disorders involving loss of WT *MAGEL2* expression. SYS pathophysiology must be considered in terms of both loss of the normal cytoplasmic functions of *MAGEL2* and the potential gain of novel, and possibly toxic, nuclear functions. Instead, in PWS, the absence of *MAGEL2* may

allow compensatory mechanisms or redundancy in endosomal trafficking pathways to maintain near-normal metabolic and trafficking function, masking disruptions in the assays we performed. Nonetheless, the exact differences between complete loss of *MAGEL2*, as in PWS, and expression of a truncated protein, as in SYS, remain unclear particularly regarding its cytoplasmic functions.

The nuclear mislocalisation of truncated *MAGEL2* opens new questions into how this affects the protein's known roles in endosomal transport, circadian regulation, and RNA metabolism. Endosomal recycling is increasingly recognised as critical for neuronal development and function, and defects in this pathway have been implicated in many neurodevelopmental disorders [167]. Retromer dysfunction has been shown to interfere with AMPA receptor recycling, affecting synaptic strength and plasticity [168]. Proper trafficking of presynaptic proteins and neurotransmitter transporters also relies on endosomal systems, and disruptions in this pathway could alter neuroendocrine signalling (reviewed in [169]). In fact, loss of *MAGEL2* has been demonstrated to reduce SG and neuropeptide release [72]. *MAGEL2* is also known to interact with components of the retromer complex and other regulators of endosomal trafficking in the endosomes, playing a role in recycling transmembrane proteins [92,94]. Therefore, mislocalised truncated *MAGEL2* proteins likely lose access to these partners, which results in a loss of function. In fact, it has been shown that the interaction between truncated *MAGEL2* and USP7 is significantly decreased compared to the WT *MAGEL2* protein [104]. Our finding of reduced A β 1-40 secretion in SYS patient-derived cells further supports that endosomal recycling is impaired in SYS. *MAGEL2* also modulates the activity of clock proteins and influences CRY1 ubiquitination, so its truncation and mislocalisation could disrupt those interactions and destabilize circadian rhythms [101,102]. In this line, *Mage12*-KO and PWS models show blunted behavioural rhythms and altered clock output [59,103]. Clinically, sleep disturbances in patients with *MAGEL2* truncations align with these findings, supporting a link between loss of *MAGEL2* function and circadian dysfunction. In our transcriptomic analyses across diver cell types, we also observed downregulation of *SIK1*, a kinase involved in circadian clock resetting, suggesting a novel layer of circadian dysregulation in SYS [170].

At the same time, the mislocalisation of truncated *MAGEL2* could lead to toxic neomorphic effects. It has been suggested that the N-terminal region of *MAGEL2* may play a role in RNA metabolism, especially through associations with components of the m⁶A RNA reader machinery (YTHDF1-3 and TNRC6 proteins) [91]. Truncated *MAGEL2* may therefore sequester YTHDF proteins or disrupt their normal nuclear-cytoplasmic

dynamics, disrupting m⁶A-dependent degradation of snoRNAs and other neurodevelopmental transcripts, and in turn altering gene expression. In fact, studies have suggested that truncated variants exhibit significantly reduced capacity to regulate transcription compared to WT MAGEL2 [105]. Reduced expression of several *SNORD* genes, *MKRN3*, and *SNRPN*, all within the PW critical region, was detected by qPCR and Northern Blot in NPCs from SYS patients. In contrast, in our transcriptomic datasets spanning fibroblasts, iPSCs, and neural cell types, *SNORD* transcripts were not detected due to low expression in both patients and controls, and we observed no significant changes in *SNRPN* or *MKRN3* mRNA. Given that some of the truncating *MAGEL2* mutations studied overlap between our models and those of Heimdörfer *et al.* [104], it is unlikely that mutation-specific effects explain the divergent results. Instead, part of the discrepancies may arise from technical factors affecting the detection of small non-coding RNAs. For instance, bulk mRNA-Seq is generally less sensitive for small non-coding RNAs than targeted qPCR, and RNA extraction methods can also influence recovery. Moreover, the fact that such effects were observed at the progenitor stage and we did not detect them in any of our cell models may point to differences in iPSC lines, culture systems, or stress conditions that were not captured in our experimental settings.

Nuclear retention has also been shown to have toxic effects in other neurological conditions. For example, in dentatorubral-pallidoluysian atrophy (MIM #125370) a neurodegenerative disorder caused by mutations in *ATN1* gene, aberrant proteins misfold and accumulate in the nucleus [171]. These aggregates drive cellular toxicity and are believed to play a central role in the neuronal degeneration observed in the disease. Additionally, mislocalisation of typically cytoplasmic or membrane-associated proteins to the nucleus is a well-established mechanism in cancer progression (reviewed in [172]). For instance, EGFR, usually localised at the plasma membrane, can translocate to the nucleus under pathological conditions, where it promotes cell proliferation and tumor growth [173]. Similarly, EBP50, a cytoplasmic scaffolding protein, has been shown to accumulate in the nucleus during colorectal carcinogenesis, where it activates oncogenic Wnt signalling [174]. Understanding how nuclear mislocalisation of truncated MAGEL2 alters cellular function will be an important focus for SYS studies.

Our work also revealed a link between the degree of nuclear mislocalisation and clinical severity, as variants associated with a severe AMC phenotype showed a more pronounced nuclear retention. This adds a new layer to our understanding of genotype-phenotype correlations in SYS, where a greater degree of nuclear mislocalisation of the truncated protein may directly contribute to a more severe clinical presentation. The

phenotypic variability even in mutations close to each other, may be explained by differences in the resulting amino acid sequence, particularly in the case of frameshift mutations. A frameshift can alter the entire downstream peptide sequence, potentially leading to different protein structures and uncovering or hiding functional domains or interaction sites compared to nonsense mutations at similar positions [175]. These differences can impact the folding, stability, and molecular interactions of the truncated MAGEL2 protein, influencing its cellular function and ultimately affecting the phenotype. Interestingly, as previously mentioned, evidence from animal models also supports this idea that not all truncating variants produce the same degree of functional disruption. In a rat model carrying the p.Ser132Glyfs*728 Magel2 mutation, and in a mouse model harbouring p.Glu564Serfs*130, the full SYS phenotype was not recapitulated, with both models showing different partial symptomatology [77,78]. A parallel can be seen in the dystrophin gene, where Duchenne muscular dystrophy (MIM #310200) patients with nonsense mutations sometimes exhibit a milder disease course than those with frameshift mutations [176]. Our observations highlight the importance of considering not only the presence of a truncating mutation, but also its location and the nature of the resulting protein, when predicting disease severity.

An important area for further investigation is the identification of the mechanism that leads to the mislocalisation of truncated MAGEL2. As the WT protein is predominantly cytoplasmic, one hypothesis is that the truncation may disrupt or eliminate existing nuclear export signals (NES) necessary for its cytoplasmic retention. Inhibition of the export factor CRM1 leads to a significant nuclear accumulation of WT MAGEL2, which may indicate that MAGEL2 normally undergoes dynamic shuttling through NES-dependent, CRM1-mediated export [104]. Currently, no experimentally validated NES motifs have been published for *MAGEL2*. Computational tools can predict potential NESs, but their functional validation through targeted mutagenesis and ICC assays would be crucial to pinpoint the exact sequence responsible for the nuclear shift. In line with this, we conducted additional experiments to explore the role of putative NESs predicted by LocNES [177], generating three plasmids truncated at specific C-terminal regions (**Table 2**). Two of these NESs were also computationally predicted in the study by Heimdörfer *et al.* [104].

Preliminary results from these ongoing experiments in which I participated, show that these truncated MAGEL2 proteins mainly localise to the cytoplasm, similar to the WT form (**Figure 8A**). This suggests that losing these predicted NES motifs alone is not sufficient to cause nuclear accumulation. For future studies, a potentially more precise approach would be to mutagenise the critical residues within the NES motifs rather than truncating the protein, allowing the specific contribution of these sequences to be assessed without disrupting overall protein structure. It would also be of interest to determine the point at which truncated MAGEL2 shifts from a WT cytoplasmic localisation, as seen in the newly generated plasmids, to nuclear accumulation, as observed in the mutations tested in our experiments (**Figure 8B**).

Table 2: Generation of *MAGEL2* truncated plasmids containing predicted nuclear export signal (NES) motifs.

Generated plasmid	NES sequence	NES position
p.Arg1107*	RPKFGLLMVVLSLIF	1107-1121
p.Leu1131*	VREDLIFNFLFKLGL	1127-1141
p.Met1195*	WGPRAFLETSKMLVL	1184-1198

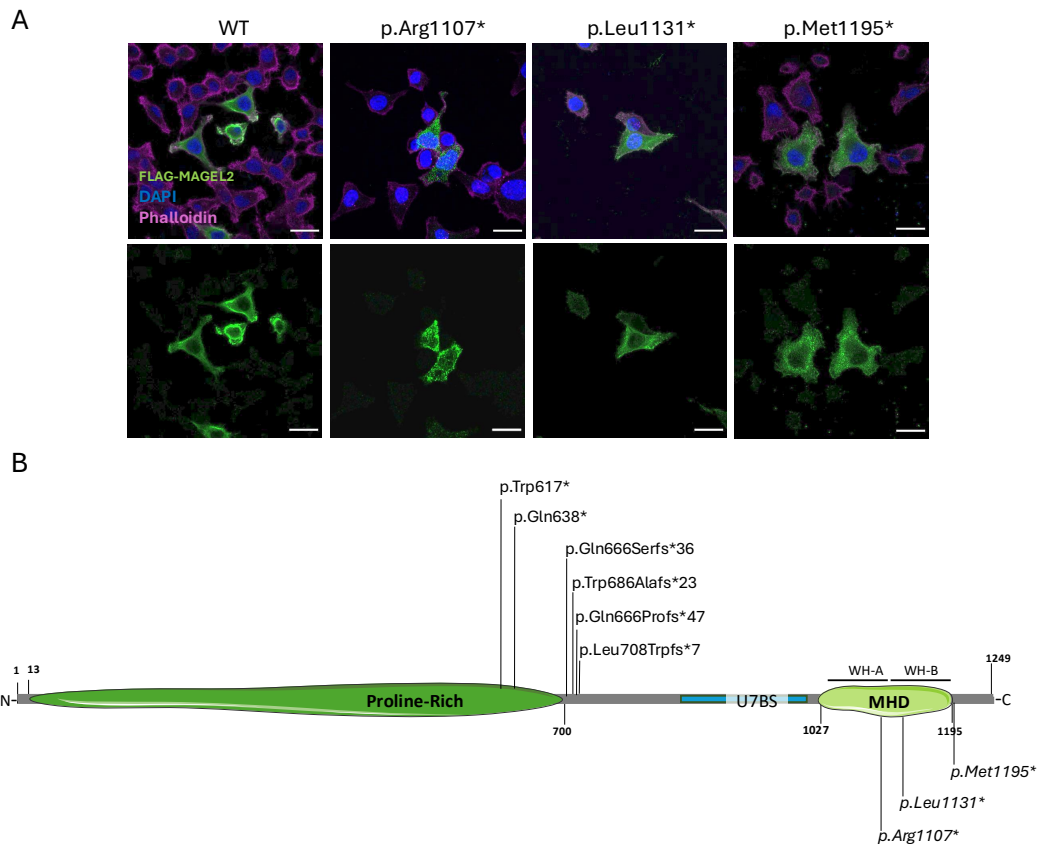


Figure 8: A) Representative immunocytochemistry stainings of HeLa cells transfected with FLAG-tagged *MAGEL2* plasmids truncated at putative NES locations (p.Arg1107*, p.Leu1131*, p.Met1195*). The upper row shows merged images (FLAG-MAGEL2, DAPI, and Phalloidin), while the lower row shows FLAG-MAGEL2 alone, illustrating its predominantly cytoplasmic localization. Nuclei are stained with DAPI (blue), and actin with Phalloidin (magenta). **B)** Schematic representation of *MAGEL2* protein domains and the location of patient-derived and experimental truncating mutations. Patient mutations associated with SYS and tested in our ICC studies are displayed above, while truncations designed to test the function of predicted NESs are shown below in italics. Domains shown include the proline-rich region, the USP7-binding site (U7BS), and the MAGE homology domain (MHD), which contains winged-helix (WH-A, WH-B) subdomains.

Interestingly, several SYS patients carrying truncating mutations near or beyond the predicted NES-containing regions have been reported, including p.Phe1150Trpfs*4, p.Met1195Cysfs*4, and p.Lys1203* [21,36,41]. These patients are mostly well characterised and exhibit a full SYS phenotype. Because the resulting truncated proteins extend beyond the tested NES motifs, it is unclear whether they would still mislocalise to the nucleus. Particularly intriguing is truncation p.Met1195Cysfs*4, where the addition of a short four-amino acid tail, compared with the tested p.Met1195* mutation, might alter localisation and drive nuclear accumulation. To address this, plasmids carrying these

patient-specific mutations should be generated and tested in ICC experiments, which would clarify whether nuclear mislocalisation contributes to their pathogenic effects.

While the aforementioned experiments are still to be replicated, several hypotheses can explain our preliminary result. i) Truncated MAGEL2 might be subject to an increased nuclear import compared to the WT protein, potentially through the exposure of nuclear localisation signal (NLS) motifs. The predicted disordered nature of the N-terminal region may facilitate these structural rearrangements, especially when C-terminal interactions are lost. While computational predictions of NLSs in MAGEL2 are inconsistent, some tools such as NLSExplorer [178] do suggest their presence at the N-terminal end of MAGEL2 (amino acids 4 to 33) [104]. ii) Patient mutations such as p.Gln666Profs*47 and p.Gln638* eliminate approximately 600 amino acids of the C-terminal region, including the entire MHD (amino acids 1027-1195) and U7BS domain. As full-length MAGEL2 interacts with other proteins via the MHD or the U7BS, loss of these domains may impair anchoring to the cytosolic endosomal machinery. Therefore, the nuclear shift may simply be a passive consequence of failed cytoplasmic retention. iii) Since the experiments were done in transfected cell lines, the nuclear retention observed in SYS-associated mutations might reflect differences in protein folding and stability only under overexpression conditions. iv) Another possibility is that the MAGEL2 protein has additional NESs that were missed by prediction tools, since these tools are limited by known consensus sequences and training datasets. Classical leucine-rich NES consensus sequences represent only one class of export signals recognised by the CRM1 export system. MAGEL2 may use non-canonical export sequences other than the established consensus motifs.

Exploring the predicted 3D structure of these truncated *MAGEL2* variants could provide insights into how the truncations affect the overall protein conformation. This may reveal novel interaction surfaces or changes in dynamics that influence their nuclear localisation and pathogenicity. AlphaFold is a computational tool that predicts the 3D structure of proteins based on their amino acid sequence, providing insights into potential folding patterns, functional domains, and interaction surfaces [179]. Predictions for the WT MAGEL2 protein show a clear difference in confidence scores across its length. As illustrated in **Figure 9**, the per-residue confidence score is notably high for the C-terminal MHD, indicating a well-predicted and likely structured region. In contrast, the N-terminal end of the protein consistently shows very low scores. This, along with other experimental findings, indicates that the N-terminal region of MAGEL2 is likely highly unstructured [91]. Thus, truncating the C-terminal end leaves behind a protein mostly

made up of this flexible N-terminal domain, which presents several challenges. Disordered proteins are difficult to model because they do not adopt a single fixed conformation (reviewed in [180]). Their flexibility also makes it harder to predict how different truncated proteins might interact with other cellular partners, as binding surfaces in disordered regions can be transient and context dependent. The unstructured nature of truncated MAGEL2 could itself contribute to its altered localisation and potential neomorphic effects. For instance, highly disordered regions can sometimes facilitate interactions with components of the nuclear import machinery or allow the protein to navigate through nuclear pores more easily without requiring specific folded structures [181]. It has been shown that while proteins with well-defined 3D structures are often associated with enzymes or integral membrane proteins, intrinsically disordered proteins predominantly participate in processes related to signalling and regulation, such as transcription control, or mRNA processing. This is consistent with MAGEL2 known roles, and it may also add a layer of interpretation to potential altered mechanisms of truncated MAGEL2 in transcriptomic regulation.

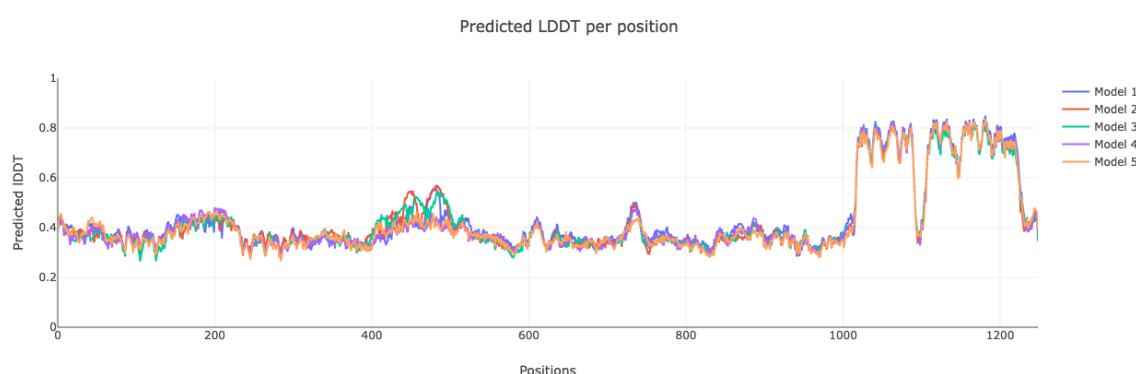


Figure 9: Predicted Local Distance Difference Test (pLDDT) for WT MAGEL2 3D structure. A per-residue confidence score ranging from 0 to 1 where higher values (typically >0.9) indicate high confidence in local atomic placement, suggesting accurate backbone and sidechain positions. Obtained using AlphaFold 3 [179].

The abnormal nuclear localisation of truncated MAGEL2, along with the correlation with clinical severity observed specifically in AMC variants, provide strong evidence for a neomorphic gain-of-function mechanism in SYS. However, one important limitation of our approach is the reliance on heterologous overexpression systems, which can introduce artifacts such as protein misfolding, aberrant localisation, or disruption of endogenous protein-partner balances [182,183]. Additionally, immortalised cell lines, which are often used in such systems, can carry alterations in cell cycle regulation, apoptosis pathways, and DNA damage responses, potentially modifying cellular

behaviour in ways that may not mirror physiological conditions (reviewed in [184]). These effects may generate artificial phenotypes that do not completely capture disease mechanisms. Therefore, to validate our findings in a more physiologically relevant context, our next goal was to study the subcellular localisation of endogenous *MAGEL2* in patient-derived cells. Given the intrinsically disordered nature of the N-terminus, no commercially available antibodies specifically target the *MAGEL2* N-terminal region, while those against the C-terminus lack specificity.

To overcome this issue, we used CRISPR/Cas9-based gene editing to introduce a hemagglutinin (HA) tag at the N-terminal end of endogenous *MAGEL2*, in both a control iPSC line and a patient iPSC line carrying a *MAGEL2* truncating mutation (p.Gln666Profs*47). We successfully generated and validated the tagged iPSC lines, which harbored the tag in-frame with endogenous *MAGEL2*, and expressed the corresponding pluripotency markers. Subsequently, we performed ICC and WB experiments on these iPSCs after differentiating them into iNs, which showed the highest *MAGEL2* expression levels among the cell types we analysed. Despite extensive troubleshooting, we were unable to obtain a robust and specific signal for *MAGEL2*. We tested various fixation protocols and antibody concentrations, but the primary HA antibody is highly non-specific in both tagged and untagged control cells, preventing a clear assessment of the protein's natural subcellular localisation. Although this antibody has previously been used to detect HA-tagged *MAGEL2* in heterologous systems, the much lower endogenous expression in iNs compared to plasmid-based overexpression likely explains these difficulties. We also explored the use of a genetically encoded probe for imaging HA-tagged proteins *in vivo* [185], but preliminary experiments were unsuccessful, probably due to limited transfection efficiency. To address this, we are now considering alternative transfection methods beyond Lipofectamine.

Several other strategies could also be explored in the future to overcome the experimental limitations. First, using alternative tagging approaches, such as a larger, more robust tag like GFP may help. GFP would additionally allow live imaging of both truncated and WT *MAGEL2*, helping unveil their dynamic shuttling between the cytoplasm and the nucleus in real-time [104]. Although a longer tag could affect protein function, its better signal-to-noise ratio might compensate for this issue in localisation studies. Alternatively, exploring multi-epitope tags (3xHA, 3xFLAG) could increase the chances of specific detection. Another strategy could be the use of proximity-dependent biotin identification (BioID), fused to the *MAGEL2* protein. This method could allow for indirect profiling of *MAGEL2*'s subcellular localisation by including nuclear or cytoplasmic

interactors through pulldown and mass spectrometry (MS). Importantly, BioID has previously been used in overexpression systems to identify proteins in proximity to MAGEL2 through transient plasmid transfection [91]. However, these studies have not yet been extended to endogenous MAGEL2. A complementary method to proximity labelling is immunoprecipitation followed by MS using the HA epitope. Even if HA antibody specificity is not ideal for imaging, it may still be effective for biochemical pulldown. These experiments could confirm the presence of the HA-tagged protein and determine its subcellular enrichment through fractioning before immunoprecipitation. Additionally, identifying co-precipitating partners could shed light on MAGEL2's nuclear functions and interaction networks. Similar approaches were taken by Hao *et al.* using heterologous overexpression systems, which revealed important MAGEL2 interactors [94]. Applying such methods in patient-derived iPSC or neuronal models would allow the study of interaction partners at endogenous expression levels in a human neuronal context, potentially uncovering disease-relevant networks that may not be detected in heterologous systems. Another antibody-based approach that may be more sensitive is the use of proximity ligation assays. This method amplifies the signal only when two antibodies recognise nearby epitopes, making it suitable for detecting protein-protein interactions or colocalisation events, such as MAGEL2's interaction with nuclear importins or nuclear pore components. Even if the HA antibody has some background noise, the need for proximity between antibody pairs may enable specific detection of nuclear interactions.

As seen above, a key step in our research involved transitioning to patient-derived cell models. At the start of the project, we considered working with fibroblasts obtained from SYS patients and their families thanks to the close collaboration with patient associations. Fibroblasts offer an accessible way to study the cellular consequences of genetic mutations, with different studies having proven their value for investigating neurological diseases [186]. They are easy to obtain from skin biopsies and can provide important insights into broad cellular processes like gene expression regulation, metabolic pathways, and protein trafficking. However, given the low endogenous expression levels of *MAGEL2* in this cell type, and their limited relevance to the neurodevelopmental features of SYS, during the course of this thesis we decided to start working with iPSC-derived neural models. To this end, our group generated four patient and two control iPSC lines from fibroblasts using an mRNA-based reprogramming method. Following successful reprogramming, we carried out the characterisation of the

iPSC lines (see **Annex**). Additional control iPSC lines were incorporated from the Coriell Institute and the BNLC, increasing the size and diversity of the control group.

The generation of iPSC-derived neural models represented the next steps. These systems enable the study of *MAGEL2* function and the impact of its truncation in cell types potentially directly involved in the syndrome's neurodevelopmental features. The analyses in iNs and iAs, allowed us to detect both cell type-specific changes and convergent molecular signatures across cell types, offering new insights into disease pathways. To achieve this, we used TF-based programming methods to generate inhibitory GABAergic iNs and iAs [155,158]. These methods help produce more homogeneous and reproducible populations, minimising variability and enabling more accurate interpretation of omics data. The rationale to generate inhibitory iNs stems from both the expression profile of *MAGEL2* and the clinical phenotype of SYS. Many SYS-related symptoms, such as sleep disturbances, feeding difficulties, and hormonal imbalances, are linked to hypothalamic dysfunction. Since hypothalamic circuits are primarily composed of inhibitory neurons, modelling this cell type may provide a more faithful representation of the disease-relevant cellular environment. Supporting this, single-cell mRNA sequencing from both cortical and ventral organoids generated by our collaborators showed that *MAGEL2* expression was enriched in inhibitory neuronal subtypes (personal communication from Dr. Isaac Canals). Data from the Human Protein Atlas also show that *MAGEL2* expression is higher in inhibitory neurons compared to excitatory ones [187]. In addition, several studies in *Mage12*-KO mouse models have shown abnormalities in GABAergic signalling [51], reinforcing the idea that inhibitory neuronal populations are particularly vulnerable to *MAGEL2* loss or truncation. In parallel, we also generated iAs, which are crucial not only for their active role in CNS function but also because they support neuronal survival and maturation *in vitro* [134]. Including astrocytes helps create a more physiologically relevant environment, enhancing neuron health and longevity during experiments. To our knowledge, only one study has directly examined astrocytes in the context of SYS, and it was performed in a mouse model rather than in human cells [66]. This highlights the novelty of investigating human iPSC-derived astrocytes in SYS and their potential contribution to disease-relevant phenotypes.

Additionally, although not directly addressed in this thesis, our research group has used the same SYS patient and control-derived iPSC lines to generate 3D brain organoids. Both 2D and 3D models provide complementary insights. The 2D cultures are useful for pinpointing cell-intrinsic molecular alterations and for conducting high-resolution imaging

or electrophysiology experiments requiring less specialised handling and reducing experimental costs. Moreover, 2D cultures, particularly TF-based protocols, can sometimes offer clearer insights into specific aspects of neuronal maturation and synaptic function in a simplified system before escalating to the complexity of organoids. In contrast, as developmental syndromes like SYS are often rooted in early brain patterning defects, organoids better access these early stages and help reveal developmental delays, structural abnormalities, or dysregulated neurogenesis [188]. By combining both systems, it becomes possible to bridge molecular findings with developmental phenotypes, enhancing the value of the results.

Across this thesis we examined SYS transcriptomes in multiple human-derived models, including primary fibroblasts, iPSCs, and iPSC-derived neurons and astrocytes, in order to capture both peripheral and neural aspects of the disease. mRNA sequencing of fibroblasts from three SYS patients and six controls identified more than a hundred DEGs, despite fibroblasts not being a tissue with high *MAGEL2* expression. In fact, *MAGEL2* expression was not even detected in our transcriptomic dataset of fibroblasts. When some of these fibroblast lines were reprogrammed into iPSCs, transcriptomic profiling was repeated in three SYS patients and seven controls. These data confirmed that the parental imprinting pattern was preserved after reprogramming, ensuring that disease-relevant *MAGEL2* expression was maintained in our iPSC models. However, only three prevalent DEGs between patients and controls were found, and no significant pathway enrichments. This may reflect that pluripotent cells, by nature, share a highly similar core transcriptional program that may mask subtle disease-specific changes. Another possibility is that *MAGEL2*-related effects require specific developmental cues or environmental triggers, which are absent in undifferentiated iPSCs. Furthermore, the reprogramming process itself may introduce transcriptional and epigenetic alterations that may overshadow subtle changes caused by *MAGEL2* truncations.

After iPSC analysis, we extended our mRNA-Seq to iPSC-derived neurons and astrocytes. Neural cells are more mature and specialised, and potentially more relevant to SYS pathophysiology. Indeed, *MAGEL2* expression was higher in both iNs and iAs compared to iPSCs (**Figure 10**). The number of dysregulated genes was similar between astrocytes and neurons, but still lower than in fibroblasts. This difference may reflect both cell-intrinsic biology and technical considerations. Neurons and astrocytes are post-mitotic and highly specialized, so their transcription is relatively stable. As a result, SYS-related changes tend to affect fewer genes, usually within specific pathways where even

small shifts can have big functional effects. iNs additionally go through a differentiation protocol that may introduce variability between samples and mask subtle disease-related changes. Fibroblasts, in contrast, are minimally manipulated, and less specialized. Their transcription is more flexible and responsive to stress or metabolic imbalance, so disease effects can be seen across many processes, leading to a broader set of detectable changes. It should also be noted that fibroblast data were obtained using an older RNA-Seq method, which may contribute additional variability compared to the current iN and iA analyses.

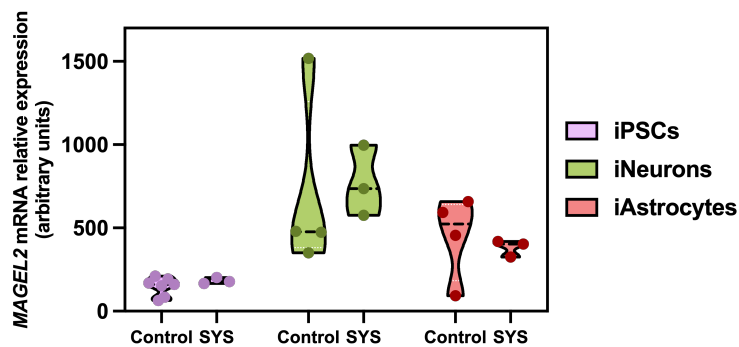


Figure 10: *MAGEL2* mRNA relative expression across SYS patient-derived and control iPSCs, iNeurons and iAstrocytes. Plots show median, and individual data points. No significant differences were detected between SYS patients and controls.

MAGEL2 expression in brain organoids is higher than in both iPSCs and iNs (unpublished data from our group included in the future PhD thesis of Aina Prat-Planas). More specifically, expression is lowest in iPSCs, increases by differentiation day 30 in brain organoids, and then progressively declines through day 90 of differentiation. This temporal pattern suggests that *MAGEL2* plays a more prominent role during early neurodevelopmental stages, potentially supporting processes such as progenitor maturation, cell fate specification, and the initial establishment of neuronal networks, while becoming less critical as cells reach a more stable, mature state. Therefore, our iNs may bypass some of these early developmental steps by directly reprogramming iPSCs into inhibitory neurons using a TF-based method. While this strategy provides robust, homogeneous neuronal populations suited for bulk omic analyses, it may underrepresent the developmental windows where *MAGEL2* expression is naturally highest. This was one consideration in our decision to also employ a small molecule-based differentiation strategy for calcium imaging studies, among other reasons that will be discussed later.

A central goal of this thesis was to identify robust and clinically relevant biomarkers for SYS across different cell types. Biomarkers play a particularly important role in the field of rare diseases, where clinical variability and small patient numbers often complicate timely diagnosis. In many cases, they can help shorten the diagnostic journey, allow for earlier clinical intervention, and enable more precise patient stratification for new treatments. In clinical trials, validated biomarkers can be used as early indicators of treatment response and help accelerate the evaluation of new therapies. Beyond their diagnostic and prognostic value, biomarkers can also provide information about disease mechanisms, pointing to more targeted therapeutic strategies. For these reasons, identifying and validating reliable biomarkers remains a priority in rare disease research.

mRNA sequencing of fibroblasts from SYS patients identified *HOTAIR*, a well-known lncRNA involved in chromatin remodelling and transcriptional silencing, as the most significantly upregulated gene [189]. Besides its role in chromatin dynamics, research also shows that *HOTAIR* can enhance the PI3K/AKT/mTOR signalling pathway [190], which was intriguing as previous reports have noted mTOR pathway dysregulation in SYS-derived fibroblasts and iNs [67]. However, *HOTAIR* was not detected in the transcriptomes of our neural models, suggesting that this lncRNA's regulatory influence might be restricted to certain cell types, or expressed at levels below detection in differentiated neurons. LncRNAs have highly context-dependent expression patterns and are often lowly expressed, and strongly dependent on cell identity and state [191]. Additionally, none of our mRNA-Seq datasets shows higher mTOR levels. This discrepancy may reflect differences in experimental conditions, including nutrient availability, temperature, or culture stress, which are known to influence basal mTOR activity and could mask subtle alterations [192]. In iPSC-derived neurons, the differences may also be caused by neuronal subtype. Whereas previous studies focused on excitatory neurons, our cultures consist of inhibitory neurons, in which *MAGEL2*-dependent mTOR regulation may follow a distinct pattern [193].

Gene enrichment analysis showed that many of the DEGs identified in SYS patient-derived fibroblasts play roles in important cellular processes, including mitosis-related Reactome categories such as 'Resolution of Sister Chromatid Cohesion' and 'Mitotic Spindle Checkpoint,' as well as developmental and neurological processes. Aberrant timing of cell cycle exit is increasingly recognised as a pathogenic mechanism in both neurodevelopmental and neurodegenerative disorders [194,195]. As *MAGEL2* is not established as a primary cell cycle regulator, the role of mitotic mechanisms was

particularly intriguing. For that reason, we decided to further explore this pathway and perform flow cytometry experiments in iPSCs. Cells were stained for Anti-Histone H3 (phospho S10), a well-established marker for mitosis, and propidium iodide to assess DNA content and cell cycle distribution. Before the analysis, cells were treated with nocodazole to synchronise the cell cycle, allowing for a more precise assessment of cells in mitosis [196]. Despite the upregulation of mitosis-related genes in the fibroblast transcriptomic data, our flow cytometry results in iPSCs did not reveal significant differences in overall mitotic rates between control and patient-derived lines (data not shown). Additionally, our transcriptomic profiling of SYS-patient derived iPSCs showed no significant gene expression differences and no pathway enrichments regarding mitotic processes. This suggested that the cell cycle-related transcriptomic signature in SYS fibroblasts is not a universal feature across all cell types but may emerge in more differentiated or tissue-specific contexts. However, since iPSCs were differentiated into post-mitotic neuronal lineages, any effects on mitosis-related pathways are likely to be subtle and not entirely relevant in this context.

It is important to highlight that pathway enrichment relies on statistical inference and has not yet been functionally validated in our models, meaning that the observed associations should be interpreted with caution until supported by experimental evidence. Within this context, several hypotheses could explain the seemingly inconsistent findings. Firstly, the transcriptomic analysis was done on fibroblasts, where *MAGEL2* expression is very low, while the flow cytometry was conducted on iPSCs. Thus, mitotic dysregulation observed at the gene expression level in fibroblasts may not manifest as altered cell division rates in iPSCs, which have tightly regulated cell cycles for maintaining pluripotency. Second, Reactome enrichment analysis reflects pathway trends that may come from subtle shifts in gene expression. Compensatory mechanisms may maintain overall cell cycle timing despite altered expression of mitosis-related genes. Third, *MAGEL2* truncation might affect specific aspects of mitosis such as mitotic fidelity or spindle orientation that could require more sensitive assays to unmask. Finally, post-transcriptional regulation or protein degradation mechanisms could buffer the effects of altered transcript levels, resulting in normal mitotic behaviour despite transcriptional changes.

In fibroblasts, genes involved in neuronal differentiation, synapse formation, and axon guidance were also upregulated, including *SLITRK4* or *PTPRD* [197,198]. This suggests aberrant activation of neurodevelopmental programs even in non-neuronal cells.

Recognising the potential significance of these findings, we next asked whether similar pathways might also be disrupted in neuronal contexts. The majority of specific gene expression changes found in fibroblasts did not replicate in inhibitory iNs or iAs. Genes such as *SLITRK4*, *CLDN1*, and *CRLF1*, which were robustly dysregulated in fibroblasts, showed no significant changes in expression in our neural datasets. In addition, a subset of fibroblast DEGs, including *ISL2*, *INMT*, or *APOD*, were not detected, possibly due to their very low or inexistent expression in the brain. Despite this lack of overlap at the single-gene level, our neuronal models did reveal some shared disruptions at the pathway level. In SYS patient-derived inhibitory iNs, transcriptomic profiling showed widespread downregulation of genes involved in aspects of neuronal development, such as synaptic organisation, neurotransmission, and neuronal projection formation. A particularly interesting contributor to these enriched pathways is *NNAT*, an imprinted hypothalamic gene that, as well as participating in neurodevelopment, is downregulated in appetite-regulating nuclei during fasting or leptin deficiency [199]. Likewise, downregulation of other genes influencing neuronal excitability, day-night firing rhythms, and motor neuron impairments, highlights potential mechanisms overlapping with *MAGEL2*-related syndromes [200,201]. Supporting the relevance of these findings, transcriptomic analyses in ASD and other NDDs have also revealed downregulation of genes involved in synapse formation, neuronal projections, and neurotransmitter transport, suggesting that similar molecular mechanisms may contribute to overlapping phenotypes in SYS [202,203].

The transcriptomic profile of fibroblasts also revealed dysregulation of genes controlling limb identity and spatial patterning. For example, genes like *PITX1*, *HOXC10*, and *HOXC11* were upregulated in SYS patient-derived fibroblasts, while *TBX5*, important for forelimb and heart development, was significantly downregulated. This imbalance indicates that patterning programs may be disrupted in SYS, which may explain the limb contractures, skeletal asymmetries, and occasional heart issues seen in SYS. Further supporting this interpretation, we also noted the abnormal upregulation of several developmental TFs, including homeobox (*HOX*) genes, in SYS patient-derived inhibitory iNs. These findings are not unique to SYS. Dysregulation of *HOX* genes is associated with a spectrum of phenotypes, reflecting their essential role in development [204,205]. Therefore, the upregulation of such genes points to a failure to repress early regional identity programs, potentially interfering with the correct spatial and functional differentiation of neurons discussed previously. In the neural context, sustained

expression of early *HOX* determinants could impair neuronal subtype specification, axon pathfinding, and connectivity [206].

In addition to neurodevelopmental pathways, another feature of the transcriptional profile of SYS patient-derived iNs was the persistent upregulation of genes related to the ECM. This signature was seen specifically in our neural models and was not enriched in fibroblasts, suggesting that ECM-related dysregulation may be a specific feature of neuronal cells. The ECM plays a central role in neural development by modulating cell adhesion, migration or synaptic density and plasticity [207,208]. ECM gene expression is characteristically high during early neurogenesis supporting axon guidance or synaptogenesis; but stabilises as neurons mature, facilitating synaptic pruning and plasticity. In SYS patient-derived iNs, however, ECM components such as collagens, or proteoglycans were persistently upregulated. This continued expression may not only create a restrictive extracellular environment that impairs synaptic refinement and circuit formation but could also represent a compensatory response to defective vesicle trafficking along the axon [209,210]. Such dysregulation may therefore impact the development of functional neural networks.

SYS iAs also showed upregulation of ECM components, particularly glycoproteins and proteoglycans, accompanied by reduced expression of ECM-modifying enzymes like *MMP9*. However, it is important to note that it is not yet known whether this represents a compensatory response or contributes directly to functional deficits. When astrocytes enter a reactive state, they markedly increase the production of ECM proteoglycans such as neurocan and brevican [211]. Thus, the ECM upregulation observed in SYS iAs at the transcript level may reflect a partial shift toward a reactive-like phenotype, thought to contribute to glial scar formation, and restrict structural plasticity. Excessive or aberrant astrocyte-produced ECM could also affect inhibitory synapse formation and contribute to the deficits indicated by the neuronal transcriptome [212,213]. This hypothesis may be supported by our preliminary calcium imaging data, which show altered network responses to GABAergic blockade despite preserved baseline activity. Reinforcing the relevance of ECM dysregulation in neurodevelopmental contexts, mRNA-Seq analyses in ASD or schizophrenia cases have similarly revealed upregulation of ECM-related genes, proving that such alterations can impair neurogenesis and neuronal connectivity [214,215].

This shift toward ECM gene expression may also have implications beyond the CNS. Aberrant ECM deposition is a well-documented contributor to musculoskeletal abnormalities in genetic disorders [216,217]. Therefore, the ECM upregulation seen in SYS may not only impair synaptic organisation but may also contribute to manifestations such as congenital joint contractures, hypotonia, and reduced bone mineral density, explaining the co-occurrence of neurological and musculoskeletal phenotypes in SYS. Moreover, as temporal distribution of ECM molecules is tightly regulated [218], failure to downregulate such transcriptional programs at the appropriate time could indicate a broader mistiming of differentiation programs, which may be mechanistically linked to the dysregulation of patterning and developmental networks observed in SYS cells. Adding to this understanding, recent studies highlight that some *HOX* genes can have a direct regulatory influence on ECM expression, governing the spatial and temporal expression necessary for proper neuronal crest migration [219].

Taken together, our data suggest the possibility that *MAGEL2* truncation in SYS neural cell types affects the transcriptional programs linked to terminal synapse maturation, although further validation is required. This impairment appears to involve continued activation of early patterning genes and sustained ECM production, ultimately affecting the formation of a permissive extracellular environment and the stabilisation of synaptic identity. Other transcriptomic studies in PWS models have consistently highlighted downregulation of pathways linked to neuronal function, including sodium and chloride channels, calcium signalling, and multiple categories related to synaptic organisation, and transmission [55,220,221]. Moreover, loss of *MAGEL2* has been shown to reduce neuropeptide release [72]. However, to our knowledge, no reports in PWS have described an upregulation of ECM-related pathways. In fact, some studies have found the opposite trend, pointing toward enhanced ECM remodelling rather than deposition [222]. Moreover, no published evidence supports dysregulation of *HOX* genes or other canonical developmental regulators in *MAGEL2*-associated contexts. This may suggest that while the loss of *MAGEL2* as in PWS may drive the neuronal pathway downregulation, the sustained ECM production and developmental gene activation we see in SYS could be a distinctive consequence of truncated *MAGEL2*, potentially contributing to more severe symptoms. Studies have suggested that while WT *MAGEL2* is involved in transcriptional regulation, truncated variants exhibit impaired capacity to regulate transcription [104]. These findings, together with the nuclear mislocalisation of truncated *MAGEL2* proteins, could be behind the inappropriate activation of embryonic

patterning genes and sustained ECM production. Furthermore, MAGEL2's involvement in endosomal recycling, particularly through the retromer complex, may be crucial for the proper trafficking of ECM components. Given that ECM proteins are large, heavily modified, and energetically costly to produce, efficient trafficking and recycling are vital to maintain cellular homeostasis. Disruption in MAGEL2 could result in the aberrant distribution of ECM components, potentially contributing to the gene dysregulation observed in SYS.

While transcriptomic differences were detected in iNs, proteomic changes were milder. This discordance between RNA and protein levels is not uncommon in neural cells due to post-transcriptional regulation [223]. However, in SYS, it may reflect an additional mechanism. As previously mentioned, it was observed that while WT *MAGEL2* significantly influences transcription regulation, when truncated, its capacity for transcription regulation is reduced [104], which could amplify the observed transcriptional differences. It is also important to note that our proteomics analyses focused on intracellular proteins; secreted proteins were not measured. Given MAGEL2's role in retrograde transport, its dysfunction may impair proper localisation or turnover of specific proteins rather than their abundance. Finally, technical limitations of proteomics may underlie the milder observed differences. While MS-based proteomics is a valuable tool for broad protein profiling and hypothesis generation, its depth of coverage remains lower than that of RNA sequencing. Proteins with low abundance, membrane localisation, or complex post-translational modifications are often missed or under-quantified [224].

Besides discussing pathway enrichment changes, it is also important to highlight a subset of genes consistently dysregulated across all SYS-derived neural cell types. Several DEGs emerged as consistently altered across iNs and iAs, including genes such as *NNAT*, *SIK1*, or *PAX8-AS1*. Furthermore, preliminary unpublished data shows that these same genes are also dysregulated in SYS-derived brain organoids generated by our group, highlighting their potential as stable molecular markers of *MAGEL2* dysfunction in neural cell types (unpublished data from our group included in the future PhD thesis of Aina Prat-Planas). Such reproducibility is rare in transcriptomic studies, where cell-type and differentiation-stage effects often dominate variability. *NNAT* has already been mentioned earlier in the discussion, and its consistent downregulation may contribute to impaired synaptic maturation in SYS [225]. *SIK1* encodes a kinase involved in circadian rhythm synchronisation, which is particularly notable given the frequency of sleep disturbances in SYS [170], a phenotype also linked to MAGEL2 function in

circadian regulation [102,103]. *PAX8-AS1* has been implicated in apoptosis regulation and linked to diseases such as osteoporosis and various cancers [226,227]. Though detailed functional studies of this gene in neural contexts are not yet available, the fact that SYS studies suggest that the N-terminal domain of MAGEL2 may interact with nuclear RNA-binding proteins involved in RNA metabolism might point to a mechanism in which truncated MAGEL2 disrupts the regulation of non-coding RNAs like *PAX8-AS1* [91]. The persistence of these changes across diverse neural systems suggests they reflect core aspects of SYS pathophysiology. These genes could be used as molecular readouts in SYS research. Future work should assess their value as biomarkers, their responsiveness to therapies, and their potential in functional rescue.

In addition to studying gene expression, we also performed metabolomic analysis to explore specific metabolic changes in SYS patient-derived fibroblasts. Metabolomics offers a functional view of cellular state, reflecting the interaction between genetic, transcriptional, and environmental influences. As a result of our analysis, we observed two prominent metabolic signatures: a significant decrease in intracellular glutamine levels and increases in various organic acids. Intracellular metabolomics has not been replicated in 2D neuronal cultures, instead these analyses are ongoing in 3D brain organoids.

The lower glutamine levels seen in fibroblasts derived from SYS patients may result from a combination of impaired uptake, altered use, or reduced synthesis. Truncation of MAGEL2, leading to loss of its cytoplasmic function, may impair retromer function, and trafficking or recycling of glutamine transporters may be affected [228]. This would reduce cellular uptake of glutamine from the extracellular environment, leading to its depletion inside cells. Our transcriptomic and proteomic analyses did not reveal significant differences in the expression levels of glutamine transporter genes across fibroblasts, iPSCs, or CNS cell types. This suggests that the overall abundance of these proteins is not altered. Instead, the functional consequences of MAGEL2 truncation may manifest mainly at the level of intracellular trafficking, leading to reduced surface availability of glutamine transporters despite unchanged transcript abundance and total protein levels.

An alternative or additional mechanism for lower glutamine levels may involve higher demand for glutamine. The upregulation of *HOTAIR* in our fibroblasts from SYS patients suggests a possible activation of anabolic programs through mTOR signalling [67,190].

While, as previously mentioned, none of our mRNA-Seq datasets showed increased expression of mTOR pathway genes, this does not exclude post-transcriptional or activity-based regulation. Activation of mTORC1 promotes protein and nucleotide synthesis, both of which require glutamine as a substrate or nitrogen donor [229,230]. In this context, intracellular glutamine may become limiting even if glutamate levels stay the same, possibly due to homeostatic regulation or replenishment through other pathways.

HOTAIR has also been found to influence the expression of GLUT1 (*SLC2A1*), a key protein for glucose uptake [231]. In our data, *SLC2A1* mRNA levels were not significantly altered in fibroblasts or CNS cell types, and later proteomic analysis in iNs confirmed no major change in total GLUT1 protein abundance. Again, even in the absence of expression changes, MAGEL2 truncation could, in theory, impair GLUT1 trafficking and reduce its presence at the plasma membrane. In fact, defects in GLUT1 trafficking have been described in other contexts of endosomal dysfunction, where its impaired plasma membrane delivery reduces glucose uptake despite unaltered protein abundance [232,233]. Further work using ICC to assess GLUT1 distribution, combined with glucose uptake assays, would help determine the actual contribution of GLUT1 trafficking to metabolic changes in SYS.

During metabolic or oxidative stress, glutamine is a substrate for both energy metabolism and antioxidant defence. In our mRNA-Seq data of SYS patient-derived fibroblasts, we observed upregulation of *ME1* (malic enzyme 1). ME1 generates NADPH by converting malate to pyruvate, and NADPH is essential for recycling glutathione, the cell's main antioxidant [234]. Therefore, in response to oxidative stress, fibroblasts may upregulate *ME1*, and to supply sufficient substrate for this pathway, glutamine may be increasingly funnelled into the TCA cycle. Because glutamate is rapidly recycled and maintained within a buffered pool, its steady-state levels may not change, even though glutamine is consumed at a higher rate. *ME1* upregulation was not observed in our transcriptomic datasets from iNs and iAs, cell types with distinct metabolic demands, redox environments, and glutamine utilisation pathways compared to fibroblasts. Such differences could reflect cell type-specific strategies for maintaining redox homeostasis, with neurons and astrocytes possibly relying more on alternative NADPH-generating enzymes or antioxidant systems.

Because MAGEL2 is a component of the retromer-associated MUST complex and contributes to endosomal recycling of membrane proteins, we investigated if SYS cell

lines presented alterations in pathways regulated by retromer function. One such pathway involves amyloid precursor protein (APP) trafficking, which directly influences the secretion of A β peptides. APP is known to play key roles in neuronal calcium homeostasis and axonal integrity [235,236]. Because A β peptides are widely studied in the context of Alzheimer's disease (AD), well-established, sensitive assays are available to quantify their levels in cellular and extracellular compartments. Using these tools, we consistently found a reduction of secreted A β 1-40 peptides in SYS patient-derived cell lines, first demonstrated in fibroblasts and then replicated in neural co-cultures. This decrease across distinct cell types strongly supports secreted A β 1-40 as a potential biomarker for SYS.

Our findings of reduced A β 1-40 secretion in SYS differ from some reports linking retromer dysfunction to increased amyloidogenic processing. Bhalla *et al.* demonstrated that VPS35 deficiency in hippocampal neurons led to elevated extracellular levels of both A β 1-40 and A β 1-42, accompanied by increased colocalisation of APP with BACE1 and enlarged early endosomes [237]. Similar increases in A β production upon retromer disruption were also observed in HeLa cells with endogenous APP expression [238]. In contrast, studies using HEK293 cells overexpressing APP695, reported a reduction in A β 1-40 secretion upon VPS35 knockdown, aligning more closely with our results [239,240]. Such differences across studies may reflect not only cell type-specific variations in APP abundance, trafficking, and endosomal composition, but also methodological differences in experimental models, APP expression levels, and detection techniques.

In contrast to the previously mentioned studies, our model introduces the presence of truncated MAGEL2 as an added factor. MAGEL2 is not a core retromer component but a regulator of its function through ubiquitin-dependent cargo selection. Therefore, MAGEL2 truncation may affect specific aspects of trafficking without fully recapitulating classical retromer deficiency phenotypes. We propose that truncation of MAGEL2 compromises the retromer pathway through loss of its interaction with USP7, and TRIM27; impairing cargo sorting efficiency without completely destabilising the retromer. In fact, it has already been suggested that MAGEL2 association with the retromer might persist even when truncated, but displaying compromised MUST complex assembly [104]. As a result, ubiquitination events normally mediated by USP7/TRIM27 may be lost, leading to inefficient trafficking of APP to compartments where amyloidogenic cleavage occurs.

The fact that in our studies, neither APP nor secretase levels were significantly altered in our proteomic and transcriptomic datasets, further supports post-translational or trafficking-based mechanisms likely causing the reduced levels of secreted A β 1-40. APP undergoes two alternative proteolytic pathways, with intracellular trafficking influencing which predominates (**Figure 11A**). In the amyloidogenic pathway (**Figure 11B**), after APP is internalised from the cell surface, it traffics through early and recycling endosomes, where it is initially cleaved by BACE1 generating a soluble sAPP β fragment and a longer membrane-embedded C-terminal fragment (C99). γ -secretases then process C99 to produce the A β peptides and an APP intracellular domain (AICD) [241]. APPs that recycle rapidly through early endosomes are more likely to encounter β -secretases (BACE1), favouring amyloidogenic processing. On the other hand, directing APP to the cell surface or to recycling pathways can bias toward non-amyloidogenic cleavage. In this line, it would be informative to measure levels of products of APP processing through the non-amyloidogenic pathway, where an increase would suggest a shift toward non-amyloidogenic processing.

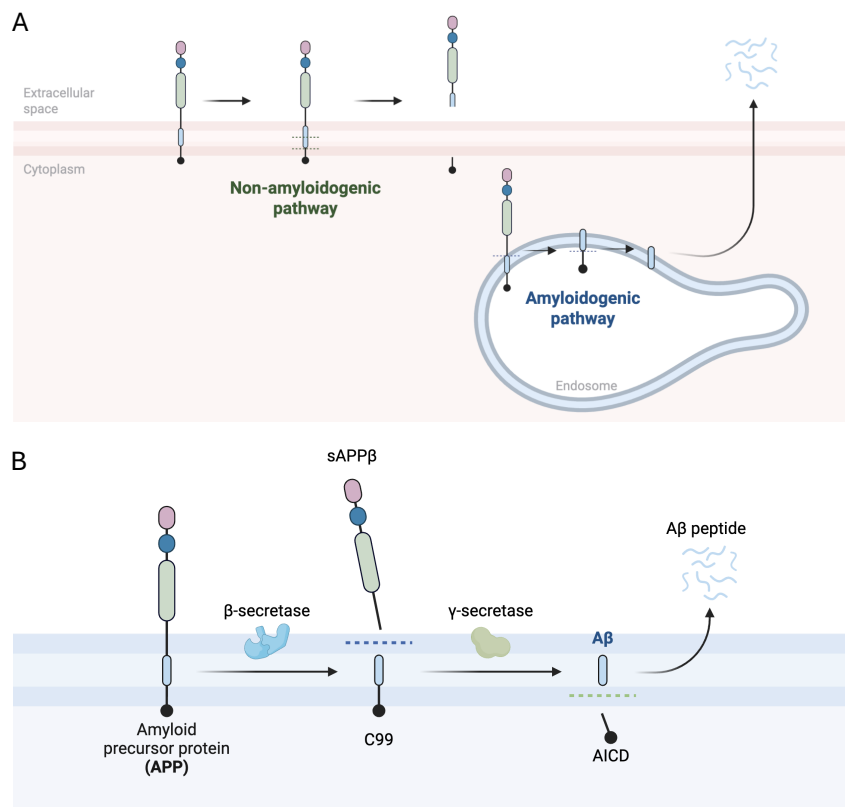


Figure 11: Processing of Amyloid Precursor Protein (APP). **A)** APP is trafficked to the plasma membrane, where α -secretase cleavage initiates the non-amyloidogenic pathway, or internalized into endosomes, favouring β -secretase cleavage and the amyloidogenic pathway. **B)** Canonical amyloidogenic processing of APP leading to A β peptide production.

Distinguishing between impaired A β production or trafficking would be key to interpret the reduction in A β 1-40 secretion. One possibility is that A β peptides are generated but retained within intracellular compartments or degraded, rather than secreted. So far, our analyses have focused on measuring A β levels in the extracellular medium, leaving the intracellular component unexplored. While, as previously mentioned, in our transcriptomic and proteomic datasets intracellular APP levels were unaltered in all cell types, individual A β isoforms cannot be resolved. Future studies using ELISA or ICC could detect A β peptide accumulation within cells and clarify whether lower extracellular A β 1-40 reflects impaired secretion or increased intracellular retention.

A β peptides are best known for their role in neurodegeneration, particularly in AD, where their accumulation contributes to toxicity and synaptic loss (reviewed in [242]). In spite of that, evidence indicates that their production in normal conditions contributes to maintaining neuronal function [243,244]. Thus, the lower extracellular levels of A β 1-40 in SYS could signal, or even contribute to, deficits in synaptic maturation or disruptions in the fine-tuning of neural circuits. Adding to this interpretation, higher A β 1-40 and 1-42 levels are associated with a more mature neuronal phenotype [245]. This idea is reinforced by our transcriptomic data, which show a widespread downregulation of genes involved in synaptic function.

While reduced A β 1-40 secretion in our SYS models is robust, the complete profile of A β isoforms, particularly A β 1-42 and their relative ratios, has yet to be characterised. A β 1-40 is the most abundant isoform and is generally considered less prone to aggregation than A β 1-42, which is more neurotoxic and widely studied in the context of AD. The ratio between A β 1-42 and A β 1-40 (A β 42:40) is widely recognised as a sensitive marker of altered APP processing used in various neurological conditions [246]. In our SYS fibroblast model, extracellular A β 1-42 levels were extremely low in both patients and controls, with no significant differences detected. Nevertheless, isoform-specific alterations could be more pronounced in iPSC-derived neural cultures, where APP expression and trafficking more closely mimic brain physiology. Measuring both A β 1-40 and A β 1-42 in this neural context could provide a more detailed understanding of how MAGEL2 truncation affects APP cleavage dynamics. This experiment is planned as part of our ongoing work with SYS neuronal models, allowing the project to move forward beyond the results presented here.

In order to assess neuronal excitability and network activity in neuronal cultures, we performed calcium imaging studies. For these experiments, we initially tested co-cultures

of excitatory iNs and iAs obtained through TF-based programming. However, these conditions failed to yield sufficient neuronal density for assessment of network connectivity, and they also lacked the inhibitory component required to model E-I interactions. This last limitation could have been addressed by combining three cell types (excitatory iNs, inhibitory iNs, and iAs), but the poor performance of the preliminary cocultures, together with the added technical complexity of establishing triple cultures, led us to explore an alternative strategy. We chose to use a small molecule-based differentiation protocol, which generates mixed populations of forebrain excitatory and inhibitory neurons, and astrocytes. This approach improved neuronal density and provided both components of the E-I balance needed for studying network dynamics. Additionally, because small molecule cultures follow developmental stages more gradually, they provide a better window into how neuronal activity evolves with maturation.

MAGEL2 expression is most prominent in the hypothalamus, but it is also detected at lower levels in telencephalic regions such as the basal ganglia and cerebral cortex. This distribution makes hypothalamic neurons theoretically the most relevant model for SYS, yet their generation is technically demanding, poorly standardised, and sometimes yields heterogeneous populations with variable maturity. In contrast, the kit used in our study specifies that it yields *FOXP1*-positive neurons, a marker of telencephalic identity. The telencephalon is the anterior-most subdivision of the forebrain, giving rise to structures such as the cerebral cortex, hippocampus, basal ganglia, and olfactory bulb. As the hypothalamus originates from the diencephalon, a different forebrain subdivision, the cultures derived with the used kit do not yield hypothalamic neuronal populations. Although this limits modelling of hypothalamic dysfunction, *MAGEL2* expression in telencephalic neurons is still high [84]. Moreover, telencephalic differentiation is well-established, generating excitatory and inhibitory neuronal populations with robust and synchronous calcium transients. These properties make them more suitable for longitudinal calcium imaging, providing a practical and physiologically meaningful system to study E-I network dynamics in SYS. Nevertheless, a limitation of our study concerns the reproducibility observed across control lines. At the time the experiments were conducted, only three control lines were available, and of these, just one consistently reproduced the expected differentiation timeline according to previously published literature and personal communications from our collaborator Dr. Tornero [247]. This raises caution in interpreting variability as disease-related, since line-specific differences may also contribute. In our preliminary calcium imaging experiments, neuronal

differentiation was performed in two different rounds with three biological replicates each; however, the NPCs used were derived from the same batch, suggesting that variability might have originated at this earlier stage. It is worth noting that the same control lines have been used in other experiments in this thesis, as well as in Aina Prat-Planas' thesis project, without showing abnormal maturation. Future work should therefore prioritise the inclusion of a larger number of control lines and the refinement of the NPC differentiation protocol to reduce variability and improve reproducibility.

These preliminary calcium imaging studies revealed that, at baseline, SYS patient-derived networks were able to generate spontaneous activity, maturing along a broadly similar trajectory to the control. This contrasts with previous reports in PWS patient-derived NPCs, where defects in neurite outgrowth and early synapse formation were observed [221], and with studies in *Mage12*-KO mice showing reduced expression of presynaptic proteins and impaired neuritogenesis [76]. These discrepancies may be explained by differences in model system and developmental stage. NPCs represent an earlier stage of lineage commitment, where deficits in differentiation are more readily observed, whereas our neurons had progressed through to functional network formation by dd55, possibly allowing partial compensation. Species factors may also contribute, as murine *Mage12*-KO neurons may not recapitulate all aspects of human *MAGEL2* function.

The differences between SYS and control lines became apparent following GABA A receptor blockade, unmasking latent hyperexcitability in SYS networks. If excitatory neurons were primarily responsible for the phenotype, differences in firing rate would be expected at baseline, yet our recordings showed no such effect. Compensation through upregulation of inhibition could in principle normalise baseline activity, but our omics data show deficits in GABA A receptor γ - and β -subunits mRNA expression, making such compensation unlikely to be effective. Thus, a more coherent interpretation is that excitatory drive is preserved, and the functional instability arises from an inability of inhibitory systems to appropriately regulate activity. GABAergic circuit maturation is critical for establishing E-I balance during early cortical development, and its disruption is a recurrent feature in neurodevelopmental disorders such as ASD, Rett syndrome, and certain forms of epilepsy [129,248].

Beyond disrupted receptor expression, other pathways may contribute to the potential inhibitory deficit. Our transcriptomic analyses, although done in neural cultures obtained through TF-mediated programming, highlighted dysregulation of ECM remodelling. ECM molecules, particularly perineuronal nets, are essential for the stabilisation and

anchoring of synapses [249]. If ECM remodelling is impaired, synapses may form but fail to mature or remain unstable, resulting in an overreliance on extrasynaptic tonic inhibitory mechanisms. Tonic inhibition modulates excitability continuously but lacks the temporal precision required to control oscillatory synchrony [250]. This makes it less effective at shaping network activity and may explain why SYS cultures maintain baseline activity but collapse into pathological synchrony once phasic inhibition is blocked. Looking ahead, it would be valuable to explore the contribution of astrocytes more directly. While the genetically encoded calcium indicator (GECI) used here is restricted to neurons via the *Syn1* promoter, astrocytic GECIs could capture complementary aspects of inhibitory signalling and ECM regulation, providing a fuller picture of how non-neuronal components contribute to network instability in SYS.

A last layer of interpretation may come from our metabolomic findings in SYS fibroblasts, where glutamine levels were significantly reduced while glutamate levels remained unchanged. Although fibroblasts are not a directly relevant cell type for neuronal function, this pattern raises the possibility of a vulnerability in glutamine metabolism that could affect inhibitory neurotransmission. In the brain, glutamine is essential for the glutamate-glutamine cycle, since GABA synthesis requires continuous conversion of glutamine to glutamate and then to GABA. Therefore, it is possible that GABAergic neurons are sensitive to a reduction in precursor supply. Still, glutamine handling in fibroblasts cannot be assumed to mirror neuronal metabolism, and peripheral glutamate levels may not accurately reflect synaptic glutamate pools. To address these limitations and test whether glutamine metabolism contributes to impaired inhibitory control in SYS, metabolomic studies in brain organoids are ongoing in our laboratory.

As previously mentioned, our group has generated SYS patient-derived brain organoids from the same iPSC lines used in 2D neural culture differentiation as part of a parallel PhD thesis by Aina Prat-Planas. Preliminary transcriptomic profiling of these organoids has revealed a partial overlap in DEGs with those identified in 2D cultures, highlighting candidate molecular markers of *MAGEL2* dysfunction. However, a comprehensive integrative analysis across all models still remains an important next step. Once organoid transcriptomic data are interpreted, comparative analyses with 2D neuronal cultures will allow us to distinguish core versus model-specific transcriptional signatures. In addition, preliminary ELISA experiments measuring A β 1-40 secretion have been performed using conditioned media from organoids, but concentration of the analyte was too high, resulting in readings outside the assay's detection range. Therefore, future validation of our 2D culture results will be carried out in organoids with appropriate dilutions of the

media. In parallel, ongoing single-cell, calcium imaging, proteomic, and metabolomic analyses in brain organoids aim to provide a more comprehensive view of SYS pathophysiology. Calcium imaging, metabolomic and proteomic analyses will complement our current findings and help validate the results from fibroblasts and 2D neuronal cultures, while single-cell analyses are expected to provide further insights and uncover additional details. Importantly, this will represent the first SYS disease modelling using brain organoids, offering a new perspective for understanding *MAGEL2*-related pathophysiology.

A relevant question is why *MAGEL2* truncating mutations, as seen in SYS, result in more severe phenotypes compared to cases where *MAGEL2* is entirely deleted. Reported cases with atypical deletions restricted to *MAGEL2*, *NDN*, and *MKRN3* have shown variable outcomes: two individuals did not even display the full spectrum of PWS features [251,252], whereas another patient presented with a complete PWS phenotype [253], but still less severe than SYS. Our findings, especially the stability and mislocation of the truncated *MAGEL2* protein, might offer an explanation for the phenotypic differences between SYS and PWS. The central hypothesis has already been discussed, and is that the truncated form of *MAGEL2*, rather than simply being a non-functional product of haploinsufficiency, actively exerts neomorphic effects. This aberrant activity could lead to novel interactions or interfere with critical processes, thus driving the more severe and distinct features of SYS.

In contrast, in patients with a complete deletion of the paternal *MAGEL2* copy, a complementary explanation for the milder phenotype could be a phenomenon of "leaky expression" of the maternal allele. The complete absence of the paternal allele and its associated regulatory elements could slightly alter the epigenetic landscape, leading to a low level of residual expression from the maternal WT allele (**Figure 12**). This minimal expression of functional *MAGEL2*, even if low, could provide some protective effect or partially compensate for the loss of the paternal copy, thereby resulting in a milder phenotype compared to SYS patients. This is supported by previous findings in the *Ndn* gene, another maternally imprinted gene in the Prader-Willi critical region. In a mouse model, low-level maternal *Ndn* expression was detected in the absence of the paternal allele. This incomplete reactivation was sufficient to rescue perinatal lethality and respiratory dysfunction in a subset of mutant mice, despite non-genetic variability across individuals [254]. Further supporting this theory, *MAGEL2/Magel2* transcripts have been detected in brain tissue from both PWS patients and a mouse model with a complete deletion of the paternal gene and its promoter [255].

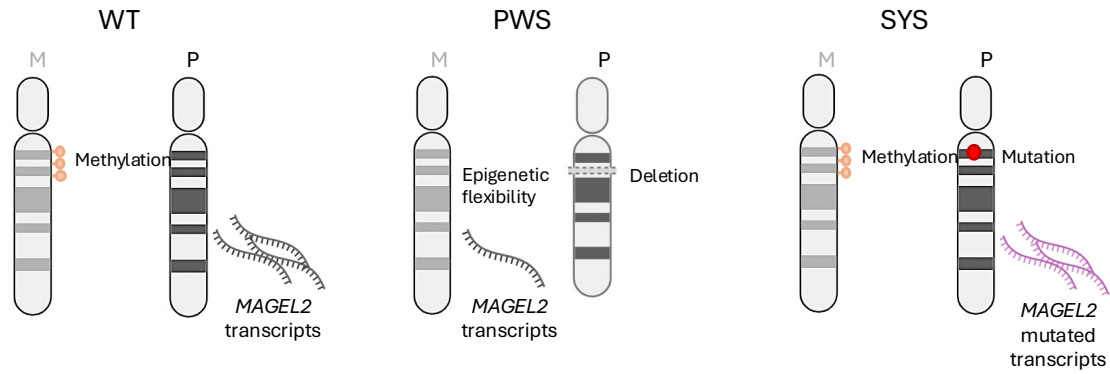


Figure 12: Schematic of *MAGEL2* expression in WT, PWS, and SYS. In WT, only the paternal allele is active due to maternal silencing. In PWS, deletion of the paternal allele may allow low-level maternal expression. In SYS, a truncating mutation in the paternal allele leads to normal levels of mutant *MAGEL2* transcripts, with continued maternal silencing.

While our research has improved the understanding of SYS pathophysiology through the use of diverse cell models, it is essential to acknowledge the limitations of our studies and to outline future directions that can address them. One issue that must be considered is the relatively small sample size used in both iPSC-derived and fibroblast experiments. Specifically, most analyses were performed on neural cell types derived from three SYS patients and four controls, while the transcriptomic fibroblast dataset included samples from three SYS patients and six controls. Although these numbers are within the range of most studies involving rare disease iPSC-derived models due to their significant technical difficulty and high cost, they limit the statistical power to detect subtle expression changes and may reduce the reproducibility of findings across cohorts [256]. This limitation is particularly relevant for calcium imaging studies, where inter-control variability was high, and only one control line consistently and timely differentiated into neural cells suitable for functional analysis. Therefore, increasing the sample size or replication by independent groups in future research will be critical for validating candidate biomarkers, and improving the robustness of molecular signatures.

Another consideration when interpreting findings from patient-derived lines is the inter-individual variability that exists even among patients with the same diagnosis. This diversity among patient lines and between patient and control lines, especially when controls are not genetically matched, can introduce confounding factors. While transcriptomic, proteomic, and metabolic differences were identified between SYS patient and control-derived cell lines, it remains possible that some of these alterations reflect normal variation between individuals, rather than direct consequences of *MAGEL2* truncating mutations. This is particularly relevant for neurodevelopmental

disorders like SYS, where disease-associated changes may be subtle and multifactorial, requiring careful distinction from background genetic noise. To address this, one promising future direction would be generating isogenic controls by using CRISPR/Cas9 to correct the *MAGEL2* mutation in patient-derived iPSC lines (**Figure 13A**). This way, most observed phenotypic or molecular differences between the isogenic pair could then be attributed to the specific disease-causing variant. It is important to consider that generating and characterising an isogenic control for each patient line significantly increases the number of experimental conditions required for every experiment, rapidly raising the technical complexity and the associated economic cost. An alternative strategy involves using iPSCs from a WT individual as the starting line and then introducing the different *MAGEL2* mutations through CRISPR/Cas9 genome editing (**Figure 13B**). This approach creates multiple disease models on a shared genetic background, simplifying comparisons and reducing the impact of inter-individual variability. However, the downside to this method is that it does not account for the unique genetic background and potential modifying factors present in the actual patients, which might influence disease manifestation *in vivo*. Both strategies offer different advantages and limitations, and their selection should be guided by the specific aims and scale of future studies.

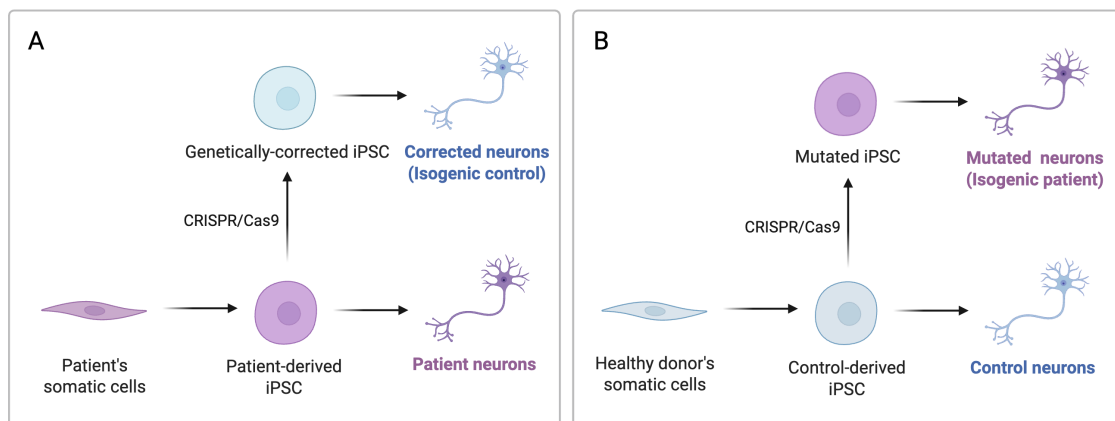


Figure 13: Generation of isogenic iPSC-derived neuron models using CRISPR/Cas9 genome editing. **(A)** Patient somatic cells are reprogrammed into iPSCs, and the pathogenic variant is corrected using CRISPR/Cas9 to generate an isogenic control line. **(B)** Somatic cells from a healthy donor are reprogrammed into iPSCs, and CRISPR/Cas9 is used to introduce a patient mutation. This results in an isogenic mutant line.

Despite these limitations, our results support a model in which SYS is not purely a loss-of-function disorder, but a “mixed-mechanism” disease where loss of normal *MAGEL2* functions coexist with nuclear neomorphic effects. This has important implications for

therapeutic strategies, as interventions aimed only at restoring *MAGEL2* expression may be insufficient or even counterproductive if they also increase levels of the mislocalised truncated form. Based on this hypothesis, a promising therapeutic strategy involves selectively reducing the expression of the mutant *MAGEL2* transcript. One powerful and precise technique for this is RNA interference (RNAi). RNAi is a cellular mechanism that regulates gene expression by sequence-specific degradation of mRNA molecules or inhibition of their translation. It can be used therapeutically with small interfering RNAs (siRNAs) or short hairpin RNAs (shRNAs), engineered to target specific mRNA sequences. When introduced into cells, these synthetic RNA molecules guide the RNAi silencing complex to complementary mRNA targets, leading to their cleavage and subsequent downregulation of the corresponding protein (reviewed in [257]). RNAi-based therapies for several genetic neurological disorders have been recently developed, allowing their exploitation for therapeutic use with FDA approval of the first RNAi therapy in 2018 [258]. In the context of SYS, the main objective would be to develop RNAi-based therapies that specifically silence the mutated *MAGEL2* mRNA. This would prevent the production of the aberrant protein, alleviating its hypothesised neomorphic effects. In fact, in ongoing experiments in our group, we have already been testing the efficacy of different *MAGEL2* siRNAs in iPSC-derived neurons and quantifying the reduction of *MAGEL2* transcript levels. So far, Lipofectamine siRNA transfection has achieved only modest knockdown in neurons. This work will be continued to further optimise delivery methods and assess therapeutic potential. Crucially, the success of these silencing strategies can be validated using the biomarkers identified and discussed earlier in this thesis, specifically, extracellular A β 1-40 levels or expression of key DEGs like *NNAT*, *SIK1*, or *PAX8-AS1*. If these profiles shifted back to normal levels, it would suggest that the cellular dysfunction caused by the truncated protein has been corrected. This therapeutic avenue represents a direct and targeted approach to SYS treatment, and its success would not only offer a potential therapy for this neurodevelopmental disorder but also provide strong validation for the neomorphic hypothesis regarding *MAGEL2* truncation.

A similar approach would involve the use of antisense oligonucleotides (ASOs). These are short, single-stranded molecules designed to bind specific RNA sequences and modulate gene expression. ASOs can act through several mechanisms, including RNase H-mediated degradation of target RNA, inhibition of translation, or alteration of pre-mRNA splicing [259]. They are especially attractive for neurological conditions due to their chemical stability, capacity for nuclear activity, and suitability for delivery. Recent

publications support their use in clinical trials focused on neurodevelopmental conditions through intrathecal administration, showing acceptable safety profiles and promising efficacy results [260]. CRISPR-based RNA targeting methods have also emerged as promising tools for allele-specific silencing. These CRISPR-guided RNA excision systems can generate site-specific deletions within transcripts, enabling selective removal of pathogenic sequences while leaving the WT allele intact [261].

Alternatively, another therapeutic strategy for SYS would involve the epigenetic reactivation of the silent *MAGEL2* allele. The presence of an intact but inactive maternal copy presents the opportunity to reverse imprinting and restore WT *MAGEL2* expression. While such approaches have considerable potential, it will be important to consider how they might influence expression of the already active paternal truncated allele. As previously mentioned, if the expression of the truncated allele was further increased, the therapeutic benefit of producing WT protein could be accompanied by higher levels of the mislocalised form, with uncertain consequences. This possibility underscores the value of developing methods that favour selective activation of the maternal copy, maximising functional rescue while minimising unintended effects. In this context, ASOs may also be designed to target regulatory elements involved in the epigenetic silencing of the maternal *MAGEL2* allele, with the aim of reactivating its expression. This strategy, similar to efforts in Angelman syndrome (MIM #105830) [262], could potentially restore a functional *MAGEL2* protein in patient cells. Recent advances in CRISPR-based editing tools have also enabled targeted modulation of gene expression. This technology has already shown results in PWS. For instance, targeted activation of the *SNRPN* promoter has been used to restore its expression from the maternal allele [263]. Furthermore, disruption of a maternally transcribed noncoding RNA, has been shown to unsilence *SNRPN* and *SNORD116* [264]. Similar strategies could, in principle, be applied to activate maternal *MAGEL2*, either by directly targeting its promoter or by disrupting upstream repressive regulatory elements. While this approach remains in early stages for SYS, it represents a promising avenue for future investigation and therapeutic development.

In summary, this thesis offers a comprehensive and multi-layered investigation into SYS. By combining clinical and genetic reviews with the development of patient-derived cell models, and integrated multi-omic and functional analyses, we shed light on the complex molecular mechanisms driving SYS. Our findings support that SYS pathogenicity does not solely arise from a loss of normal *MAGEL2* function, but rather from a toxic neomorphic effect due to the stability and nuclear localisation of the truncated protein.

This mislocalisation is linked to clinical severity and likely contributes to the widespread transcriptional, translational, and metabolic dysregulation observed in our patient-derived models. Notably, we identified a consistent reduction in A β 1-40 secretion across different SYS cell types, possibly serving as a valuable biomarker for the diagnosis, and therapeutic monitoring of SYS. Furthermore, the generation and characterisation of disease-relevant CNS cell types allowed us to uncover novel molecular signatures that may play a role in SYS pathophysiology, opening new avenues for investigation. Looking forward, the discoveries presented here provide a strong foundation for further studies aimed at unravelling disease mechanisms, validating candidate biomarkers, and exploring therapeutic strategies including gene silencing or epigenetic reactivation of *MAGEL2*.

Finally, I would like to emphasise that this work has been made possible through the close collaboration of our research group with patient associations, most notably AESYS and Associació Síndrome Opitz C. Their support has been essential not only in securing the financial resources that fuel our science, but also in facilitating the donation of fibroblast samples, which were the foundation of this research. I am deeply grateful to the patients and families who have contributed to this work, often under difficult personal circumstances. Their generosity and trust are the driving force behind our commitment. The humble contribution of this research is dedicated to them, with the hope that it may ultimately help improve diagnosis, understanding, and care for those living with SYS. While important steps have been taken, it is clear that a long road still lies ahead. Continued research will be crucial to deepen our understanding of disease mechanisms and to move closer to personalised therapeutic strategies that can meaningfully impact patients' lives. This shared goal must continue to guide and inspire the scientific and clinical communities.

CONCLUSIONS

- A comprehensive phenotypic review allowed the characterisation of key clinical features of SYS and the establishment of the first multilingual clinical guidelines for the syndrome, providing a standardised framework for diagnosis and management, and serving as a practical roadmap for both patients and clinicians.
- Truncating mutations in *MAGEL2* do not lead to protein degradation but instead generate stable mutant proteins that mislocalise the nucleus, supporting a toxic neomorphic mechanism in SYS. This nuclear mislocalisation correlates with clinical severity.
- Patient-derived fibroblasts show significant alterations in transcriptomic and metabolomic profiles. Key findings include the upregulation genes involved in mitotic processes, alongside a decrease in intracellular glutamine levels and an accumulation of organic acids.
- Extracellular levels of A β 1-40 peptide are reduced in SYS patient-derived fibroblasts and cocultures of iNeurons and iAstrocytes. This reinforces A β 1-40 as a robust and consistent biomarker for SYS across different cell types, highlighting its potential for diagnostic and monitoring applications.
- The comprehensive characterisation of SYS patient-derived iPSC lines provides a robust *in vitro* platform for modelling SYS, enabling the differentiation into neural cell types.
- Transcriptomic profiling of SYS inhibitory iNeurons showed suppression of synaptic and neuronal identity programs alongside upregulation of ECM remodelling and developmental regulators. This suggests a shift away from a mature synaptic phenotype toward an immature, morphogen-driven state.
- Proteomic analysis revealed subtle alterations, particularly in pathways linked to PI3K signalling, cytoskeletal organisation, and collagen dynamics. Integrative transcriptome-proteome analysis highlighted a subset of commonly dysregulated genes, like *ALDH1A2*, *SRPX2*, or *CRLF1*, that may represent candidates for functional follow-up.
- SYS patient-derived iAstrocytes displayed pronounced upregulation of ECM components, potentially pointing to impaired astrocyte-neuron communication and extracellular environment regulation.

- A subset of genes, including *NNAT*, *SIK1*, and *PAX8-AS1*, emerged as consistently dysregulated across neural cell types and brain organoids, making them strong candidates for further functional study and biomarker development.
- Preliminary calcium imaging analyses of patient-derived neurons showed that SYS networks form functional excitatory circuits but display altered responses to GABA A blockade, potentially due to impaired excitatory-inhibitory balance. Validation in additional lines is needed to confirm these findings.

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ANNEX

ANNEX 1: CHARACTERISATION OF THE iPSC LINES REPROGRAMMED BY OUR RESEARCH GROUP

BANCO NACIONAL DE LÍNEAS CELULARES (TRONCALES) *National Bank of Stem Cell Lines* IMPRESO DE SOLICITUD DE REGISTRO Y DEPÓSITO DE UNA LÍNEA iPSC HUMANA *Application Form to Register and Deposit of an human iPSC cell line*

FECHA: 07-06-2024

DOCUMENTOS QUE DEBEN ACOMPAÑAR LA SOLICITUD:*Attached documents:*

- ☒ **Copia de la autorización del proyecto en el cual se genera la línea celular, junto con informe favorable del Comité de Ética de la Investigación del centro de procedencia.**
A copy of the project authorization in which the cell line is obtained along with a favourable report of the Clinical Research Ethics Committee
- ☐ **Copia de cualquier publicación científica relacionada con la línea iPS generada.**
A copy of any relevant published scientific papers related to the iPS cell line generated
- ☒ **C. V. del investigador principal (una página; formato libre).**
A one page CV for the Principal Investigator
- ☒ **Número de registro del proyecto**

SECCIÓN 1-INFORMACIÓN DE LA MUESTRA ORIGINAL Y DE LA iPS GENERADA.*Section 1-Information of the original cell line and the generated iPS*

Nombre de la línea iPSC <i>Name of the iPSC line:</i>	SYS FiPS63-mR6F-1	
Nº de registro en el Human Pluripotent Stem Cell Registry (1)		
Muestra original donada. Detallar tipo de célula, tejido de origen y localización anatómica de la muestra biológica de la que se obtiene la línea original. Si son células comerciales, detallar nombre, referencia y distribuidor comercial <i>Original sample donated. Detail cell type, tissue of origin and anatomic location of the biological sample from which the original line is obtained. If cells are commercial, detail name, reference and trade distributor.</i>	Fibroblastos de biopsia de piel abdominal. <i>Fibroblasts from abdominal skin biopsy</i>	
Sexo y edad del donante. <i>Sex and age of the donor</i>	Femenino, 20 <i>Female, 20</i>	
¿El donante tiene alguna patología? <i>Has the donor any pathological condition?</i>	NO ☐ <i>No</i>	SÍ ☒ (especificar) Schaaf-Yang syndrome <i>Yes (specify)</i>
¿La patología es de origen genético? <i>Is the pathological condition of genetic origin?</i>	NO ☐ <i>No</i>	SÍ ☒ (especificar) MAGEL2 c.1912C>T; p.Gln638* <i>Yes (specify)</i>

Documento de Solicitud de registro y depósito de una línea iPS humana. Versión enero 2021

Los apartados que requieran entrada de texto, deben rellenarse tanto en castellano como en inglés

Text items should be filled in both Spanish and English

Muestra biológica recibida <i>Biological sample</i>	Fresco ☒ <i>Fresh</i> Crioconservado ☐ <i>Cryopreserved</i>
Fecha de la donación de la muestra biológica <i>Date of donation of the biological sample</i>	15-11-2016
Fecha del uso o descongelación <i>(si congelado)</i> <i>Date used or thawed (if frozen)</i>	01-02-2021
Identificación celular: Huella genética por análisis de microsatélites/STR/otros marcadores de las células de origen <i>Cell Identity: Genetic fingerprinting by microsatellite analysis / STR/ other markers of the original cells.</i>	La huella genética de los fibroblastos se analizó mediante microsatélites (Anexo 1). <i>The genetic fingerprint of the fibroblasts was analyzed using microsatellites (Annex 1).</i>
Método utilizado en la generación de la línea iPSC. (Integrativa/ No-integrativa) Especificar factores y plásmidos de reprogramación utilizados. <i>Method used for the generation of iPSC line (Integrative / Non-integrative)</i> <i>Specify factors and plasmids used for reprogramming</i>	Se utilizó el kit de reprogramación StemRNA 3rd Gen (Stemgent®) para establecer las líneas de iPSC transfectando un cóctel de ARN que contiene ARNm de reprogramación no modificados (OCT4, SOX2, KLF4, cMYC, NANOG y LIN28 (OSKMNL)), ARNm de evasión inmune (E3, K3, B18) y microARN bicatenarios del grupo 302/367 en fibroblastos. <i>The StemRNA 3rd Gen Reprogramming Kit (Stemgent®) was used to establish the iPSCs lines by transfecting an RNA cocktail containing non-modified reprogramming mRNAs (OCT4, SOX2, KLF4, cMYC, NANOG and LIN28 (OSKMNL)), immune evasion mRNAs (E3, K3, B18) and double-stranded microRNAs from the 302/367 cluster into fibroblasts.</i>
Condiciones de cultivo de la línea de iPSC generada. (si se describen en publicación, indicar referencia) <i>iPSC Culture conditions (if they are described in a publication, please indicate the reference)</i>	Las colonias se recogieron y se sembraron en placas de 4 pocillos recubiertas con laminina en medio mTeSR Plus™. Las células se pasaron usando TrypLE™ Select Enzyme (ThermoFisher Scientific) a placas de 6 pocillos recubiertas con Matrigel (Corning, Inc.) para la expansión, el medio se complementó con inhibidor de Rock (Y-27632) (StemCell Technologies) durante 24 horas después de cada pase. <i>Colonies were picked and plated in laminin-coated 4-well plates in mTeSR Plus™ medium. Cells were passaged using TrypLE™ Select Enzyme (ThermoFisher Scientific) to 6-well plates coated with Matrigel (Corning, Inc.) for expansion, medium was supplemented with Rock inhibitor (Y-27632) (StemCell Technologies) for 24 hours after every passage.</i>
Criopreservación de la línea celular (Describir método de congelación/descongelación) <i>Cryopreservation of the cell line (Describe freezing / thawing method)</i>	La criopreservación de colonias se ha realizado en FBS(90%) + DMSO(10%), mediante contenedor de isopropanol a -80°C. Los viales se han descongelado a temperatura ambiente. <i>Cryopreservation of colonies was carried out in FBS (90%) + DMSO (10%), using an isopropanol container at -80°C. The vials have been thawed at room temperature.</i>
Pase de la línea celular en el momento del banqueo/registro. (Máximo: Pase 15) <i>Passage at the time of the banking/registration (Max: Passage 15)</i>	10

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Text items should be filled in both Spanish and English

¿Ha sido la línea modificada genéticamente? <i>Has the line been genetically modified?</i>	Sí Yes ☐ No No ☒ Especificar: <i>Specify:</i>
--	--

SECCIÓN 2 RESULTADOS DE LA CARACTERIZACIÓN DE LA LÍNEA iPSC.

Adjuntar resultados (imágenes o gráficos) como anexo

Section 2 iPSC Cell Line characterization results. Attach results (images and graphics) as an annex

Test de pluripotencia <i>Pluripotency test</i>	Método <i>Method</i>		Nº pase <i>Passage n.</i>	Resultado <i>Results</i>	Comentarios <i>Comments</i>
Se informará de al menos 5 de los siguientes marcadores <i>At least 5 of the following test will be reported</i>	Oct 4	ICC	9	+	
	Nanog	ICC	9	+	
	Sox 2	ICC	9	+	
	SSEA3				
	SSEA4	ICC	15	+	
	TRA-1-60				
	TRA-1-81	ICC	15	+	
	Fosfatasa. Alk				
Test de diferenciación in vitro <i>In vitro differentiation test</i>	Método <i>Method</i>	Marcador <i>Marker</i>	Nº pase <i>Passage n</i>	Resultado <i>Results</i>	Comentarios <i>Comments</i>
Cuerpos embrioides <i>Embryoid bodies</i>	Ectodermo <i>Ectoderm</i>	TUJ1	7	+	
	Mesodermo <i>Mesoderm</i>	AFP	7	+	
	Endoderm <i>Endoderm</i>	α-SMA	7	+	
	<i>Ver Anexo 2</i>				

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Test de diferenciación in vivo <i>In vivo differentiation test</i>	Método <i>Method</i>	Marcador <i>Marker</i>	Nº pase <i>Passage n</i>	Resultado <i>Results</i>	Comentarios <i>Comments</i>
Teratomas <i>Teratomas</i>	Ectodermo <i>Ectoderm</i> Mesodermo <i>Mesoderm</i> Endodermo <i>Endoderm</i>				
Cariotipo (pase) <i>Karyotype (passage)</i>	46, XX (Anexo 3) 46, XX (Annex 3)				
Identificación celular: Huella genética por análisis de microsatélites/STR/ otros marcadores de la línea celular/ Cell Identity: Genetic fingerprinting by microsatellite analysis / STR/ other cell line markers	Los marcadores de microsatélites de la muestra inicial de fibroblastos coinciden con los de la línea de iPS generada. (Anexo 1). <i>Microsatellite markers of the initial fibroblasts sample are identical to the markers of the iPS line (Annex 1).</i>				
Test de integración <i>Integration Test</i>	No procede, ya que se ha usado una metodología no integrativa basada en la transfección de RNA. <i>Not required, since the methodology used was non integrative and based on transfection of RNA.</i>				
Test de silenciamiento <i>Silencing Test</i>	No procede, ya que se ha usado una metodología no integrativa basada en la transfección de RNA. <i>Not required, since the methodology used was non integrative and based on transfection of RNA.</i>				
Confirmación de la presencia de la mutación de las células de origen <i>Confirmation of the mutation in the original cells</i>	La presencia de la mutación de las células de origen fue confirmada en las iPSCs generadas mediante secuenciación Sanger (Anexo 4). <i>The presence of the mutation of the original cells was confirmed in the generated iPSCs by Sanger sequencing (Annex 4).</i>				
Test de micoplasma <i>Mycoplasma Test</i>	Negativo por PCR (Anexo 5) <i>Negative by PCR (Annex 5)</i>				

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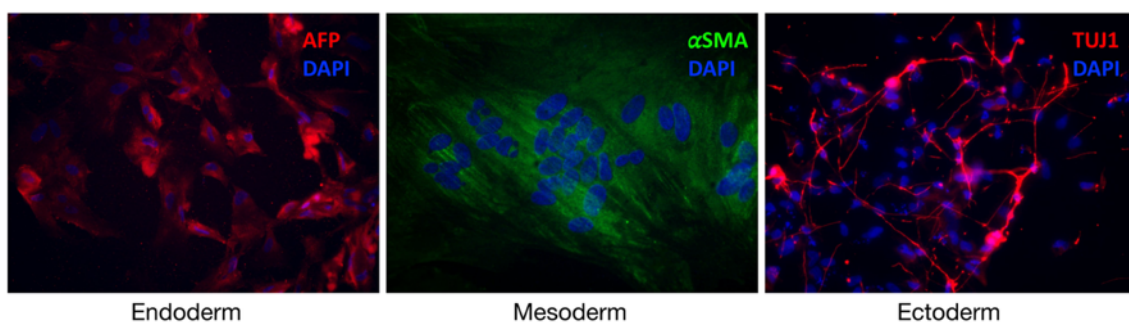
ANEXOS (ANNEXES) al documento de depósito de la línea iPSC SYS FiPS63

ANEXO 1: Análisis de microsatélites	<i>ANNEX 1: Microsatellite analysis</i>
ANEXO 2: Test de diferenciación <i>in vitro</i>	<i>ANNEX 2: in vitro differentiation test</i>
ANEXO 3: Cariotipo	<i>ANNEX 3: Karyotype</i>
ANEXO 4: Genotipado	<i>ANNEX 4: Genotyping</i>
ANEXO 5: Test de mycoplasma	<i>ANNEX 5: Mycoplasma test</i>
ANEXO 6: ICC marcadores pluripotencia	<i>ANNEX 6: ICC pluripotency markers</i>

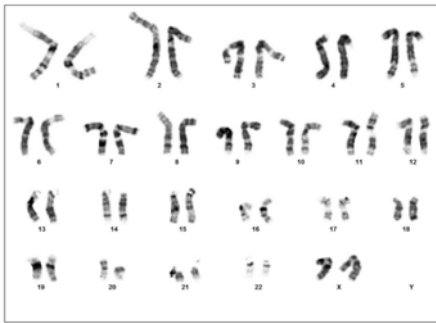
ANEXO 1: Análisis de microsatélites

ANNEX 1: Microsatellite analysis

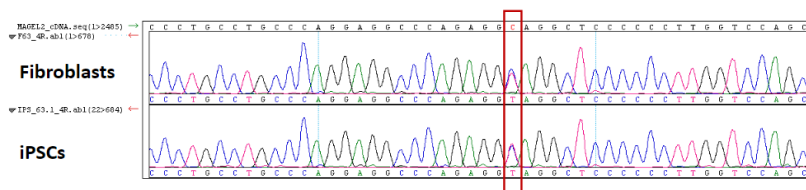
STR	Size range	iPSC	Fibroblast
AMEL	104-112 bps	104/104	104/104
D13S317	166-204 bps	187/187	187/187
D16S539	258-310 bps	287/291	287/291
D18S51	279-364 bps	299/318	299/318
D21S11	195-262 bps	215/233	215/233
D5S818	108-158 bps	130/134	130/134
D7S820	208-252 bps	220/224	220/224
D8S1179	198-252 bps	226/230	226/230
FGA	305-466 bps	329/333	329/333
Penta D	365-448 bps	412/418	412/418
Penta E	368-480 bps	423/439	423/439
TH01	148-194 bps	169/173	169/173
TPOX	255-303 bps	269/269	269/269
vWA	120-184 bps	146/158	146/158

ANEXO 2: Test de diferenciación *in vitro**ANNEX 2: in vitro differentiation test*

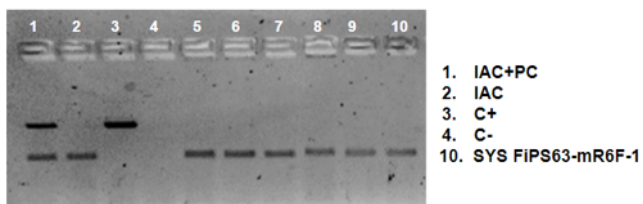
ANEXO 3: Cariotipo
ANNEX 3: Karyotype



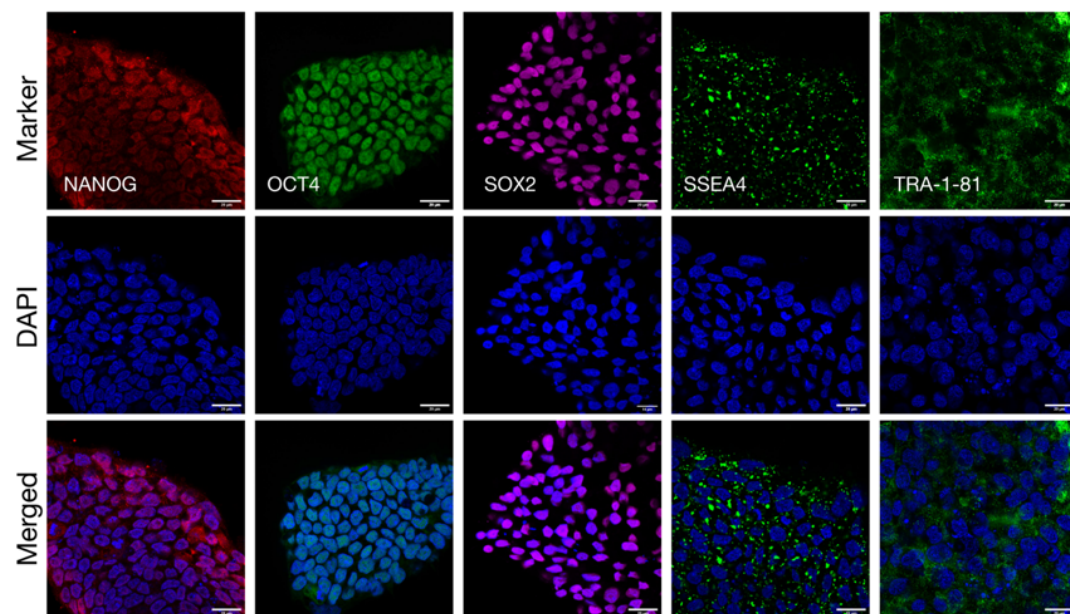
ANEXO 4: Genotipado
ANNEX 4: Genotyping



ANEXO 5: Test de micoplasma
ANNEX 5: Mycoplasma test



ANEXO 6: ICC marcadores pluripotencia
ANNEX 6: ICC pluripotency markers



BANCO NACIONAL DE LÍNEAS CELULARES (TRONCALES)
National Bank of Stem Cell Lines
IMPRESO DE SOLICITUD DE REGISTRO Y DEPÓSITO DE UNA LÍNEA iPSC HUMANA
Application Form to Register and Deposit of an human iPSC cell line

FECHA: 07-06-2024

DOCUMENTOS QUE DEBEN ACOMPAÑAR LA SOLICITUD:

Attached documents:

- ☒ **Copia de la autorización del proyecto en el cual se genera la línea celular, junto con informe favorable del Comité de Ética de la Investigación del centro de procedencia.**
A copy of the project authorization in which the cell line is obtained along with a favourable report of the Clinical Research Ethics Committee
- ☐ **Copia de cualquier publicación científica relacionada con la línea iPS generada.**
A copy of any relevant published scientific papers related to the iPS cell line generated
- ☒ **C. V. del investigador principal (una página; formato libre).**
A one page CV for the Principal Investigator
- ☒ **Número de registro del proyecto**

SECCIÓN 1-INFORMACIÓN DE LA MUESTRA ORIGINAL Y DE LA iPS GENERADA.

Section 1-Information of the original cell line and the generated iPS

Nombre de la línea iPSC <i>Name of the iPSC line:</i>	SYS FiPS66-mR6F-1
Nº de registro en el Human Pluripotent Stem Cell Registry (1)	
Muestra original donada. Detallar tipo de célula, tejido de origen y localización anatómica de la muestra biológica de la que se obtiene la línea original. Si son células comerciales, detallar nombre, referencia y distribuidor comercial <i>Original sample donated. Detail cell type, tissue of origin and anatomic location of the biological sample from which the original line is obtained. If cells are commercial, detail name, reference and trade distributor.</i>	Fibroblastos de biopsia de piel abdominal. <i>Fibroblasts from abdominal skin.</i>
Sexo y edad del donante. <i>Sex and age of the donor</i>	Masculino, 6 <i>Male, 6</i>
¿El donante tiene alguna patología? <i>Has the donor any pathological condition?</i>	NO ☐ <i>No</i> SÍ ☒ (especificar) <i>Yes (specify)</i> Schaaf-Yang syndrome

Documento de Solicitud de registro y depósito de una línea iPS humana. Versión enero 2021
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¿La patología es de origen genético? <i>Is the pathological condition of genetic origin?</i>	NO ☐ SÍ ☒ (especificar) <i>MAGEL2 c.1996dupC;</i> p.Gln666Profs*47 No Yes (specify)
Muestra biológica recibida <i>Biological sample</i>	Fresco ☒ Crioconservado ☐ <i>Fresh</i> <i>Cryopreserved</i>
Fecha de la donación de la muestra biológica <i>Date of donation of the biological sample</i>	15/11/2016
Fecha del uso o descongelación <i>(si congelado)</i> <i>Date used or thawed (if frozen)</i>	01/02/2021
Identificación celular: Huella genética por análisis de microsatélites/STR/otros marcadores de las células de origen <i>Cell Identity: Genetic fingerprinting by microsatellite analysis / STR/ other markers of the original cells.</i>	La huella genética de los fibroblastos se analizó mediante microsatélites (Anexo 1). <i>The genetic fingerprint of the fibroblasts was analyzed using microsatellites (Annex 1).</i>
Método utilizado en la generación de la línea iPSC. (Integrativa/ No-integrativa) Especificar factores y plásmidos de reprogramación utilizados. <i>Method used for the generation of iPSC line (Integrative / Non-integrative)</i> <i>Specify factors and plasmids used for reprogramming</i>	Se utilizó el kit de reprogramación StemRNA 3rd Gen (Stemgent®) para establecer las líneas de iPSC transfectando un cóctel de ARN que contiene ARNm de reprogramación no modificados (OCT4, SOX2, KLF4, cMYC, NANOG y LIN28 (OSKMNL)), ARNm de evasión inmune (E3, K3, B18) y microARN bicatenarios del grupo 302/367 en fibroblastos. <i>The StemRNA 3rd Gen Reprogramming Kit (Stemgent®) was used to establish the iPSCs lines by transfecting an RNA cocktail containing non-modified reprogramming mRNAs (OCT4, SOX2, KLF4, cMYC, NANOG and LIN28 (OSKMNL)), immune evasion mRNAs (E3, K3, B18) and double-stranded microRNAs from the 302/367 cluster into fibroblasts.</i>
Condiciones de cultivo de la línea de iPSC generada. (si se describen en publicación, indicar referencia) <i>iPSC Culture conditions (if they are described in a publication, please indicate the reference)</i>	Las colonias se recogieron y se sembraron en placas de 4 pocillos recubiertas con laminina en medio mTeSR Plus™. Las células se pasaron usando TrypLE™ Select Enzyme (ThermoFisher Scientific) a placas de 6 pocillos recubiertas con Matrigel (Corning, Inc.) para la expansión, el medio se complementó con inhibidor de Rock (Y-27632) (StemCell Technologies) durante 24 horas después de cada pase. <i>Colonies were picked and plated in laminin-coated 4-well plates in mTeSR Plus™ medium. Cells were passaged using TrypLE™ Select Enzyme (ThermoFisher Scientific) to 6-well plates coated with Matrigel (Corning, Inc.) for expansion, medium was supplemented with Rock inhibitor (Y-27632) (StemCell Technologies) for 24 hours after every passage.</i>

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Criopreservación de la línea celular (Describir método de congelación/descongelación) <i>Cryopreservation of the cell line (Describe freezing / thawing method)</i>	La criopreservación de colonias se ha realizado en FBS(90%) + DMSO(10%), mediante contenedor de isopropanol a -80°C. Los viales se han descongelado a temperatura ambiente. <i>Cryopreservation of colonies was carried out in FBS (90%) + DMSO (10%), using an isopropanol container at -80°C. The vials have been thawed at room temperature.</i>
Pase de la línea celular en el momento del banqueo/registro. (Máximo: Pase 15) <i>Passage at the time of the banking/registration (Max: Passage 15)</i>	11
¿Ha sido la línea modificada genéticamente? <i>Has the line been genetically modified?</i>	Sí Yes ☐ No No ☒ Especificar: Specify:

SECCIÓN 2 RESULTADOS DE LA CARACTERIZACIÓN DE LA LÍNEA iPSC.

Adjuntar resultados (imágenes o gráficos) como anexo

Section 2 iPSC Cell Line characterization results. Attach results (images and graphics) as an annex

Test de pluripotencia <i>Pluripotency test</i>	Método <i>Method</i>	Nº pase <i>Passage n.</i>	Resultado <i>Results</i>	Comentarios <i>Comments</i>
Se informará de al menos 5 de los siguientes marcadores <i>At least 5 of the following test will be reported</i>	Oct 4	ICC	9	+
	Nanog	ICC	9	+
	Sox 2	ICC	9	+
	SSEA3			
	SSEA4	ICC	15	+
	TRA-1-60			
	TRA-1-81	ICC	15	+
	Fosfatasa. Alk			

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Los apartados que requieran entrada de texto, deben rellenarse tanto en castellano como en inglés

Text items should be filled in both Spanish and English

Test de diferenciación in vitro <i>In vitro differentiation test</i> Cuerpos embrioides <i>Embryoid bodies</i>						
	Ectodermo <i>Ectoderm</i>	Método <i>Method</i>	Marcador <i>Marker</i>	Nº pase <i>Passage n</i>	Resultado <i>Results</i>	Comentarios <i>Comments</i>
		ICC	TUJ1	7	+	
	Mesodermo <i>Mesoderm</i>	ICC	AFP	7	+	
	Endodermo <i>Endoderm</i>	ICC	α-SMA	7	+	
	<i>Ver Anexo 2</i>					
Test de diferenciación in vivo <i>In vivo differentiation test</i> Teratomas <i>Teratomas</i>						
	Ectodermo <i>Ectoderm</i>	Método <i>Method</i>	Marcador <i>Marker</i>	Nº pase <i>Passage n</i>	Resultado <i>Results</i>	Comentarios <i>Comments</i>
	Mesodermo <i>Mesoderm</i>					
	Endodermo <i>Endoderm</i>					
Cariotipo (pase) <i>Karyotype (passage)</i>	46, XY (Anexo 3)					
	46, XY (<i>Annex 3</i>)					
Identificación celular: Huella genética por análisis de microsatélites/STR/ otros marcadores de la línea celular/ Cell Identity: Genetic fingerprinting by microsatellite analysis / STR/ other cell line markers	Los marcadores de microsatélites de la muestra inicial de fibroblastos coinciden con los de la línea de iPS generada. (Anexo 1). <i>Microsatellite markers of the initial fibroblasts sample are identical to the markers of the iPS line (Annex 1).</i>					
Test de integración <i>Integration Test</i>	No procede, ya que se ha usado una metodología no integrativa basada en la transfección de RNA. <i>Not required, since the methodology used was non integrative and based on transfection of RNA.</i>					

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Text items should be filled in both Spanish and English

Test de silenciamiento) <i>Silencing Test)</i>	No procede, ya que se ha usado una metodología no integrativa basada en la transfección de RNA. <i>Not required, since the methodology used was non integrative and based on transfection of RNA.</i>
Confirmación de la presencia de la mutación de las células de origen <i>Confirmation of the mutation in the original cells</i>	La presencia de la mutación de las células de origen fue confirmada en las iPSCs generadas mediante secuenciación Sanger (Anexo 4). <i>The presence of the mutation of the original cells was confirmed in the generated iPSCs by Sanger sequencing (Annex 4).</i>
Test de micoplasma <i>Mycoplasma Test</i>	Negativo por PCR (Anexo 5) <i>Negative by PCR (Annex 5)</i>

ANEXOS (ANNEXES) al documento de depósito de la línea iPSC SYS FiPS66-mR6F-1

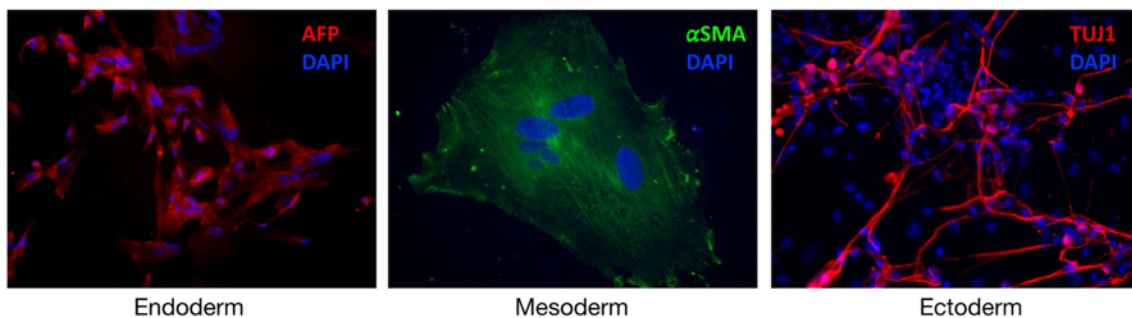
ANEXO 1: Análisis de microsatélites
 ANEXO 2: Test de diferenciación *in vitro*
 ANEXO 3: Cariotipo
 ANEXO 4: Genotipado
 ANEXO 5: Test de mycoplasma
 ANEXO 6: ICC marcadores pluripotencia

ANNEX 1: Microsatellite analysis
ANNEX 2: in vitro differentiation test
ANNEX 3: Karyotype
ANNEX 4: Genotyping
ANNEX 5: Mycoplasma test
ANNEX 6: ICC pluripotency markers

ANEXO 1: Análisis de microsatélites
 ANNEX 1: *Microsatellite analysis*

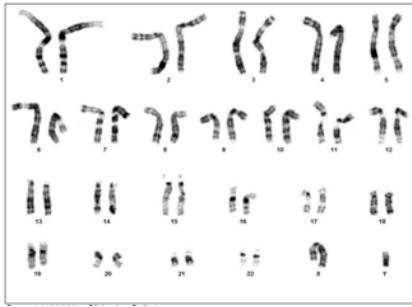
STR	Size range	iPSC	Fibroblast
AMEL	104-112 bps	104/110	104/110
CSF1PO	312-361 bps	337/341	337/341
D13S317	166-204 bps	175/191	175/191
D16S539	258-310 bps	274/286	274/286
D18S51	279-364 bps	313/314	313/314
D21S11	195-262 bps	215/219	215/219
D3S1358	94-148 bps	124/136	124/136
D7S820	208-252 bps	220/244	220/244
D8S1179	198-252 bps	214/230	214/230
FGA	305-466 bps	345/358	345/358
Penta D	365-448 bps	399/403	399/403
Penta E	368-480 bps	387/392	387/392
TH01	148-194 bps	165/176	165/176
TPOX	255-303 bps	269/281	269/281
vWA	120-184 bps	146/158	146/158

ANEXO 2: Test de diferenciación *in vitro*
 ANNEX 2: *in vitro differentiation test*



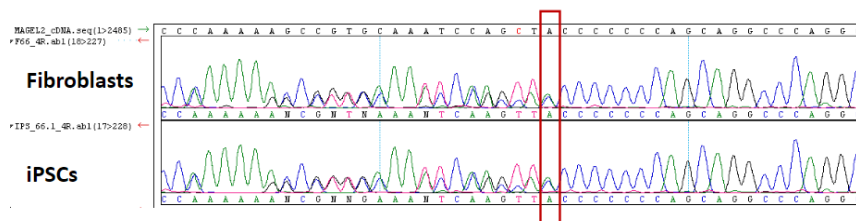
ANEXO 3: Cariotipo

ANNEX 3: Karyotype



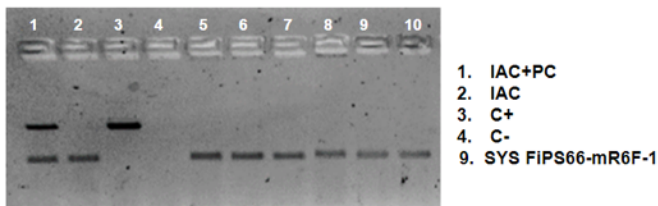
ANEXO 4: Genotipado

ANNEX 4: Genotyping



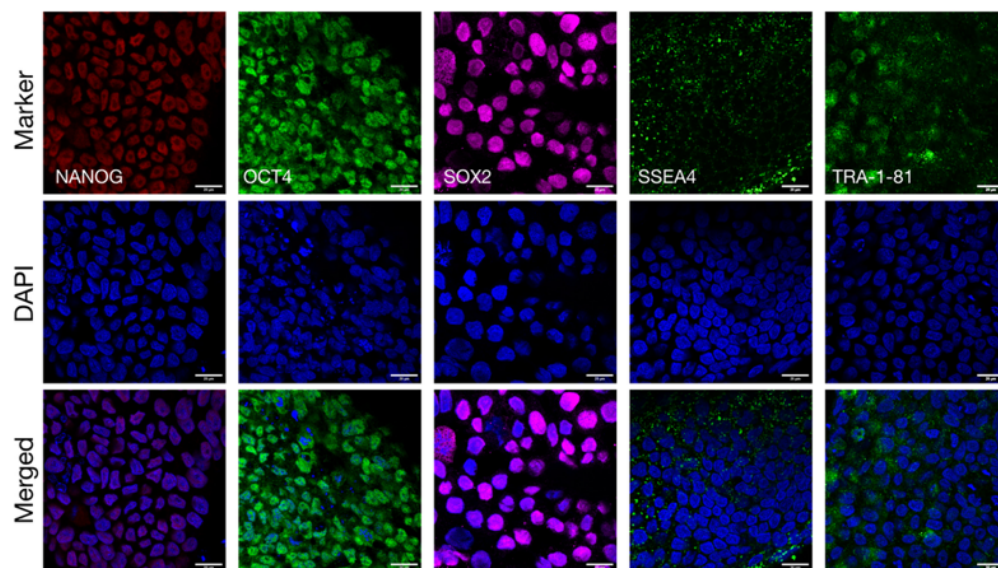
ANEXO 5: Test de micoplasma

ANNEX 5: Mycoplasma test



ANEXO 6: ICC marcadores pluripotencia

ANNEX 6: ICC pluripotency markers



BANCO NACIONAL DE LÍNEAS CELULARES (TRONCALES)
National Bank of Stem Cell Lines
IMPRESO DE SOLICITUD DE REGISTRO Y DEPÓSITO DE UNA LÍNEA iPSC HUMANA
Application Form to Register and Deposit of an human iPSC cell line

FECHA: 07/06/2024

DOCUMENTOS QUE DEBEN ACOMPAÑAR LA SOLICITUD:

Attached documents:

- ☒ **Copia de la autorización del proyecto en el cual se genera la línea celular, junto con informe favorable del Comité de Ética de la Investigación del centro de procedencia.**
A copy of the project authorization in which the cell line is obtained along with a favourable report of the Clinical Research Ethics Committee
- ☐ **Copia de cualquier publicación científica relacionada con la línea iPS generada.**
A copy of any relevant published scientific papers related to the iPS cell line generated
- ☒ **C. V. del investigador principal (una página; formato libre).**
A one page CV for the Principal Investigator
- ☒ **Número de registro del proyecto**

SECCIÓN 1-INFORMACIÓN DE LA MUESTRA ORIGINAL Y DE LA iPS GENERADA.

Section 1-Information of the original cell line and the generated iPS

Nombre de la línea iPSC <i>Name of the iPSC line:</i>	SYS FiPS68-mR6F-3
Nº de registro en el Human Pluripotent Stem Cell Registry (1)	
Muestra original donada. Detallar tipo de célula, tejido de origen y localización anatómica de la muestra biológica de la que se obtiene la línea original. Si son células comerciales, detallar nombre, referencia y distribuidor comercial <i>Original sample donated. Detail cell type, tissue of origin and anatomic location of the biological sample from which the original line is obtained. If cells are commercial, detail name, reference and trade distributor.</i>	Fibroblastos de biopsia de piel abdominal. <i>Fibroblasts from abdominal skin.</i>
Sexo y edad del donante. <i>Sex and age of the donor</i>	Femenino, 6 <i>Female, 6</i>
¿El donante tiene alguna patología? <i>Has the donor any pathological condition?</i>	NO ☐ <i>No</i> SÍ ☒ (especificar) Schaaf-Yang syndrome <i>Yes (specify)</i>

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Text items should be filled in both Spanish and English

¿La patología es de origen genético? <i>Is the pathological condition of genetic origin?</i>	NO ☐ SÍ ☒ (especificar) <i>MAGEL2 c.1996dupC;</i> p.Gln666Profs*47 No Yes (specify)
Muestra biológica recibida <i>Biological sample</i>	Fresco ☒ Crioconservado ☐ <i>Fresh</i> <i>Cryopreserved</i>
Fecha de la donación de la muestra biológica <i>Date of donation of the biological sample</i>	15/11/2016
Fecha del uso o descongelación <i>(si congelado)</i> <i>Date used or thawed (if frozen)</i>	01/03/2021
Identificación celular: Huella genética por análisis de microsatélites/STR/otros marcadores de las células de origen <i>Cell Identity: Genetic fingerprinting by microsatellite analysis / STR/ other markers of the original cells.</i>	La huella genética de los fibroblastos se analizó mediante microsatélites (Anexo 1). <i>The genetic fingerprint of the fibroblasts was analyzed using microsatellites (Annex 1).</i>
Método utilizado en la generación de la línea iPSC. (Integrativa/ No-integrativa) Especificar factores y plásmidos de reprogramación utilizados. <i>Method used for the generation of iPSC line (Integrative / Non-integrative)</i> <i>Specify factors and plasmids used for reprogramming</i>	Se utilizó el kit de reprogramación StemRNA 3rd Gen (Stemgent®) para establecer las líneas de iPSC transfectando un cóctel de ARN que contiene ARNm de reprogramación no modificados (OCT4, SOX2, KLF4, cMYC, NANOG y LIN28 (OSKMNL)), ARNm de evasión inmune (E3, K3, B18) y microARN bicatenarios del grupo 302/367 en fibroblastos. <i>The StemRNA 3rd Gen Reprogramming Kit (Stemgent®) was used to establish the iPSCs lines by transfecting an RNA cocktail containing non-modified reprogramming mRNAs (OCT4, SOX2, KLF4, cMYC, NANOG and LIN28 (OSKMNL)), immune evasion mRNAs (E3, K3, B18) and double-stranded microRNAs from the 302/367 cluster into fibroblasts.</i>
Condiciones de cultivo de la línea de iPSC generada. (si se describen en publicación, indicar referencia) <i>iPSC Culture conditions (if they are described in a publication, please indicate the reference)</i>	Las colonias se recogieron y se sembraron en placas de 4 pocillos recubiertas con laminina en medio mTeSR Plus™. Las células se pasaron usando TrypLE™ Select Enzyme (ThermoFisher Scientific) a placas de 6 pocillos recubiertas con Matrigel (Corning, Inc.) para la expansión, el medio se complementó con inhibidor de Rock (Y-27632) (StemCell Technologies) durante 24 horas después de cada pase. <i>Colonies were picked and plated in laminin-coated 4-well plates in mTeSR Plus™ medium. Cells were passaged using TrypLE™ Select Enzyme (ThermoFisher Scientific) to 6-well plates coated with Matrigel (Corning, Inc.) for expansion, medium was supplemented with Rock inhibitor (Y-27632) (StemCell Technologies) for 24 hours after every passage.</i>

Documento de Solicitud de registro y depósito de una línea IPS humana. Versión enero 2021
Los apartados que requieran entrada de texto, deben rellenarse tanto en castellano como en inglés
Text items should be filled in both Spanish and English

Criopreservación de la línea celular (Describir método de congelación/descongelación) <i>Cryopreservation of the cell line (Describe freezing / thawing method)</i>	La criopreservación de colonias se ha realizado en FBS(90%) + DMSO(10%), mediante contenedor de isopropanol a -80°C. Los viales se han descongelado a temperatura ambiente. <i>Cryopreservation of colonies was carried out in FBS (90%) + DMSO (10%), using an isopropanol container at -80°C. The vials have been thawed at room temperature.</i>
Pase de la línea celular en el momento del banqueo/registro. (Máximo: Pase 15) <i>Passage at the time of the banking/registration (Max: Passage 15)</i>	7
¿Ha sido la línea modificada genéticamente? <i>Has the line been genetically modified?</i>	Sí Yes ☐ No No ☒ Especificar: Specify:

SECCIÓN 2 RESULTADOS DE LA CARACTERIZACIÓN DE LA LÍNEA iPSC.

Adjuntar resultados (imágenes o gráficos) como anexo

Section 2 iPSC Cell Line characterization results. Attach results (images and graphics) as an annex

Test de pluripotencia <i>Pluripotency test</i>	Método <i>Method</i>	Nº pase <i>Passage n.</i>	Resultado <i>Results</i>	Comentarios <i>Comments</i>
Se informará de al menos 5 de los siguientes marcadores <i>At least 5 of the following test will be reported</i>	Oct 4	ICC	12	+
	Nanog	ICC	12	+
	Sox 2	ICC	12	+
	SSEA3			
	SSEA4	ICC	12	+
	TRA-1-60			
	TRA-1-81	ICC	12	+
	Fosfatasa. Alk			

Test de diferenciación in vitro <i>In vitro differentiation test</i> Cuerpos embrioides <i>Embryoid bodies</i>						
	Ectodermo <i>Ectoderm</i>	Método <i>Method</i>	Marcador <i>Marker</i>	Nº pase <i>Passage n</i>	Resultado <i>Results</i>	Comentarios <i>Comments</i>
		ICC	TUJ1	9	+	
	Mesodermo <i>Mesoderm</i>	ICC	AFP	9	+	
Test de diferenciación in vivo <i>In vivo differentiation test</i> Teratomas <i>Teratomas</i>	Endodermo <i>Endoderm</i>	ICC	α-SMA	9	+	
	Ver Anexo 2					
	Ectodermo <i>Ectoderm</i>					
	Mesodermo <i>Mesoderm</i>					
Cariotipo (pase) <i>Karyotype (passage)</i>	Endodermo <i>Endoderm</i>					
	46, XX; pase 8 (Anexo 3)					
	46, XX; passage 8 (Annex 3)					
Identificación celular: Huella genética por análisis de microsatélites/STR/ otros marcadores de la línea celular/ Cell Identity: Genetic fingerprinting by microsatellite analysis / STR/ other cell line markers	Los marcadores de microsatélites de la muestra inicial de fibroblastos coinciden con los de la línea de iPS generada. (Anexo 1). <i>Microsatellite markers of the initial fibroblasts sample are identical to the markers of the iPS line (Annex 1).</i>					
Test de integración <i>Integration Test</i>	No procede, ya que se ha usado una metodología no integrativa basada en la transfección de RNA. <i>Not required, since the methodology used was non integrative and based on transfection of RNA</i>					

Documento de Solicitud de registro y depósito de una línea iPS humana. Versión enero 2021

Los apartados que requieran entrada de texto, deben rellenarse tanto en castellano como en inglés

Text items should be filled in both Spanish and English

Test de silenciamiento) <i>Silencing Test)</i>	No procede, ya que se ha usado una metodología no integrativa basada en la transfección de RNA. <i>Not required, since the methodology used was non integrative and based on transfection of RNA</i>
Confirmación de la presencia de la mutación de las células de origen <i>Confirmation of the mutation in the original cells</i>	La presencia de la mutación de las células de origen fue confirmada en las iPSCs generadas mediante secuenciación Sanger (Anexo 4). <i>The presence of the mutation of the original cells was confirmed in the generated iPSCs by Sanger sequencing (Annex 4).</i>
Test de micoplasma <i>Mycoplasma Test</i>	Negativo por PCR (Anexo 5) <i>Negative by PCR (Annex 5)</i>

ANEXOS (ANNEXES) al documento de depósito de la línea iPSC SYS FiPS68

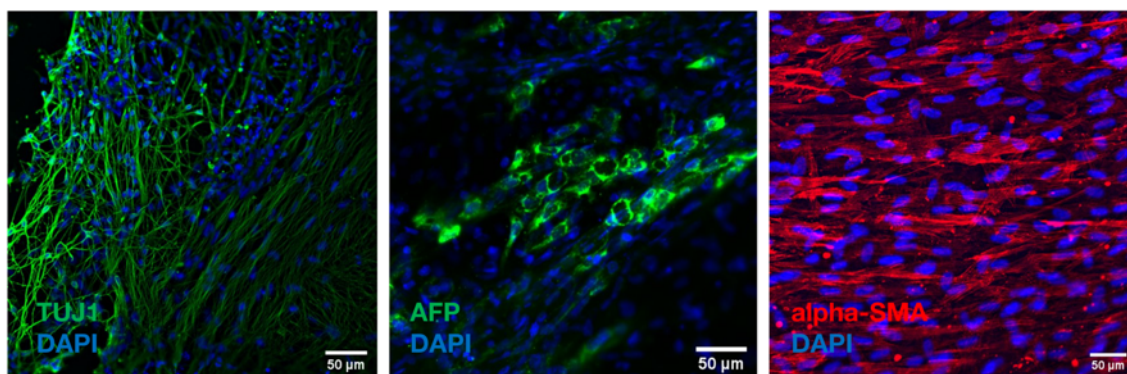
ANEXO 1: Análisis de microsatélites
 ANEXO 2: Test de diferenciación *in vitro*
 ANEXO 3: Cariotipo
 ANEXO 4: Genotipado
 ANEXO 5: Test de mycoplasma
 ANEXO 6: ICC marcadores pluripotencia

ANNEX 1: Microsatellite analysis
ANNEX 2: in vitro differentiation test
ANNEX 3: Karyotype
ANNEX 4: Genotyping
ANNEX 5: Mycoplasma test
ANNEX 6: ICC pluripotency markers

ANEXO 1: Análisis de microsatélites
ANNEX 1: Microsatellite analysis

STR	Size range	iPSC	Fibroblast
AMEL	104-112 bps	103/103	103/103
CSF1PO	312-361 bps	333/341	333/341
D13S317	166-204 bps	175/187	175/187
D16S539	258-310 bps	286/294	286/294
D18S51	279-364 bps	298/298	298/298
D21S11	195-262 bps	219/223	219/223
D5S818	108-158 bps	134/134	134/134
D8S1179	198-252 bps	230/234	230/234
FGA	305-466 bps	337/341	337/341
Penta D	365-448 bps	417/417	417/417
Penta E	368-480 bps	423/433	423/433
TH01	148-194 bps	173/176	173/176
TPOX	255-303 bps	268/268	268/268
vWA	120-184 bps	142/150	142/150

ANEXO 2: Test de diferenciación *in vitro*
ANNEX 2: in vitro differentiation test

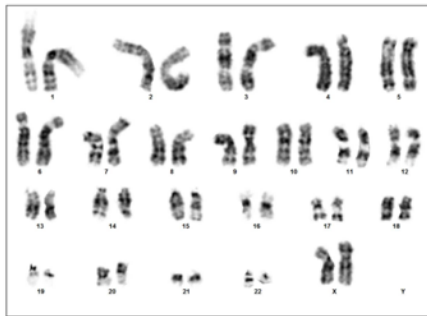


Ectoderm

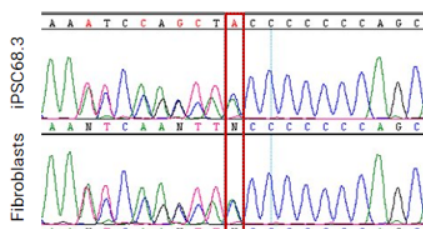
Endoderm

Mesoderm

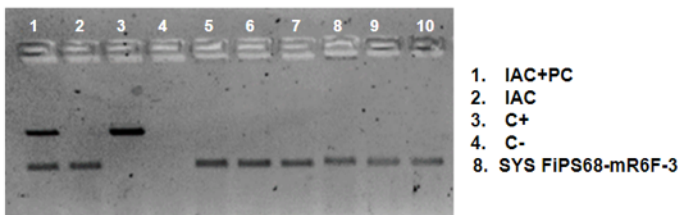
ANEXO 3: Cariotipo
ANNEX 3: Karyotype



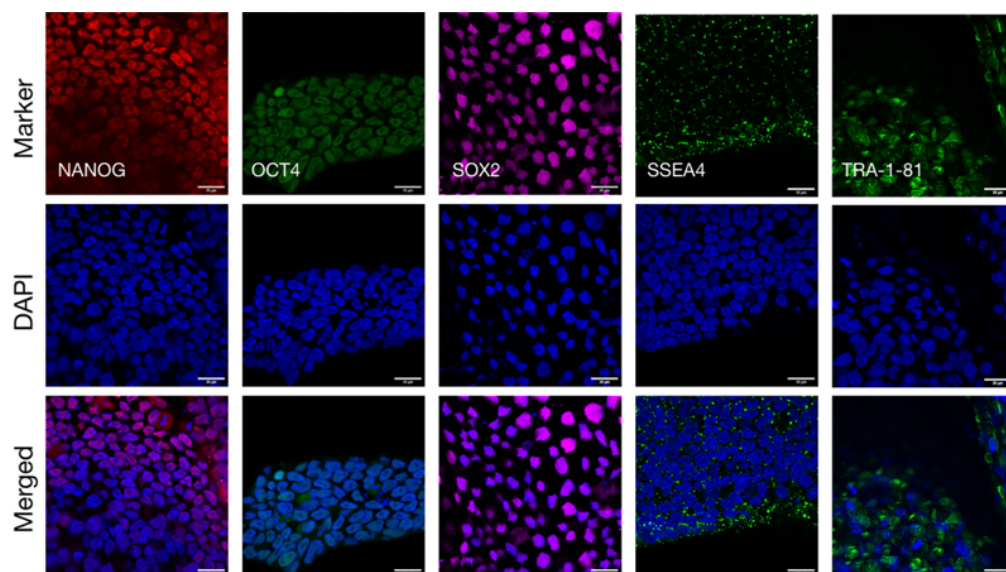
ANEXO 4: Genotipado
ANNEX 4: Genotyping



ANEXO 5: Test de micoplasma
ANNEX 5: Mycoplasma test



ANEXO 6: ICC marcadores pluripotencia
ANNEX 6: ICC pluripotency markers



BANCO NACIONAL DE LÍNEAS CELULARES (TRONCALES)
National Bank of Stem Cell Lines
IMPRESO DE SOLICITUD DE REGISTRO Y DEPÓSITO DE UNA LÍNEA iPSC HUMANA
Application Form to Register and Deposit of an human iPSC cell line

FECHA: 07-06-2024

DOCUMENTOS QUE DEBEN ACOMPAÑAR LA SOLICITUD:

Attached documents:

- ☒ **Copia de la autorización del proyecto en el cual se genera la línea celular, junto con informe favorable del Comité de Ética de la Investigación del centro de procedencia.**
A copy of the project authorization in which the cell line is obtained along with a favourable report of the Clinical Research Ethics Committee
- ☐ **Copia de cualquier publicación científica relacionada con la línea iPS generada.**
A copy of any relevant published scientific papers related to the iPS cell line generated
- ☒ **C. V. del investigador principal (una página; formato libre).**
A one page CV for the Principal Investigator
- ☒ **Número de registro del proyecto**

SECCIÓN 1-INFORMACIÓN DE LA MUESTRA ORIGINAL Y DE LA iPS GENERADA.

Section 1-Information of the original cell line and the generated iPS

Nombre de la línea iPSC <i>Name of the iPSC line:</i>	SYS FiPS120-mR6F-2
Nº de registro en el Human Pluripotent Stem Cell Registry (1)	
Muestra original donada. Detallar tipo de célula, tejido de origen y localización anatómica de la muestra biológica de la que se obtiene la línea original. Si son células comerciales, detallar nombre, referencia y distribuidor comercial <i>Original sample donated. Detail cell type, tissue of origin and anatomic location of the biological sample from which the original line is obtained. If cells are commercial, detail name, reference and trade distributor.</i>	Fibroblastos de biopsia de piel de antebrazo. <i>Fibroblasts from forearm skin biopsy.</i>
Sexo y edad del donante. <i>Sex and age of the donor</i>	Masculino, 12 <i>Male, 12</i>
¿El donante tiene alguna patología? <i>Has the donor any pathological condition?</i>	NO ☒ <i>No</i> SÍ ☐ (especificar) <i>Yes (specify)</i>

Documento de Solicitud de registro y depósito de una línea iPS humana. Versión enero 2021
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Text items should be filled in both Spanish and English

¿La patología es de origen genético? <i>Is the pathological condition of genetic origin?</i>	NO ☐ SÍ ☐ (especificar) <i>No Yes (specify)</i>
Muestra biológica recibida <i>Biological sample</i>	Fresco ☒ Crioconservado ☐ <i>Fresh Cryopreserved</i>
Fecha de la donación de la muestra biológica <i>Date of donation of the biological sample</i>	Enero 2019
Fecha del uso o descongelación <i>(si congelado)</i> <i>Date used or thawed (if frozen)</i>	Marzo 2021
Identificación celular: Huella genética por análisis de microsatélites/STR/otros marcadores de las células de origen <i>Cell Identity: Genetic fingerprinting by microsatellite analysis / STR/ other markers of the original cells.</i>	La huella genética de los fibroblastos se analizó mediante microsatélites (Anexo 1). <i>The genetic fingerprint of the fibroblasts was analyzed using microsatellites (Annex 1).</i>
Método utilizado en la generación de la línea iPSC. (Integrativa/ No-integrativa) Especificar factores y plásmidos de reprogramación utilizados. <i>Method used for the generation of iPSC line (Integrative / Non-integrative)</i> <i>Specify factors and plasmids used for reprogramming</i>	Se utilizó el kit de reprogramación StemRNA 3rd Gen (Stemgent®) para establecer las líneas de iPSC transfectando un cóctel de ARN que contiene ARNm de reprogramación no modificados (OCT4, SOX2, KLF4, cMYC, NANOG y LIN28 (OSKMNL)), ARNm de evasión inmune (E3, K3, B18) y microARN bicatenarios del grupo 302/367 en fibroblastos. <i>The StemRNA 3rd Gen Reprogramming Kit (Stemgent®) was used to establish the iPSCs lines by transfecting an RNA cocktail containing non-modified reprogramming mRNAs (OCT4, SOX2, KLF4, cMYC, NANOG and LIN28 (OSKMNL)), immune evasion mRNAs (E3, K3, B18) and double-stranded microRNAs from the 302/367 cluster into fibroblasts.</i>
Condiciones de cultivo de la línea de iPSC generada. (si se describen en publicación, indicar referencia) <i>iPSC Culture conditions (if they are described in a publication, please indicate the reference)</i>	Las colonias se recogieron y se sembraron en placas de 4 pocillos recubiertas con laminina en medio mTeSR Plus™. Las células se pasaron usando TrypLE™ Select Enzyme (ThermoFisher Scientific) a placas de 6 pocillos recubiertas con Matrigel (Corning, Inc.) para la expansión, el medio se complementó con inhibidor de Rock (Y-27632) (StemCell Technologies) durante 24 horas después de cada pase. <i>Colonies were picked and plated in laminin-coated 4-well plates in mTeSR Plus™ medium. Cells were passaged using TrypLE™ Select Enzyme (ThermoFisher Scientific) to 6-well plates coated with Matrigel (Corning, Inc.) for expansion, medium was supplemented with Rock inhibitor (Y-27632) (StemCell Technologies) for 24 hours after every passage.</i>

Documento de Solicitud de registro y depósito de una línea iPS humana. Versión enero 2021
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Text items should be filled in both Spanish and English

Criopreservación de la línea celular (Describir método de congelación/descongelación) <i>Cryopreservation of the cell line (Describe freezing / thawing method)</i>	La criopreservación de colonias se ha realizado en FBS(90%) + DMSO(10%), mediante contenedor de isopropanol a -80°C. Los viales se han descongelado a temperatura ambiente. <i>Cryopreservation of colonies was carried out in FBS (90%) + DMSO (10%), using an isopropanol container at -80°C. The vials have been thawed at room temperature.</i>
Pase de la línea celular en el momento del banqueo/registro. (Máximo: Pase 15) <i>Passage at the time of the banking/registration (Max: Passage 15)</i>	7
¿Ha sido la línea modificada genéticamente? <i>Has the line been genetically modified?</i>	Sí Yes ☐ No No ☒ Especificar: <i>Specify:</i>

SECCIÓN 2 RESULTADOS DE LA CARACTERIZACIÓN DE LA LÍNEA iPSC.

Adjuntar resultados (imágenes o gráficos) como anexo

Section 2 iPSC Cell Line characterization results. Attach results (images and graphics) as an annex

Test de pluripotencia <i>Pluripotency test</i>	Método <i>Method</i>	Nº pase <i>Passage n.</i>	Resultado <i>Results</i>	Comentarios <i>Comments</i>
Se informará de al menos 5 de los siguientes marcadores <i>At least 5 of the following test will be reported</i>	Oct 4	ICC	12	+
	Nanog	ICC	12	+
	Sox 2	ICC	12	+
	SSEA3			
	SSEA4	ICC	12	+
	TRA-1-60			
	TRA-1-81	ICC	12	+
	Fosfatasa. Alk			

Documento de Solicitud de registro y depósito de una línea iPS humana. Versión enero 2021

Los apartados que requieran entrada de texto, deben rellenarse tanto en castellano como en inglés

Text items should be filled in both Spanish and English

Test de diferenciación in vitro <i>In vitro differentiation test</i> Cuerpos embrioides <i>Embryoid bodies</i>						
	Ectodermo <i>Ectoderm</i>	Método <i>Method</i>	Marcador <i>Marker</i>	Nº pase <i>Passage n</i>	Resultado <i>Results</i>	Comentarios <i>Comments</i>
		ICC	TUJ1	9	+	
	Mesodermo <i>Mesoderm</i>	ICC	AFP	9	+	
	Endodermo <i>Endoderm</i>	ICC	α-SMA	9	+	
	<i>Ver Anexo 2</i>					
Test de diferenciación in vivo <i>In vivo differentiation test</i> Teratomas <i>Teratomas</i>	Ectodermo <i>Ectoderm</i>	Método <i>Method</i>	Marcador <i>Marker</i>	Nº pase <i>Passage n</i>	Resultado <i>Results</i>	Comentarios <i>Comments</i>
	Mesodermo <i>Mesoderm</i>					
	Endodermo <i>Endoderm</i>					
Cariotipo (pase) <i>Karyotype (passage)</i>	46, XY (Anexo 3) 46, XY (Annex 3)					
Identificación celular: Huella genética por análisis de microsatélites/STR/ otros marcadores de la línea celular/ Cell Identity: Genetic fingerprinting by microsatellite analysis / STR/ other cell line markers	Los marcadores de microsatélites de la muestra inicial de fibroblastos coinciden con los de la línea de iPS generada. (Anexo 1). <i>Microsatellite markers of the initial fibroblasts sample are identical to the markers of the iPS line (Annex 1).</i>					
Test de integración) <i>Integration Test)</i>	No procede, ya que se ha usado una metodología no integrativa basada en la transfección de RNA. <i>Not required, since the methodology used was non integrative and based on transfection of RNA.</i>					

Documento de Solicitud de registro y depósito de una línea iPS humana. Versión enero 2021
 Los apartados que requieran entrada de texto, deben rellenarse tanto en castellano como en inglés
Text items should be filled in both Spanish and English

Test de silenciamiento) <i>Silencing Test)</i>	No procede, ya que se ha usado una metodología no integrativa basada en la transfección de RNA. <i>Not required, since the methodology used was non integrative and based on transfection of RNA.</i>
Confirmación de la presencia de la mutación de las células de origen <i>Confirmation of the mutation in the original cells</i>	No procede <i>Not required</i>
Test de micoplasma <i>Mycoplasma Test</i>	Negativo por PCR (Anexo 4) <i>Negative by PCR (Annex 4)</i>

ANEXOS (*ANNEXES*) al documento de depósito de la línea iPSC SYS FiPS120

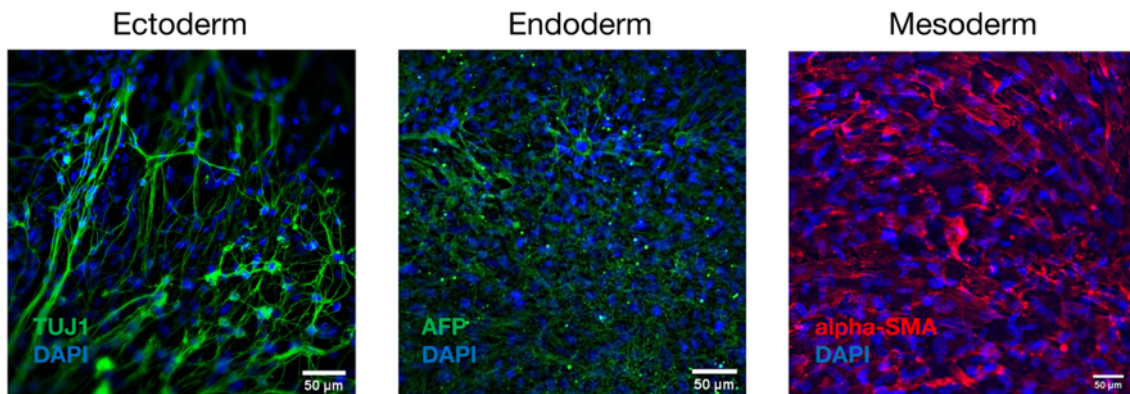
ANEXO 1: Análisis de microsatélites
 ANEXO 2: Test de diferenciación *in vitro*
 ANEXO 3: Cariotipo
 ANEXO 4: Genotipado
 ANEXO 5: ICC marcadores pluripotencia

ANNEX 1: Microsatellite analysis
ANNEX 2: in vitro differentiation test
ANNEX 3: Karyotype
ANNEX 4: Genotyping
ANNEX 5: ICC pluripotency markers

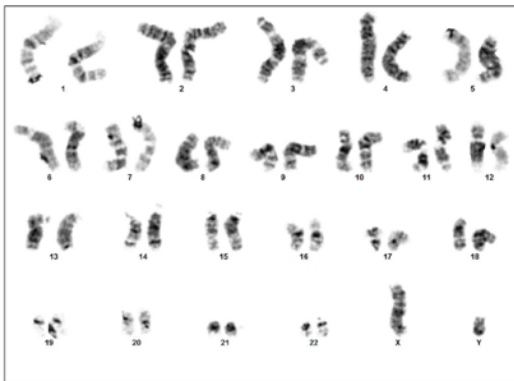
ANEXO 1: Análisis de microsatélites
ANNEX 1: Microsatellite analysis

STR	Size range	iPSC	Fibroblast
AMEL	104-112 bps	104/109	104/109
D13S317	166-204 bps	175/175	175/175
D16S539	258-310 bps	290/294	290/294
D18S51	279-364 bps	223/223	222/222
D3S1358	94-148 bps	136/136	135/135
D5S818	108-158 bps	130/130	130/130
D7S820	208-252 bps	232/236	232/236
D8S1179	198-252 bps	226/234	226/234
Penta D	365-448 bps	398/408	398/408
TPOX	255-303 bps	269/281	269/281
vWA	120-184 bps	150/150	150/150

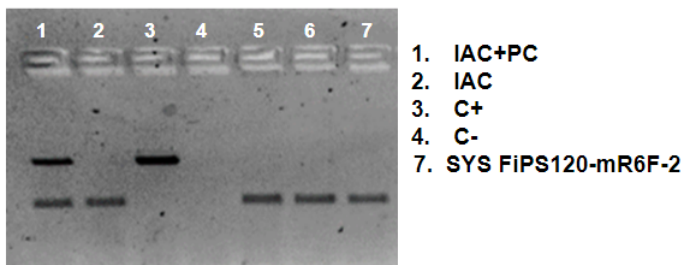
ANEXO 2: Test de diferenciación *in vitro*
ANNEX 2: in vitro differentiation test



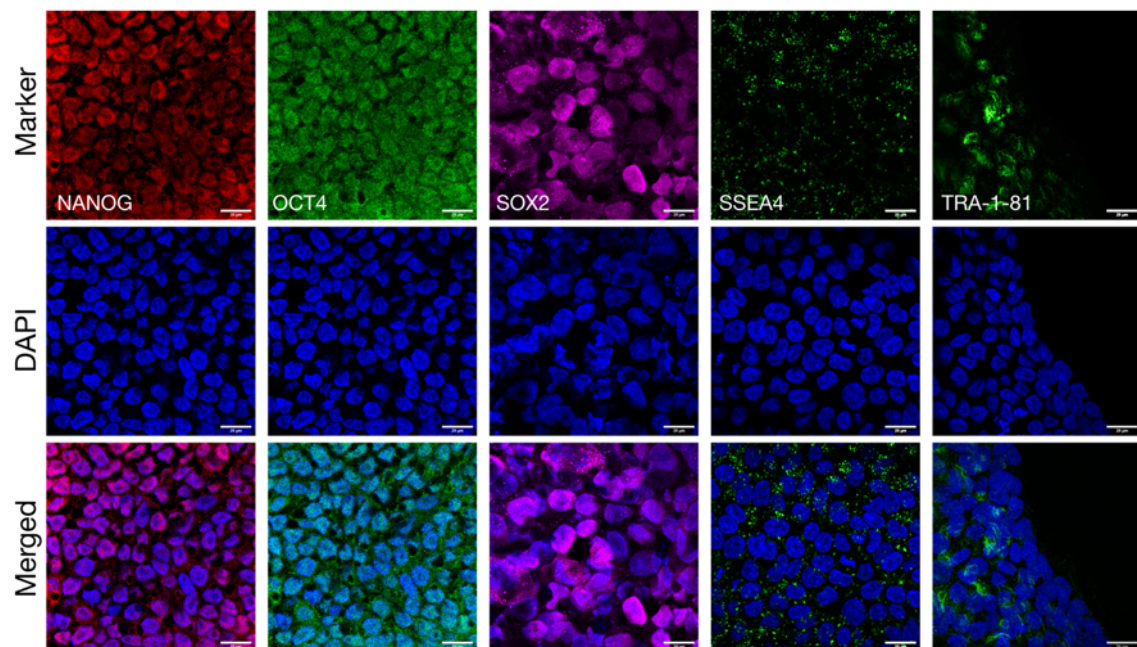
ANEXO 3: Cariotipo
ANNEX 3: Karyotype



ANEXO 4: Test de micoplasma
ANNEX 4: Mycoplasma test



ANEXO 5: ICC marcadores pluripotencia
ANNEX 5: ICC pluripotency markers



BANCO NACIONAL DE LÍNEAS CELULARES (TRONCALES)
National Bank of Stem Cell Lines
IMPRESO DE SOLICITUD DE REGISTRO Y DEPÓSITO DE UNA LÍNEA iPSC HUMANA
Application Form to Register and Deposit of an human iPSC cell line

FECHA: 07-06-2024

DOCUMENTOS QUE DEBEN ACOMPAÑAR LA SOLICITUD:

Attached documents:

- ☒ **Copia de la autorización del proyecto en el cual se genera la línea celular, junto con informe favorable del Comité de Ética de la Investigación del centro de procedencia.**
A copy of the project authorization in which the cell line is obtained along with a favourable report of the Clinical Research Ethics Committee
- ☐ **Copia de cualquier publicación científica relacionada con la línea iPS generada.**
A copy of any relevant published scientific papers related to the iPS cell line generated
- ☒ **C. V. del investigador principal (una página; formato libre).**
A one page CV for the Principal Investigator
- ☒ **Número de registro del proyecto**

SECCIÓN 1-INFORMACIÓN DE LA MUESTRA ORIGINAL Y DE LA iPS GENERADA.

Section 1-Information of the original cell line and the generated iPS

Nombre de la línea iPSC <i>Name of the iPSC line:</i>	SYS FiPS132-mR6F-4
Nº de registro en el Human Pluripotent Stem Cell Registry (1)	
Muestra original donada. Detallar tipo de célula, tejido de origen y localización anatómica de la muestra biológica de la que se obtiene la línea original. Si son células comerciales, detallar nombre, referencia y distribuidor comercial <i>Original sample donated. Detail cell type, tissue of origin and anatomic location of the biological sample from which the original line is obtained. If cells are commercial, detail name, reference and trade distributor.</i>	Fibroblastos de biopsia de piel del antebrazo. <i>Fibroblasts from forearm skin biopsy.</i>
Sexo y edad del donante. <i>Sex and age of the donor</i>	Femenino, 13 <i>Female, 13</i>
¿El donante tiene alguna patología? <i>Has the donor any pathological condition?</i>	NO ☐ <i>No</i> SÍ ☒ (especificar) <i>Yes (specify)</i> Schaaf-Yang syndrome

Documento de Solicitud de registro y depósito de una línea iPS humana. Versión enero 2021
 Los apartados que requieran entrada de texto, deben rellenarse tanto en castellano como en inglés
Text items should be filled in both Spanish and English

¿La patología es de origen genético? <i>Is the pathological condition of genetic origin?</i>	NO ☐ SÍ ☒ (especificar) MAGEL2 c.3019C>T, p.Gln1007* No Yes (specify)
Muestra biológica recibida <i>Biological sample</i>	Fresco ☒ Crioconservado ☐ Fresh Cryopreserved
Fecha de la donación de la muestra biológica <i>Date of donation of the biological sample</i>	Mayo 2019
Fecha del uso o descongelación <i>(si congelado)</i> <i>Date used or thawed (if frozen)</i>	01-03-2021
Identificación celular: Huella genética por análisis de microsatélites/STR/otros marcadores de las células de origen <i>Cell Identity: Genetic fingerprinting by microsatellite analysis / STR/ other markers of the original cells.</i>	La huella genética de los fibroblastos se analizó mediante microsatélites (Anexo 1). <i>The genetic fingerprint of the fibroblasts was analyzed using microsatellites (Annex 1).</i>
Método utilizado en la generación de la línea iPSC. (Integrativa/ No-integrativa) Especificar factores y plásmidos de reprogramación utilizados. <i>Method used for the generation of iPSC line (Integrative / Non-integrative)</i> <i>Specify factors and plasmids used for reprogramming</i>	Se utilizó el kit de reprogramación StemRNA 3rd Gen (Stemgent®) para establecer las líneas de iPSC transfectando un cóctel de ARN que contiene ARNm de reprogramación no modificados (OCT4, SOX2, KLF4, cMYC, NANOG y LIN28 (OSKMNL)), ARNm de evasión inmune (E3, K3, B18) y microARN bicatenarios del grupo 302/367 en fibroblastos. <i>The StemRNA 3rd Gen Reprogramming Kit (Stemgent®) was used to establish the iPSCs lines by transfecting an RNA cocktail containing non-modified reprogramming mRNAs (OCT4, SOX2, KLF4, cMYC, NANOG and LIN28 (OSKMNL)), immune evasion mRNAs (E3, K3, B18) and double-stranded microRNAs from the 302/367 cluster into fibroblasts.</i>
Condiciones de cultivo de la línea de iPSC generada. (si se describen en publicación, indicar referencia) <i>iPSC Culture conditions (if they are described in a publication, please indicate the reference)</i>	Las colonias se recogieron y se sembraron en placas de 4 pocillos recubiertas con laminina en medio mTeSR Plus™. Las células se pasaron usando TrypLE™ Select Enzyme (ThermoFisher Scientific) a placas de 6 pocillos recubiertas con Matrigel (Corning, Inc.) para la expansión, el medio se complementó con inhibidor de Rock (Y-27632) (StemCell Technologies) durante 24 horas después de cada pase. <i>Colonies were picked and plated in laminin-coated 4-well plates in mTeSR Plus™ medium. Cells were passaged using TrypLE™ Select Enzyme (ThermoFisher Scientific) to 6-well plates coated with Matrigel (Corning, Inc.) for expansion, medium was supplemented with Rock inhibitor (Y-27632) (StemCell Technologies) for 24 hours after every passage.</i>

Documento de Solicitud de registro y depósito de una línea iPS humana. Versión enero 2021

Los apartados que requieran entrada de texto, deben rellenarse tanto en castellano como en inglés

Text items should be filled in both Spanish and English

Criopreservación de la línea celular (Describir método de congelación/descongelación) <i>Cryopreservation of the cell line (Describe freezing / thawing method)</i>	La criopreservación de colonias se ha realizado en FBS(90%) + DMSO(10%), mediante contenedor de isopropanol a -80°C. Los viales se han descongelado a temperatura ambiente. <i>Cryopreservation of colonies was carried out in FBS (90%) + DMSO (10%), using an isopropanol container at -80°C. The vials have been thawed at room temperature.</i>
Pase de la línea celular en el momento del banqueo/registro. (Máximo: Pase 15) <i>Passage at the time of the banking/registration (Max: Passage 15)</i>	7
¿Ha sido la línea modificada genéticamente? <i>Has the line been genetically modified?</i>	Sí Yes ☐ No No ☒ Especificar: Specify:

SECCIÓN 2 RESULTADOS DE LA CARACTERIZACIÓN DE LA LÍNEA iPSC.

Adjuntar resultados (imágenes o gráficos) como anexo

Section 2 iPSC Cell Line characterization results. Attach results (images and graphics) as an annex

Test de pluripotencia <i>Pluripotency test</i>	Método <i>Method</i>	Nº pase <i>Passage n.</i>	Resultado <i>Results</i>	Comentarios <i>Comments</i>
Se informará de al menos 5 de los siguientes marcadores <i>At least 5 of the following test will be reported</i>	Oct 4	ICC	12	+
	Nanog	ICC	12	+
	Sox 2	ICC	12	+
	SSEA3			
	SSEA4	ICC	12	+
	TRA-1-60			
	TRA-1-81	ICC	12	+
	Fosfatasa. Alk			

Documento de Solicitud de registro y depósito de una línea iPS humana. Versión enero 2021
 Los apartados que requieran entrada de texto, deben rellenarse tanto en castellano como en inglés
Text items should be filled in both Spanish and English

Test de diferenciación in vitro <i>In vitro differentiation test</i> Cuerpos embrioides <i>Embryoid bodies</i>						
	Ectodermo <i>Ectoderm</i>	Método <i>Method</i>	Marcador <i>Marker</i>	Nº pase <i>Passage n</i>	Resultado <i>Results</i>	Comentarios <i>Comments</i>
		ICC	TUJ1	9	+	
	Mesodermo <i>Mesoderm</i>	ICC	AFP	9	+	
	Endodermo <i>Endoderm</i>	ICC	α-SMA	9	+	
	<i>Ver Anexo 2</i>					
Test de diferenciación in vivo <i>In vivo differentiation test</i> Teratomas <i>Teratomas</i>	Ectodermo <i>Ectoderm</i>	Método <i>Method</i>	Marcador <i>Marker</i>	Nº pase <i>Passage n</i>	Resultado <i>Results</i>	Comentarios <i>Comments</i>
	Mesodermo <i>Mesoderm</i>					
	Endodermo <i>Endoderm</i>					
Cariotipo (pase) <i>Karyotype (passage)</i>	46, XX; pase 8 (Anexo 3)					
	46, XX; passage 8 (Annex 3)					
Identificación celular: Huella genética por análisis de microsatélites/STR/ otros marcadores de la línea celular/ Cell Identity: Genetic fingerprinting by microsatellite analysis / STR/ other cell line markers	Los marcadores de microsatélites de la muestra inicial de fibroblastos coinciden con los de la línea de iPS generada. (Anexo 1). <i>Microsatellite markers of the initial fibroblasts sample are identical to the markers of the iPS line (Annex 1).</i>					
Test de integración) <i>Integration Test)</i>	No procede, ya que se ha usado una metodología no integrativa basada en la transfección de RNA. <i>Not required, since the methodology used was non integrative and based on transfection of RNA</i>					
Test de silenciamiento) <i>Silencing Test)</i>	No procede, ya que se ha usado una metodología no integrativa basada en la transfección de RNA. <i>Not required, since the methodology used was non integrative and based on</i>					

Documento de Solicitud de registro y depósito de una línea iPS humana. Versión enero 2021

Los apartados que requieran entrada de texto, deben rellenarse tanto en castellano como en inglés

Text items should be filled in both Spanish and English

	<i>transfection of RNA</i>
Confirmación de la presencia de la mutación de las células de origen <i>Confirmation of the mutation in the original cells</i>	La presencia de la mutación de las células de origen fue confirmada en las iPSCs generadas mediante secuenciación Sanger (Anexo 4). <i>The presence of the mutation of the original cells was confirmed in the generated iPSCs by Sanger sequencing (Annex 4).</i>
Test de micoplasma <i>Mycoplasma Test</i>	Negativo por PCR (Anexo 5) <i>Negative by PCR (Annex 5)</i>

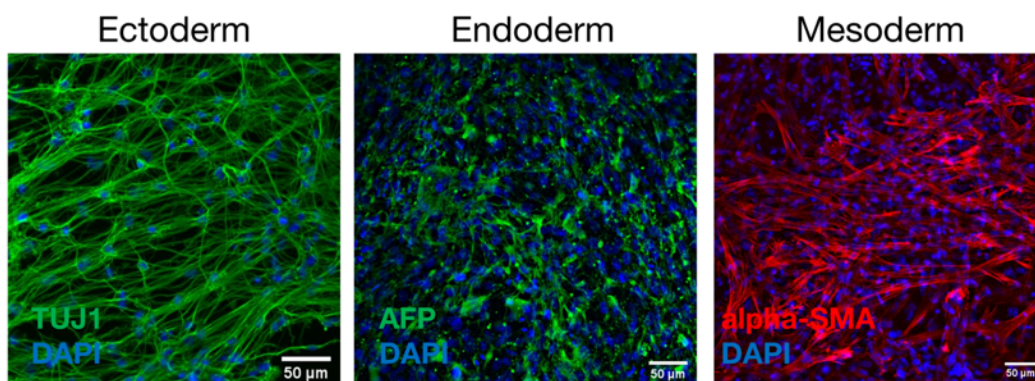
ANEXOS (ANNEXES) al documento de depósito de la línea iPSC SYS FiPS132

ANEXO 1: Análisis de microsatélites	<i>ANNEX 1: Microsatellite analysis</i>
ANEXO 2: Test de diferenciación <i>in vitro</i>	<i>ANNEX 2: in vitro differentiation test</i>
ANEXO 3: Cariotipo	<i>ANNEX 3: Karyotype</i>
ANEXO 4: Genotipado	<i>ANNEX 4: Genotyping</i>
ANEXO 5: Test de mycoplasma	<i>ANNEX 5: Mycoplasma test</i>
ANEXO 6: ICC marcadores pluripotencia	<i>ANNEX 6: ICC pluripotency markers</i>

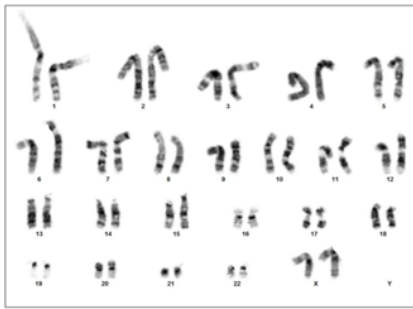
ANEXO 1: Análisis de microsatélites
ANNEX 1: Microsatellite analysis

STR	Size range	iPSC	Fibroblast
AMEL	104-112 bps	104/104	104/104
D13S317	166-204 bps	175/187	175/187
D16S539	258-310 bps	286 /294	286/294
D18S51	279-364 bps	314/325	314/325
D21S11	195-262 bps	223/223	223/223
D5S818	108-158 bps	126/130	125/130
D7S820	208-252 bps	220/232	220/232
D8S1179	198-252 bps	214/226	214/226
FGA	305-466 bps	337/337	337/337
Penta D	365-448 bps	403/417	403/417
Penta E	368-480 bps	392/439	392/439
TH01	148-194 bps	169/176	169/176
TPOX	255-303 bps	269/269	269/269
vWA	120-184 bps	146/146	146/146

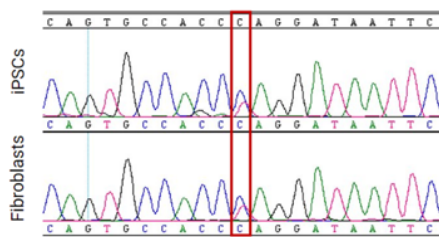
ANEXO 2: Test de diferenciación *in vitro*
ANNEX 2: in vitro differentiation test



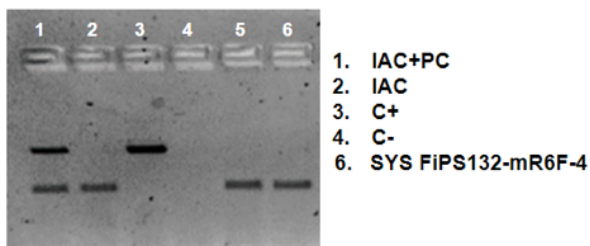
ANEXO 3: Cariotipo
ANNEX 3: Karyotype



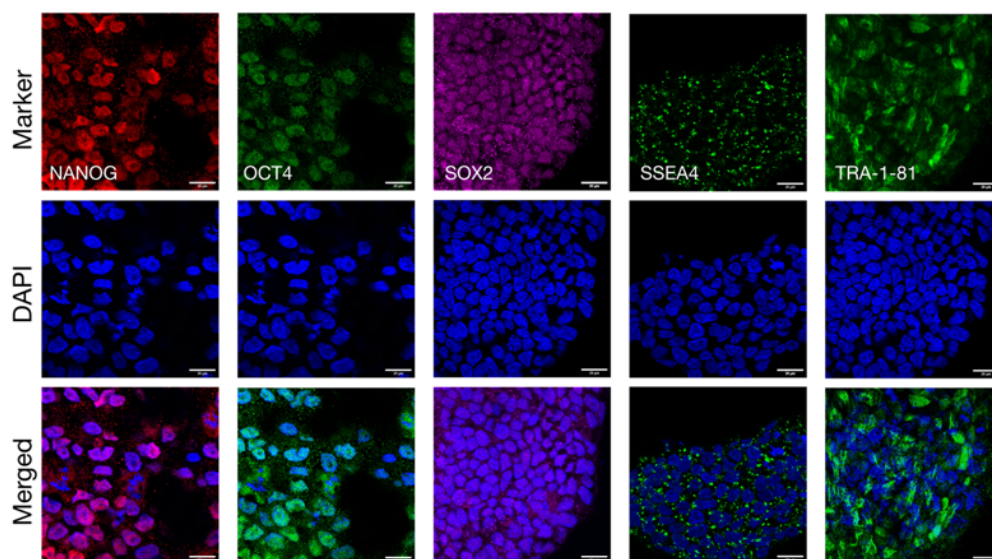
ANEXO 4: Genotipado
ANNEX 4: Genotyping



ANEXO 5: Test de micoplasma
ANNEX 5: Mycoplasma test



ANEXO 6: ICC marcadores pluripotencia
ANNEX 6: ICC pluripotency markers



BANCO NACIONAL DE LÍNEAS CELULARES (TRONCALES)
National Bank of Stem Cell Lines
IMPRESO DE SOLICITUD DE REGISTRO Y DEPÓSITO DE UNA LÍNEA iPSC HUMANA
Application Form to Register and Deposit of an human iPSC cell line

FECHA: 07-06-2024

DOCUMENTOS QUE DEBEN ACOMPAÑAR LA SOLICITUD:

Attached documents:

- ☒ **Copia de la autorización del proyecto en el cual se genera la línea celular, junto con informe favorable del Comité de Ética de la Investigación del centro de procedencia.**
A copy of the project authorization in which the cell line is obtained along with a favourable report of the Clinical Research Ethics Committee
- ☐ **Copia de cualquier publicación científica relacionada con la línea iPS generada.**
A copy of any relevant published scientific papers related to the iPS cell line generated
- ☒ **C. V. del investigador principal (una página; formato libre).**
A one page CV for the Principal Investigator
- ☒ **Número de registro del proyecto**

SECCIÓN 1-INFORMACIÓN DE LA MUESTRA ORIGINAL Y DE LA iPS GENERADA.

Section 1-Information of the original cell line and the generated iPS

Nombre de la línea iPSC <i>Name of the iPSC line:</i>	SYS FiPS135-mR6F-5
Nº de registro en el Human Pluripotent Stem Cell Registry (1)	
Muestra original donada. Detallar tipo de célula, tejido de origen y localización anatómica de la muestra biológica de la que se obtiene la línea original. Si son células comerciales, detallar nombre, referencia y distribuidor comercial <i>Original sample donated. Detail cell type, tissue of origin and anatomic location of the biological sample from which the original line is obtained. If cells are commercial, detail name, reference and trade distributor.</i>	Fibroblastos de biopsia de piel abdominal. <i>Fibroblasts from abdominal skin biopsy</i>
Sexo y edad del donante. <i>Sex and age of the donor</i>	Femenino, 13 <i>Female, 13</i>
¿El donante tiene alguna patología? <i>Has the donor any pathological condition?</i>	NO ☒ <i>No</i> SÍ ☐ (especificar) <i>Yes (specify)</i>

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Text items should be filled in both Spanish and English

¿La patología es de origen genético? <i>Is the pathological condition of genetic origin?</i>	NO ☐ SÍ ☐ (especificar) <i>No Yes (specify)</i>
Muestra biológica recibida <i>Biological sample</i>	Fresco ☒ Crioconservado ☐ <i>Fresh Cryopreserved</i>
Fecha de la donación de la muestra biológica <i>Date of donation of the biological sample</i>	Mayo 2019
Fecha del uso o descongelación <i>(si congelado) Date used or thawed (if frozen)</i>	1 marzo 2021
Identificación celular: Huella genética por análisis de microsatélites/STR/otros marcadores de las células de origen <i>Cell Identity: Genetic fingerprinting by microsatellite analysis / STR/ other markers of the original cells.</i>	La huella genética de los fibroblastos se analizó mediante microsatélites (Anexo 1). <i>The genetic fingerprint of the fibroblasts was analyzed using microsatellites (Annex 1).</i>
Método utilizado en la generación de la línea iPSC. (Integrativa/ No-integrativa) Especificar factores y plásmidos de reprogramación utilizados. <i>Method used for the generation of iPSC line (Integrative / Non-integrative) Specify factors and plasmids used for reprogramming</i>	Se utilizó el kit de reprogramación StemRNA 3rd Gen (Stemgent®) para establecer las líneas de iPSC transfectando un cóctel de ARN que contiene ARNm de reprogramación no modificados (OCT4, SOX2, KLF4, cMYC, NANOG y LIN28 (OSKMNL)), ARNm de evasión inmune (E3, K3, B18) y microARN bicatenarios del grupo 302/367 en fibroblastos. <i>The StemRNA 3rd Gen Reprogramming Kit (Stemgent®) was used to establish the iPSCs lines by transfecting an RNA cocktail containing non-modified reprogramming mRNAs (OCT4, SOX2, KLF4, cMYC, NANOG and LIN28 (OSKMNL)), immune evasion mRNAs (E3, K3, B18) and double-stranded microRNAs from the 302/367 cluster into fibroblasts.</i>
Condiciones de cultivo de la línea de iPSC generada. (si se describen en publicación, indicar referencia) <i>iPSC Culture conditions (if they are described in a publication, please indicate the reference)</i>	Las colonias se recogieron y se sembraron en placas de 4 pocillos recubiertas con laminina en medio mTeSR Plus™. Las células se pasaron usando TrypLE™ Select Enzyme (ThermoFisher Scientific) a placas de 6 pocillos recubiertas con Matrigel (Corning, Inc.) para la expansión, el medio se complementó con inhibidor de Rock (Y-27632) (StemCell Technologies) durante 24 horas después de cada pase. <i>Colonies were picked and plated in laminin-coated 4-well plates in mTeSR Plus™ medium. Cells were passaged using TrypLE™ Select Enzyme (ThermoFisher Scientific) to 6-well plates coated with Matrigel (Corning, Inc.) for expansion, medium was supplemented with Rock inhibitor (Y-27632) (StemCell Technologies) for 24 hours after every passage.</i>

Documento de Solicitud de registro y depósito de una línea IPS humana. Versión enero 2021
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Text items should be filled in both Spanish and English

Criopreservación de la línea celular (Describir método de congelación/descongelación) <i>Cryopreservation of the cell line (Describe freezing / thawing method)</i>	La criopreservación de colonias se ha realizado en FBS(90%) + DMSO(10%), mediante contenedor de isopropanol a -80°C. Los viales se han descongelado a temperatura ambiente. <i>Cryopreservation of colonies was carried out in FBS (90%) + DMSO (10%), using an isopropanol container at -80°C. The vials have been thawed at room temperature.</i>
Pase de la línea celular en el momento del banqueo/registro. (Máximo: Pase 15) <i>Passage at the time of the banking/registration (Max: Passage 15)</i>	7
¿Ha sido la línea modificada genéticamente? <i>Has the line been genetically modified?</i>	Sí Yes ☐ No No ☒ Especificar: <i>Specify:</i>

SECCIÓN 2 RESULTADOS DE LA CARACTERIZACIÓN DE LA LÍNEA iPSC.

Adjuntar resultados (imágenes o gráficos) como anexo

Section 2 iPSC Cell Line characterization results. Attach results (images and graphics) as an annex

Test de pluripotencia <i>Pluripotency test</i>	Método <i>Method</i>	Nº pase <i>Passage n.</i>	Resultado <i>Results</i>	Comentarios <i>Comments</i>
Se informará de al menos 5 de los siguientes marcadores <i>At least 5 of the following test will be reported</i>	Oct 4	ICC	12	+
	Nanog	ICC	12	+
	Sox 2	ICC	12	+
	SSEA3			
	SSEA4	ICC	12	+
	TRA-1-60			
	TRA-1-81	ICC	12	+
	Fosfatasa. Alk			

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 Los apartados que requieran entrada de texto, deben rellenarse tanto en castellano como en inglés
Text items should be filled in both Spanish and English

Test de diferenciación in vitro <i>In vitro differentiation test</i> Cuerpos embrioides <i>Embryoid bodies</i>						
	Ectodermo <i>Ectoderm</i>	Método <i>Method</i>	Marcador <i>Marker</i>	Nº pase <i>Passage n</i>	Resultado <i>Results</i>	Comentarios <i>Comments</i>
		ICC	TUJ1	9	+	
	Mesodermo <i>Mesoderm</i>	ICC	AFP	9	+	
	Endodermo <i>Endoderm</i>	ICC	α-SMA	9	+	
	<i>Ver Anexo 2</i>					
Test de diferenciación in vivo <i>In vivo differentiation test</i> Teratomas <i>Teratomas</i>	Ectodermo <i>Ectoderm</i>	Método <i>Method</i>	Marcador <i>Marker</i>	Nº pase <i>Passage n</i>	Resultado <i>Results</i>	Comentarios <i>Comments</i>
	Mesodermo <i>Mesoderm</i>					
	Endodermo <i>Endoderm</i>					
Cariotipo (pase) <i>Karyotype (passage)</i>	46, XX (Anexo 3) 46, XX (<i>Annex 3x</i>)					
Identificación celular: Huella genética por análisis de microsatélites/STR/ otros marcadores de la línea celular/ Cell Identity: Genetic fingerprinting by microsatellite analysis / STR/ other cell line markers	Los marcadores de microsatélites de la muestra inicial de fibroblastos coinciden con los de la línea de iPS generada. (Anexo 1). <i>Microsatellite markers of the initial fibroblasts sample are identical to the markers of the iPS line (Annex 1).</i>					
Test de integración <i>Integration Test</i>	No procede, ya que se ha usado una metodología no integrativa basada en la transfección de RNA. <i>Not required, since the methodology used was non integrative and based on transfection of RNA</i>					
Test de silenciamiento <i>Silencing Test</i>	No procede, ya que se ha usado una metodología no integrativa basada en la transfección de RNA. <i>Not required, since the methodology used was non integrative and based on</i>					

Documento de Solicitud de registro y depósito de una línea iPS humana. Versión enero 2021
 Los apartados que requieran entrada de texto, deben rellenarse tanto en castellano como en inglés
Text items should be filled in both Spanish and English

	<i>transfection of RNA</i>
Confirmación de la presencia de la mutación de las células de origen <i>Confirmation of the mutation in the original cells</i>	No procede <i>Not required</i>
Test de micoplasma <i>Mycoplasma Test</i>	Negativo por PCR (Anexo 4) <i>Negative by PCR (Annex 4)</i>

ANEXOS (*ANNEXES*) al documento de depósito de la línea iPSC SYS FiPS135

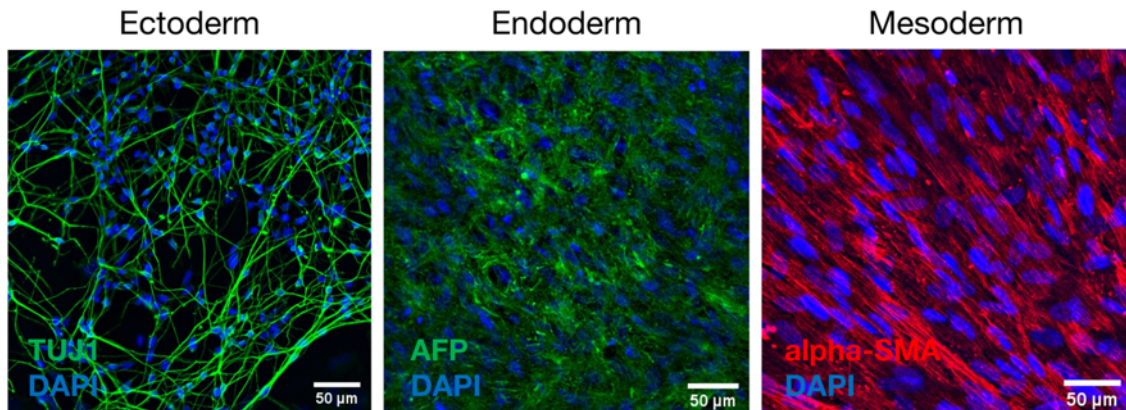
ANEXO 1: Análisis de microsatélites
 ANEXO 2: Test de diferenciación *in vitro*
 ANEXO 3: Cariotipo
 ANEXO 4: Genotipado
 ANEXO 5: ICC marcadores pluripotencia

ANNEX 1: Microsatellite analysis
ANNEX 2: in vitro differentiation test
ANNEX 3: Karyotype
ANNEX 4: Genotyping
ANNEX 5: ICC pluripotency markers

ANEXO 1: Análisis de microsatélites
ANNEX 1: Microsatellite analysis

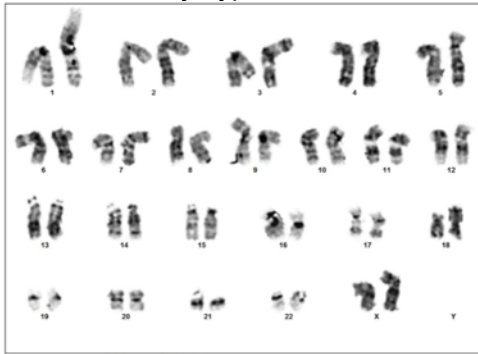
STR	Size range	iPSC	Fibroblast
AMEL	104-112 bps	104/104	104/104
D13S317	166-204 bps	179/187	179/187
D16S539	258-310 bps	286/294	286/294
D18S51	279-364 bps	302/310	302/310
D21S11	195-262 bps	215/233	215/233
D3S1358	94-148 bps	132/132	132/132
D7S820	208-252 bps	224/232	224/232
D8S1179	198-252 bps	222/226	222/226
FGA	305-466 bps	333/349	333/349
Penta D	365-448 bps	418/427	418/427
Penta E	368-480 bps	418/428	418/428
TH01	148-194 bps	169/169	169/169
TPOX	255-303 bps	269/285	269/285
vWA	120-184 bps	150/154	150/154

ANEXO 2: Test de diferenciación *in vitro*
ANNEX 2: in vitro differentiation test



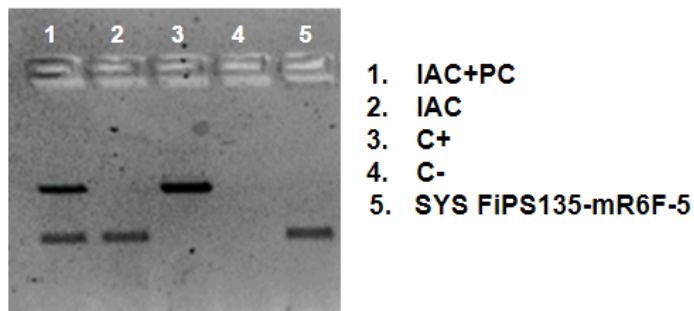
ANEXO 3: Cariotipo

ANNEX 3: Karyotype



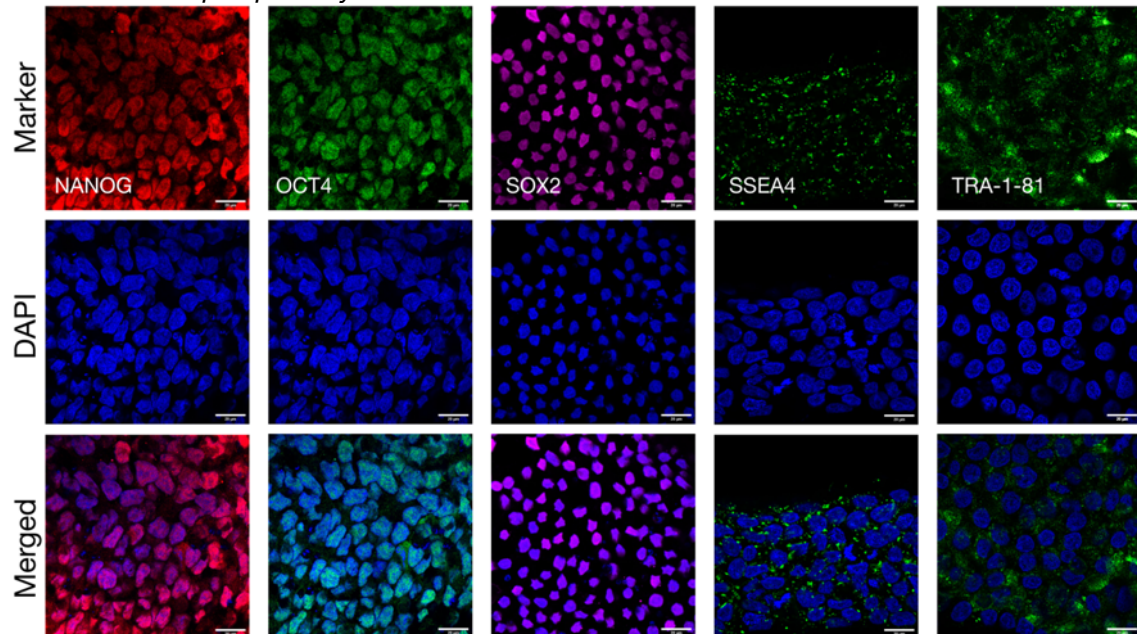
ANEXO 4: Test de micoplasma

ANNEX 4: Mycoplasma test



ANEXO 5: ICC marcadores pluripotencia

ANNEX 5: ICC pluripotency markers



During the development of this thesis, I simultaneously participated in another research line within our group, focused on the study of TRAF7 syndrome. This resulted in the publication of the article presented in Annex 2.

ANNEX 2: EXPANDING THE PHENOTYPIC SPECTRUM OF TRAF7-RELATED CARDIAC, FACIAL, AND DIGITAL ANOMALIES WITH DEVELOPMENTAL DELAY: REPORT OF 11 NEW CASES AND LITERATURE REVIEW

Summary:

Background: TRAF7-related cardiac, facial, and digital anomalies with developmental delay (CAFDADD), a multisystemic neurodevelopmental disorder caused by germline missense variants in the TRAF7 gene, exhibits heterogeneous clinical presentations.

Methods: We present a detailed description of 11 new TRAF7-related CAFDADD cases, featuring eight distinct variants, including a novel one.

Results: Phenotypic analysis and a comprehensive review of the 58 previously reported cases outline consistent clinical presentations, emphasizing dysmorphic features, developmental delay, endocrine manifestations, and cardiac defects. In this enlarged collection, novelties include a wider range of cognitive dysfunction, with some individuals exhibiting normal development despite early psychomotor delay. Communication challenges, particularly in expressive language, are prevalent, necessitating alternative communication methods. Autistic traits, notably rigidity, are observed in the cohort. Also, worth highlighting are hearing loss, sleep disturbances, and endocrine anomalies, including growth deficiency. Cardiac defects, frequently severe, pose early-life complications. Facial features, including arched eyebrows, contribute to the distinct gestalt. A novel missense variant, p.(Arg653Leu), further underscores the complex relationship between germline TRAF7 variants and somatic changes linked to meningiomas.

Conclusions: Our comprehensive analysis expands the phenotypic spectrum, emphasizing the need for oncological evaluations and proposing an evidence-based schedule for clinical management. This study contributes to a better understanding of TRAF7-related CAFDADD, offering insights for improved diagnosis, intervention, and patient care.

Reference:

Palma-Milla C, Prat-Planas A, Soengas-Gonda E, *et al.* Expanding the Phenotypic Spectrum of TRAF7-Related Cardiac, Facial, and Digital Anomalies with Developmental Delay: Report of 11 New Cases and Literature Review. *Pediatr Neurol.* 2024 Jun;155:8-17. doi: 10.1016/j.pediatrneurol.2024.03.008.



Contents lists available at ScienceDirect

Pediatric Neurology

journal homepage: www.elsevier.com/locate/pnu

Research Paper

Expanding the Phenotypic Spectrum of TRAF7-Related Cardiac, Facial, and Digital Anomalies With Developmental Delay: Report of 11 New Cases and Literature Review



Carmen Palma-Milla, PhD ^{a, b, 1, *}, Aina Prat-Planas, MSc ^{c, d, e, 1},
 Emma Soengas-Gonda, MSc ^{a, d, 1}, Mónica Centeno-Pla, MSc ^{c, d, e, f},
 Jaime Sánchez-Pozo, MD ^{a, g}, Irene Lazaro-Rodríguez, MD ^{a, g},
 Juan F. Quesada-Espinosa, MSc ^{a, b}, Ana Arteché-Lopez, PhD ^{a, b}, Jonathan Olival, PhD ^h,
 Marta Pacio-Míguez, MSc ⁱ, María Palomares-Bralo, PhD ^{d, i, j},
 Fernando Santos-Simarro, MD, PhD ^k, Ramón Cancho-Candela, MD, PhD ^l,
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ABSTRACT

Background: TRAF7-related cardiac, facial, and digital anomalies with developmental delay (CAFDADD), a multisystemic neurodevelopmental disorder caused by germline missense variants in the TRAF7 gene, exhibits heterogeneous clinical presentations.

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Keywords:

TRAF7-related CAFDADD
Neurodevelopment
Behavioral presentation
Sleep disturbances
Cardiac complications

Methods: We present a detailed description of 11 new TRAF7-related CAFDADD cases, featuring eight distinct variants, including a novel one.

Results: Phenotypic analysis and a comprehensive review of the 58 previously reported cases outline consistent clinical presentations, emphasizing dysmorphic features, developmental delay, endocrine manifestations, and cardiac defects. In this enlarged collection, novelties include a wider range of cognitive dysfunction, with some individuals exhibiting normal development despite early psychomotor delay. Communication challenges, particularly in expressive language, are prevalent, necessitating alternative communication methods. Autistic traits, notably rigidity, are observed in the cohort. Also, worth highlighting are hearing loss, sleep disturbances, and endocrine anomalies, including growth deficiency. Cardiac defects, frequently severe, pose early-life complications. Facial features, including arched eyebrows, contribute to the distinct gestalt. A novel missense variant, p.(Arg653Leu), further underscores the complex relationship between germline TRAF7 variants and somatic changes linked to meningiomas.

Conclusions: Our comprehensive analysis expands the phenotypic spectrum, emphasizing the need for oncological evaluations and proposing an evidence-based schedule for clinical management. This study contributes to a better understanding of TRAF7-related CAFDADD, offering insights for improved diagnosis, intervention, and patient care.

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Introduction

TRAF7-related CAFDADD (also known as CAFDADD; cardiac, facial, and digital anomalies with developmental delay; MIM #618164; ORPHA: 592570) is a multisystemic neurodevelopmental disorder caused by heterozygous germline pathogenic variants in the TRAF7 gene (tumor necrosis factor receptor-associated factor 7; MIM *606692). Since its initial description in 2018¹ and further delineation,² a total of 58 subjects with TRAF7-related CAFDADD have been published, carrying 26 different variants.^{3–8} Although the clinical presentation of the syndrome appears to be highly variable, some common features have been identified, including a range of neurological and developmental abnormalities, such as intellectual disability, speech and language delays, and motor impairments, as well as epilepsy in some individuals. Dysmorphic facial features (blepharophimosis, hypertelorism, and micrognathia, among others) and cardiovascular abnormalities are also seen in most individuals with TRAF7-related CAFDADD.² Additionally, skeletal anomalies like digital anomalies, scoliosis, or other spine deformities have been reported.^{1,2}

In humans, the tumor necrosis factor receptor-associated factor (TRAF) family consists of seven genes, TRAF7 being the last member to be identified. All of them share a domain organization consisting of a modular structure, which allows them to convey signals from different receptors, but each plays a unique and well-defined role in cellular biology. Specifically, TRAF7 is a multifunctional intracellular protein that acts as a key mediator of the nuclear factor-κB, the mitogen-activated protein kinases, and extracellular signal-regulated kinase signaling pathways, playing a crucial role in the control of cell survival, apoptosis, proliferation, and differentiation.^{9–14}

The TRAF7 gene encodes a 670-amino acid protein (the E3 ubiquitin-protein ligase TRAF7) containing an N-terminal RING finger domain, an adjacent TRAF-type zinc finger domain, a centrally situated coiled-coil motif, and seven WD40 repeats in the C-terminal domain. The WD40 repeats are unique to TRAF7 within the TRAF family, with all other members containing a C-terminal TRAF domain instead.¹⁰ TRAF7 syndromic variants are germline pathogenic missense variants that cluster mostly at the WD40 repeats of the TRAF7 protein, some of them being recurrent (p.Arg655Gln, p.Arg524Trp, and p.Phe617Leu being the most frequently identified).

Somatic TRAF7 variants have also been described in a wide range of tumors: meningiomas, mesotheliomas, intraneural perineuriomas, and adenomatoid tumors of the genital tract, usually in concurrence with KLF4, AKT1, or PIK3CA variants.^{11,15–19} In these

tumors missense variants affecting the WD40 domain are predominant, but loss-of-function variants are also present. So far, a large number of somatic cancer-related variants and an increasing number of germline syndromic-associated variants have been described, mostly overlapping in type and distribution, even affecting the same residue. However, the exact same variants have not been identified in both contexts, with the exception of a recently reported individual with mild syndromic features presenting with multiple meningiomas and a variant changing Arg641 residue to cysteine in a mosaic status.⁵

Here, we report 11 subjects with a clinical and molecular diagnosis of TRAF7-related CAFDADD, harboring *de novo* germline variants in TRAF7, one of which is novel. In addition, we conducted an extensive review of the literature, which allowed us to expand the clinical and genetic delineation of TRAF7-related CAFDADD. Based on this analysis, we have developed recommendations that may contribute to better clinical management of this ultrarare syndrome.

Material and Methods

Subjects

Eleven individuals from different Spanish hospitals with a confirmed diagnosis of TRAF7-related CAFDADD have been included in this study. These individuals were recruited through a nationwide collaboration focused on TRAF7-related CAFDADD, fostered by the CIBERER consortium.²⁰ The study has been conducted in accordance with the Declaration of Helsinki and has been reviewed and approved by the ethics committee at each institution. All adult participants or guardians of minors or incapacitated subjects provided written informed consent for participation.

Literature review

We performed a Cochrane Library search and a PubMed database search from the date of the first clinical description¹ of pathology associated with variants in TRAF7 until September 15, 2023, using the terms: “TRAF7” and “CAFDADD.” In addition, the HGMD Professional 2023.3 database has been consulted covering the same period. Although the scope of this review focused on germline pathogenic variants leading to TRAF7-related CAFDADD, the review also included literature referring to somatic mutations, especially focusing on the locations of TRAF7-related CAFDADD variants. Data extraction included first author, publication year, molecular data

(identified variant and residue changes, *de novo* or inherited condition), pregnancy and perinatal information, multiorgan clinical data, information about complementary examinations, neuroimaging, and age and cause of death.

Clinical assessment

Anthropometric measures and a comprehensive dysmorphic physical examination were performed; these included the individual's facial features; length, symmetry, and presence of any abnormalities in the neck, upper and lower limbs, back, and chest; and cardiopulmonary function. Palpation of the abdomen for abnormalities, comprehensive genital assessment including pubertal stage, and a comprehensive skin examination for cutaneous manifestations or abnormal pigmentation patterns were also performed. Cardiac anomalies, visual or auditory impairment, as well as available brain neuroimages were assessed. Neurodevelopmental milestones were evaluated with Denver Developmental Screening Test.²¹ Specific Autism Diagnostic Observational Schedule-2 was addressed in Patient 6. Sleep disorders were assessed by the Sleep Disturbance Scale for Children (SDSC) scale.²² Findings were encoded using Human Phenotype Ontology codes.²³

Genetics

Informed consent was obtained from the patients' parents according to International Ethical Guidelines and Declaration of Helsinki.

Whole exome sequencing was performed in multiple Spanish reference centers (Hospital 12 de Octubre, Hospital la Paz, Hospital Gregorio Marañón, Madrid; Hospital Sant Joan de Déu, Barcelona) according to established protocols and bioinformatic analyses following current recommendations. Variant interpretation was made according to American College of Medical Genetics and Genomics (ACMG) guidelines.²⁴ All variants were validated and segregated by Sanger sequencing and are referenced to the canonical

transcript ENST00000326181.11 (NM_032271.3). Patient 10 was previously published as Patient 33 in Castilla-Vallmanya et al.²

Results

Clinical findings

Eleven individuals (eight male and three female) were recruited, ranging in age from one month to 26 years. Their clinical description is summarized in Table 1. Detailed description of each subject is presented in the Supplementary Clinical Information file and in Supplementary Table 1. Most individuals presented unspecific prenatal complications such as single umbilical artery (three of 11), polyhydramnios (three of 11), or intrauterine growth retardation (two of 11).

Neurological features

At birth, all patients showed hypotonia (11 of 11) with poor sucking (seven of 11), some of them requiring a feeding tube. Global developmental delay was common (eight of 10), ranging from mild to moderate. Most patients in our cohort exhibited delayed language acquisition and development (nine of 10), with expressive language being more severely affected than receptive language. Despite some improvement over time, all presented with some communicative difficulties. Currently, Subjects P4 and P10 are communicating by sign language. Regarding neurodevelopmental comorbidities, autistic traits, lack of cognitive flexibility, or emotional disorders were present in half of the cases (five of 10), with one patient fulfilling autism spectrum disorder criteria evaluated by Autism Diagnostic Observational Schedule-2. Concerning behavioral phenotype, they were commonly described as having a happy demeanor or overfriendliness (seven of 10).

On brain magnetic resonance imaging, most of the patients (nine of 11) presented with structural findings such as ventriculomegaly, periventricular cysts, or hydrocephalus, with no other characteristic features.

TABLE 1.
Clinical Overview of the 11 Patients Presented Here

Clinical Characteristics	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10*	P11	TOTAL
Age at last evaluation	1	11	17	2	18	8	12	16	9 months	26	21	
Gender	Male	Female	Male	Male	Male	Male	Male	Male	Female	Female	Male	8 Males:3 females
Variant	K346E	S558F	H570D	L595V	S629N	V646L	R653L	R655Q	R655Q	R655Q	R655Q	
Perinatal complications	–	+	+	+	+	+	+	+	+	+	+	10/11
Short stature	–	+	+	–	+	+	+	+	–	+	+	8/11
Motor delay	–	+	+	–	+	+	+	+	+	+	+	9/11
Speech delay	+	(Mild)	+	(Mild)	+	+	+	+	NA	+	+	10/10
GDD/ID	–	+	+	(Mild)	+	–	+	+	NA	+	+	8/10
Autism spectrum traits	–	–	+	–	–	+	–	–	NA	+	+	4/10
Overfriendliness	+	+	+	NA	–	–	+	+	–	+	–	6/10
Hypotonia	+	+	+	+	+	+	+	+	+	+	+	11/11
CNS anomalies	+	–	+	–	+	+	+	+	+	+	+	9/11
Sleep disturbances	+	–	–	–	+	+	+	+	NA	+	+	7/10
Skull shape anomalies	+	+	+	–	+	–	+	+	+	+	+	9/11
Blepharophimosis	+	+	+	+	+	+	+	+	+	+	+	11/11
Strabismus	–	+	+	+	+	+	–	–	+	+	+	8/11
Abnormality of the palate/mouth	–	+	+	+	+	+	+	+	+	+	+	10/11
Cardiac anomalies	+	+	+	+	+	+	+	+	+	+	–	10/11
Skeletal/limb anomalies	+	+	+	+	+	+	+	+	+	+	–	11/11
Hearing loss	+	+	+	+	+	+	+	+	+	+	+	11/11
Visual abnormalities	–	–	–	+	+	+	+	–	+	–	–	5/11

Abbreviations:

CNS = Central nervous system

GDD = Global developmental delay

HPO = Human Phenotype Ontology

ID = Intellectual disability

NA = Not assessable

Detailed information and HPO codes are presented in Supplementary Table 1.

* Patient 10 has been previously published (Castilla-Vallmanya et al., 2020) and has been thoroughly reviewed in this work.

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Skull shape anomalies	+	+	+	–	+	–	+	+	+	+	+	9/11
Blepharophimosis	+	+	+	+	+	+	+	+	+	+	+	11/11
Strabismus	–	+	+	+	+	+	–	–	+	+	+	8/11
Abnormality of the palate/mouth	–	+	+	+	+	+	+	+	+	+	+	10/11
Cardiac anomalies	+	+	+	+	+	+	+	+	+	+	–	10/11
Skeletal/limb anomalies	+	+	+	+	+	+	+	+	+	+	+	11/11
Hearing loss	+	+	+	+	+	+	+	+	+	+	+	11/11
Visual abnormalities	–	–	–	+	+	+	+	–	+	–	–	5/11

Abbreviations:

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Detailed information and HPO codes are presented in Supplementary Table 1.

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Epilepsy was present in only one patient (Patient 10); it appeared in the second month of life and was well controlled with monotherapy.

As in other neurodevelopmental conditions, some kind of sleep disorder was reported for most of the patients (seven of 10) as assessed by the SDSC scale. The most frequent were sleep-wake transition disorders (five of 10), requiring medication in some patients, followed by difficulties with initiating or maintaining sleep (three of 10) and disorders of excessive somnolence (three of 10). Two of the patients showed abnormalities in three of the evaluated sleep areas.

Dysmorphic features

Dysmorphic features were common in all cases, with facial features similar to other patients with *TRAF7*-related CAFDADD (Fig 1). Skull shape anomalies were frequent but heterogeneous (nine of 11), trigonocephaly being the most common. Ocular examination revealed notable abnormalities with ptosis (11 of 11), blepharophimosis (11 of 11), hypertelorism (11 of 11), and epicanthal folds (nine of 11) being the most characteristic. Ear abnormalities were common but inconsistent, with low-set position being the most common finding (10 of 11). Abnormalities of the nose were present in all patients, consisting of a round nasal tip with a prominent columella. Abnormalities of the palate/mouth were frequently seen, including micrognathia and/or retrognathia (nine of 11) and narrow, high, or cleft palate (nine of 10). Dental anomalies were present in all the five patients assessed; these anomalies included malpositioned teeth and oligodontia. Broad and/or short neck was also common (10 of 11). Other dysmorphic features included hypoplastic, low-set, or widely spaced nipples (six of 10) and hypospadias in half of the male cases (four of eight).

Cardiac features

All patients except Patient 11 presented with congenital heart defects (10 of 11), the most frequent cardiac malformation being patent ductus arteriosus (six of 11). Other cardiac defects included

aortic coarctation (four of 11) and valvular or septal defects (ventricular septal defect; four of 11), bicuspid aortic valve (three of 11), and atrioventricular canal and atrial septal defect (one of 11). Additionally, three patients presented a persistent left superior vena cava, and another two, peripheral pulmonary artery stenosis. Aside from congenital heart defects, four patients had cardiac insufficiency (four of 11) and four had syncope of unknown origin (four of 11).

Skeletal features

Digital anomalies were detected in all patients with substantial variability in the types of anomalies observed (Fig 2). Clinodactyly was present in five cases (five of 11) and brachydactyly in four cases. Other digital anomalies included overlapping toes (four of 11), syndactyly (three of 11), and metatarsus adductus (three of 11). Furthermore, a single patient displayed arachnodactyly.

Pectus carinatum was present in most patients (seven of 11). Scoliosis (six of 11) and kyphosis (four of 11) were also present in a range of patients. Additionally, five patients had pes planus and valgus and one had pes cavus. Two patients had 11 pairs of ribs, and in two patients a marfanoid habitus was described. Joint hypermobility (four of 10) or joint contractures (three of 10) were also present in some cases.

Endocrinological features

Most patients had normal anthropometric parameters at birth, but all of them tended to lower body mass indexes during their subsequent growth (Supplementary Figure 1). Short stature and low weight were common, and most patients presented values of height below -2 S.D. (eight of 11) (Fig 3), despite presenting normal insulin-like growth factor 1 (IGF-1) and Insulin-like growth factor-binding protein 3 (IGFBP-3) hormonal levels when they were analyzed. This trait is more notable if midparental height is considered (Clinical Supplementary Data). Bone age has been assessed in four patients, and two showed delayed bone age (two of four). Bone mineral density has been assessed in only one patient (Patient 10, at age

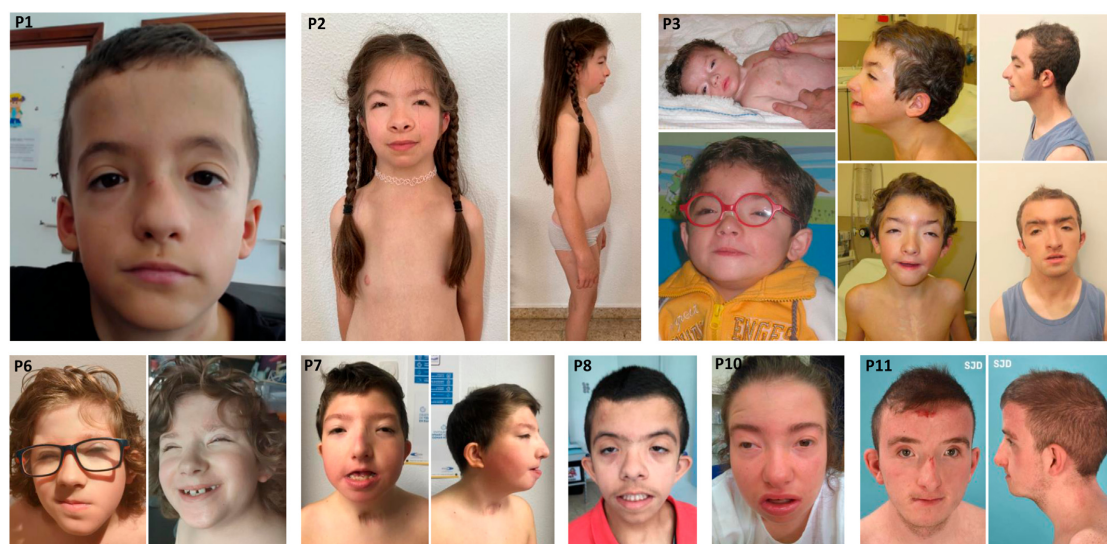


FIGURE 1. Facial features of the patients identified in this study with variants in *TRAF7*. Patient numbers are indicated at the top of each panel. See text for description of the major features. In Patient 3, photographs taken at different ages are shown. The color version of this figure is available in the online edition.



FIGURE 2. Anomalies of the extremities of the patients identified in this study with variants in *TRAF7*. Patient numbers are indicated at the top of each panel. See text for description of the major features. The color version of this figure is available in the online edition.

23 years), and lumbar spine osteopenia (T-score of -1.3) and femoral neck osteoporosis (T-score of -2.5) were noted.

Other symptoms

All patients presented hearing loss attributed to diverse etiologies: conductive (three of 11), sensorineural (three of 11), and mixed (three of 11). Visual abnormalities were also common (nine of 11), including strabismus in most patients (eight of 11), myopia (three of 11), and optic nerve atrophy (two of 11).

Six patients had digestive problems, including gastroesophageal reflux and dysphagia (three patients needed feeding through a nasogastric tube or a gastrostomy). Two patients presented hepatomegaly. Five patients had renal problems, and two presented hernias. Two patients had recurrent respiratory infections, and one had recurrent urinary tract infections.

Proposed guidelines for the clinical management of *TRAF7*-related CAFDADD

Following a thorough clinical review of the individuals presented in this study and a systematic analysis of the 57 previously published subjects with *TRAF7*-related CAFDADD (Table 2), we have developed a set of guidelines for the clinical management of this condition (Fig 4 and Supplementary Figure 2), highlighting the relevance of a multidisciplinary and personalized assessment and follow-up. Considering the recent publication of the first patient with a mosaic variant in *TRAF7* who developed a meningioma,⁶ the presence of meningiomas in one adult patient (43 years old),¹ and one endometrioid adenocarcinoma in a 36-year-old woman,² oncological consult should also be considered for patients with *TRAF7*-related CAFDADD, especially after puberty.

Genetic findings

All patients are heterozygous carriers of pathogenic missense variants in *TRAF7*. All variants were confirmed to have occurred *de*

novo, except for P1, conceived by ovidonation, for whom the paternal sample was negative. Eight different variants have been detected (Table 3), seven of them previously associated with *TRAF7*-related CAFDADD^{1,2,5,7} including the recurrent variant p.Arg655Gln, which was detected in four unrelated patients (Patients 8 to 11). Patient 7 harbors a novel variant, c.1958G>T [p.(Arg653Leu)], in a residue where other amino acid substitutions have been previously reported in meningiomas.²⁶ As in previous studies, all variants are localized in the WD40 domains at the C-terminus of *TRAF7*, except for variant c.1036A>G [p.(Lys346Glu)], which affects a residue in the coiled coil domain. None of these variants were detected in populational databases (gnomAD v4; accessed November 2023); all of them affect evolutionary conserved residues and are predicted to have deleterious effects by several *in silico* tools, and, with the exception of variant c.1958G>T [p.(Arg653Leu)], all have been previously identified in patients with *TRAF7*-related CAFDADD. This novel variant is classified as pathogenic according to ACMG criteria²⁴ (PS2, PM1, PM2, PP2, PP3).

Discussion

Here we provide a detailed description of 11 new *TRAF7*-related CAFDADD cases, harboring eight different *TRAF7* variants, one of them novel. We also provide a detailed phenotypic description and a comprehensive review of the clinical aspects of this disorder including the previously published cases and propose recommendations for clinical follow-up.

Clinical presentation was mostly consistent with the phenotypic spectrum previously described in *TRAF7*-related CAFDADD, the leitmotif being the coexistence of four findings: dysmorphic features, developmental delay, growth issues and short stature, and cardiac defects.

Despite developmental delay and hypotonia being constant during infancy, some individuals showed normal cognitive function later on. All patients presented good communicative engagement, but they all suffered some delay in speech acquisition and language

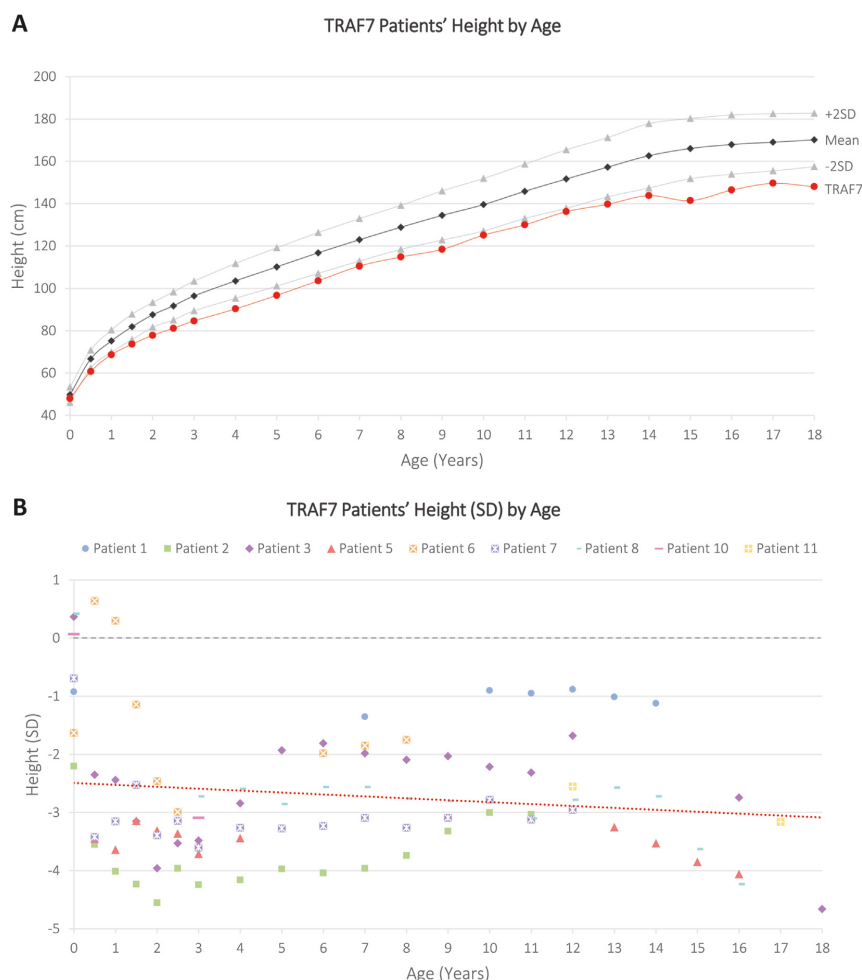


FIGURE 3. TRAF7 syndrome patients' height by age. (A) Plot of the mean height of the 9 Spanish patients reported here (red) in comparison with the Spanish population mean (average of boys and girls; black) and ± 2 S.D. (gray). (B) Plot of the height S.D. by age of the 9 Spanish patients corrected by sex. The red line indicates the mean S.D. for all 9 TRAF7 syndrome patients represented here. Patient 4 is excluded here due to his origin (Bulgaria) and Patient 9 is excluded due to the early age at death (9 mo). The color version of this figure is available in the online edition.

development, with particular impact on expressive language rather than receptive language. As with intellectual disability, language problems were highly variable within the group. Although some patients showed a good evolution reaching almost normal communication in adulthood, others remained highly impaired, requiring alternative communications such as signs or pictograms. Considering these results, the use of alternative or augmentative communication methods is recommended for TRAF7-related CAFDADD.

Hearing loss, both conductive and neurosensory, is also a hallmark that may importantly interfere with communication skills. Therefore it is paramount to perform periodic hearing assessment as indicated in the suggested clinical guidelines (Fig 4) since an early detection may facilitate therapeutic approaches such as tympanic drainages or the use of audiology devices, leading to an

improvement in hearing and, consequently, optimizing communication skills.

Autistic traits were present in some of the cases in our cohort, with a particular emphasis on rigidity and lack of cognitive flexibility. However, only one patient fulfilled autism spectrum disorder diagnostic criteria. Although in some of the others a formal diagnosis was not reached, the symptomatology is still deserving of attention.²⁷ Future challenges are to achieve an accurate diagnosis and to set up appropriate intervention and management actions to optimize the functional prognosis of the patients and families with TRAF7-related CAFDADD. Many of the patients here had a happy and very friendly character, although they presented certain difficulties in social skills, with cognitive rigidity, as well as obsession with routines, blockages, and tantrums due to frustration. These traits may collectively constitute a distinctive behavioral phenotype

TABLE 2.
Clinical Summary of All TRAF7 Syndrome Cases

Clinical Characteristics	HPO Term	This Publication (n = 11)	%	Previous Reports* (n = 57)	%	Total (n = 68)	%
Gender (male:female ratio)		8:3	72.7%	36:21	63.16%	44:24	64.7%
Perinatal findings							
Abnormality of prenatal development or birth	HP:0001197	7/11	63.6%	26/48	54.2%	33/59	55.9%
Postnatal concerns		9/11	81.8%	31/49	63.3%	40/60	66.7%
Biometry at diagnosis							
Low weight	HP:0004325	4/11	36.4%	11/43	25.6%	15/54	27.8%
Short stature	HP:0004322	8/11	72.7%	17/41	41.5%	25/52	48.1%
Development							
Motor delay	HP:0001270	9/11	81.8%	45/52	86.5%	54/63	85.7%
Speech delay	HP:0000750	9/10	90.0%	38/42	90.5%	47/52	90.4%
Global developmental delay/intellectual disability	HP:0001263/HP:0001249	9/10	90.0%	30/33	90.9%	39/43	90.7%
Autism spectrum traits	HP:0000729	4/10	40.0%	7/52	13.5%	11/62	17.7%
Behavior phenotype	HP:0000708	9/10	90.0%	14/52	26.9%	23/62	37.1%
Neurological features							
Hypotonia	HP:0001252	11/11	100.0%	17/22	77.3%	28/33	84.8%
Seizures	HP:0001250	1/11	9.1%	10/47	21.3%	11/58	19.0%
Structural findings on neuroimaging	HP:0410263	9/11	81.8%	37/47	78.7%	46/58	79.3%
Dysmorphic features							
Skull shape anomalies	HP:0002683	9/11	81.8%	21/48	43.8%	30/59	50.8%
Ptosis	HP:0000508	11/11	100.0%	30/53	56.6%	41/64	64.1%
Blepharophimosis	HP:0000581	11/11	100.0%	37/53	69.8%	48/64	75.0%
Epicanthal folds	HP:0000286	9/11	81.8%	28/53	52.8%	37/64	57.8%
Hypertelorism	HP:0000316	11/11	100.0%	17/53	32.1%	28/64	43.8%
Strabismus	HP:0000486	8/11	72.7%	7/48	14.6%	15/59	25.4%
Abnormally set or dysplastic ears	HP:0000357/HP:0000377	10/11	90.9%	37/54	68.5%	47/65	72.3%
Abnormality of the nose	HP:0000366	10/11	90.9%	32/53	60.4%	42/64	65.6%
Micrognathia/retrognathia	HP:0000347/HP:0000278	9/11	81.8%	15/53	28.3%	24/64	37.5%
Abnormality of the palate/mouth	HP:0000163	9/10	90.0%	26/37	70.3%	35/47	74.5%
Dental anomalies	HP:0000164	5/5	100.0%	11/37	29.7%	16/42	38.1%
Abnormality of the neck	HP:0000464	10/11	90.9%	26/44	59.1%	36/55	65.5%
Abnormality of the nipples	HP:0004404	6/10	60.0%	15/44	34.1%	21/54	38.9%
Hypospadias	HP:0000047	4/8	50.0%	3/19	15.8%	7/27	25.9%
Cardiac features							
Abnormality of the cardiovascular system	HP:0001626	10/11	90.9%	47/56	83.9%	57/67	85.1%
Patent ductus arteriosus	HP:0001643	6/11	54.5%	33/56	58.9%	39/67	58.2%
Coarctation of the aorta	HP:0001680	4/11	36.4%	2/56	3.7%	4/67	6.0%
Peripheral pulmonary artery stenosis	HP:0004969	2/11	18.2%	0/56	0.0%	2/67	3.0%
Valvular and/or septal defects	HP:0001654/HP:0001671	5/11	45.5%	31/56	55.4%	36/67	53.7%
Persistent left superior vena cava	HP:0005301	3/11	27.3%	2/56	1.9%	3/67	4.5%
Skeletal findings							
Marfanoid habitus	HP:0001519	2/10	20.0%	0/54	0.0%	2/64	3.1%
Joint contractures	HP:0034392	3/10	30.0%	18/54	33.3%	21/64	32.8%
Hypermobility of the upper limbs	HP:0001382	4/10	40.0%	4/54	7.4%	8/64	12.5%
Digital anomalies	HP:0011297	11/11	100.0%	39/54	72.2%	50/65	76.9%
Palmar crease abnormalities	HP:0010490	2/11	18.2%	13/54	24.1%	15/65	23.1%
Pectus carinatum	HP:0000768	7/11	63.6%	18/44	40.9%	25/55	45.5%
11 Pairs of ribs	HP:0000878	2/11	18.2%	1/52	1.9%	3/63	4.8%
Scoliosis	HP:0002650	6/11	54.5%	7/54	13.0%	13/65	20.0%
Kyphosis	HP:0002808	4/11	36.4%	3/54	5.6%	7/65	10.8%
Pes planus and valgus	HP:0001763/HP:0008081	6/9	66.7%	15/54	27.8%	21/63	33.3%
Other findings							
Hearing loss	HP:0000365	11/11	100.0%	25/48	52.1%	36/59	61.0%
Visual abnormalities	HP:0000478	5/11	45.5%	29/48	60.4%	34/59	57.6%

Abbreviation:

HPO = Human Phenotype Ontology

* Tokita et al.¹; Castilla-Vallmanya et al.²; Accogli et al.³; Chaisrisawadisuk et al.⁴; Song et al.²⁵; Paprocka et al.⁵; Kaidonis et al.⁶; Collieran et al.⁸. Owing to the lack of information regarding the patients with TRAF7 syndrome in Scott et al., 2022 this case has been omitted in this scrutiny.

in patients with *TRAF7*, and some patients would require follow-up by psychologists or mental health professionals. Previous literature regarding these behavioral traits is scarce, but as it frequently alters the family's functionality and well-being, they should be highlighted.^{28,29}

Although sleep disturbances were not universal or severe among these patients, almost half of the patients present with some kind of affection, especially referring to the sleep-wake transition. We observed this kind of parasomnia, which occurs during the transition from wakefulness to sleep or between sleep stages, in five of the 10 patients. Of note, increased sleepiness,

observed here in three patients, was also mentioned in two previously published subjects.¹ Sleep assessment and follow-up is relevant and should be taken into consideration in patients with *TRAF7* as sleep disturbances lead to a decline in cognitive performance and behavior.

Regarding endocrine characteristics, although short stature is an early sign, at birth it may not be striking enough unless accompanied by other signs. During childhood, short stature becomes more notorious and is usually accompanied by proportionate or low weight. Owing to the scarcity of data, it is unclear whether this is genetically determined stature or it may be explained by a



FIGURE 4. Schematic guidelines for TRAF7 syndrome clinical management. Perinatal period: Boxes include the medical area of disease. Medical problems and management requirements are detailed below. Therapies are indicated in italics. Childhood and adolescence: Boxes include medical area of disease. Medical problems, management recommendations, and complementary examinations are detailed below. Therapies are indicated in italics. An A in a black dot states that the recommended periodicity for clinical evaluation is annually, for every specialty and for all the individuals. In contrast, a P in a burgundy dot means that those complementary examinations and management tools need to be personalised, and applied only if patient's personal characteristics require them, always following the medical team criteria. ASD, autism spectrum disorder; EEG, electroencephalogram; ECG, electrocardiogram; PDA, patent ductus arteriosus; PSG, polysomnography. A Spanish version of this figure is included in [Supplementary Figure 1](#). The color version of this figure is available in the online edition.

hormonal deficiency during puberty. Hormonal factors have not been adequately studied in TRAF7 cases and overall somatometric data are insufficient, but in spite of the small number of individuals taken into consideration, our observations suggest that this deficiency worsened after pubertal age (Fig 3). It is important to take this factor into account in future reviews of patients with TRAF7-related CAFDADD since it may have therapeutic implications, as it has been shown in some genetic syndromes combining endocrine and neurological disturbances.³⁰

As in the previously described patients, structural congenital heart defects were present in a majority of patients with TRAF7. These defects trigger the most severe complications, especially during their early life. In our cohort, patent ductus arteriosus was the most prevalent manifestation together with a combination of severe cardiac, valvular, and vessel pathologies including life-threatening abnormalities such as hypoplastic aortic arch leading to cardiac insufficiency in four patients in the cases presented here. The involvement of TRAF7 in endothelial tissues, and particularly

TABLE 3.
Molecular Data of Individuals With TRAF7 Variants Identified in This Study

Variant	p.(Lys346Glu)	p.(Ser558Phe)	p.(His570Asp)	p.(Leu595Val)	p.(Ser629Asn)	p.(Val646Leu)	p.(Arg653Leu)	p.Arg655Gln
cDNA	c.1036A>G	c.1673C>T	c.1708C>G	c.1783C>G	c.1886G>A	c.1936G>C	c.1958G>T	c.1964G>A
Patient number	1	2	3	4	5	6	7	8–11
Zygosity	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous
Origin	De novo*	De novo	De novo	De novo	De novo	De novo	De novo	De novo
Domain	Coiled-coil	WD40	WD40	WD40	WD40	WD40	WD40	WD40
References	Tokita et al. ¹	Castilla-Vallmanya et al. ²	Castilla-Vallmanya et al. ²	Paprocka et al. ⁵	Scott et al. ⁷	Castilla-Vallmanya et al. ²	This study	Tokita et al. ¹ ; Castilla-Vallmanya et al. ² ; Accogli et al. ³ ; Song et al., 2021

Abbreviation:

cDNA = Complementary DNA

* Ovodonation.

regarding vascular complications, has been recently demonstrated in a murine model.¹⁴

As in previous reports, patients described here share the gestalt of the patients with TRAF7-related CAFDADD² especially on the upper face, including blepharophimosis, ptosis, hypertelorism, and a wide or flat nasal bridge. Additional facial features are a bulbous nasal tip and dysplastic or low-set ears. Further revision of previously published pictures together with the cases presented here highlights that arched eyebrows are also relatively common in this syndrome.

A novel variant has been described in this work, p.(Arg653Leu), identified in Patient 7; it is a missense variant affecting the Arg653 residue, where other missense somatic changes associated with meningioma tumors have been described. In fact, p.Arg653Gln and p.Arg653Pro are recurrent variants in those tumors.

This is not the first time that germline changes associated with TRAF7-related CAFDADD and somatic changes in meningioma have been identified in the same residue, as is the case with the germline variant p.(Leu519Phe) associated with TRAF7 disorder and somatic variants p.(Leu519Pro) and p.(Leu519Arg) described in meningioma tumors. This apparent division where some missense changes in germline lead to TRAF7-related CAFDADD, whereas similar changes in somatic tissue give rise to tumor conditions is something that needs to be clarified. Furthermore, Kaidonis et al. (2022) recently described an individual with bilateral optic nerve sheath meningiomas and TRAF7-related CAFDADD who had the c.1921C>T p.(Arg641Cys) variant in mosaicism, previously associated with meningiomas.⁶ Two additional individuals with TRAF7-related CAFDADD have been reported to develop meningiomas.^{1,2} This finding raises the question of whether individuals with germline variants in TRAF7 may have an increased risk of developing malignancies such as meningiomas. We believe it is important to keep this fact in mind as new evidence on this relationship is gathered.

Diagnosis of TRAF7-related CAFDADD might be hampered by the presence of features overlapping with other syndromes, which resulted in different initial diagnosis orientations. FAT1, KAT6B, RASopathies, or connective tissue disorders were considered as likely players in initial diagnosis. KAT6B-related Ohdo syndrome (SBBYSS; MIM #603736) has common characteristics with TRAF7-related CAFDADD, such as motor and speech delay, digital anomalies, cardiac defects, and craniofacial features, including blepharophimosis and ptosis.³¹ Also, connective disorders and RASopathies (e.g., Costello syndrome) typically suspected in patients with TRAF7 might be due to a correlation in the pathomechanisms of both RASopathies and TRAF7-related CAFDADD involving the regulation of RAS/mitogen-activated protein kinase signaling pathways.^{1,5}

This new cohort of TRAF7-related patients with CAFDADD expands the phenotypic spectrum of this disorder that presents with variable but recognizable features, particularly referring to behavior or sleep disturbances. In this work, we address growing issues that need more attention in future studies such as autistic traits, short stature, and hearing loss. An in-depth revision led to the first proposal of a based-on-evidence schedule to help in the clinical management and monitoring of patients with TRAF7-related CAFDADD.

CRedit authorship contribution statement

Carmen Palma-Milla: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Investigation, Formal analysis, Data curation, Conceptualization. **Aina Prat-Planas:** Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. **Emma Soengas-Gonda:**

Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Investigation, Formal analysis, Data curation. **Mónica Centeno-Pla:** Writing – review & editing, Visualization. **Jaime Sánchez-Pozo:** Writing – review & editing, Formal analysis. **Irene Lazaro-Rodríguez:** Writing – review & editing, Writing – original draft, Supervision, Resources, Formal analysis. **Juan F. Quesada-Espinosa:** Writing – review & editing, Software, Methodology, Formal analysis. **Ana Arteché-Lopez:** Writing – review & editing, Formal analysis. **Jonathan Olival:** Writing – review & editing, Methodology. **Marta Pacio-Míguez:** Formal analysis, Data curation. **María Palomares-Bralo:** Writing – review & editing, Investigation. **Fernando Santos-Simarro:** Writing – review & editing, Investigation, Formal analysis. **Ramón Cancho-Candela:** Writing – review & editing, Investigation, Formal analysis. **María Vázquez-López:** Writing – review & editing, Investigation, Formal analysis. **Veronica Seidel:** Writing – review & editing. **Antonio F. Martínez-Monseny:** Writing – review & editing, Investigation, Formal analysis. **Didac Casas-Alba:** Writing – review & editing, Investigation, Formal analysis. **Daniel Grinberg:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Susanna Balcells:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Mercedes Serrano:** Writing – review & editing, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Raquel Rabionet:** Writing – review & editing, Supervision, Conceptualization. **Miguel A. Martín:** Writing – review & editing, Supervision. **Roser Urreiziti:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary Data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.pediatrneurol.2024.03.008>.

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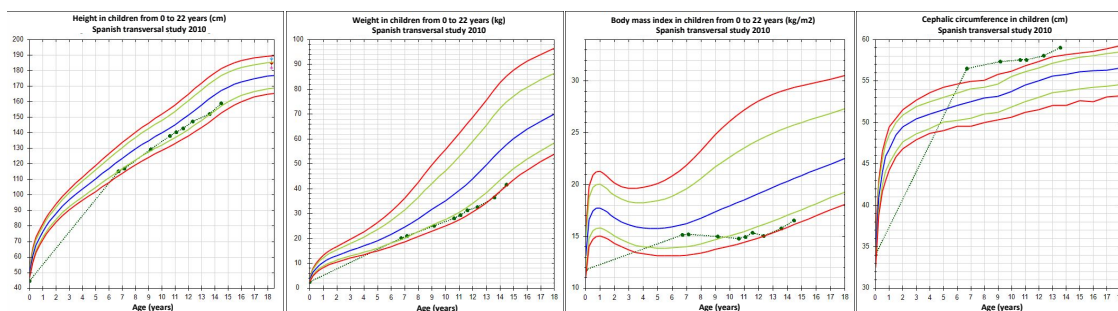
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Supplementary information for:

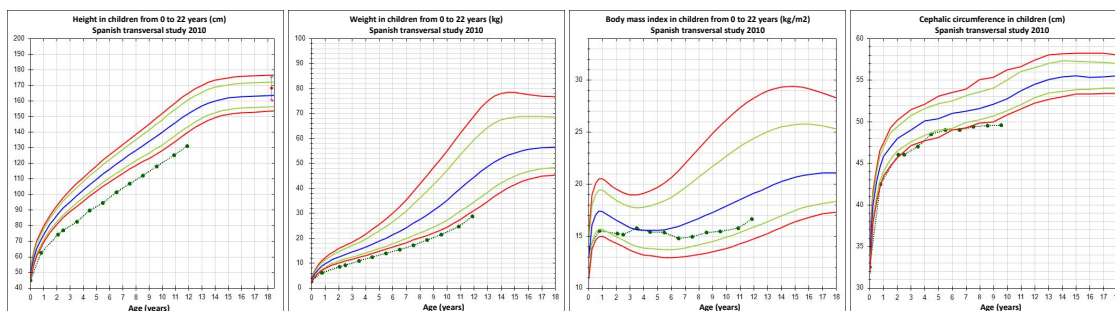
Expanding the Phenotypic Spectrum of TRAF7-Related Cardiac, Facial, and Digital Anomalies with Developmental Delay: Report of 11 New Cases and Literature Review

Carmen Palma-Milla*; Aina Prat-Planas*; Emma Soengas-Gonda*; Mónica Centeno-Pla; Jaime Sánchez-Pozo; Irene Lazaro-Rodriguez; Juan F. Quesada-Espinosa; Ana Arteché-Lopez; Jonathan Olival; Marta Pacio-Miguez; María Palomares-Bralo; Fernando Santos-Simarro; Ramón Cancho-Candela; María Vázquez-López; Veronica Seidel; Antonio F Martínez-Monseny; Didac Casas-Alba; Daniel Grinberg; Susanna Balcells; Mercedes Serrano; Raquel Rabionet; Miguel A. Martín*; Roser Urreizti*

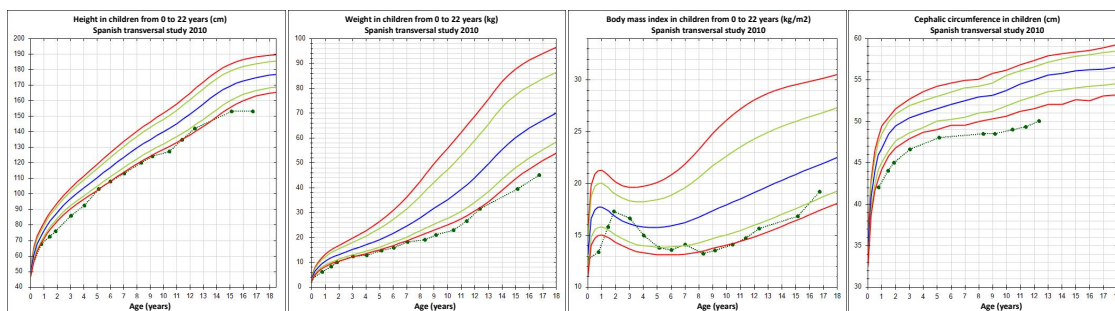
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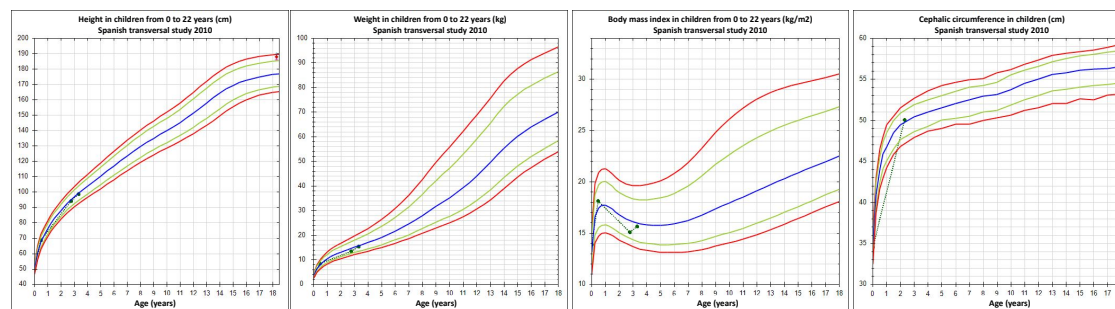
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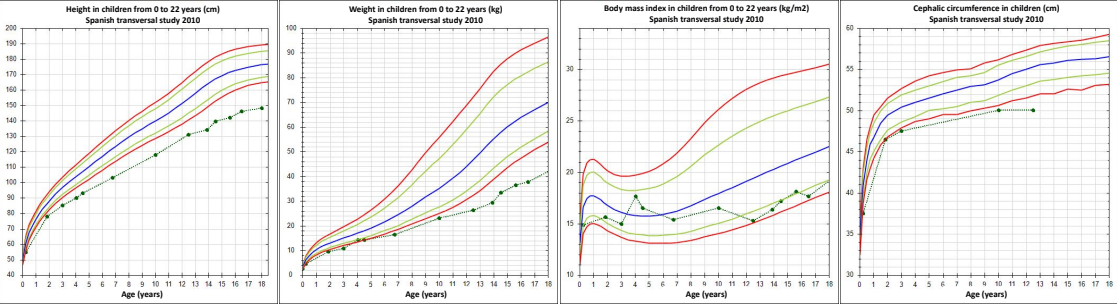
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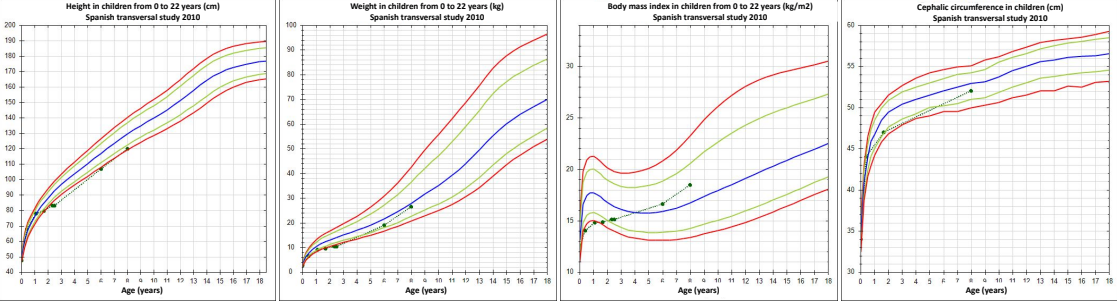
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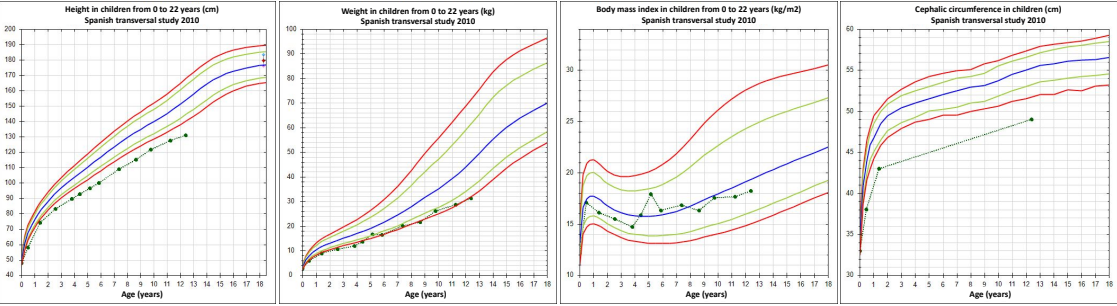
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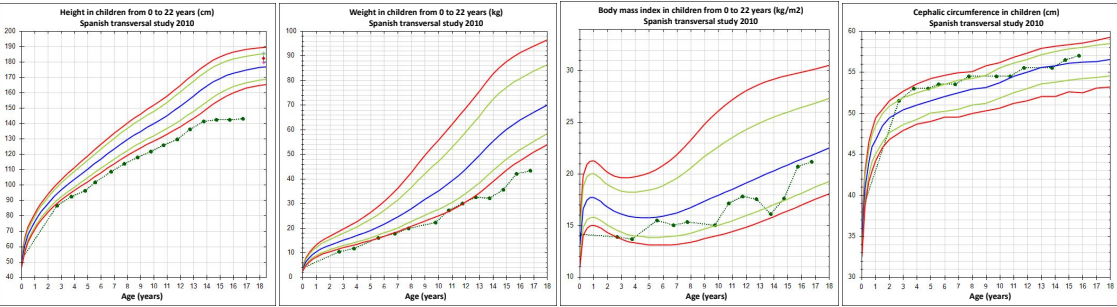
Patient 6



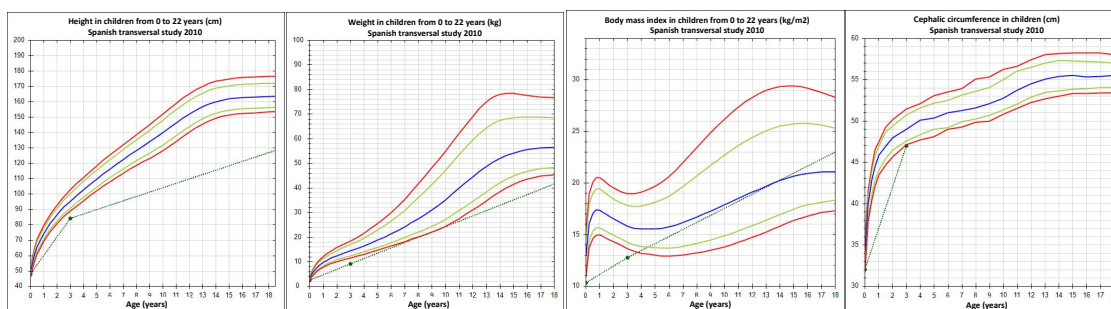
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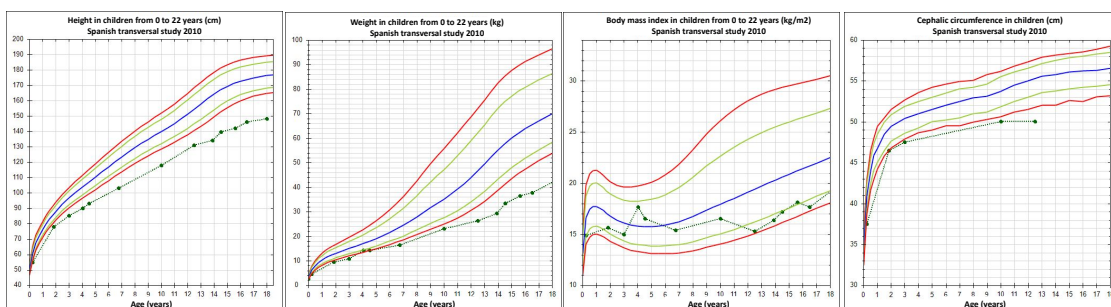
Patient 8



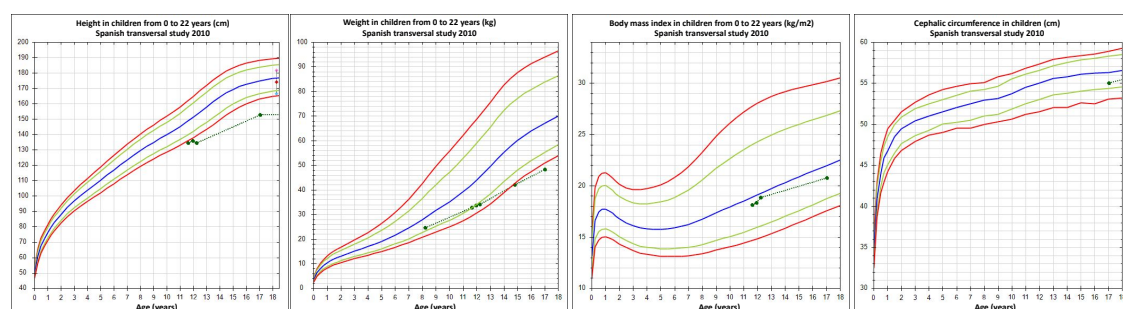
Patient 9



Patient 10



Patient 11



Suppl. Figure 1. Anthropometric curves of the TRAF7 syndrome patients identified in this study. Anthropometric curves showing the P50 (blue), P10/P90 (light green) and P3/P97 (red) percentiles. Patient measurements are indicated by a dark green dot. The mid parental height is shown using a red diamond (paternal and maternal height in blue and pink diamonds respectively).



Suppl. Figure 2. Directrices esquemáticas para el manejo clínico del síndrome TRAF7.

Figure 5 but in Spanish. Período perinatal: Los recuadros incluyen el área médica de la enfermedad. Los problemas médicos y los requisitos de manejo se detallan a continuación. Las terapias están indicadas en cursiva. Infancia y adolescencia: Los recuadros incluyen el área médica de la enfermedad. A continuación, se detallan los problemas médicos, recomendaciones de manejo y exámenes complementarios. Las terapias están indicadas en cursiva. Una A dentro de un punto negro indica que la periodicidad recomendada para la evaluación clínica es anual, para cada especialidad y para todos los individuos. Por el contrario, una P dentro de un punto granate significa que esas exploraciones y herramientas de gestión complementarias deben personalizarse y aplicarse sólo si las características personales del paciente lo requieren, siempre siguiendo el criterio del equipo médico. DAP, ductus arterioso persistente; EEG, electroencefalograma; ECG, electrocardiograma; PSG, polisomnografía; TEA, trastorno del espectro autista.

Suppl. Table 1. Clinical description of all cases.

	HPO term	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Age of diagnosis		13	10	15	2	16	6
Age at last evaluation		14	11	17	2	18	8
Gender		Male	Female	Male	Male	Male	Male
Pathogenic Variant							
cDNA (NM_032271.2)		c.1036A>G	c.1673C>T	c.1708C>G	c.1783C>G	c.1886G>A	c.1936G>C
Protein (Q6Q0C0)		p.Lys346Glu	p.(Ser558Phe)	p.(His570Asp)	p.(Leu595Val)	p.(Ser629Asn)	p.(Val646Leu)
Prenatal findings							
Abnormality of prenatal development or birth	HP:0001197	-	Single umbilical artery, Cystic hygroma	-	-	Intrauterine growth retardation	-
Biometry at birth							
Gestational age		35 + 1 w	38 w	UNK (Term)	40 + 6 w	40 w	39 + 4 w
Weight (Kg) (SD)		2.33 (-0.36)	2.8 (-0.3)	3.3 (0.02)	3.47 (-0.1)	2.65 (-1.88)	2.8 (-1.42)
Length (cm) (SD)		44.5 (-0.93)	45 (-2.2)	51 (0.54)	50 (-0.26)	UNK	47.5 (-0.35)
OFC (cm) (SD)		34 (1)	32.5 (-0.9)	UNK	34.5 (-0.16)	UNK	34 (1.3)
Postnatal concerns		-	-	Congenital heart disease	Poor suck	Poor suck, Congenital heart disease, Heart failure	Poor suck
Biometry at diagnosis							
Weight (Kg) (SD)		36.4 (-1.6)	24.6 (-1.9)	39 (-2)	12.5 (-0.4)	36 (-2.41)	19 (-1.2)
Height (cm) (SD)		152 (-1.2)	125 (-3.1)	153 (-2.3)	90.5 (0.6)	143.6 (-4.05)	106 (-2.3)
Development							
Motor delay	HP:0001270	-	+	+	-	+	+
Speech delay	HP:0000750	+	+	+	+	+	+
Global developmental delay/Intellectual disability	HP:0001263/ HP:0001249	-	+	+	+	+	-
Autism Spectrum Traits	HP:0000729	-	-	+	-	-	+
Behaviour Phenotype	HP:0000708	Overfriendliness	Overfriendliness	Overfriendliness, cognitive rigidity and obsessions	NA	Emotional and conduct disorder	Friendly, shy, cognitive rigidity and obsessions
Neurologic features							
Hypotonia	HP:0001252	+	+	+	+	+	+
Seizures	HP:0001250	-	-	-	-	-	-

Structural findings on neuroimaging	HP:0410263	Residual ventriculomegaly, Thinning of the corpus callosum	-	Mild delayed myelination	-	<i>Ex vacuo</i> hydrocephalus	Periventricular cysts
Sleep disturbances*							
Disorders of initiating and maintaining sleep	HP:0031354/ HP:0031355	-	-	-	-	+	+
Sleep-wake transition disorders	HP:0006979	-	-	-	-	+	+
Sleep breathing disorders	HP:0010535	-	-	-	-	+	-
Sleep hyperhydrosis	HP:0000975	-	-	-	-	-	-
Disorders of arousal	HP:0025235	-	-	-	-	-	-
Disorders of excessive somnolence	HP:0001262	+	-	-	-	-	+
Dysmorphic features							
Skull shape anomalies	HP:0002683	+	+	Trigonocephaly	-	Craniosynostosis	-
Ptosis	HP:0000508	+	+	+	+	+	+
Blepharophimosis	HP:0000581	+	+	+	+	+	+
Epicanthal folds	HP:0000286	+	+	+	-	+	+
Hypertelorism	HP:0000316	+	+	+	+	+	+
Strabismus	HP:0000486	-	+	+	+	+	+
Abnormally set or dysplastic ears	HP:0000357/ HP:0000377	+	+	+	-	+	+
Abnormality of the nose	HP:0000366	+	+	-	+	+	+
Micrognathia/Retrognathia	HP:0000347/ HP:0000278	-	+	+	+	+	+ (Retrognathia)
Abnormality of the palate/mouth	HP:0000163	-	+	+	+	+	+

Dental anomalies	HP:0000164	ND	ND	+	NA	UNK	Oligodontia
Abnormality of the neck	HP:0000464	+	+	+	+	+	+
Abnormality of the nipples	HP:0004404	-	+	-	-	UNK	+
Hypospadias	HP:0000047	-	NA	-	+	+	-
Cardiac features							
Patent ductus arteriosus	HP:0001643	-	+	-	-	+	+
Coarctation of the aorta	HP:0001680	+	+	+	-	-	-
Peripheral pulmonary artery stenosis	HP:0004969	-	+	-	-	-	-
Valvular and/or septal defects	HP:0001654/ HP:0001671	Bicuspid aortic valve, Perimembranous ventricular septal	-	Perimembranous ventricular septal	-	Atrioventricular canal, Atrial septal	-
Persistent left superior vena cava	HP:0005301	-	-	-	+	-	-
Cardiac insufficiency	HP:0001635	-	-	-	-	+	-
Syncopes	HP:0001279	-	-	+	-	+	+
Other		Double superior vena cava			Hypoplastic aortic arch		
Skeletal findings							
Marfanoid habitus	HP:0001519	+	+	-	-	UNK	-
Joint contractures	HP:0034392	-	+	-	-	UNK	-
Hypermobility of the upper limbs	HP:0001382	+	+	+	-	UNK	+
Digital anomalies	HP:0011297	Long thin fingers	Metatarsus adductus	Brachydactyly, Clinodactyly, Thumb with low implantation	Abnormality of the thumbs, Promient fingertrip pads	Abnormality of the thumbs, Clinodactyly, Camptodactyly	Syndactyly, Clinodactyly, Overlapping toes
Palmar crease abnormalities	HP:0010490	-	-	+	-	+	-
Pectus carinatum	HP:0000768	+	+	+	-	+	+

11 pairs of ribs	HP:0000878	-	+	-	-	-	-
Scoliosis	HP:0002650	+	+	+	-	+	-
Kyphosis	HP:0002808	-	-	-	+	+	-
Pes planus and valgus	HP:0001763/ HP:0008081	+	-	+	NA	UNK	+
Others findings							
Hearing loss	HP:0000365	Conductive	Mixed	+	+	Mixed	Conductive
Visual abnormalities	HP:0000478	-	-	-	Myopia	Myopia	Myopia, Astigmatism,
Strabismus	HP:0000486	-	+	+	+	+	+
Others		Multiple hair whorls	Gastroesophageal reflux, Nephroptosis, Hypoplastic labia majora	Gastrostomy fed (up to 6 years), Gastroesophageal reflux, Celiac disease, Recurrent respiratory infections in infancy (required oxygen), Nephrolithiasis, Hypercalciuria	Cutis laxa, Thin nails	Digestive problems, Vertebral anomalies, Hepatomegaly, Glomerulonephritis, Cryptorchidism, Hydrocele	Dysphagia, Subclinical hypothyroidism, Congenital diaphragmatic hernia, Recurrent respiratory infections, Cryptorchidism, Delayed bone age

	HPO term	Patient 7	Patient 8	Patient 9	Patient 10	Patient 11
Age of diagnosis		11	15	1 month	19	21
Age at last evaluation		12	16	9 months	26	21
Gender		Male	Male	Female	Female	Male
Pathogenic Variant						
cDNA (NM_032271.2)		c.1958G>T	c.1964G>A	c.1964G>A	c.1964G>A	c.1964G>A
Protein (Q6Q0C0)		p.(Arg653Leu)	p.Arg655Gln	p.Arg655Gln	p.Arg655Gln	p.Arg655Gln
Prenatal findings						
Abnormality of prenatal development or birth	HP:0001197	Polyhydramnios	Single umbilical artery, Macrocephaly, Polyhydramnios	Single umbilical artery, Increased nuchal translucency	Intrauterine growth retardation, Fetal akinesia, Polyhydramnios	Megabladder
Biometry at birth						
Gestational age		37 + 5 w	41 w	40 + 1 w	37 + 5 w	37 w
Weight (Kg) (SD)		2.8 (-0.68)	3.75 (0.67)	2.7 (-1.57)	2.3 (-1.99)	2.5 (-0.92)
Length (cm) (SD)		48 (-0.69)	51.5 (0.41)	45.5 (-2.64)	48 (-0.65)	UNK
OFC (cm) (SD)		33 (-0.5)	38 (1.7)	33.5 (-0.76)	32 (-1.9)	UNK
Postnatal concerns		Poor suck	Poor suck, Congenital heart disease	Poor suck	Poor suck, Apgar 4/6, Apnea crisis, Severe hypotonia	Hydrocephalus (at 9 months)
Biometry at diagnosis						
Weight (Kg) (SD)		28.7 (-1.4)	42 (-1.92)	6.55 (-1.3) (7 months)	52.4 (-0.62)	45 (-2.6)
Height (cm) (SD)		127.6 (-3)	142.4 (-4.19)	63 (-1.6) (7 months)	141 (-3.87)	153 (-3.82)
Development						
Motor delay	HP:0001270	+	+	+	+	+
Speech delay	HP:0000750	+	+	NA	+	+
Global developmental delay/Intellectual disability	HP:0001263/ HP:0001249	+	+	NA	+	+
Autism Spectrum Traits	HP:0000729	-	-	NA	+	+
Behaviour Phenotype	HP:0000708	Overfriendliness	Overfriendliness	-	Emotional and conduct disorder, Overfriendliness	Obsessions (Agressivity only during adolescence)
Neurologic features						
Hypotonia	HP:0001252	+	+	+	+	+
Seizures	HP:0001250	-	-	-	+	-

Structural findings on neuroimaging	HP:0410263	Ventriculomegaly, Chiari malformation	Triventricular hydrocephalus (normal IV ventricle)	Periventricular cysts, Cerebellar anomalies, Cortical malformations, diffuse cerebral atrophy, Communicating hydrocephalus	Ventriculomegaly, Abnormality of the posterior fossa	Hydrocephalus, Complex malformation of the occipito-cervical junction, Bilateral cochlear malformation
Sleep disturbances*						
Disorders of initiating and maintaining sleep	HP:0031354/ HP:0031355	-	-	NA	+	-
Sleep-wake transition disorders	HP:0006979	+	+	NA	+	-
Sleep breathing disorders	HP:0010535	-	-	NA	-	+
Sleep hyperhydrosis	HP:0000975	-	-	NA	-	-
Disorders of arousal	HP:0025235	-	-	NA	-	-
Disorders of excessive somnolence	HP:0001262	-	-	NA	-	+
Dysmorphic features						
Skull shape anomalies	HP:0002683	Trigonobrachycephaly	+	Craniosynostosis, Trigonocephaly	Craniosynostosis, Trigonocephaly, Progressive microcephaly	Relative macrocephaly
Ptosis	HP:0000508	+	+	+	+	+
Blepharophimosis	HP:0000581	+	+	+	+	+
Epicanthal folds	HP:0000286	-	+	+	+	+
Hypertelorism	HP:0000316	+	+	+	+	+
Strabismus	HP:0000486	-	-	+	+	+
Abnormally set or dysplastic ears	HP:0000357/ HP:0000377	+	+	+	+	+
Abnormality of the nose	HP:0000366	+	+	+	+	+

Micrognathia/Retrognathia	HP:0000347/ HP:0000278	+	+	+	+ (Retrognathia)	-
Abnormality of the palate/mouth	HP:0000163	+	+	+	+	+
Dental anomalies	HP:0000164	Malpositioned teeth	ND	NA	+	Malpositioned teeth
Abnormality of the neck	HP:0000464	-	+	+	+	+
Abnormality of the nipples	HP:0004404	+	+	+	-	+
Hypospadias	HP:0000047	+	+	NA	NA	-
Cardiac features						
Patent ductus arteriosus	HP:0001643	+	+	+	-	-
Coarctation of the aorta	HP:0001680	+	-	+	-	-
Peripheral pulmonary artery stenosis	HP:0004969	-	-	-	+	-
Valvular and/or septal defects	HP:0001654/ HP:0001671	Bicuspid aortic valve, Ventricular septal	-	Bicuspid aortic valve, Ventricular septal	-	-
Persistent left superior vena cava	HP:0005301	+	-	+	-	-
Cardiac insufficiency	HP:0001635	+	+	+	-	-
Syncopes	HP:0001279	-	-	-	+	-
Other				Hypoplastic aortic arch Severe dilatation of right chambers	Premature ventricular contraction, Ventricular septal hypertrophy	
Skeletal findings						
Marfanoid habitus	HP:0001519	-	-	-	-	-
Joint contractures	HP:0034392	+	-	-	+ (Arthrogryposis multiplex congenita)	-
Hypermobility of the upper limbs	HP:0001382	-	-	-	-	-

Digital anomalies	HP:0011297	Brachydactyly, Syndactyly, Clinodactyly, Metatarsus adductus	Brachydactyly, Syndactyly, Metatarsus adductus, Arachnodactyly	Long fingers, First toe with low implantation, Overlapping toes	Clinodactyly, Flexion deformity, Overlapping toes, Hallux valgus	Brachydactyly, Prominent interphalangeal joints in the hands, Sandal gap, Hypoplasia of the 4th toe, Overlapping toes
Palmar crease abnormalities	HP:0010490	-	-	-	-	-
Pectus carinatum	HP:0000768	-	+	-	+	-
11 pairs of ribs	HP:0000878	-	-	-	+ (Thin ribs)	-
Scoliosis	HP:0002650	-	+	-	+	-
Kyphosis	HP:0002808	-	+	-	-	+
Pes planus and valgus	HP:0001763/ HP:0008081	+	+	-	-	+ (Pes cavus)
Others findings						
Hearing loss	HP:0000365	Sensorineural	Sensorineural	Conductive	Sensorineural	Mixed
Visual abnormalities	HP:0000478	Optic atrophy	-	Optic nerve anomalies	-	-
Strabismus	HP:0000486	-	-	+	+	+
Others		Gastrostomy fed	Left nephroptosis	Nasogastric tube, Hepatomegaly, Pyelic duplicity of the right kidney, Death at 9 months (decompensated hydrocephalus with intracranial hypertension)	Clitoral hypertrophy, Delayed bone age	Recurrent urinary tract infections, Neurogenic bladder, Hernia

UNK: Unknown; ND: Not Determined; NA: Not Assessable; ASD: Autism Spectrum Disorder. Patient 9 has been previously published. In this table, clinical information of this case has been excluded from the previous publication and is depicted, more detailed, here. *The Sleep Disturbance Scale for Children (SDSC). Construction and validation of an instrument to evaluate sleep disturbances in childhood and adolescence. J Sleep Res. 1996 Dec;5(4):251-61. doi: 10.1111/j.1365-2869.1996.00251.x. PMID: 9065877.

