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Characterization of a *Drosophila* Model of Ewing Sarcoma

Serena Mahnoor



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Serena Mahnoor

Barcelona, 2025



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Universitat de Barcelona

Faculty of Biology

Doctoral Programme in Biomedicine

Characterizing a Drosophila Model of Ewing Sarcoma

**Caracterització d'un model de Drosophila del
sarcoma d'Ewing**

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SUMMARY

Ewing sarcoma (EwS) is a cancer that arises in the bones and soft tissues, and is driven by a single genetic lesion, most commonly the Ewing's sarcoma breakpoint region 1-Friend leukaemia virus integration 1 (EWS::FLI1) fusion oncogene. Implementation of genetically modified animal models of EwS has proved difficult largely owing to EWS::FLI1's high toxicity. The EWS::FLI1_{1FS} frameshift variant that circumvents toxicity but is still able to perform key oncogenic functions provided the first study model in *Drosophila*. However, the quest for *Drosophila* lines expressing full-length, unmodified EWS::FLI1 remained open. Here, we show that EWS::FLI1_{1FS}'s lower toxicity is owed to reduced protein levels caused by its frameshifted C-terminal peptide, and report new strategies through which we have generated *Drosophila* lines that express full-length, unmodified EWS::FLI1. Using these lines, we have found that the upregulation of transcription from GGAA-microsatellites (GGAA μ Sats) presents a positive linear correlation within a wide range of EWS::FLI1 protein concentrations. In contrast, rather counterintuitively, GGAA μ Sats-independent transcriptomic dysregulation presents relatively minor differences across the same range, suggesting that GGAA μ Sat-dependent and -independent transcriptional upregulation present different kinetics of response with regards to changing EWS::FLI1 protein concentration. Our results underpin the functional relevance of varying EWS::FLI1 expression levels and provide experimental tools to investigate, in *Drosophila*, the effect of the EWS::FLI1 'high' and 'low' states that have been reported and are suspected to be important for EwS in humans.

Investigations into EWS::FLI1 biology have found substantial roles for "chromatin" proteins, i.e. those that either bind or modify the chromatin, as key facilitators of EWS::FLI1 function. However, a systematic evaluation the role of these chromatin proteins in EwS has not been conducted. Using the characterized transgenic lines, we conducted genetic screens to interrogate all *Drosophila* chromatin proteins with the aim to identify those that can modify EWS::FLI1₁-driven transcription from GGAA μ Sats. We identified 6 RNAi lines as high confidence hits, each of which target genes whose human orthologs have reported relevance in EwS cell lines, demonstrating that the mechanism for EWS::FLI1-induced neoenhancer formation is highly conserved in the fly. Amongst the identified hits is protein phosphatase, *alphabet* (*alph*). *PPM1A*, the mammalian orthologs of *alph*, was previously reported to facilitate EWS::FLI1-driven transcription from GGAA μ Sats in EwS cells. My results further suggest EWS::FLI1₁-driven transcription from GGAA μ Sats is dependent on the phosphatase activity of *alph*.

Keywords: Ewing sarcoma, EWS::FLI1, GGAA microsatellite, Ewing sarcoma study model

RESUM

El sarcoma d'Ewing (EwS) és un càncer que s'origina als ossos i als teixits tous, i que està impulsat per una única lesió genètica, essent la més comuna l'oncogen de fusió Ewing's sarcoma breakpoint region 1-Friend leukemia virus integration 1 (EWS::FLI1). La implementació de models animals modificats genèticament d'EwS ha resultat difícil sobretot a causa de l'alta toxicitat d'EWS::FLI1. La variant de canvi de pauta de lectura EWS::FLI1_{1FS}, que evita la toxicitat però és encara capaç de dur a terme funcions oncogèniques clau, va proporcionar el primer model d'estudi en *Drosophila*. Tot i així, la recerca de línies de *Drosophila* amb l'expressió de l'oncogen EWS::FLI1₁ sencer i no modificat persistia. En aquesta tesi mostrem que la menor toxicitat d'EWS::FLI1_{1FS} es deu als nivells reduïts de proteïna, causats pel seu corresponent pèptid C-terminal amb canvi de pauta, i reportem noves estratègies amb les quals hem generat línies de *Drosophila* que expressen EWS::FLI1₁ sencer i no modificat. Amb l'ús d'aquestes línies hem descobert que la regulació a l'alça de la transcripció de microsatèl·lits GGAA (GGAA μ Sats) presenta una correlació lineal positiva dins d'un ampli rang de concentracions de proteïna EWS::FLI1₁. En canvi, de manera força contraintuïtiva, la desregulació transcriptòmica independent dels GGAA μ Sats mostra diferències relativament menors dins el mateix rang, suggerint que la regulació transcriptòmica dependent i independent dels GGAA μ Sats presenta diferents cinètiques de resposta respecte els canvis en la concentració de la proteïna EWS::FLI1₁. Els nostres resultats donen suport a la rellevància funcional que suposen les variacions en els nivells d'expressió d'EWS::FLI1₁, i proporcionen eines experimentals per investigar, en *Drosophila*, l'efecte dels estats "alts" i "baixos" d'EWS::FLI1₁ que s'han reportat i que se sospita que són importants per a l'EwS en humans.

Diverses investigacions sobre la biologia d'EWS::FLI1 han descrit rols substancials per a les proteïnes de la cromatina, és a dir, aquelles que s'uneixen o modifiquen la cromatina, com a facilitadores clau de la funció d'EWS::FLI1. No obstant, no s'ha dut a terme una avaluació sistemàtica sobre el paper d'aquestes proteïnes de la cromatina en l'EwS. Mitjançant l'ús de les línies transgèniques caracteritzades, hem realitzat cribratges genètics per interrogar totes les proteïnes de la cromatina de *Drosophila* amb l'objectiu d'identificar aquelles que poden modificar la transcripció impulsada per EWS::FLI1₁ des dels GGAA μ Sats. Hem identificat 6 línies d'ARNi com a resultats d'alta confiança, cadascuna de les quals té com a diana gens els ortòlegs dels quals s'ha reportat una rellevància en línies cel·lulars d'EwS, demostrant que el mecanisme de formació d'amplificadors genètics *neo* induïda per EWS::FLI1 està altament conservat en la mosca. Entre els resultats identificats hi ha la fosfatasa proteica *alphabet* (*alph*). *PPM1A*, l'ortòleg mamífer d'*alph*, ja havia estat reportat com a facilitador de la transcripció impulsada per EWS::FLI1 des dels GGAA μ Sats en cèl·lules d'EwS. Els meus resultats suggereixen, a més, que la transcripció impulsada per EWS::FLI1₁ des dels GGAA μ Sats depèn de l'activitat fosfatasa d'*alph*.

Paraules clau: El sarcoma d'Ewing, EWS::FLI1, microsatèl·lit GGAA, Model d'estudi del sarcoma d'Ewing

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List of Abbreviations

A.U.	Arbitrary Units
Act5C	Actin 5C
AEL	After Egg Laying
ALL	Acute Lymphoblastic Leukaemia
AML	Acute Myeloid Leukemia
ANOVA	Analysis Of Variance
APL	Acute Promyelocytic Leukemia
ARMS	Alveolar RMS
BAF	BRG1/BRM-Associated Factor
BDSC	Bloomington Drosophila Stock Centre
BFDR	Bayesian False Discovery Rate
CAYA	Childhood, Adolescence And Young Adulthood
CLD	Compact Letter Display
COMPASS	Complex Of Proteins Associated With Set1
CoO	Cell Of Origin
CTRL	Control
da	Daughterless
DAPI	4,6-Diamidino-2-Phenylindole
DAVID	Database For Annotation, Visualization and Integrated Discovery
DIPG	Diffuse Intrinsic Pontine Glioma
DNA-BD	DNA Binding Domain
ETS	E26 Transformation Specific
EwS	Ewing Sarcoma
EWS/EWSR1	Ewing's Sarcoma Breakpoint Region 1
ey	eyeless
FC	Fold Change
FDR	False Discovery Rate
FET	Fus, Ewsr1 & Taf15
FLI1	Friend Leukaemia Integration 1
FStail	Frameshifted Tail
GFP	Green Fluorescent Protein
GGAAμSats	GGAA Microsatellite
GMR	Glass Multimer Reporter

GO	Gene Ontology
HDAC	Histone Deacetylases
IRES	Internal Ribosome Entry Site
LCD	Low Complexity Domain
mCherry	Monomeric Cherry
Mef2	Myocyte Enhancer Factor 2
Mgtor	Megator
MLL	Mixed Lineage Leukemia
NARs	NUP-Associated Regions
NPC	Nuclear Pore Complex
nub	nubbin
NUP	Nucleoporin
NuRD	Nucleosome Remodelling And Deacetylase
ORF	Open Reading Frame
OS	Osteosarcomas
PCR	Polymerase Chain Reaction
PDX	Patient-Derived Xenograft
P-val	P Value
px	Pixel
RBP	RNA Binding Protein
RMS	Rhabdomyosarcoma
RNAi	Ribonucleic Acid Interference
RT-qPCR	Reverse Transcription-Quantitative Polymerase Chain Reaction
sal	salm
SD	Standard Deviation
SE	Super-Enhancer
sev	sevenless
TPR	Translocated Promoter Region
TRiP	Transgenic Rnai Project
tubP	Alphatubulin84b Promoter
twi	Twist
UAS	Upstream Activating Sequence
Utx	Ubiquitously Transcribed Tetratricopeptide Repeat, X Chromosome
WHO	World Health Organization

1. INTRODUCTION

1.1 Brief overview of Paediatric Cancer

1.1.1 Definition of Paediatric Cancer

Paediatric cancer refers to a heterogenous group of solid and haematological neoplasms that predominantly occur during childhood, adolescence and young adulthood (CAYA)- a period that spans the 0–20-year age group- and is the leading cause of disease-related paediatric death (Steliarova-Foucher et al., 2017; Pfister et al., 2022). Cancer in this age group is considered a “rare” occurrence, with approximately 200,000 cases of childhood cancer reported globally in 2022, in contrast to nearly 20 million total cancer cases diagnosed worldwide the same year (Huang et al., 2022; Bray et al., 2024).

Paediatric cancers mainly arise from tissues undergoing growth and differentiation (i.e. developing tissue) and are considered developmental diseases (Scotting et al., 2005; Mora, 2012; Amatruda, 2021; Behjati et al., 2021). The most commonly diagnosed types are derived from the mesoderm or neuroectoderm, namely leukaemias, Hodgkin and non-Hodgkin lymphomas, and brain and nervous system tumours (Pfister et al., 2022; Siegel et al., 2025). When looking at all age groups, however, leukaemias rank 11th, while Hodgkin and non-Hodgkin lymphomas rank 26th and 8th, respectively (according to the NCI’s Surveillance Epidemiology and End Results (SEER) Database). These and other observations, detailed in the following sections, suggest that paediatric cancers are not merely early inceptions of cancers typically arising later in life, but that they are a different class of disease.

1.1.2 Differences between Paediatric and Adult Cancers

Adult cancers are the result of a linear, multistep process of mutation accumulation that can span up to decades and can be influenced by environmental factors. Consequently, adult cancer is often referred as a disease of aging, with cancer incidence following a dramatic increase with increasing age. The extent of mutational burden in adult cancers can often complicate efforts to identify the primary mutation driving the cancer, with cases of hypermutation littered across the spectrum (Campbell et al., 2017).

Indeed, even among paediatric tumours, mutational burden increases with age (Gröbner et al., 2018). However, in comparison with adult tumours, the mutation burden in paediatric tumours is substantially lower, making it easy to spot the genetic insults that initiate the disease. Consequently, we know that paediatric cancers are typically driven by few, and often single mutations (Gröbner et al., 2018; Ma et al., 2018). This includes paediatric tumours presenting with hypermutation caused by germline mutations in *TP53* (Li-Fraumeni syndrome)

or mismatch repair genes (Constitutional Mismatch Repair Deficiency) (Bouffet et al., 2016). In fact, most paediatric cancers have remarkably “silent” genetic landscapes (Gröbner et al., 2018; Ma et al., 2018). This strongly suggests that epigenetic instability, defined as the dysregulation of gene expression due to disruption of epigenetic information, plays a key role in driving such paediatric cancers (Sarkies and Sale, 2012). Posterior fossa ependymomas, for instance, are an extreme example that lack recurrent genetic aberrations and appear to be entirely epigenetically driven (Lin and Monje, 2020).

There is little overlap between the top-driver mutations found in adult and paediatric cancers, which can be broadly categorized for both groups into those affecting transcription regulation, epigenetic machinery, signalling pathways, and the cell cycle (Gröbner et al., 2018; Ma et al., 2018; Chen et al., 2024). Notably, driver mutations affecting transcription regulation and epigenetic machinery are disproportionately more common in paediatric cancers as compared to adult cancers (Chen et al., 2024). Furthermore, some of the genes frequently mutated in both adult and paediatric cancers are likely to differ in their evolutionary trajectory. For instance, somatic mutations in *KRAS* are predicted to be late, subclonal events in paediatric cancers such as acute lymphoblastic leukaemia (Ma et al., 2015; Kempter et al., 2025), whereas in adult cancers they are considered early clonal events (Gerstung et al., 2020).

Finally, the ontogenetic state of the tissues from which adult and paediatric cancers arise delineates another key distinction. Adult cancers largely arise in tissues that have completed their developmental program, with 85% of adult cancers arising in self-renewing epithelia in various organs including the breast, lung, colon and prostate. In contrast, paediatric cancers arise from embryonal tissues during fetal or postnatal development with only 9% arising from the epithelia (Hewitt et al., 2003; Marshall et al., 2014). This distinction is discussed further in the following section.

1.1.3 Developmental Origin of Paediatric Cancers

Development is tightly regulated by programmed cellular processes, facilitating progression through transient cell states that vary in competence to produce differentiated cell types. There are three strong indications that the origin of paediatric cancers lies in such transient developmental states that are uniquely susceptible to malignant transformation by driver mutations.

The first indication, derived from phenotypic characterizations of paediatric vs adult cancer, is that most paediatric cancers are specific to CAYA and are either absent or exceedingly rare

in adults. For example, retinoblastomas are initiated by mutations in the retinal development gene *RB1* and almost exclusively occur within the first five years of postnatal life. Only about 40 cases of adult retinoblastoma have been reported in the literature to date (Garg et al., 2018; Zhou et al., 2022; Verma et al., 2023). When a cancer type does occur in both adults and children, they display marked differences with respect to tumour biology. For instance, diffuse intrinsic pontine glioma (DIPG), a rare and aggressive brain tumour peaking in incidence between the ages of 6-9, presents with histone 3.3 or 3.1 mutations in 80% of paediatric cases (Lapin et al., 2017). In contrast, these mutations represent less than 3% of their adult counterparts (Schroeder et al., 2014). Another example is acute myeloid leukaemia (AML) that can affect any age group, but presents with age-dependent molecular alterations: paediatric cases most often harbour chromosomal translocations, often involving *lysine methyltransferase 2A (KMT2A)*, while adult AML most commonly features mutations in *DNA methyltransferase 3A (DNMT3A)* that are rare in paediatric AML (Bolouri et al., 2018). These differences suggest that the cell-of-origin (CoO) of paediatric cancers is either absent in adults or lacks the unique environmental conditions that promote oncogenic transformation.

A second major line of evidence, derived from animal models of paediatric cancers, is that the tumour-inducing capacity of paediatric cancer driver mutations is restricted to specific developmental windows. For instance, the DIPG-driving histone 3.3 mutations cause embryonic lethality when introduced too early in development into mouse embryonic stem cells, while postnatal neural progenitor cells occur “too late” in development to introduce these mutations as these cells are refractory to their oncogenic effects. Only the *in-utero* introduction of these mutations in neural progenitor cells of the developing hindbrain induces tumorigenic growth (Pathania et al., 2017). Another example is that of the tumour suppressor *SMARCB1*. Germline mutations for *SMARCB1* predispose to the development of rhabdoid tumours, which are highly aggressive paediatric tumours that most often occur in the brain. In mice, *SMARCB1* loss early in development in neural crest cells is necessary to yield tumours with rhabdoid features, whereas its loss at later stages of development either fails to initiate tumorigenesis, causes lethality, or yields lymphoma-like phenotypes (Han et al., 2016; Vitte et al., 2017).

Finally, further in support of the developmental origin of paediatric cancers are observations made in human patients of childhood acute lymphoblastic leukaemia (ALL). Specifically, studies of ALL patients with healthy twin siblings suggest that a first, necessary (initiating) mutation is acquired during pre-natal development to form clinically silent pre-leukemic cells, which are prerequisite for the postnatal development of ALL (Greaves, 2018). Altogether, these examples are part of a growing plethora of observations that suggest paediatric cancers arise as a result of “arrested development” (Behjati et al., 2021; Chen et al., 2015).

1.2 Ewing sarcoma (EwS)

1.2.1 General background paediatric sarcomas

Sarcomas, a heterogeneous group of cancers arising from bone and soft tissues that develop from the mesenchyme, account for nearly 13% of paediatric solid malignant tumours, and less than 1% in adult cancers (Hewitt et al., 2003). The most common soft tissue sarcoma is rhabdomyosarcoma (RMS), while the most common bone sarcomas are osteosarcomas (OS) and Ewing sarcoma (EwS).

Though traditionally classified based on histological features, sarcomas are increasingly being defined by their molecular alterations (Pfister et al., 2022). These alterations can be roughly subdivided into two groups according to the complexity of their alterations. The first group is characterized by few genetic alterations such as chromosomal translocations (e.g. EwS), other specific mutations that result in oncogene activation (e.g. embryonal RMS), or tumour suppressor loss of function (e.g. rhabdoid tumours), whereas the second display karyotypically complex profiles marked by numerous chromosomal imbalances (e.g. leiomyosarcoma). Karyotypically complex sarcomas typically afflict adult patients (Guillou and Aurias, 2010), while translocations are observed more in paediatric cases and account for around 15% of all sarcoma cases (Anderson et al., 2012).

Translocations can result in a gene fusion involving an oncogene whose proper activation or function is deregulated by its gene partner. In paediatric sarcomas, such gene fusions most often encode aberrant transcription factors that incur catastrophic transcriptional dysregulation (Mertens et al., 2009), of which EwS is a prototypical example. Alternatively, translocations can deregulate genes at breakpoint regions through an exchange in regulatory sequences, as is seen in chondromyxoid fibroma where the glutamate receptor *GRM1* is juxtaposed to regulatory segments of different partner genes altogether causing *GRM1* upregulation (Nord et al., 2014). The work in this thesis is focused on EwS.

1.2.2 Definition of EwS

EwS is an undifferentiated small round cell sarcoma that harbours gene fusions between a member of the FET (*EUS*, *EWSR1* & *IAF15*) family of RNA-binding proteins (RBP) and a member of the ETS (*E26 transformation specific*) family of transcription factors. This sarcoma is a highly aggressive malignant neoplasm that arises from bone and soft tissue, with CAYA patients mainly presenting with bone lesions while older patients primarily present with extraosseous EwS (Jahanseir et al., 2020; Karski et al., 2013). EwS incidences are observed more in males, with a male:female ratio of about 3:2, and are more diagnosed in patients of

white European descent than those of African descent (Paulussen et al., 2008; Worch et al., 2010; Monument et al., 2014).

1.2.3 Histopathology

EwS is histologically composed of small, undifferentiated-looking round cells with round nuclei, with extended areas of haemorrhage and tumour necrosis. Less common cases of “atypical” EwS specimens can harbour large cells with prominent nuclei, or present with a higher degree of differentiation littered with ill-defined rosettes of up to 10 cells (WHO Classification of Tumours Editorial Board, 2020). Immunohistochemically, the most relevant diagnostic marker for EwS is CD99, a cell-surface glycoprotein that is diffusely expressed in about 95% of EwS. As CD99 is also found on haematopoietic cells including endothelial cells and lymphocytes, as well as other tumours, the definitive diagnosis of EwS requires genetic confirmation for presence of the FET::ETS gene fusions (Machado et al., 2018) (see 1.2.5).

1.2.4 Treatment and Prognosis

EwS tumours display an aggressive tendency towards distant metastasis and recurrence; 20-30% of patients present systemic metastasis at diagnosis (Ye et al., 2019; Zhan et al., 2021), and 20-43% of patients have occult EwS cells in peripheral blood and bone marrow at diagnosis (Avigad et al., 2004; Fagnou et al., 1998; Peter et al., 1995; Pfleiderer et al., 1995; Sumerauer et al., 2001; West et al., 1997; Zoubek et al., 1998).

The aggressive nature of EwS and the lack of spontaneous regression necessitates correspondingly intensive therapies that start with a regimen of combination chemotherapy to limit primary tumour size and progression of metastasis. This is followed by local treatment via surgery, which may at times imply amputation and/or high-dose radiotherapy that places patients at risk of long-term medical complications (Gaspar et al., 2015). Treatment is finalized with multi-agent cytotoxic chemotherapy to eradicate minimal residual disease, the major cause of death.

There are no EwS-specific drugs. As such, the improvement in disease prognosis is limited to multi-agent intensive chemotherapies and localized control, which currently come at the cost of secondary malignant neoplasms and other late effects that burden long-term survival and quality of life (Hustu et al., 1968; Pinkel, 1962; Sutow and Sullivan, 1962; Marina et al., 2017; Shulman et al., 2022). Metastasis remains the strongest prognostic factor to date, with the 5 year survival rates reported varying between 20-45% for patients with metastasis as compared to 60-70% for those with localized disease (Rodríguez-Galindo et al., 2008; Shukla et al., 2013; Gaspar et al., 2015; Zhan et al., 2021; Shulman et al., 2022). Approximately one fourth of patients with initially localized disease experience relapse after the completion of their

treatment regimens, a rate that is far higher for patients with metastatic disease (Mater and Wagner, 2019), and the 5-year survival rate for relapsed patients is 15% (Gupta et al., 2024). These are dismally poor prognoses, underlining the urgency for identifying druggable EwS dependencies, as well as the means to stratify the disease to better direct clinical trials for EwS (Apfelbaum et al., 2022a; Shulman et al., 2022).

1.2.5 The recurrent FET::ETS fusions of EwS

The definitive diagnosis of EwS depends on the presence of a FET::ETS gene fusion. In 85% of Ewing sarcoma (EwS) tumours, the gene fusion occurs between the *Ewing's sarcoma breakpoint region 1* (*EWSR1*) gene from the FET family and the *Friend leukaemia integration 1* (*FLI1*) gene from the ETS family, yielding *EWSR1::FLI1* that is commonly abbreviated to *EWS::FLI1* as used henceforth. In the remaining 15% of cases, the fusions involve other members from the FET and ETS groups, namely *Fused In Sarcoma* (*FUS*) or *TATA-Box Binding Protein Associated Factor 15* (*TAF15*) from the FET family, and *ETS-related gene* (*ERG*), *ETS variant 1* (*ETV1*), *ETS variant 4* (*ETV4*), or *Fifth Ewing Variant Protein* (*FEV*) from the ETS family (Table 1.1) (Delattre et al., 1992; Zucman et al., 1992; Aurias et al., 1984; Becroft, 1984; Whang-Peng et al., 1984; Zucman et al., 1993; Whang-Peng et al., 1986; Zoubek et al., 1996; Turc-Carel et al., 1988; de Alava et al., 1998; Sorensen et al., 1994; Jeon et al., 1995; May et al., 1993; Picard et al., 2022).

Owing to the high incidence of *EWS::FLI1* in EwS tumours, this fusion in particular has been the main focus of the vast majority of EwS research, guiding efforts towards the identification of new therapies. Correspondingly, the following sections will largely highlight *EWS::FLI1*'s role in EwS.

Table 1.1: FET::ETS fusions in EwS. The abundance of FET::ETS fusions as encountered in EwS tumour samples, adapted from (Grünewald et al., 2018) *The *TAF15::ETV4* fusion has been reported only once (Picard et al., 2022).

FET member	ETS member	Gene Fusion	Chromosomal translocation	Frequency
FUS	FEV	<i>FUS::FEV</i>	t(2;16)(q35;p11)	<1%
	ERG	<i>FUS::ERG</i>	t(16;21)(p11;q22)	<1%
EWSR1	FLI1	<i>EWS::FLI1</i>	t(11;22)(q24;q12)	≈85%
	ERG	<i>EWS::ERG</i>	t(21;22)(q22;q12)	≈10%
	ETV1	<i>EWS::ETV1</i>	t(7;22)(p22;q12)	<1%
	ETV4	<i>EWS::ETV4</i>	t(17;22)(q21;q12)	<1%
	FEV	<i>EWS::FEV</i>	t(2;22)(q33;q12)	<1%
TAF15	ETV4	<i>TAF15::ETV4</i>	t(17;17)(q12;21)	<1%*

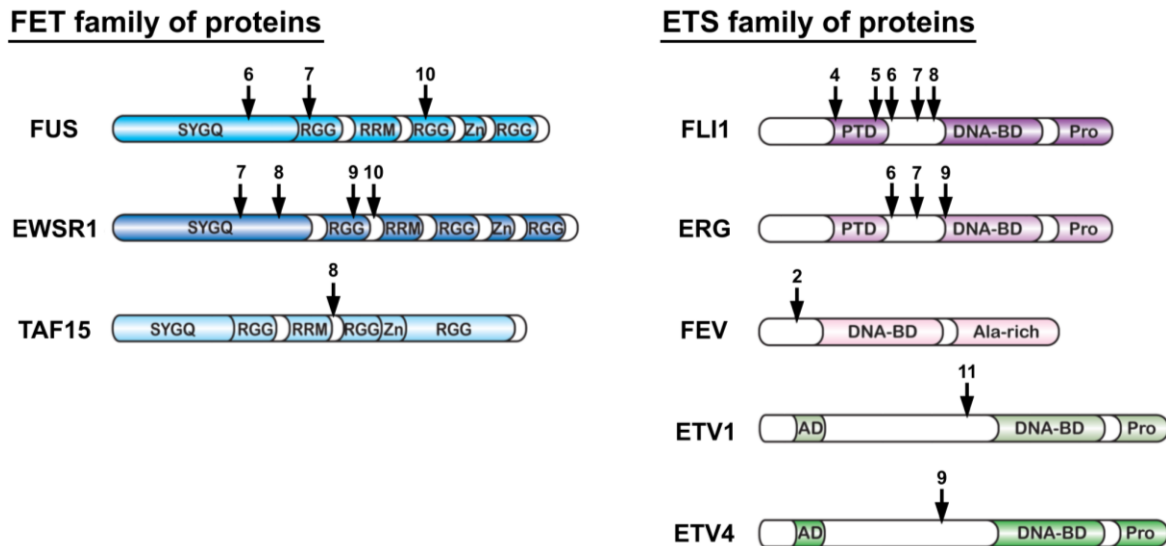


Figure 1.1: Protein domain structures of wild-type FET and ETS members. Protein domains of wild-type FET members FUS and EWSR1, and wild-type ETS members FLI1, FEV, ETV1 and ETV4. Arrows mark breakpoint regions that occur in the introns (indicated by numbers) of member genes encountered in EwS. All fusions share a portion of the serine-tyrosine-glycine-glutamine (SYGQ) rich region of the FET N-terminus and the DNA binding domain (DNA-BD). RGG, arginine-glycine-glycine rich region; RRM, RNA-recognition motif; Zn, putative zing finger; PTD, pointed domain. Adapted from (Sankar and Lessnick, 2011).

The fusion transcripts are quite structurally heterogeneous, with at least 18 in-frame chimeric transcripts having been encountered for the *EWS::FLI1* rearrangement *in-vivo*. The most prevalent subtypes, however, are the *EWS::FLI1* type 1 and type 2 (*EWS::FLI1₁* and *EWS::FLI1₂*, henceforth). *EWS::FLI1₁* consists of EWSR1 exons 1-7 fused to FLI1 exons 6-9, accounting for roughly 60% of *EWS::FLI1* translocations. *EWS::FLI1₂* involves EWSR1 exons 1-7 fused to FLI1 exons 5-9, making up for almost 20% of *EWS::FLI1* translocations (de Alava et al., 1998). The unifying hallmark for all fusions is that their amino (N)-terminal consists of the FET member's low complexity domain (LCD), also known as its prion-like domain (PrLD), which harbours [glycine/serine]-tyrosine-[glycine/serine] motifs followed by glutamine (SYGQ), while their carboxy (C)-terminus consists of the DNA-binding domain (DNA-BD) of the ETS member (Figure 1.1).

Though structurally heterogeneous, FET::ETS fusion proteins converge in their functions. They act as aberrant transcription factors, interacting with the cell's transcriptional and epigenetic machinery to drive transcription from normally inaccessible, "silent" chromatin as well as dysregulating existing transcriptional programs, and further interact with alternative splicing machinery to modulate alternative splicing (see Figure 1.2A-D, on page 14).

EWS::FLI1-induced transcriptional dysregulation is characterized by a core "signature" of up- and down-regulated genes (Hancock and Lessnick, 2008; Kauer et al., 2009). The continuous expression of *EWS::FLI1* is essential for EwS tumour formation and growth (Kovar

et al., 1996; Tanner et al., 2017; Martinez-Lage et al., 2020), and many EWS::FLI1 dysregulated genes have been found necessary to maintain EWS::FLI1-induced phenotypes. As such the following sections will elaborate on the mechanisms and characteristics of EWS::FLI1-driven transcriptional dysregulation, a topic of central focus in this thesis.

1.2.6 EWS::FLI1-mediated transcriptional dysregulation

1.2.6.1 GGAA microsatellites (GGAA μ Sat)-dependent dysregulation

Microsatellites are tracts of tandemly repeated DNA composed of short motifs that are 1-6 base pairs long (Ellegren, 2004; Coenye and Vandamme, 2005). In comparison to unique, non-repetitive DNA, polymorphisms in microsatellites present with high variability in the number of repetitions. This variation is brought about by slipped-strand mispairing during DNA replication, which occurs as a result of the tertiary structure of repetitive DNA promoting the misalignment of neighbouring repeats, and leads to the insertion or deletion of repeat motifs by DNA polymerase at mutation rates far higher than non-repetitive DNA (Leclercq et al., 2010).

Most microsatellites are embedded in non-coding regions, where a lack of negative selective pressure results in high levels of polymorphism within species (Budowle and Sajantila, 2024), and consequently low synteny across species. While the majority of such microsatellites have unknown functional relevance, some microsatellites embedded within or near promoter regions have been shown to function as enhancers, with their polymorphisms influencing gene expression (Gymrek et al., 2016). One such example is the pentanucleotide microsatellite, (TG[C/T]CC)_n, located in the p53-induced gene 3 (*PIG3*) gene promoter that is necessary for the binding and subsequent transcriptional activation by p53. The number of repeats, ranging between 10-17 in this case, are directly correlated to the extent of transcriptional activation by p53, with higher number of repetitions correlating with higher transcription levels (Contente et al., 2002).

In EwS, tetranucleotide microsatellites composed of GGAA repeat motifs hold a central role in the transcriptional dysregulation brought about by FET::ETS fusions. These sequences, henceforth referred to as GGAA μ Sats, are normally transcriptionally silent (Riggi et al., 2014). In EwS cells, FET::ETS fusions behave as pioneer factors that binds to GGAA μ Sats and lead to an enrichment for active chromatin marks H3 lysine 27 acetylation (H3K27ac) and H3 lysine 4 monomethylation (H3K4me1) in these regions. This results in the repurposing of these microsatellites as de-novo enhancers or promoters depending on their proximity to the respective dysregulated gene (Gangwal et al., 2010; Patel et al., 2012; Riggi et al., 2014; Boulay et al., 2018; Dallmayer et al., 2019; Harlow et al., 2019).

The loss of certain EWS::FLI1 gene targets upregulated through GGAA μ Sat-harbours promoters has been shown to abrogate the oncogenic phenotypes induced by EWS::FLI1 expression, such as colony formation in some cell lines or tumour formation in xenograft models. These genes include transcription factors such as orphan nuclear receptor *NR0B1* (Kinsey et al., 2006; Gangwal et al., 2010; Johnson et al., 2017), zinc finger transcription factor *EGR2* (Postel-Vinay et al., 2012; Grünewald et al., 2015), *FEZ family zinc finger protein 1* (*FEZF1*) (García-García et al., 2021) and *SRY-box transcription factor 2* (*SOX2*) (Boulay et al., 2018).

Some of the abovementioned transcription factors upregulated by EWS::FLI1 can participate in reinforcing the EWS::FLI1 transcriptional program. *FEZF1* drives the ectopic expression of a subset of neural cell-specific genes, which is a hallmark feature of the upregulated transcriptome in EwS (Rorie et al., 2004; Hu-Lieskovan et al., 2005; García-García et al., 2021). *NR0B1* and transcription factor *NKX2.2* (another GGAA μ Sat-associated EWS::FLI1 target) contribute as transcriptional repressors (Owen et al., 2008; Kinsey et al., 2009), and their combined effect is the downregulation of genes involved with mesenchymal phenotype (Tirode et al., 2007; Lin et al., 2010; Fadul et al., 2015; Khoogar et al., 2022).

Like other microsatellites in non-coding regions, GGAA μ Sats are highly polymorphic (Ellegren, 2004; Eckert and Hile, 2009). Investigations into the functional relevance of GGAA μ Sats polymorphisms found GGAA μ Sats length to be correlated with the extent of transcription that EWS::FLI1 can drive from them. Specifically, an increasing number of consecutive motifs correlates with increased EWS::FLI1 binding intensity and subsequent transcription, with the optimal lengths, or “sweet spots”, occurring at 18-26 repeats and 50-60 repeats (Monument et al., 2012, 2014; Johnson et al., 2017).

Given the correlation between GGAA μ Sats length and transcriptional activation and the necessity of GGAA μ Sat-regulated genes for oncogenic growth in EwS, GGAA μ Sats are interesting to investigate as predictors of disease biology and susceptibility. To this end, there are only three reports that provide insight into GGAA μ Sat relevance. The first is from a genome-wide association study of EwS and non-EwS genomes that identified an EwS risk allele with a longer GGAA μ Sat near the *EGR2* locus that caused significantly higher *EGR2* expression. This allele was found far more infrequently in African populations as compared to European populations (Postel-Vinay et al., 2012; Grünewald et al., 2015). A second study investigated differences in the GGAA μ Sat of the *NR0B1* gene between white European and African populations to find that despite both populations being highly polymorphic for *NR0B1* GGAA μ Sats, the African populations generally had longer GGAA μ Sats (Beck et al., 2012). The correlation between longer GGAA μ Sats and lower incidence of EwS in African populations

may hypothetically be explained by the EwS CoO not being permissive to greater levels of EWS::FLI1-driven NR0B1 expression. Finally, a third study that investigated clinical relevance of the *NR0B1* GGAA μ Sat polymorphisms found that though EwS patients presented with a strong enrichment for *NR0B1* GGAA μ Sats containing either 21-25 or 56-60 GGAA motifs, paralleling the “sweet spots” for maximal EWS::FLI1-driven expression, these polymorphisms did not correlate with clinical outcome measured with regards to 5-year event free survival (Monument et al., 2014). Altogether these studies have suggested that GGAA μ Sat polymorphisms for some EWS::FLI1 target genes might be predictors of EwS incidence.

1.2.6.2 GGAA μ Sat-independent dysregulation

In addition to transcriptional dysregulation through GGAA-microsatellites, EWS::FLI1 can bind to non-GGAA μ Sat regions, including ETS family binding motifs, to dysregulate transcription (Mao et al., 1994). ETS binding motifs share a core 5'-GGA(AT)-3', and were identified among the most highly enriched motifs in genomic loci that are transcriptionally repressed upon EWS::FLI1 binding (Guillon et al., 2009; Riggi et al., 2014; Shimizu et al., 2018), which results in the downregulation of genes, such as the cyclin-dependent kinase inhibitor, *CDKN1A* (Nakatani et al., 2003) that is normally activated by ETS factor ELK-1 (Shin et al., 2011). Conversely, EWS::FLI1 also activates transcription at other ETS binding sites in the promoters of genes such as *phospholipase D2 (PLD2)*, and polycomb repressive complex 2 subunit *Enhancer Of Zeste Drosophila Homolog 2 (EZH2)* (Kikuchi et al., 2007; Richter et al., 2009), which are normally silenced by ETS factors ETS1 (Kang et al., 2014) and ERG (Kunderfranco et al., 2010), respectively. The dual effect of EWS::FLI1 at ETS-binding sites (encompassing both gene activation and repression) is attributed to the fusion's ability to displace wild-type ETS family members and opposes their regulatory function (Riggi et al., 2014).

Evidence suggests that EWS::FLI1 interaction with transcription factors other than ETS family members also contributes to transcriptional dysregulation in EwS. For instance, forkhead box (FOX) transcription factors, whose binding motifs are also enriched in EWS::FLI1 repressed sites, can interact with the EWS-portion of EWS::FLI1 and increase enhancer activity at EWS::FLI1-target genes (Niedan et al., 2014; Shimizu et al., 2018). Another such cooperative interaction is necessary for the upregulation of the EWS::FLI1-target gene uridine phosphorylase (*UPP*), which relies on EWS::FLI1's binding with the activator protein 1 (AP-1 or Fos-Jun) transcription factor at AP-1 sites in the *UPP* promoter (Kim et al., 2006). Collectively, EWS::FLI1's cooperative and antagonistic interactions with endogenous transcription factors broaden the spectrum of genes impacted by EWS::FLI1 and underscore the complexity of the regulatory network that gets subverted as a result.

Oncogenic transformation in EwS requires the misexpression of EWS::FLI1 target genes dysregulated through GGAA μ Sat-independent mechanisms just as much as those dependent on GGAA μ Sats. For instance, the suppressive activity of EZH2 enables oncogenic transformation in EwS, which is lost upon its inhibition (Richter et al., 2009). Similarly, the restoration of EWS::FLI1 down-regulated targets *Lysyl Oxidase (LOX)*, *TGF- β type II receptor (TGFB2)* and *Protein tyrosine phosphatase L1 (PTPL1)* blocks tumorigenicity in EwS (Hahm et al., 1999; Abaan et al., 2005; Sankar et al., 2013; Agra et al., 2013).

1.2.6.3 Epigenetic reprogramming by EWS::FLI1

While the full picture of how EWS::FLI1 mediates transcriptional dysregulation is still emerging, work over the last decade has established that both GGAA μ Sat-dependent and -independent transcriptional dysregulation by EWS::FLI1 rely upon the fusion's ability to interact with the epigenetic machinery of the cell.

At EWS::FLI1-activated sites, EWS::FLI1 binding leads to an acquisition of histone marks associated with open and actively transcribed chromatin, including H3K4me1, H3K4me3, H3K27ac, and a depletion of repressive marks including H3K9me3 and H3K27me3 (Shimizu et al., 2018; Svoboda et al., 2014; Tomazou et al., 2015; Grünwald et al., 2015; Tak et al., 2022). At GGAA μ Sats specifically, EWS::FLI1 promotes chromatin opening by recruiting chromatin remodelling complexes, specifically the BRG1/BRM-associated factor (BAF) and Complex of proteins associated with Set1 (COMPASS)-like complexes, through its interaction with their core components (including BRG1, SMARCC1 and SMARCC2 of the BAF complex (Boulay et al., 2017; Lindén et al., 2019), and WDR5 and ASH2 of the COMPASS complex) (Riggi et al., 2014). EWS::FLI1 further recruits other components of transcriptional machinery, including RNA polymerase II (Petermann et al., 1998), transcriptional coactivator histone acetyltransferases CREB binding protein (CBP) and E1A Binding Protein P300 (P300) (Ramakrishnan et al., 2004; Wei et al., 2024), and RNA Helicase A (Toretsky et al., 2006). These interactions and recruitment events collectively promote EWS::FLI1-mediated transcriptional activation (Figure 1.2-A).

Conversely, at EWS::FLI1-repressed sites, EWS::FLI1 employs two different mechanisms (Figure 1.2-B,C). It “passively” represses gene expression by displacing endogenous ETS transcription factors as previously detailed. Alternatively, it “actively” represses by interacting with repressive chromatin remodelling machinery such as lysine specific demethylase 1 (LSD1) (Sankar et al., 2014, 2013; Theisen et al., 2021), components of the Nucleosome Remodelling and Deacetylase (NuRD) complex (CHD4, HDAC2 and RBP7) (Selvanathan et al., 2015), and associated HDACs (Richter et al., 2009; Hernandez-Muñoz et al., 2016; Sánchez-Molina et al., 2020). Altogether, these mechanisms demonstrate EWS::FLI1's

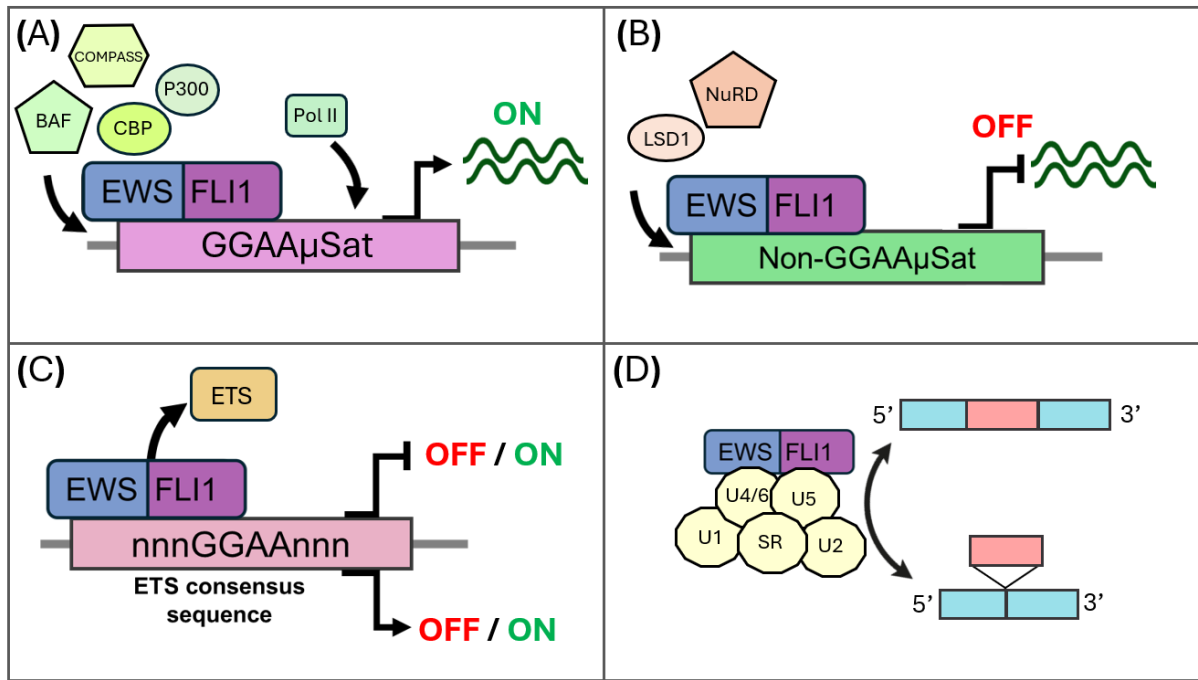


Figure 1.2: EWS::FLI1 oncogenic functions in EwS. (A-B) EWS::FLI1 actively dysregulates transcription by (A) converting heterochromatic regions harbouring GGAAμSats into neoenhancers through the recruitment of epigenetic modifiers, such as the BAF and COMPASS complex, and the histone acetyltransferases CBP and P300, and RNA Pol II, or (B) repressing transcription from euchromatic regions of the genome through the recruitment of repressive chromatin remodelling machinery such as LSD1 and the NuRD complex. (C) EWS::FLI1 passively dysregulates transcription by opposing the regulatory functions of endogenous transcription factors (TF), such as ETS members, by outcompeting them from their consensus sequences. (D) EWS::FLI1 dysregulates alternative splicing by interacting with members of the spliceosome machinery.

extensive capacity to reprogram the chromatin landscape, making chromatin modifiers that interact with EWS::FLI1 attractive targetable dependencies for the development of EwS therapies (Sankar et al., 2013, 2014; García-Domínguez et al., 2018; Sánchez-Molina et al., 2022).

1.2.7 The regulation of EWS::FLI1 activity

As previously mentioned, EwS tumour formation and growth depends on the continuous expression of EWS::FLI1. However, only a few cells types have been found permissive to EWS::FLI1 expression, with most undergoing either cell cycle arrest or death upon in response (Deneen and Denny, 2001; Lessnick et al., 2002; Sohn et al., 2010). This in turn suggests that tolerance to EWS::FLI1 expression must be dependent on cell autonomous mechanisms. Accordingly, mechanisms that restrict EWS::FLI1 activity have been identified in permissive cells.

Endogenous transcription factors can function as direct “limiters” of EWS::FLI1-driven changes to transcription. For instance, the ETS member ETV6 competes with EWS::FLI1 to

bind short GGAA repeat sequences, antagonizes its transcription activating effect (Gao et al., 2023). The loss of ETV6 increases EWS::FLI1 binding to the chromatin and suppresses EwS cell proliferation. A similar limiting function might be expected of all ETS transcription factors that are outcompeted by EWS::FLI1. Another “limiter” is transcription factor homeobox D13 (HOXD13), which colocalizes to EWS::FLI1-bound sites and promotes the upregulation of EWS::FLI1-repressed genes. HOXD13 is also an upregulated target of EWS::FLI1, and HOXD13 loss inhibits the EwS cell proliferation, as well as reduces metastasis (Heyking et al., 2016; Apfelbaum et al., 2022b).

At the post-translational level, EWS::FLI1 protein stability is regulated by E3 Ubiquitin ligases tripartite motif-containing 8 (TRIM8) and Speckle-type POZ protein (SPOP), and the deubiquitinases OTU domain-containing protein 7A (OTUD7A), ubiquitin specific protease 7 (USP7) and USP19 (Seong et al., 2021; Su et al., 2021). Interestingly, both the elevation of EWS::FLI1 levels following TRIM8 loss and the decrease in EWS::FLI1 levels by OTUD7A loss causes cell death. These observations have fuelled the hypothesis for a “Goldilocks” principle in EwS, whereby EWS::FLI1 is maintained at “just-right” levels (Figure 1.3-A).

At the post-transcriptional level, EWS::FLI1 mRNA transcripts are regulated by an RNA-binding protein Lin-28 homolog (LIN28B) whose loss decreases EWS::FLI1 mRNA stability (Keskin et al., 2020). However, LIN28B is expressed in a minority of EwS tumours, indicating that it may function as a mediator that regulates EWS::FLI1 transcripts in response to specific cellular contexts. Another regulator of EWS::FLI1 transcript levels is miRNA mir-145, which exerts its regulatory effect by binding to multiple sites in the EWS::FLI1 3' untranslated region (Riggi et al., 2010; Ban et al., 2011). mir-145 transcription is itself repressed by EWS::FLI1: their mutual regulation is in turn mirrored by an inverse correlation between their expression levels that, when tipped towards elevated mir-145, halts EwS cell proliferation (Riggi et al., 2010; Ban et al., 2011).

Finally, at the level of transcription, EWS::FLI1 expression is controlled by HDAC6, which upregulates EWS::FLI1 expression through the recruitment of P300 to the EWSR1 promoter (García-Domínguez et al., 2021). While inhibitors of EWS::FLI1 transcription have not been explicitly identified, negative regulators of HDAC6 activity such as epidermal growth factor receptor (EGFR) (Lissanu Deribe et al., 2009), or extracellular signal-regulated kinase 1 (ERK1) (Williams et al., 2013), could act as limiters of EWS::FLI1 activity.

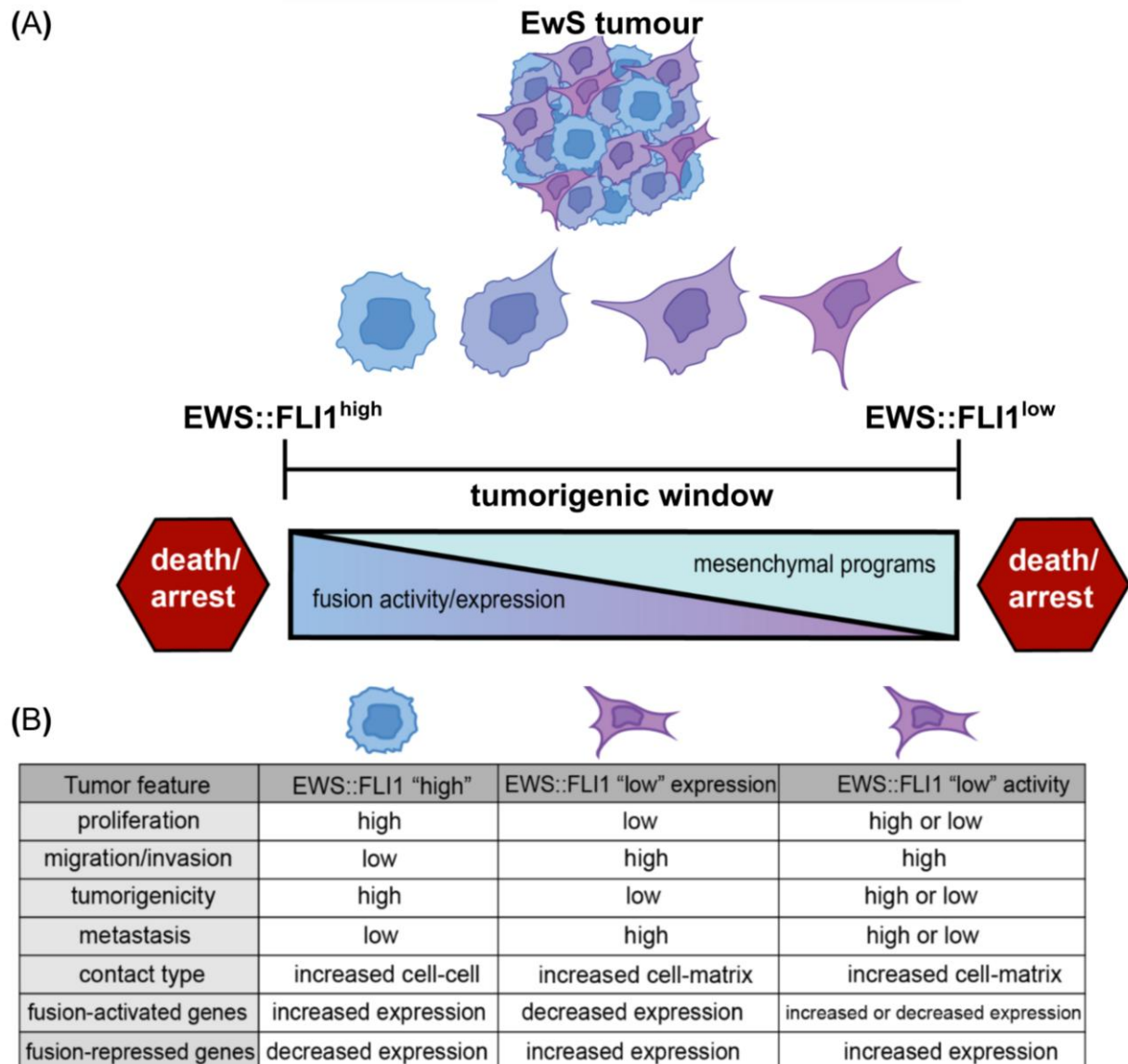


Figure 1.3: Consequences of EWS::FLI1 heterogeneity on EwS cell fates. (A) EwS tumours are composed of cells displaying varying levels of EWS::FLI1 activity levels that is maintained within tolerable windows. (B) The variation of EWS::FLI1 activity within this tolerable range can in turn drive shifts in cell states ranging between more proliferative to more mesenchymal-like phenotypes as outlined in. Adapted from Apfelbaum, Wrenn, et al., 2022

1.2.8 Intratumour heterogeneity in EwS

Intratumour heterogeneity drives tumour evolution and contributes to drug resistance and therapy failure (McGranahan and Swanton, 2017), both of which are withstanding challenges in EwS (Scotlandi et al., 2009). EwS tumours are composed of cell populations that are highly heterogenous with respect to their translational, transcriptional, and epigenetic landscapes (Bühnemann et al., 2014; Apfelbaum et al., 2022b; Goodspeed et al., 2025; Sheffield et al., 2017). Characterizing these profiles and understanding their biological implications is critical for the development of EwS-specific therapies.

Studies of EwS tumour DNA methylome profiles have revealed that EwS cells within the same tumour exist along a broad continuum of epigenetic cell states that range from more stem-like to more mesenchymal-like (Sheffield et al., 2017). Correspondingly, transcriptional profiles are also highly heterogeneous within both EwS cell lines (Apfelbaum et al., 2022b) and tumours (Keskin et al., 2021). Interestingly, a collection of recent studies suggests that the levels of EWS::FLI1 activity can determine shifts between cell states observed intratumorally (Figure 1.3-B).

Elevated EWS::FLI1 expression, termed the “EWS::FLI1^{high}” state, is characteristic of the bulk of EwS cells. These cells are marked by a proliferative, immotile phenotype, and a dysregulation of genes related with the actin cytoskeleton and the extracellular matrix (Chaturvedi et al., 2012; Franzetti et al., 2017; Aynaud et al., 2020; Khoogar et al., 2022). On the other end, the “EWS::FLI1^{low}” state is characterized by the increased ability to migrate, invade, and metastasize, at the expense of proliferative capacity (Chaturvedi et al., 2014; Franzetti et al., 2017; Apfelbaum et al., 2022b; Bierbaumer et al., 2021). Finally, the induced transition from an EWS::FLI1^{low} to EWS::FLI1^{high} state can promote metastasis *in vivo* (Franzetti et al., 2017). Altogether, these studies show that both low and high EWS::FLI1 activity levels are relevant for EwS progression.

An important question raised by these studies concerns the source of heterogeneity in EWS::FLI1 activity levels. In brief, there are two main plausible sources for this heterogeneity. The first source is EWS::FLI1 itself given that some of its gene targets can function as inhibitors of its activity (see section 1.2.7.). Second, it may be differences in the developmental context of EwS initiation, i.e. the transformation of distinct cell types descending from the initial cell-of-mutation that maintain EWS::FLI1 activity at correspondingly distinct levels. These two features are tightly intertwined, making the true source of heterogeneity difficult to disentangle (Kovar, 2005; Kovar et al., 2016). A closely related issue is the unresolved identity of the EwS's cell-of-origin (CoO), which retrospective studies have suggested is to be of mesenchymal/neuroectodermal origin. The definitive path to understanding both EwS, and whether heterogeneity is established at EwS initiation, is through the generation of models for EwS initiation, which has been largely unsuccessful so far (section 1.2.9).

The biology of cancer initiation can greatly inform therapy development, as it has for the development of differentiation therapy for neuroblastoma based on targeting pathways inducing differentiation in the neuroblastoma CoO, neural-crest cells (Reynolds et al., 2003; Sell, 2004; Coorens and Behjati, 2022). Knowledge of the cancer CoO further help understanding variable drug sensitivity; for instance, leukaemia stem cells that originate from hematopoietic stem cells are more resistant to chemotherapy as compared to those originating

from more committed progenitors (Krivtsov et al., 2013). Such examples underscore the importance of developing models to study EwS initiation.

1.2.9 The struggle to model EwS initiation

Our knowledge of EwS and FET::ETS biology is entirely based on extensive research performed using EwS tumours, patient-derived EwS cell lines, patient-derived xenograft (PDX) models, and human and mouse cells transformed to express EWS::FLI1. However, these models cannot fully recapitulate *de novo* EwS initiation, the elucidation of which is crucial to fully understand its biology.

Attempts to develop EWS::FLI1-expressing transgenic mouse models that could spontaneously develop EwS have been unsuccessful (Minas et al., 2017). Specifically EWS::FLI1 expression, more often than not, results in embryonic lethality (Minas et al., 2017). The mouse cells tested so far that do not die following EWS::FLI1 expression fail to develop EwS: EWS::FLI1 expression in the primitive mesenchyme results in abnormal muscle, cartilage, and bone development, but no tumours (Lin et al., 2008), in mesenchymal stem cells EWS::FLI1 expression yields fibrosarcoma-like tumours but not EwS (Minas et al., 2017), and EWS::FLI1 expression in neuronal tissue does not induce any evident aberrant phenotypes let alone tumours (Minas et al., 2017).

On the other hand, EwS-like phenotypes have been observed in zebrafish transgenic animals expressing EWS::FLI1. The earliest studies with zebrafish showed that the injection of EWS::FLI1 mRNA into zebrafish embryos induces mitotic defects and cell death, precluding the further identification of stable transgenic lines and the study of EWS::FLI1 effect during embryonic development (Embree et al., 2009). These limitations were overcome by the transposon-mediated expression of EWS::FLI1 (Leacock et al., 2012). In this latter model, EWS::FLI1 expression induces, with low penetrance, malignant peripheral nerve sheath tumours and, less frequently, diffuse leukaemia-like and EwS-like undifferentiated round cell tumours. The low penetrance of EwS-like phenotypes was only overcome in a p53 deficient background (Leacock et al., 2012). The necessity for a p53-loss was later circumvented in a Cre-inducible model in which EWS::FLI1 is expressed under the ubiquitin promoter. EwS-like tumours induced in this model further expressed zebrafish orthologs of EWS::FLI1 target genes including *NR0B1*, and presented with a significant enrichment for human EwS tumour transcriptional signatures (Vasileva et al., 2022) in apparent contrast to EWS::FLI1 expressing mouse cells (Braunreiter et al., 2006; Monument et al., 2014).

A very interesting observation derived from the zebrafish model concerns the relevance of GGAA μ Sats for tumour initiation. The inability to obtain EwS-like tumours in mouse has been

attributed to low synteny in GGAA μ Sats, as a result of which the mouse *NR0B1* that lacks GGAA μ Sats is not upregulated in murine-derived NIH3T3 cells expressing EWS::FLI1 (Braunreiter et al., 2006; Monument et al., 2014). Though the zebrafish genome does harbour GGAA μ Sats (Vasileva et al., 2024), the zebrafish *NR0B1* gene is not located near any GGAA μ Sats: only 8 instances of two consecutive GGAA motifs occur in isolation within 1Mb upstream of the transcription start site, and one instance of 3 GGAA motifs separated by single nucleotides occur around 30 kb upstream (GRCz11 reference genome). In comparison, the human *NR0B1* harbours up to 11 consecutive GGAA motifs in its promoter region (Monument et al., 2014). Whether EwS-like tumours arise in zebrafish as a direct result of zebrafish *nr0b1* upregulation remains to be investigated as such a dependency could explain, in part, the discrepancy between EWS::FLI1's effect in zebrafish vs murine cells. However, the low synteny between zebrafish, murine and human GGAA μ Sats strongly suggests that GGAA μ Sat-independent transcription might have a more prominent role than GGAA μ Sat-dependent transcription in mediating EwS tumour phenotypes (Vasileva et al., 2024).

Recognizing the challenges of generating EWS::FLI1-expressing transgenic models, and with the initial goal of testing whether a *Drosophila* model expressing EWS::FLI1 could be generated, our group developed a *Drosophila* transgenic line harbouring EWS::FLI1₁.

1.3 *Drosophila* as a model to investigate EWS::FLI1 function

1.3.1 *Drosophila melanogaster* as a cancer model

The fruit fly, *Drosophila melanogaster* (henceforth referred to as simply *Drosophila*) is a holometabolous that presents four developmental stages: embryo, larva (divided into 3 instar stages) pupa, and adult. In brief, development to adulthood takes about 10 days at 25°C (Figure 1.4). Growth through these developmental stages is driven either through proliferation, as seen in diploid cells in the adult primordia, or through growth via cell enlargement, polyploidization and polytenization, which is the case for most larval organs, such as salivary glands. The fly genome is composed of 4 pairs of chromosomes encoding over 13,000 genes (Stapleton et al., 2002), a relatively simple karyotype that is rendered easy to maintain through the use of synthetic balancer chromosomes.

A wide array of genetic tools developed over the past century, coupled with intrinsic features of fly biology such as a short lifespan, ease of maintenance, and a simple karyotype, have made the fly a pivotal model organism. From the earliest works by T.H. Morgan and colleagues defining the chromosomal theory of heredity, the mutagenizing effects of ionizing radiation on

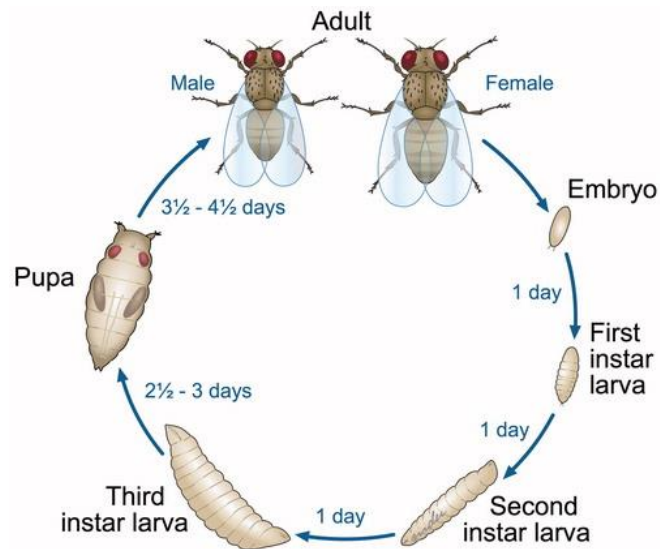


Figure 1.4: Developmental stages of *Drosophila melanogaster*. *Drosophila* passes four main stages of development: embryonic, larval, pupal, and adult stages. Image from Ong et al., 2015.

the genome, and the mapping of gene loci (Morgan, 1910; Bateson, 1916; Muller, 1927, 1928; Morgan, 1942), *Drosophila* has consistently proven to be a paradigm of a genetically tractable model. This has ultimately led to a century of discoveries made using the fly that have elucidated several key pathways in development, immunity, and disease, including cancer.

1.3.1.1 Advantages

A growing list of close to 100 tumours have been described in *Drosophila*, confirming that pro-tumorigenic programs can be triggered and investigated in the fly (Gateff, 1994; Brumby and Richardson, 2005; Gonzalez, 2013; Gong et al., 2021). These tumours arise in tissues that harbour the capacity to proliferate such as the larval brain, adult primordia and haematopoietic precursor cells, of which the most often investigated is the larval imaginal wing discs. Study of these tumours has led to the identification of cancer-related genes and the elucidation of tumorigenic functions, such as the role of cell polarity genes *lethal (2) giant larvae (lgl)*, *scribble (scrib)* and *discs large (dlg)* in maintaining apicobasal polarity to suppress neoplastic growth, and their function in cooperative oncogenesis with *Ras* and *Notch* mutations (Brumby, 2003; Humbert et al., 2008; Wu et al., 2010).

Additionally, the fly possesses functional orthologs of over 60% of human disease-associated genes, including many oncogenes and tumour suppressors (Fortini et al., 2000; Rubin et al., 2000). The conservation of pathways often dysregulated in cancer, such as RAS-MAPK, PI3K-AKT, JAK-STAT, and the insulin signalling axes, allows to generate models by recreating the genetic conditions causative of human cancers (Scanga et al., 2000; Therrien et al., 2000; Zeidler et al., 2000; Viola et al., 2023). Examples include the multigenic models of colon cancer types (Bangi et al., 2016), the EGFR and PI3K overexpression model to induce

glioma-like tumours (Read et al., 2009), and more recently a model for paediatric DIPG based on *H3K27* and *Myc* overexpression (Pablo and Casas-Tintó, 2023).

The conservation of genes and disease-relevant pathways has also enabled for the generation of “humanized” *Drosophila* strains, which carry a human gene that can functionally replace its *Drosophila* ortholog (Fernández-Hernández et al., 2016). This approach has yielded valuable *in-vivo* models for various cancer types, including those often associated with fusions oncogenes such as Alveolar RMS (ARMS) driven by PAX::FOXO1 (Galindo et al., 2006), AML driven by AML1::ETO and NUP98::HOXA9 (Osman et al., 2009; Sinenko et al., 2010; Gavory et al., 2021), and Mixed lineage leukemia (MLL) driven by MLL::AF4 (Johannessen et al., 2023). The generation of these models in particular relies on the use of the bipartite Gal4/UAS system (Brand and Perrimon, 1993), which enables to temporally and spatially control the ectopic expression of the fusion oncogenes in cells and tissues of interest.

The availability of thousands of ready-to-use strains from fly stock centres carrying mutants, enhancer and protein traps, or double-stranded RNAs for RNA interference allows to conduct large scaled genetic screens for modifiers of oncogene-driven phenotypes in *Drosophila* models. Such screens have led to, for instance, the identification of the *Drosophila* gene *rolling pebbles* (*rols*) as a downstream mediators of PAX::FOXO1-mediated lethality (Avirneni-Vadlamudi et al., 2012). Accordingly, the mammalian ortholog of *rols* was discovered as a dependency for neoplastic phenotype of ARMS cells (Avirneni-Vadlamudi et al., 2012).

Importantly, while the study of cancer in *Drosophila* typically entails studying tumours, pro-tumorigenic programs can also be triggered and studied in cells that do not yield tumours. Indeed, the ARMS *Drosophila* model is based on the ectopic expression of PAX::FOXO1, which affects the proper differentiation of muscle tissue but does not induce tumours. Instead, the fusion causes abnormal muscle fibre development, a function that *rols* was found to mediate. Another example is the cells of the adult accessory glands. Accessory glands share functional similarities with the mammalian prostate, on the basis of which the investigations have found parallels between gene expression profiles of human prostate cancer, often characterized by elevated levels of *MYC* expression, and accessory glands overexpressing *Drosophila Myc* without inducing proliferation (Church et al., 2025). Overall, the modelling of oncogenic signalling in the fly can be extended to any tissue permissive to the expression of cancer-related genes, rather than being restricted to those that present with tumours.

1.3.1.2 Limitations

As with any model organism, it is crucial to acknowledge the physiological differences between *Drosophila* and humans that preclude the modelling of key aspects of cancer. The most obvious difference is development: *Drosophila* develop through holometabolous

metamorphosis, which severely confounds drawing parallels to the developmental context in which a modelled tumour arises- of high relevance to modelling paediatric tumours. Another is the anatomical and physiological differences: *Drosophila* lacks many tissues present in mammals, such as the bone and cartilage that are particularly relevant for the modelling of bone sarcomas such as EwS. Flies harbour an open circulatory system that precludes the modelling of angiogenesis and metastatic intravasation and extravasation occurring in the closed system of humans. *Drosophila* has also not evolved adaptive immune mechanisms similar to those in mammals: relatively recent work has suggested that the fly can mount adaptive responses against viral infections, however the identified mechanisms vastly differ from mammalian adaptive immunity (Tassetto et al., 2017). This limits the use of *Drosophila* cancer models to the study of tumour-induced innate immune responses.

1.3.2 EWS::FLI1_{1FS}

In 2022, our group reported the results of our efforts to express EWS::FLI1 in the fly (Molnar et al., 2022). As in humans, the expression of EWS::FLI1 in *Drosophila* is associated with lethal toxicity that initially precluded the recovery of transgenic flies harbouring either of the two most prevalent EWS::FLI fusions, EWS::FLI1₁ and EWS::FLI1₂, downstream of a UAS promoter. The lethality was presumably due to exceedingly low expression rates that leak in absence of a Gal4. Such high toxicity has only been observed for UAS transgenes encoding exceedingly potent cell toxins like Diphtheria Toxin A, Ricin A and bacterial DNA adenine methylase (Moffat et al., 1992; Sweeney et al., 2012; Southall et al., 2013).

Subsequent transgenesis efforts carried out at lower temperatures to minimize leakiness still could not recover UAS>EWS::FLI1₂ transgenic animals. However, from a total of a single fly harbouring a frameshift that replaced 69 amino acids from the EWS::FLI1₁ C-terminus with a new sequence was recovered that we named EWS::FLI1_{1FS}. This UAS>EWS::FLI1_{1FS} harbouring fly was fully viable and fertile, allowing for establishing a stable line carrying the transgene.

As with EWS::FLI1, full-length versions of other toxic FET::ETS fusions, namely EWS::ERG, EWS::FEV, and FUS::ERG, could not be recovered through transgenesis. The same held true for mutant versions of each FET::ETS that lacked the last 69 amino acids of their C-terminus. However, replacing these last 69 amino acids with the unique 69 amino acid sequence of EWS::FLI1_{1FS}, henceforth referred to as the FStail, allowed for the recovery of transgenic animals at the standard rate of transgenesis, suggesting that lower toxicity is a neomorphic property of the FStail. However, EWS::FLI1_{1FS} retains toxicity as few cell types were found to be permissive to its expression, including larval salivary glands, and epithelial cells of the wing disc, eye disc and neuroepithelium.

Incredibly, EWS::FLI1_{1FS} interacts with orthologs of many EWS::FLI1's human protein partners, including RNA Pol II subunits, BAF complex components brm and mor (orthologous to BRG1 and SMARCC1/2, respectively) as well as NuRD complex components orthologous to CHD4. Furthermore, the fusion localizes to transcriptionally active regions of polytene chromosomes in salivary glands (Figure 1.5-A) and induces a massive dysregulation of the transcriptome (Figure 1.5-B). The transcriptional dysregulation induced by EWS::FLI1_{1FS} is significantly enriched for Drosophila orthologs of human EwS signature genes (Hancock and Lessnick, 2008; Kauer et al., 2009). The dysregulated genes are enriched for Drosophila ETS target genes, suggesting EWS::FLI1_{1FS} can outcompete endogenous ETS transcription factors, as well as ectopically upregulated genes associated with neural development, a hallmark of human EwS transcriptional signatures. A highly interesting conclusion drawn from this data is that the massive transcriptional dysregulation observed in Drosophila following EWS::FLI1_{1FS} expression must be largely GGAA μ Sat-independent because, unlike the human genome that contains thousands of GGAA μ Sats of optimal length, the Drosophila genome contains only 83 GGAA μ Sats longer than 2 consecutive GGAA motifs, of which the longest has only 9 repeats. As such, these results demonstrate that EWS::FLI1 can induce an EwS-like transcriptional signature in the absence of human-specific GGAA μ Sats.

Using transgenic lines carrying GGAA repeats upstream a heat-shock minimal promoter (HSP) followed by the yellow fluorescent protein (YFP)-coding sequence, we further showed that EWS::FLI1_{1FS} retains the ability to convert silent GGAA μ Sats into neoenhancers in Drosophila (Figure 1.5-C). This conversion is most optimal for GGAA μ Sats with 20 $\times$ (GGAA) repeats as compared to 10x, 3x and 1x repeats, confirming the “sweet spot” that closely follows reports from EwS cells. Finally, EWS::FLI1_{1FS} also interacts core subunits of the Drosophila spliceosome, orthologous to human proteins previously identified as EWS::FLI1 interactors, as well as modulates alternative splicing. These results altogether demonstrate that EWS::FLI1_{1FS} retains some of the neomorphic functions of human EWS::FLI1 in Drosophila.

Notably, driving EWS::FLI1 expression in the wing disc epithelia yields wing disc tumours that present with malignant neoplastic growth in allograft assays. While these tumours are epithelia-derived, and therefore are expected to recapitulate EwS tumours, their growth and malignancy demonstrates that GGAA μ Sat-independent transcriptional dysregulation is sufficient for EWS::FLI1-driven oncogenic transformation, suggesting these mechanisms are of greater importance than is generally acknowledged.

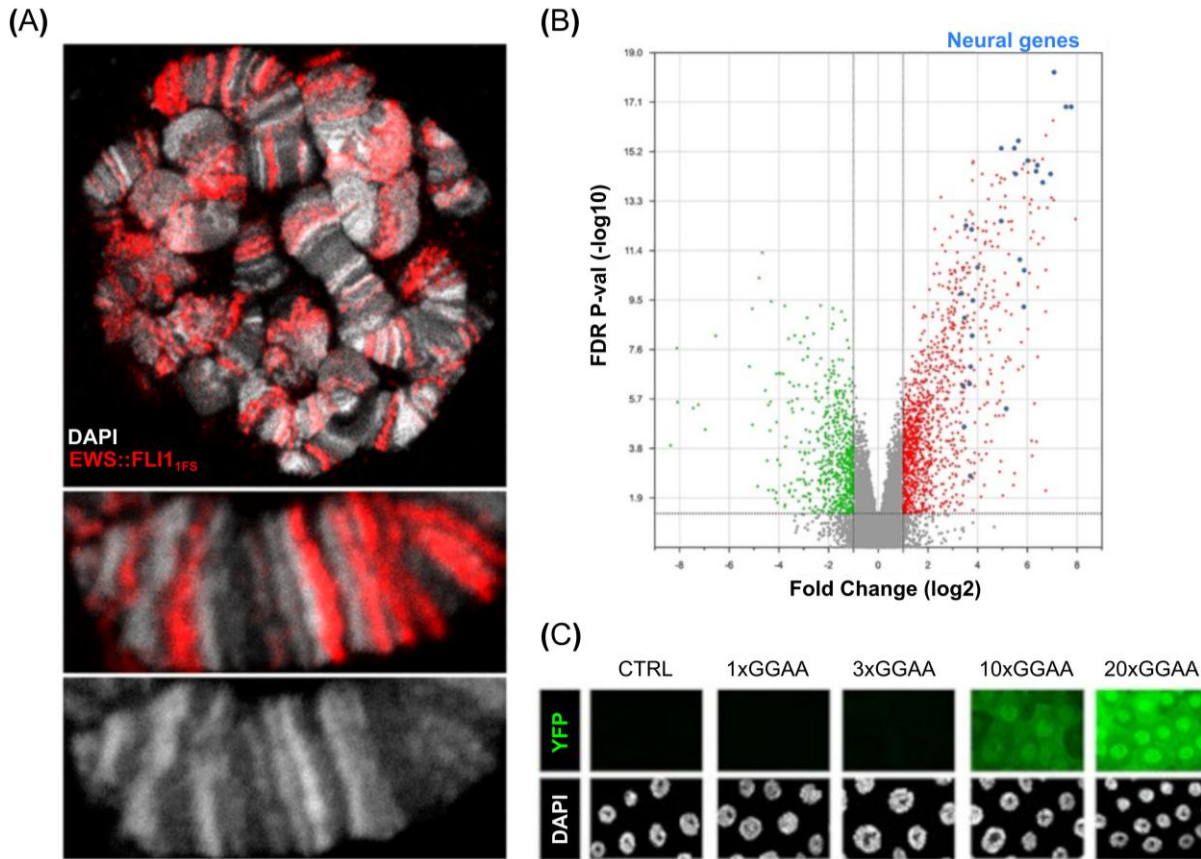


Figure 1.5: EWS::FLI1_{FS} recapitulates in *Drosophila* known neomorphic functions that induce EwS in humans. (A) Polytene chromosomes of *nub>Gal4/UAS>EWS::FLI1_{FS}* salivary glands, stained with anti-EWS antibody in red, co-stained with DAPI. **(B)** Volcano plot showing the massive transcriptional dysregulation induced by EWS::FLI expression in *nub>Gal4/UAS>EWSFLI1_{FS}* salivary glands, including ectopic upregulation of neural genes (blue dots). **(C)** Panels of a representative sections from salivary glands expressing EWS::FLI1_{FS}, showing YFP levels reflecting transcription from GGAA μ Sats of varying numbers of GGAA repeat as indicated. Adapted from (Molnar et al., 2022)

2. OBJECTIVES

Having shown that EWS::FLI1_{1FS} recapitulates known neomorphic functions that initiate EwS in humans, we questioned whether the unmodified fusion could perform better than the frameshifted counterpart. In this context, we hypothesized that the investigation of how the FStail circumvents EWS::FLI1 toxicity could contribute towards the establishment of transgenic lines expressing the unmodified, full-length EWS::FLI1 protein.

Furthermore, *Drosophila* presents itself as a unique platform to investigate GGAA μ Sats-independent dysregulation, which is difficult to accomplish in mammalian and fish model systems that harbour numerous GGAA μ Sats. To double down on these advantages, my thesis work has focused on the 3 following objectives.

- 1) To establish and characterize *Drosophila* transgenic models expressing full length, unmodified EWS::FLI1₁.
- 2) To investigate the functional relevance of varying EWS::FLI1₁ levels with respect to the following:
 - A. Transcriptional upregulation from GGAA μ Sats.
 - B. GGAA μ Sat-independent transcriptional dysregulation.
- 3) To search for new dependencies in EwS through genetic screens for modifiers of EWS::FLI1₁-dependent upregulation of transcription from GGAA μ Sats.

3. MATERIALS AND METHODS

3.1 Fly stocks

We used the GAL4/UAS system (Brand and Perrimon, 1993) to drive the expression of the RNAi lines (Appendix C) and EWS::FLI1₁ transgenes used in this study: *UAS>EWS::FLI1_{1FS}* (Molnar et al., 2022), *UAS>EWS::FLI1_{1+FStail}* (this work), *UAS>uORF-EWS::FLI1₁* (*UAS>BC-EWS::FLI1₁*) (this work), *P[UAS>EWS::FLI1₁]* (*PEWS::FLI1₁*) (this work). The 20×(GGAA) μ Sat>YFP construct (Molnar et al., 2022) was used to report for EWS::FLI1₁-driven transcription from GGAA μ Sats.

All RNAi lines used in the screen were obtained from Bloomington Drosophila Stock Centre (BDSC). For RNAi screens, the expression of UAS transgenes were driven by *sal^{EPV}>Gal4* (Cruz et al., 2009), *sev>Gal4* (BDSC ID: 5793), *longGMR>Gal4* (BDSC ID: 8605).

Alph phosphatase mutants (Baril et al., 2009) were obtained from BDSC: *alph^{XS-88}* (BDSC ID: 59007), *alph^{S-331}* (BDSC ID: 59009), *alph^{S-355}* (BDSC ID: 59008). The wild-type strain used was *w¹¹¹⁸*.

3.2 Genotypes and crossing schemes

All crosses described below were maintained at 25°C.

To evaluate EWS::FLI1 toxicity following expression in different Drosophila tissues: females of all tested Gal4 lines were crossed to males *w*; *UAS>EWS::FLI1_{1FS}/CyO*, *w*; *UAS>BC-EWS::FLI1₁/CyO*, *w*; *UAS>EWS::FLI1_{1FS}* and *w¹¹¹⁸*; *PEWS::FLI1₁/CyO*, *S*, *Tb*. To test embryonic viability, eggs were collected on apple agar plates (2% w/v agar, 1.35% w/v sucrose, 25% v/v apple juice) and the number of eggs hatching into larvae was counted for 200 eggs. To test viability at pupal/adult stages, 24-hour egg laying was performed on tubes with wholemeal fly media. Pupal lethality was evaluated by counting the number of eclosed adults from at least 150 pupae.

For the microarray expression profiling experiment: females *w*; *sal^{EPV}>Gal4* were crossed to males *w¹¹¹⁸*, to males *w*; *UAS>EWS::FLI1_{1FS}/CyO*, and to males *w*; *UAS>BC-EWS::FLI1₁/CyO* to obtain *sal^{EPV}>Gal4/+* (CTRL), *sal^{EPV}>Gal4/UAS>EWS::FLI1_{1FS}*, and *sal^{EPV}>Gal4/UAS>BC-EWS::FLI1₁* samples. The *PEWS::FLI1₁+Gal80@25* individuals were obtained by crossing females *w*; *sal^{EPV}>Gal4*; *tubP>GAL80^{ts}* to males *w¹¹¹⁸*; *PEWS::FLI1₁/CyO*, *S*, *Tb*. All samples were obtained from individuals aged between 4 and 6 days.

For phenotypic characterization of each EWS::FLI1 transgene's effect in larval salivary glands: females *w*; *sal^{EPV}>Gal4*; 20×(GGAA) μ Sat>YFP/ *TM6B* were crossed to either males *yw*,

UAS>EWS::FLI1_{1FS} /CyO, to males; *UAS>BC-EWS::FLI1₁*, or to males *w*; *PEWS::FLI1₁/CyO,S,Tb*, to obtain *sal^{EPV}>Gal4/UAS>EWS::FLI1_{1FS}; 20×(GGAA)μSat>YFP/ +*, *sal^{EPV}>Gal4/UAS>BC-EWS::FLI1₁; 20×(GGAA)μSat>YFP/ +*, and *sal^{EPV}>Gal4/ PEWS::FLI1₁; 20×(GGAA)μSat>YFP/ +*, respectively. For *PEWS::FLI1₁+Gal80@25*, females *w*; *sal^{EPV}>Gal4; P[tubP>GAL80^{ts}]* were crossed to males *PEWS::FLI1₁/CyO; 20×(GGAA)μSat>YFP/ TM6B* to obtain *sal^{EPV}>Gal4/P[UAS>EWS::FLI1₁]; 20×(GGAA)μSat>YFP/ P[tubP>GAL80^{ts}]*. All individuals analysed were between 4 and 6 days old.

For RNAi screen, males of each of the transgenic *yv*; *UAS>RNAi* strains tested were crossed with the following females *w*; *sal^{EPV}>Gal4/UAS>BC-EWS::FLI1₁, 20×(GGAA)μSat>YFP* or *w*; *sal^{EPV}>Gal4/ UAS>EWS::FLI1_{1FS}, 20×(GGAA)μSat>YFP* for the evaluation of salivary gland phenotypes. Similarly, these females were also crossed with males *alph^{XS-88}*, *alph^{S-331}*, and *alph^{S-355}/TM6B* to assess effect of abrogated *alph* phosphatase activity on transcription from *20×(GGAA)μSat>YFP*. RNAi males were also crossed with females *w*; *sev>Gal4/ UAS>EWS::FLI1_{1FS}*, *w*; *sev>Gal4/ UAS>PEWS::FLI1₁*, or *w*; *longGMR>Gal4, UAS>EWS::FLI1_{1FS}* for eye phenotypes. Owing to the X chromosome *vermillion* (*v*), female progeny from these crosses have darker eyes than males. The effect of each RNAi was compared to a control *UAS>RNAi* targeting a germline gene, *cup*, that is not expressed in salivary glands (Krause et al., 2022), nor upregulated by the *EWS::FLI1* transgenes (Molnar et al., 2022; Mahnoor et al., 2024). Salivary gland phenotypes were evaluated in individuals aged between 4 and 6 days. Adult eyes were analysed in individuals 11-13 days of age (within 1-2 days of eclosion).

3.3 Immunohistochemistry and antibodies

Immunostaining of salivary glands and wing discs was performed as follows: salivary glands and wing discs were dissected in phosphate-buffered saline (PBS), fixed for 30 min in 4% formaldehyde with 0.3% TritonX-100, washed 3 times in PBS-0.3% Triton X-100 (PBST) for 10 minutes per wash, then blocked for 90 min in PBST with 10% fetal calf serum (PBSTF). DNA was stained with DAPI. Salivary glands and wing discs were mounted in Vectashield (Molecular Probes). Primary and secondary antibodies were incubated in PBST overnight at 4°C. Primary antibodies used in this study are with rabbit anti-FLI primary antibody (1:200, ab133485, Abcam) and mouse anti-EWS (1:500, sc-48404, Santa Cruz Biotechnology). We used Alexa Fluor secondary antibodies (1:1000, Life Technologies).

3.4 Microscopy

Immunostaining images were acquired with a Leica TCS SP8 scan unit coupled to a microscope and managed by Leica Application Suite X (LAS X) software. The objective used was HC PL APO CS2 40x/1.30 OIL and images were acquired at zoom 1. Fluorophores 405 and 488 were excited with lasers Diode and Argon respectively. Image processing was carried out with Fiji. All shown immunofluorescence images correspond to a single Z.

To generate high-resolution images of adult *Drosophila* eyes, 5–6 photographs were captured for each adult at incremental focal depths using a Leica MZFLIII stereomicroscope equipped with a digital Zeiss AxioCam MRc camera. All images were acquired at zoom 6. These images were subsequently processed using Helicon Focus (v8.2.2) software to produce a single extended-depth-of-field image via Method C (pyramid) focus stacking.

3.5 Quantification and Statistical Analysis

Quantification of fluorescence levels was carried out by processing images acquired with an SP8 Leica confocal image microscope in Fiji. The mean grey values for region of Interests (ROI) were calculated to measure fluorescence levels. ROI for nuclear EWS::FLI1 signal were generated using DAPI masks that delineated salivary gland nuclei. The same masks were used to generate separate ROIs that would only span the cytoplasm to quantify cytoplasmic EWS::FLI1 signal.

Quantification of salivary gland size was performed in Inkscape 1.2.2 using Bezier curves to manually outline individual salivary glands from distal end till the meeting point of the individual ducts at the proximal end and measuring the area of the resulting shape through the Visualise Path extension.

Both salivary gland size and YFP intensity are represented in boxplots

Statistical tests and graphical representation: *P* values for salivary gland size and fluorescence levels for comparisons among all EWS::FLI1₁ transgenes were calculated in R version 4.2.3. by Dunn's test with Holm's method for p-value adjustment after rejection of Kruskal Wallis one-way ANOVA and compact letter display (CLD) was used to represent *P* values on boxplots. *P* values for Western blot band intensities were calculated by Tukey's HSD after rejection of one-way ANOVA. *P* values for comparisons of fluorescence levels in RNAi screen to compare each RNAi to its batch-matched control were calculated using two sample Student's t-test.

3.6 Cloning and transgenesis

The *pUAS_t p-element EWS::FLI1₁ construct* was made by cloning the *EcoRI-XhoI* fragment from pUC-GW-Kan EWS::FLI1₁ TI that contains the full EWS::FLI1₁ ORF in the original pUAS_t vector (Brand and Perrimon, 1993).

The *pUAS_t-EWS::FLI1₁+F_{Stop} construct* was made by introducing a *BbsI* site into the pUC-GW-Kan EWS::FLI1₁ TI. This was achieved by replacing the *BsrGI-XhoI* fragment with a gBlock (IDT) containing the *BbsI* site at the terminus of the ORF. Subsequently, another gBlock (IDT) with the FS sequence was inserted in-frame with EWS::FLI1₁ into the *BbsI* and *XhoI* sites. The resulting fragment was subcloned into the *EcoRI* and *XhoI* sites of *pUAS_t-attB* (DGRC Stock 1419, (Bischof et al., 2007)).

The *pUAS_t-uORF-EWS::FLI1₁ construct* was made by PCR amplification of the mCherry ORF from the *pLT3-Dam* vector (Southall, 2013), and the EWS::FLI1₁ ORF from pUC-GW-Kan EWS::FLI1₁ TI. Subsequently, both fragments were fused and cloned by In-Fusion (Takara) into *EcoRI* and *XhoI* sites of the *pUAS_t-attB* vector.

All PCR amplifications were done using KOD polymerase (Merck). Cloning was done by using In-Fusion Snap Assembly (Takara). Primers were ordered to Sigma. DNA fragments were ordered to IDT.

Transgenic fly lines were generated by BestGene Inc. using the Bloomington stock 24482 (ϕ C31 integrase-mediated transgenesis), and by the IRB Barcelona Drosophila Injection Service using the *w¹¹¹⁸* line (P-element-mediated transgenesis).

All transgenic lines used for experiments were sequenced to confirm that they carry the correct transgene.

gBlock DNA fragments used in this thesis:

BbsI.FS.XhoI:

```
TCAGAAGAAGACCCTACTACGAGCTACCATGCCCACCAGCAGAAGGTCAACTTCGTGCC  
ACCACATCCGTCCTCCATGCCAGTGACGTCCAGCAGCTTCTTTGGCGCTGCTTCCCAGT  
ATTGGACGTCCCCGACCGGCGGCATCTACCCAAACCCAAACGTCCCACGCCATCCGAA  
CACCCACGTCCCATCCCATCTGGGCTCCTACTACTGACTCGAGTAAGCA
```

EWS::FLI1₁ BbsI-XhoI:

```
GTCCAGCATGTACAAGTACCCGTCCGACATTAGCTACATGCCGAGCTACCATGCCACCA  
GCAGAAGGTCAACTTCGTGCCACCACATCCGTCCTCCATGCCAGTGACGTCCAGCAGC  
TTCTTTGGCGCTGCTTCCCAGTATTGGACGTCCCCGACCGGCGGCATCTACCCAAACCC
```

AAACGTCCCACGCCATCCGAACACCCACGTCCCATCCCATCTGGGCTCCTACTACGTCT
TCCTCGAGTATTAG

3.7 Transcriptomics

3.7.1 Microarray processing

Sample collection: Dissected *Drosophila* salivary glands were collected in 45 µl of a lysis buffer containing 20 mM DTT, 10 mM Tris-HCl pH 7.4, 0.5% SDS, and 0.5µg/µl proteinase K, incubated at 65 °C for 15 minutes and immediately frozen until processing. RNA extraction and cDNA generation was done at the IRB Barcelona Functional Genomics Core Facility. Briefly, RNA was treated with DNase I and purified using magnetic beads (RNAClean XP, Beckman Coulter). RNA was quantitated with Qubit RNA HS Assay kit (Invitrogen), and RNA integrity was assessed with the Bioanalyzer 2100 RNA Pico assay (Agilent). 25 ng of RNA were reverse transcribed and amplified using the Whole Transcriptome Amplification method (WTA2, Sigma Aldrich) with 17 cycles of amplification. cDNA was further purified using a spin column (PureLink Quick PCR Purification Kit, Invitrogen) and quantified using a microvolume spectrophotometer (Nanodrop ND-1000, Thermo-Fisher Scientific).

Microarray processing: 8 µg of cDNA were fragmented and labelled according to the manufacturer instructions (GeneChip Mapping 250K Nsp Assay Kit, Affymetrix). Array hybridization was performed using the GeneChip Hybridization, Wash, and Stain Kit (Applied Biosystems). Briefly, libraries were denatured at 99°C for 2 min prior to incubation into the *Drosophila* Genome 2.0 arrays (Applied Biosystems). Libraries were hybridized on the arrays for 16 h at 45 °C for 60 rpm at GeneChip Hybridization Oven 645 (Affymetrix/ThermoFisher Scientific). Washing and Stain steps were performed using a GeneChip Fluidics Station 450 following the *Drosophila* Genome 2.0 protocol (Affymetrix/ThermoFisher Scientific). Finally, arrays were scanned with a GeneChip Scanner GCS3000 (Affymetrix/ThermoFisher Scientific).

Data analysis: The CEL files containing the microarray data were generated with the Command Console software (Affymetrix/ThermoFisher Scientific) and were used for probeset-based gene expression measurements using robust multichip average (RMA) normalization. Results were analyzed using the Transcriptome Analysis Console 4.0 (TAC) software. Genes with an absolute FC of >2 and an p-value of <0.05 were considered differentially expressed.

3.7.2 Gene Set Enrichment Analysis (GSEA)

The GSEA preRanked algorithm was used to compare the human EWS::FLI signatures from (Hancock and Lessnick, 2008; Kauer et al., 2009) to all genes in the *Drosophila* microarrays ranked by average log2FC. Genesets with an FDR q-value of <0.25 were accepted as a

significant enrichment. *Drosophila* orthologs of these human signatures were identified using the DIOPT ortholog mapping online resource (Hu et al., 2011).

3.7.3 Gene Ontology (GO) analysis

Functional annotation of Gene Ontology (GO) terms was performed using the online tool Database for Annotation, Visualization and Integrated Discovery (DAVID; <http://david.abcc.ncifcrf.gov/>) (Huang et al., 2009; Sherman et al., 2022). DAVID (version 2021) and GO terms for biological process (GOTERM_BIOLOGICAL_PROCESS_4), with a p-value of <0.05 were accepted as a significant enrichment of nervous system-related genes.

3.8 Quantitative real-time PCR

Dissected *Drosophila* larval salivary glands were collected in 45 µl of a lysis buffer containing 20 mM DTT, 10 mM Tris-HCl pH 7.4, 0.5% SDS, and 0.5µg/µl proteinase K, incubated at 65 °C for 15 minutes and immediately frozen until processing. RNA extraction and cDNA generation was done at the IRB Barcelona Functional Genomics Core Facility. Briefly, RNA was treated with DNase I and purified using magnetic beads (RNAClean XP, Beckman Coulter). RNA was quantitated with Qubit RNA HS Assay kit (Invitrogen), and integrity assessed with the Bioanalyzer 2100 RNA Nano assay (Agilent). 25 ng of RNA was reverse transcribed and amplified using the Whole Transcriptome Amplification method (WTA2, Sigma Aldrich) with 17 cycles of amplification. cDNA was further purified using a spin column (PureLink Quick PCR Purification Kit, Invitrogen) and quantified using a microvolume spectrophotometer (Nanodrop ND-1000, Thermo-Fisher Scientific). cDNA yield ranged from 18 to 44 µg. PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) was used for quantitative real-time PCR following manufacturer's instructions. The real time assays were conducted in a QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific) using SYBR Green as the detection system and ROX as reference dye. Two different pairs of primers were designed to amplify YFP cDNA. mRNA levels were assessed from three independent RNA extractions and three technical replicates were performed on each sample. RNA levels were normalized to *RpL32* housekeeping gene. The primers employed are shown in Table 3.1.

3.9 Western blot

Dissected *Drosophila* larval salivary glands or wing discs were collected in 50µL of cold PBS. PBS were removed after dissection, and the samples were frozen at -20°C. For detection of YFP^{+FStail} and YFP, 8 pairs of salivary glands or 10 wing discs were used per sample from individuals with genotypes *nub>Gal4/UAS-YFP^{+FStail}* and *nub>Gal4/UAS-YFP*, respectively. For detection of EWS::FLI1₁, salivary gland pairs were collected from individuals of the

genotype $sa^{EPV}>Gal4/P[UAS>EWS::FLI1_1]; 20\times(GGAA)\mu Sat>YFP/ +$,
 $sa^{EPV}>Gal4/P[UAS>EWS::FLI1_1]; 20\times(GGAA)\mu Sat>YFP/ P[tubP>GAL80^{ts}]$,
 $sa^{EPV}>Gal4/UAS>EWS::FLI1_{1FS}; 20\times(GGAA)\mu Sat>YFP/ +$, $sa^{EPV}>Gal4/UAS>BC EWS::FLI1_1$;
 $20\times(GGAA)\mu Sat>YFP/ +$, or $sa^{EPV}>Gal4/+; 20\times(GGAA)\mu Sat>YFP/ +$ and loaded protein
 quantity was adjusted to be equal to that from 4 $sa^{EPV}>Gal4/+$ salivary gland pairs.

Samples were resuspended in 30 μ L of 2x SDS NuPAGE® LDS sample buffer with 100mM DTT, lysed via manual grinding and sonication with Bioruptor. Boiled lysates were resolved by 4-12% SDS-PAGE (Bolt™ 4-12% Bis-Tris, 1.0 mm, REF: NW04120BOX; Invitrogen), and proteins were transferred to nitrocellulose membranes (0.2 μ m Pore Size, Invitrogen). YFP^{+FStail} and YFP proteins were detected by incubating with anti-GFP primary antibodies overnight at 4°C (mouse anti-GFP (1:250, REF: 11814460001; Roche, Basel, Switzerland). α -Tubulin (Clone Dm1A) protein was detected by incubating with rat anti-TubDM antibody (1:500, REF: T6199; Sigma Aldrich) and was used as the loading control. EWS::FLI1₁ proteins were detected by incubating with rabbit anti-FLI1 primary antibody (1:200, ab133485, Abcam) and mouse anti-EWS (1:500, sc-48404, Santa Cruz Biotechnology) overnight at 4°C. Total protein was detected with REVERT™ Total Protein Stain Kit (LI-COR) and was used as a loading control.

Immunoblots were developed using Alexa Fluor®790 and Alexa Fluor®680 secondary antibodies (1:2500, Jackson ImmunoResearch) or IRDye 800CW (1:2500, LI-COR) with the Odyssey Infrared Imaging System (LI-COR).

Table 3.1: Primers used in this paper.

Name	Sequence
Cloning	
EI-Cherry-FW	AGGGAATTGGGAATTCATGGCAACTAGC
Cherry-RV	GGCCATGTTATTATGCGGTACCAGAACCTTTG
EWS::FLI-FW	TAATAACATGGCCTCCACCGATTATTCCACGTAC
EWS::FLI-XhoI-RV	TAGAGGTACCCTCGAGTCAGTAGTAGGAGCCCAG
qPCR	
EYFP-1-FW	AAGCAGAAGAACGGCATCAA
EYFP-1-RV	GGGGGTGTTCTGCTGGTAGT
EYFP-2-FW	ATGGTGAGCAAGGGCGAG
EYFP-2-RV	TGAACTTGTGGCCGTTTACGT
RpL32-b-FW	TGTGAATTTTCCTTGTCGCGT
RpL32-b-RV	TGGATAGAGATACATTACGCATA
Sequencing	
Hsp-Fw	GCAACTACTGAAATCTGCCAAG
EWS500-FW	ACCCGATGCAACCAAGTGA
EWS1100-RV	CAATGCCATGGAAGTGG
SV40	GGCATTCCACCACTGCTCCC

4. RESULTS

4.1 The C-terminal peptide of EWS::FLI1_{1FS} results in decreased protein levels

As described earlier, functional assays showed that the lower toxicity of EWS::FLI1_{1FS} was due to an unknown neomorphic property of the new FStail rather than to the loss of the 69 amino acid C-terminal peptide of EWS::FLI1₁ (Molnar et al., 2022). We did not know the nature of such a neomorphic property, but a simple hypothesis was that it could be owing to reduced protein expression and/or stability.

To test this hypothesis, we quantified the effect of FStail tagging on YFP. To this end, we generated transgenic animals carrying UAS>YFP and UAS> YFP_{+FStail}, a fusion of the FStail to the C-terminal end of YFP. By driving UAS>YFP and UAS> YFP_{+FStail} expression from the nub>Gal4 driver, we found that both YFP_{+FStail} green fluorescence intensity, as measured by confocal microscopy, as well as YFP_{+FStail} protein levels, as quantified by western blot, are extremely reduced compared with those of unmodified YFP in salivary glands and wing imaginal discs (Figure 4.1-A, B).

Because the YFP and YFP_{+FStail} transgenes were inserted at the same genomic site (attP40 - Markstein et al., 2008), by ϕ C31 integrase-mediated transgenesis (Bateman et al., 2006) and were both expressed by nub>Gal4, transcription levels are expected to be comparable. That is unless the FStail-coding sequence affects transcription, which could indeed explain the differences observed at the protein level. To test this hypothesis, we quantified YFP and YFP_{+FStail} transcripts by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) in salivary glands and wing imaginal discs. We found no significant differences in the level of mRNA from each variant in either tissue (Figure 4.1-C). Altogether, these results show that the FStail has no significant effect on transcription but affects protein expression and/or stability such that it results in a strong reduction in the concentration of a protein carrying this C-terminal tag. These results substantiate the hypothesis that EWS::FLI1_{1FS}'s reduced toxicity is owing to a significant reduction in protein concentration caused by the FStail.

To further substantiate this conclusion, and to get a direct estimate of the effect of the FStail in EWS::FLI1₁, we generated the EWS::FLI1_{1+FStail} transgene, an FStail-tagged version of full-length EWS::FLI1₁. We found that in stark contrast with unmodified EWS::FLI1₁, EWS::FLI1_{1+FStail} transgenes were obtained at the standard rate of more than five per 200 injected embryos (Figure 4.2, page 44), thus confirming that the sole addition of the FStail can circumvent the toxicity issue of unmodified EWS::FLI1₁.

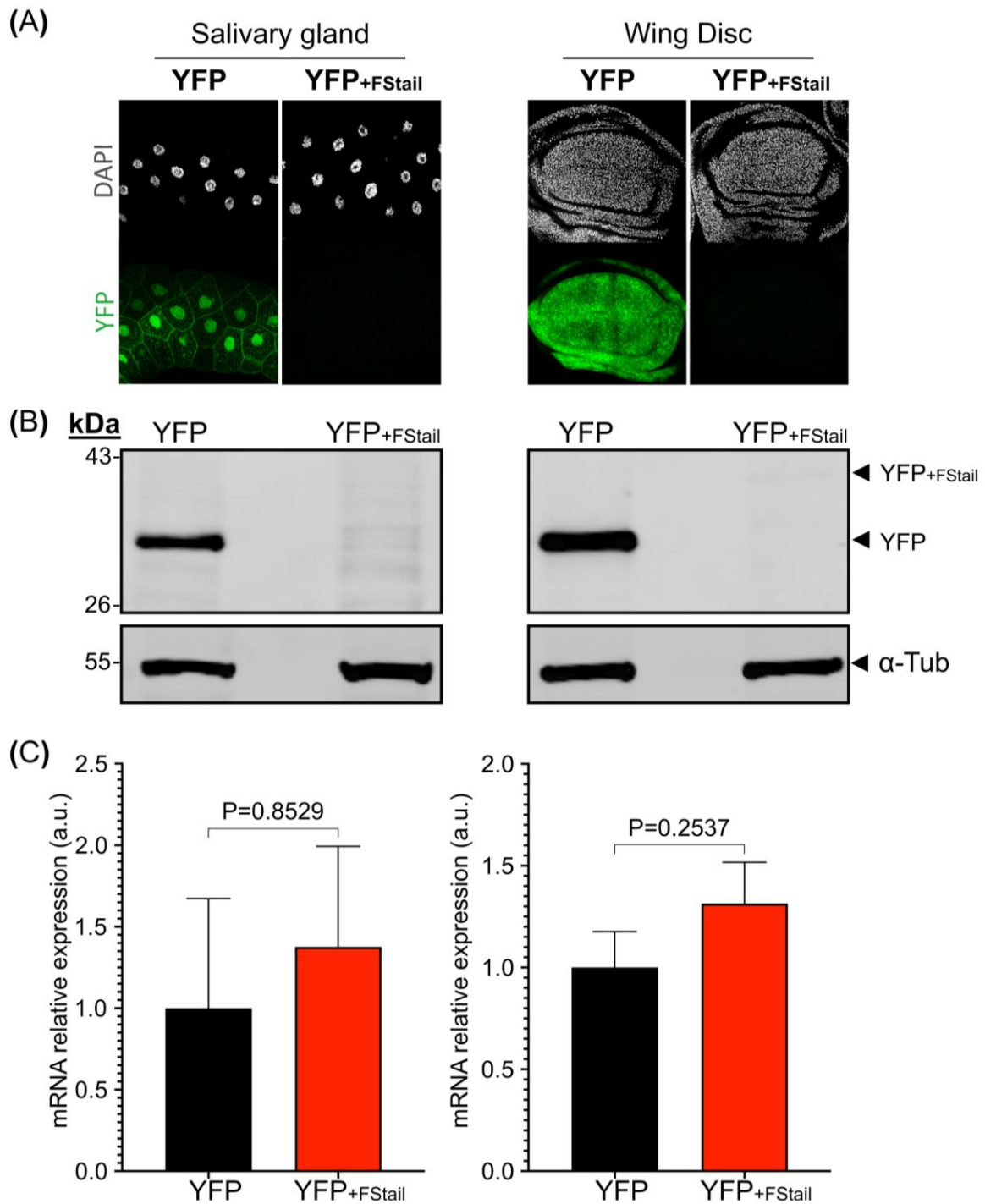


Figure 4.1: The FStail reduces protein levels without affecting transcription. YFP protein levels observed by fluorescence (A) and western blot (B), and YFP mRNA quantified by RT-qPCR (C) in salivary glands (left) and wing imaginal discs (right) from *nub>Gal4/UAS>YFP*; (YFP) and *nub>Gal4/UAS>YFP_{FStail}* (YFP_{FStail}) larvae. Scale bar = 50 μ m

4.2 Generating *Drosophila* strains carrying unmodified, full-length EWS::FLI1₁ transgenes.

Having shown that EWS::FLI1_{1FS}'s reduced toxicity is due to a significant reduction in protein concentration caused by the FStail, two approaches were employed to test if the same effect could be achieved with transgenes designed to downregulate translation or transcription, so that full length, untagged EWS::FLI1₁ could be expressed.

The first approach was to downregulate translation by placing an EWS::FLI1₁ coding sequence lacking its own internal ribosome entry site (IRES) as the secondary open reading frame (ORF) in a bicistronic transcript encoding mCherry as primary ORF. Under these conditions, translation of the secondary ORF is so severely compromised that it has been estimated to be essentially null in the absence of Gal4, hence avoiding potential toxicity issues caused by leaky transcription during transgenesis (Child et al., 1999; Luukkonen et al., 1995; Southall et al., 2013; Van Blokland et al., 2011).

The second approach was based on P-element-mediated transgenesis (Rubin and Spradling, 1982; Spradling and Rubin, 1982). Unlike precise targeting to predetermined genomic sites by ϕ C31 integrase (Bateman et al., 2006), P-element transgenes insert nearly randomly in the genome and are therefore subjected to unpredictable position effects that may significantly affect transcription. This approach was based on the reasoning that this inherent drawback could be to our advantage because it may be used to select for insertions in genomic regions in which leaky *UAS>EWS::FLI1₁* expression is downregulated below toxic levels.

Both approaches worked well to generate *Drosophila* lines carrying unmodified, full-length EWS::FLI1₁ transgenes. ϕ C31 integrase-mediated transgenic lines carrying the bicistronic construct bcEWS::FLI1₁ were obtained at the standard rate of more than 5 per 200 injected embryos, thus showing that this strategy abrogates EWS::FLI1₁ embryo toxicity. P-element-mediated transgenesis was less efficient as compared to the control, producing a single pEWS::FLI transgenic line out of 180 injected embryos (<1%), an order of magnitude below the normal rate of P-element-mediated transgenesis (>10%) (Fig.4-2). Such a low rate is not surprising, however, since only the small fraction of P-element insertions subjected to position effects strong enough to abrogate toxic, leaky EWS::FLI1₁ expression can be expected to generate viable transgenic animals. DNA sequencing of the new transgenic lines obtained from both approaches confirmed the presence of non-mutated, full length EWS::FLI1₁, as intended, thus validating the two strategies.

			E/T
ϕ C31 integrase	EWS::FLI1 ₁	UAS  EWS::FLI1 ₁	200 / 0
	EWS::FLI1 _{1FS}	UAS  EWS::FLI1 ₁ shift	200 / >5
	EWS::FLI1 _{1+FS} tail	UAS  EWS::FLI1 ₁ shift	200 / >5
	bcEWS::FLI1 ₁	UAS  mCherry  EWS::FLI1 ₁	200 / >5
P-element	empty vector	UAS	130 / 20
	pEWS::FLI1 ₁	UAS  EWS::FLI1 ₁	180 / 1

Figure 4.2: Drosophila transgenic lines expressing full-length human EWS::FLI1 type 1 protein (EWS::FLI1₁). The number of injected embryos ‘E’ and transgenic animals recovered ‘T’ in one round of injection. Transgenesis experiments with full-length EWS::FLI1₁, the published frameshift variant (UAS>EWS::FLI1_{1FS}), full-length EWS::FLI1 fused with the C-terminal peptide created by the EWS::FLI1_{1FS} frameshift (UAS>EWS::FLI1_{1+FS}tail), and a bicistronic construct carrying mCherry followed by full-length EWS::FLI1 (UAS>bcEWS::FLI1₁) were carried out using ϕ C31 integrase. The P-element expressing full-length EWS::FLI1 (UAS>pEWS::FLI1₁) was obtained by P-element-mediated transgenesis.

4.3 The collection of full-length EWS::FLI transgenes causes a range of developmental phenotypes.

As a first step to characterise the new transgenic lines, I investigated their effect on development upon expression from a variety of Gal4 drivers (Fig. 4-3-A). I found that, as far as lethality phase, wing disc tumours, and cuticular phenotypes in adult flies are concerned, the new variants cause a wide range of expressivity levels.

Top of the range is pEWS::FLI1₁ that is lethal when expressed from any of the selected Gal4 drivers, including *longGMR>Gal4* and *sal^{EPV}>Gal4* with which all of the other transgenes are viable. At the bottom of the range is the bcEWS::FLI1₁ variant that yields pupal lethality with *da>Gal4*, *Act5C>Gal4* and *48Y>Gal4*, and adult viability with *Mef2>Gal4*, and *twi>Gal4*, while the same Gal4 drivers result in embryonic lethality with all other EWS::FLI1₁ versions. Moreover, surviving adults carrying bcEWS::FLI1₁ and *Mef2>Gal4* or *twi>Gal4* present no noticeable cuticular or lifespan phenotypes. EWS::FLI1_{1FS} and EWS::FLI1_{1+FS}tail have an intermediate effect, stronger than bcEWS::FLI1₁ and weaker than pEWS::FLI1₁ (Fig. 4-3-A). EWS::FLI1_{1FS} and EWS::FLI1_{1+FS}tail are largely indistinguishable except for the effect on eye roughness and size when driven from *sev>Gal4* that is stronger in the case of EWS::FLI1_{1+FS}tail (Figure 4.3-B, row 1).

(A)

GAL4	Expression	bcEWS::FLI1 ₁	EWS::FLI1 _{1FS}	EWS::FLI1 _{1+FS} tail	pEWS::FLI1 ₁
<i>da>GAL4</i>	Ubiquitous	Pupal Lethal	Embryonic Lethal	Embryonic Lethal	Embryonic Lethal
<i>act5C>Gal4</i>	Ubiquitous	Pupal Lethal	Embryonic Lethal	Embryonic Lethal	Embryonic Lethal
<i>48Y>GAL4</i>	Endoderm	Pupal Lethal	Embryonic Lethal	Embryonic Lethal	Embryonic Lethal
<i>Mef2>GAL4</i>	Mesoderm	Viable	Embryonic Lethal	Embryonic Lethal	Embryonic Lethal
<i>twi>GAL4</i>	Mesoderm	Viable	Embryonic Lethal	Embryonic Lethal	Embryonic Lethal
<i>nub>GAL4</i>	Wing discs / CNS	Viable very short lived	Early Pupal Lethal Tumorous wing disc	Early Pupal Lethal Tumorous wing disc	Embryonic Lethal
<i>ey>GAL4</i>	Eye discs	Viable	Pupal Lethal Headless/eyeless escapers	Pupal Lethal Headless/eyeless escapers	Larval/Pupal Lethal Headless/eyeless escapers
<i>longGMR>GAL4</i>	Eye discs	Viable	Viable Rough and pigmentless eye	Viable Rough and pigmentless eye	Larval Lethal
<i>sal^{EPV}>GAL4</i>	Wing discs	Viable	Viable Abnormal wing	Viable Abnormal wing	Early Pupal Lethal Tumorous wing disc
<i>sev>GAL4</i>	Eye discs	Viable %	Viable Rough eye	Viable Rough eye	Viable Extremely rough eye

(B)

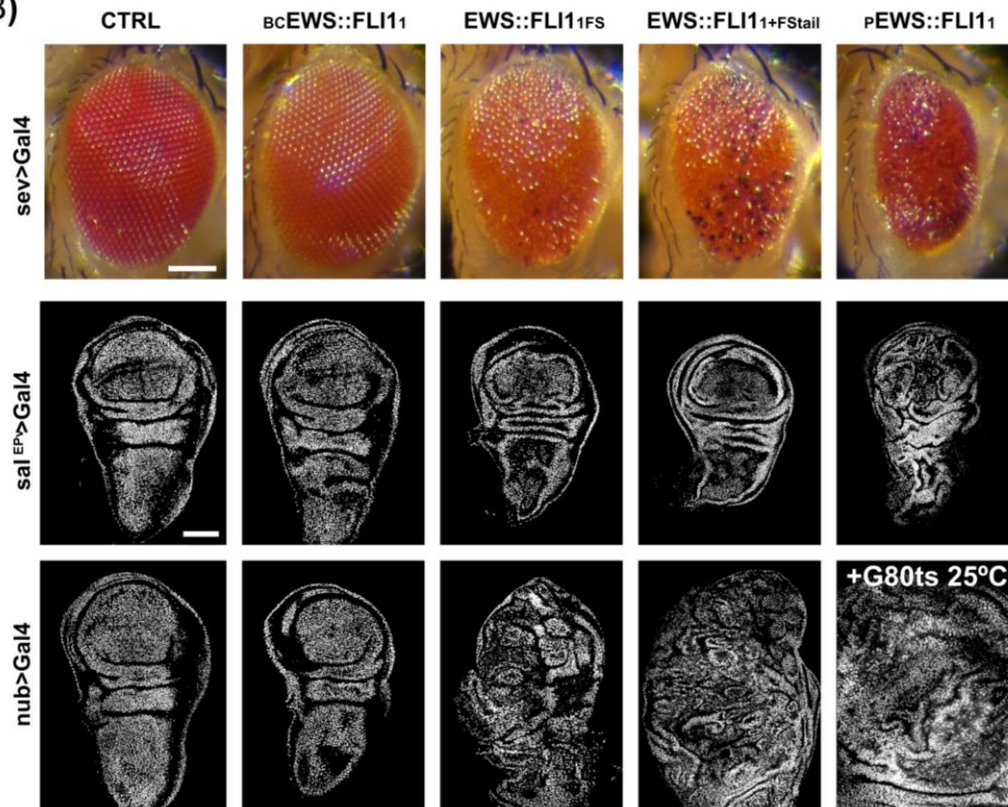


Figure 4.3: Drosophila transgenic lines expressing full-length human EWS::FLI1 generate a wide range of developmental phenotypes. (A) Summary of developmental phenotypes observed in each EWS::FLI1 transgenic line, colour coded with red for embryonic lethal, yellow for larval or pupal lethal and cyan for adult viable. (B) Adult eye and third instar wing imaginal discs from individuals expressing the indicated versions of EWS::FLI1 in ommatidia (*sev>Gal4*), central region of the wing pouch (*sal^{EPV}>Gal4*) or wing pouch and hinge region (*nub>Gal4*). The *nub>Gal4/UAS>EWS::FLI1_{1FS}* and *nub>Gal4/UAS>EWS::FLI1_{1+FS}tail* wing disc tumours shown are extreme examples to demonstrate the extent of transformation that can be induced by these transgenes; milder phenotypes have been observed. Most *nub>Gal4/UAS>pEWS::FLI1₁*; *P[tubP>GAL80ts]/+* individuals reared at 25°C die at early stages of development; only two third instar larval escapers that we have observed presented large wing disc tumours (+Gal80ts 25°C). Scale bars: 0.1 mm or 100 µm for adult eyes and wing discs, respectively.

The stronger effect of pEWS::FLI1_1 compared to that of $\text{EWS::FLI1}_{1\text{FS}}$ and $\text{EWS::FLI1}_{1+\text{FS}\text{tail}}$ is clearly reflected in their tumorigenic potential in wing imaginal discs. As published (Molnar et al., 2022), $\text{EWS::FLI1}_{1\text{FS}}$ causes wing imaginal discs tumours when driven by $nub>\text{Gal4}$, but not by $sa^{EPV}>\text{Gal4}$, which is expressed at a later stage in development and over a much smaller area in the wing disc. The same is true for $\text{EWS::FLI1}_{1+\text{FS}\text{tail}}$. In contrast, $sa^{EPV}>\text{Gal4}$ -driven expression is sufficient for pEWS::FLI1_1 to cause wing imaginal discs tumours (Figure 4.3-B, row 2), whereas its expression is lethal when driven by $nub>\text{Gal4}$.

To ameliorate the strong lethal effect of pEWS::FLI1_1 , we also generated individuals carrying this construct together with a second transgene, $P[tubP>\text{GAL80}^{ts}]$, which expresses the temperature sensitive allele of the yeast Gal80 protein from the *alphatubulin84B* promoter. Gal80 is an inhibitor of the transcriptional activation domain of Gal4. When expressed in *Drosophila*, Gal80^{ts} is fully functional at 17°C, but it is inactive at 29°C. By raising individuals carrying the pEWS::FLI1_1 and $P[tubP>\text{GAL80}^{ts}]$ transgenes at 25°C we hoped to achieve a partial inactivation of Gal80 function that would in turn partially inhibit Gal4, hence lowering EWS::FLI1_1 expression from the pEWS::FLI1_1 transgene. We refer to this combination as $\text{pEWS::FLI1}_1+\text{Gal80@25}$. The $nub>\text{Gal4}$ -driven expression of $\text{pEWS::FLI1}_1+\text{Gal80@25}$ is pupal lethal and yields tumours in the rare escapers that are even larger than that induced by $sa^{EPV}>\text{Gal4}$ -driven expression of pEWS::FLI1_1 (Figure 4.3-B, row 3)

4.4 Upregulation of transcription from GGAA μ Sat and tissue toxicity present a positive linear correlation with a wide range of EWS::FLI protein concentrations.

To further characterise the new full-length EWS::FLI1_1 lines, I quantified the efficiency of each transgene at activating transcription from GGAA μ Sats that, as said earlier, is one of the hallmarks of the EWS::FLI oncogene and is known to be critical for Ewing sarcoma tumorigenesis. $20\times(\text{GGAA})\mu\text{Sat}>\text{YFP}$, a transgene carrying 20 GGAA repeats upstream a heat-shock minimal promoter followed by the YFP coding sequence, was utilized to track GGAA μ Sat activation (Molnar et al., 2022). We chose to carry out these studies on third instar larval salivary glands because they are among the organs that are more resilient to the detrimental effects of EWS::FLI1_1 (Molnar et al., 2022). EWS::FLI1_1 transgenes were expressed from $sa^{EPV}>\text{Gal4}$ that, like other Gal4 drivers derived from the P{GawB} vector (Flybase FBtp0000352), contains a cryptic larval salivary-gland-specific enhancer element (Costantino et al., 2008).

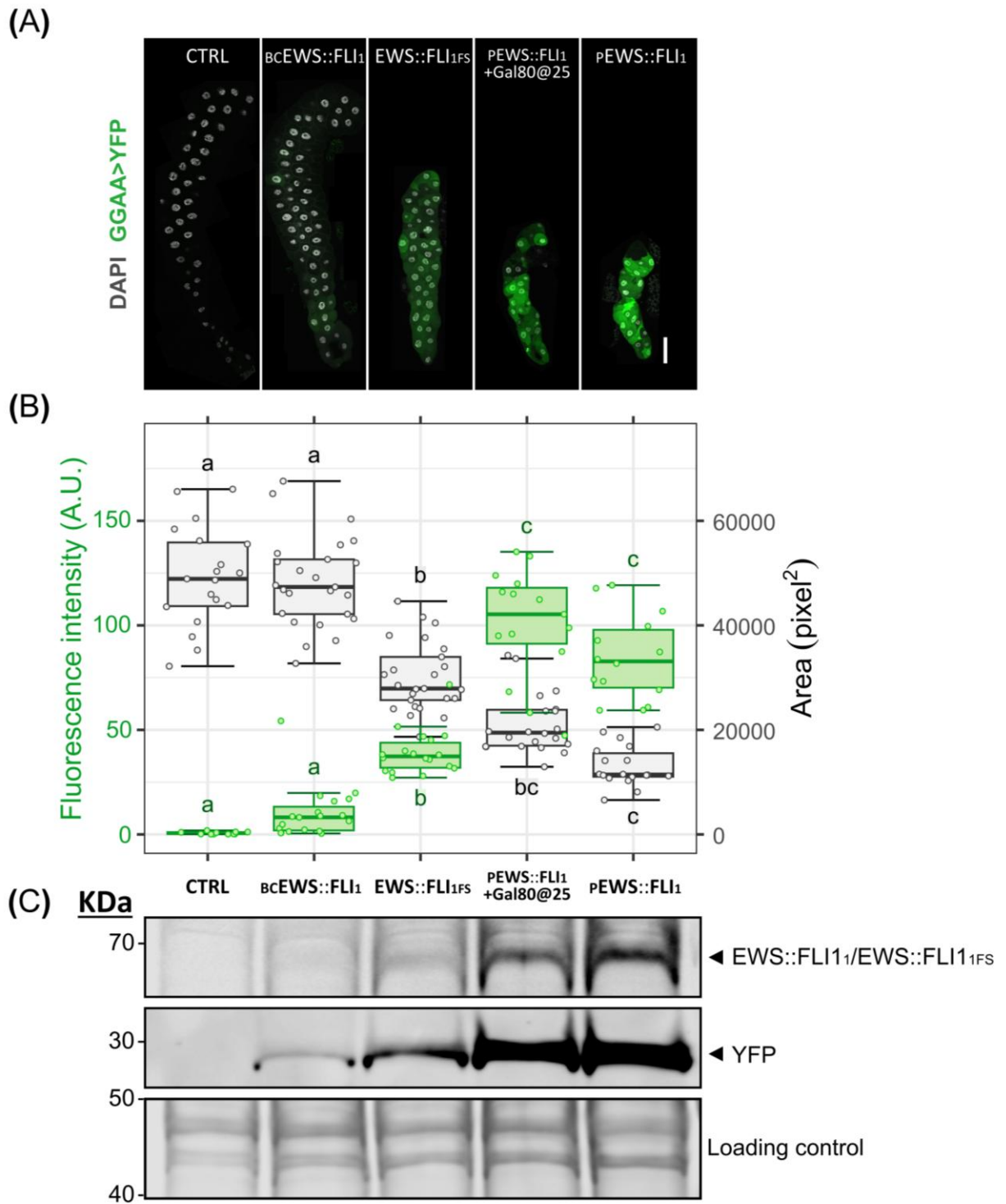


Figure 4.4 The expressivity of EWS::FLI1-induced phenotypes correlates with protein concentration. (A) Representative salivary glands showing YFP expression from 20x(GGAA)>YFP (green) and co-stained with DAPI (grey). Scale bar: 100 μ m. (B) Quantification of YFP fluorescence (green) and salivary gland size (grey) (arb. units). Letters indicate statistically significant differences between group means (Kruskal-Wallis and Dunn's test, $p < 0.05$). (C) Western blot showing protein levels of EWS::FLI1 and YFP. Letters indicate statistically significant differences between group means (ANOVA and Tukey's HSD, $p < 0.05$). Experimental conditions include: control ($sa^{EPV}>Gal4/+$), BC-EWS::FLI1 ($sa^{EPV}>Gal4/UAS>BC-EWS::FLI1$; 20x(GGAA)>YFP/+), EWS::FLI1_{FS} ($sa^{EPV}>Gal4/UAS>EWS::FLI1_{FS}$; 20x(GGAA)>YFP/+), PEWS::FLI1 ($sa^{EPV}>Gal4/UAS>EWS::FLI1$; 20x(GGAA)>YFP/+) and pEWS::FLI1+Gal80@25 ($sa^{EPV}>Gal4/UAS>EWS::FLI1$; $P[tubP>GAL80ts]/20x(GGAA)>YFP$).

Using confocal microscopy, I found that the extent of YFP upregulation induced by the collection of EWS::FLI1₁ transgenes from the $20\times(GGAA)\mu Sat>YFP$ construct spans over a wide range (Figure 4.4-A,B). At one extreme, $\rho EWS::FLI1_1$ and $\rho EWS::FLI1_1+Gal80@25$ cause the strongest effect (with *means* $\pm$ *SDs* of 85 ± 20 a.u., and 101 ± 26 a.u., respectively). At the other extreme, $\rho EWS::FLI1_1$ induces very low, but measurable levels (10 ± 12 a.u.). EWS::FLI1_{1FS} results in intermediate levels of GGAA μ Sat transcriptional activation (39 ± 11 a.u.): 386% (*P-val* = 4.4E-2) and 46% (*P-val* = 3.6E-2) of that caused by $\rho EWS::FLI1_1$ and $\rho EWS::FLI1_1$, respectively. These results show that the extent of YFP activation induced by each transgene can be ordered into a range as follows: control < $\rho EWS::FLI1_1$ << EWS::FLI1_{1FS} << $\rho EWS::FLI1_1 + Gal80@25 \approx \rho EWS::FLI1_1$.

By quantifying salivary gland sizes, measured as the area of the largest confocal z-section, as a proxy for tissue toxicity (Figure 4.4-A,B; Appendix A) I found that salivary gland size is indistinguishable between wild-type and $\rho EWS::FLI1_1$ larvae ($49k \pm 10k$ px² and $48k \pm 9k$ px², respectively; *P-val* = 0.9), but is severely compromised by $\rho EWS::FLI1_1$, alone or in combination with $P[tubP>GAL80^{ts}]$ ($13k \pm 4k$ px² and $22k \pm 7k$ px², respectively). Differences between $\rho EWS::FLI1_1$ and $\rho EWS::FLI1_1+Gal80@25$ are not significant, although $\rho EWS::FLI1_1+Gal80@25$ yields a notable number of salivary glands with an intermediary size between that of $\rho EWS::FLI1_1$ and EWS::FLI1_{1FS}. As before, EWS::FLI1_{1FS} results in intermediate levels of salivary gland sizes ($30k \pm 7k$ px²) that are about 62% (*P-val* = 5.6E-4) and 230% (*P-val* = 3.7E-4) the size of those from $\rho EWS::FLI1_1$ and $\rho EWS::FLI1_1$ individuals, respectively. The absence of detrimental effects of $\rho EWS::FLI1_1$ on salivary glands is interesting considering that it is sufficient for driving transcription from the $20\times(GGAA)\mu Sat>YFP$. These results show that, altogether, the transgenes induce toxicity as follows: control $\approx \rho EWS::FLI1_1$ << EWS::FLI1_{1FS} < $\rho EWS::FLI1_1 + Gal80@25 \leq \rho EWS::FLI1_1$.

I then carried out Western blots to quantify the level EWS::FLI1₁ protein expressed through each of the EWS::FLI1₁ transgenes (Fig. 4-4-C). As compared to EWS::FLI1_{1FS}, the levels of EWS::FLI1₁ protein are about 1.5 and 2.0-times higher in $\rho EWS::FLI1_1+Gal80@25$, and $\rho EWS::FLI1_1$, respectively, thereby validating the approach of raising $P[tubP>GAL80^{ts}]$ -carrying individuals at 25°C to tune down EWS::FLI1₁ expression from $\rho EWS::FLI1_1$. In contrast, EWS::FLI1₁ is hardly detectable in $\rho EWS::FLI1_1$ individuals. Thus, we found that EWS::FLI levels linearly increase along the following range: control < $\rho EWS::FLI1_1$ << EWS::FLI1_{1FS} << $\rho EWS::FLI1_1+Gal80@25 < \rho EWS::FLI1_1$.

Using the same Western blots, I re-assessed the levels of YFP expression from the $20\times(GGAA)\mu Sat>YFP$ brought about by each of the EWS::FLI1₁ transgenes and found them to be largely consistent with those observed by confocal microscopy. This includes the fact that

despite approximately 40% higher EWS::FLI1₁ levels in *PEWS::FLI1₁* compared to *PEWS::FLI1₁+Gal80@25* individuals, YFP expression levels are similar, hence suggesting that, as far as GGAA μ Sat transcriptional activation is concerned, EWS::FLI1₁ levels are near saturation in *PEWS::FLI1₁+Gal80@25* individuals.

Altogether, these results demonstrate a tight correlation of EWS::FLI protein levels to both their detrimental effect on salivary gland development and their effect on upregulating transcription from (GGAA) μ Sats.

4.5 GGAA μ Sat-independent transcriptome dysregulation is not linearly correlated with EWS::FLI protein levels.

In spite of its frame-shifted C-terminal tail, EWS::FLI1_{1FS} expression recapitulates in *Drosophila* key oncogenic functions of EWS::FLI1₁ like transcriptional dysregulation of hundreds of genes including a fraction of the orthologs of known EWS::FLI's targets in human cells (Molnar et al., 2022). The question, however, remains open as to whether unmodified full-length EWS::FLI1₁ protein could bring about a GGAA μ Sat-independent transcriptomic signature that is closer to that of Ewing sarcoma tumours than the EWS::FLI1_{1FS}'s signature, and whether this dysregulation would correspond linearly to EWS::FLI1₁ protein levels similarly to GGAA μ Sat-dependent dysregulation.

To address these questions, we used Affymetrix microarrays to perform a genome-wide transcriptomic analysis of salivary glands carrying the *bcEWS::FLI1₁*, and *PEWS::FLI1₁+Gal80@25* transgenes that express very different levels of full-length EWS::FLI1₁ (Appendix B). EWS::FLI1_{1FS}, salivary glands were also included as reference. For each condition, we determined the total number of dysregulated transcripts, quantified the enrichment of two published Ewing sarcoma signatures, and assessed the upregulation of neural genes, hallmarks of EWS::FLI1 activity.

We found that although the genes affected in *PEWS::FLI1₁+Gal80@25* (*n*= 879) and EWS::FLI1_{1FS} (*n*= 849) are greater in number and in their overall fold changes (with lower *p*-values for the latter) than in *bcEWS::FLI1₁* (*n*= 561), all three conditions cause a strong dysregulation of transcription (Figure 4.5-A) and the resulting transcriptomes are all highly significantly enriched for the published EWS::FLI1_{1FS} *Drosophila* signature (Molnar et al., 2022) (Figure 4.5-B). Moreover, with regards to upregulated genes, approximately one-third of the 404 genes of the Hancock and Lessnick signature (Hancock and Lessnick, 2008) are significantly enriched at the top of all three GSEA-ranked *Drosophila* datasets: EWS::FLI1_{1FS} (*n*= 115; *FDR q-val* = 0,032), *bcEWS::FLI1₁* (*n*= 117; *FDR q-val* = 0.004) and *PEWS::FLI1₁+Gal80@25* (*n*= 125; *FDR q-val* = 0.030) (Figure 4.5-C). Similar results were

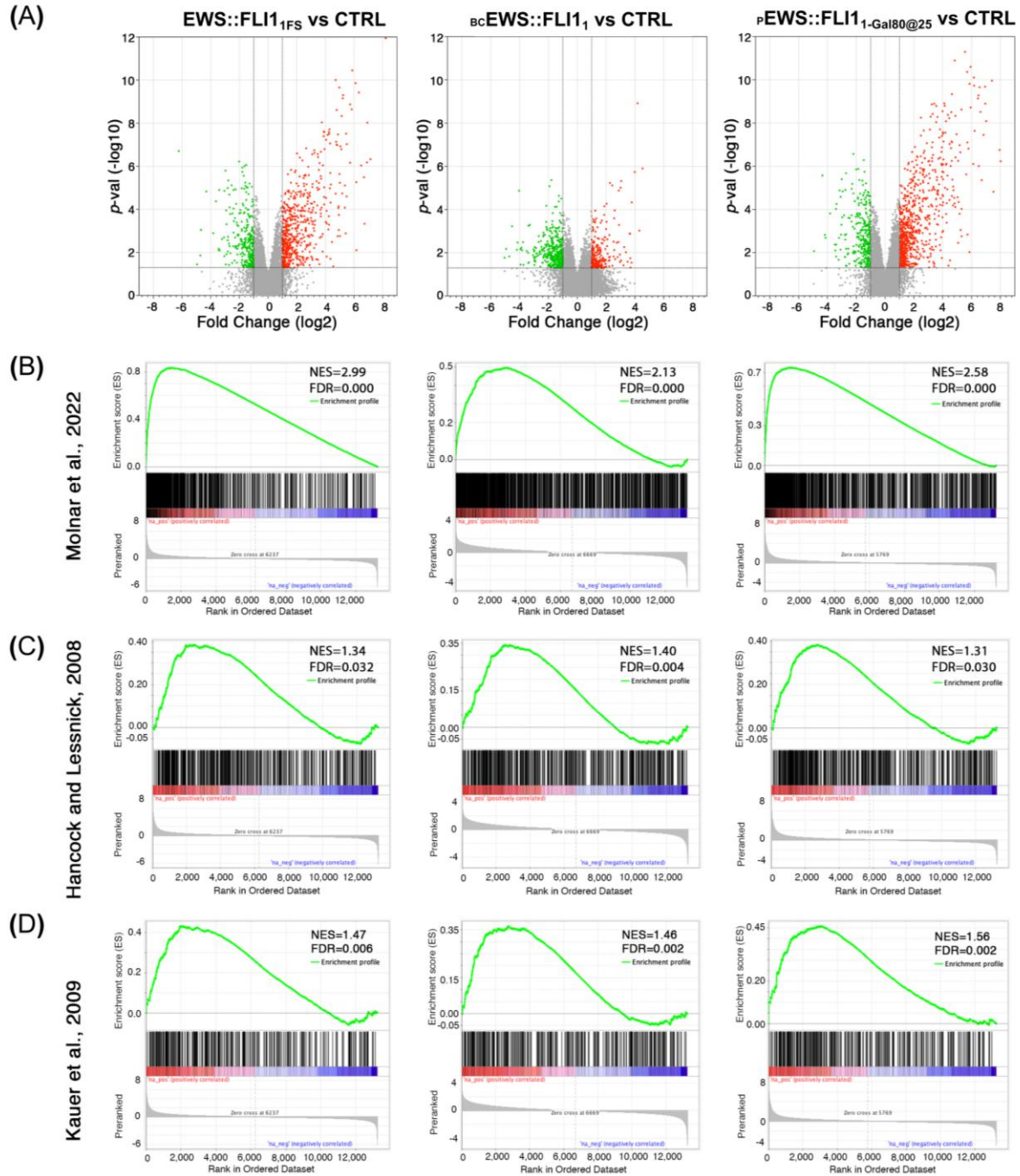


Figure 4.5: GGAμSat-independent transcriptome dysregulation is not linearly correlated with EWS::FLI1 protein levels. (A) Volcano plots showing changes in gene expression levels in EWS::FLI1_{1FS} (*sal^{EPV}>Gal4/UAS>EWS::FLI1_{1FS}*), BCEWS::FLI1₁ (*sal^{EPV}>Gal4/UAS> BCEWS::FLI1₁*), and PEWS::FLI1₁+Gal80@25 (*sal^{EPV}>Gal4/UAS> EWS::FLI1₁; P[*tubP* GAL80_{ts}]/+*) compared with control (*sal^{EPV}>Gal4/+*) samples. Red and green dots represent genes that are significantly ($p < 0.05$) up ($FC > 2$) and downregulated ($FC < -2$) genes, respectively. (B-D) Gene set enrichment analysis showing the *Drosophila* Molnar et al., 2022 (B) and the human (Hancock and Lessnick, 2008) (C) and (Kauer et al., 2009) (D) EWS signatures to be significantly enriched ($FDR < 0.25$) in the ranked datasets of EWS::FLI1_{1FS}, BCEWS::FLI1₁ and PEWS::FLI1₁+Gal80@25 compared with control.

obtained with the 277 genes of the Kauer et al. signature (Kauer et al., 2009): EWS::FLI1_{1FS} (n= 74; *FDR q-val* = 0,006), bcEWS::FLI1₁ (n= 82; *FDR q-val* = 0.002) and pEWS::FLI1₁+Gal80@25 (n= 101; *FDR q-val*= 0.02). However, differences between bcEWS::FLI1₁ and the other conditions became apparent with regards to the upregulation of genes associated with neural development, a trait of EWS::FLI1₁ activity in human cells that is also observed in *Drosophila* salivary glands expressing EWS::FLI1_{1FS} (Molnar et al., 2022). Gene Ontology (GO) biological process “nervous system” is well represented in both the pEWS::FLI1₁+Gal80@25 and the EWS::FLI1_{1FS} transcriptomes (n=121, *P-val*=6.8E-18; and n=69, *P-val*=8.2E-5) but is not significantly enriched in the bcEWS::FLI1₁ transcriptome with only 13 genes from the same GO being upregulated.

These are remarkable results as far as bcEWS::FLI1₁ is concerned, taking into account the extremely low levels of EWS::FLI1₁ protein and the lack of toxicity caused by this construct that nonetheless is nearly as good as EWS::FLI1_{1FS} at driving GGAA μ Sat-independent transcriptome dysregulation.

Altogether, these results show that, (1) the dysregulation induced by full length EWS::FLI1₁ expression is not much closer to the human EwS signature than that induced by EWS::FLI1_{1FS}, and (2) unlike GGAA μ Sat-dependent transcriptional upregulation, GGAA μ Sat-independent transcriptome dysregulation, including ectopic expression of orthologs of human Ewing sarcoma signature genes, is not linearly correlated with EWS::FLI1₁ protein levels.

The results presented in sections 4.1-4.5 have been published in Open Biology (Mahnoor et al., 2024) (see Appendix E).

4.6 *In-vivo* RNAi screen identifies suppressors of EWS::FLI1-driven neoenhancer formation

Most of what we know regarding EWS::FLI1 biology point towards a substantial involvement of “chromatin” proteins, which either bind to or modify the chromatin structure, as regulators of EWS::FLI1-driven transcription. However, a systematic study of all such proteins has not been undertaken. Taking advantage of the various EWS::FLI1₁-harbouring *Drosophila* transgenic lines established thus far, we screened all genes whose function related even loosely to the chromatin with the aim of identifying chromatin proteins that either (i) have already been described in the literature to be of relevance to EwS, or (ii) have a previously unknown role in EwS.

To this end, we acquired RNAi lines targeting all 424 *Drosophila* genes categorized under the Gene Ontology (GO) groups "Chromatin Genes" and "Chromatin Binding". Additionally, we incorporated 116 *Drosophila* orthologs of genes reported in the literature with predicted or

established relevance to EwS. This included known or predicted EWS::FLI1 interactors, likely modifiers of EWS::FLI1-target upregulation, and orthologs of genes associated with EWS::FLI1-mediated alternative splicing reported across various studies (Sankar et al., 2013, 2014; Selvanathan et al., 2015; Grohar et al., 2016; Sánchez-Molina et al., 2020; Molnar et al., 2022). In total, a comprehensive collection of 599 UAS>hairpin-RNA lines, targeting 540 distinct candidate genes, was acquired from the Drosophila Transgenic RNAi Project (TRiP) collection at the Bloomington Drosophila Stock Centre (BDSC).

The YFP levels obtained through 20×(GGAA) μ Sat>YFP following *EWS::FLI1_{1FS}* expression driven by *sal^{EPv}>Gal4* are sufficiently high for clear, *in-situ*, detection of YFP in larval salivary glands under a fluorescence stereoscope. Therefore, through a rapid visual assessment of YFP intensity *in-situ* from 6-10 individual flies per RNAi line, we identified 63 RNAi lines as hits that consistently lower YFP levels to varying extents as compared to controls lacking a UAS>RNAi. Notably, no enhancers (lines increasing YFP levels) were detected in this screen. The lack of identified enhancers may reflect a difficulty in distinguishing YFP from control samples *in-situ* rather than a lack of enhancers

The abrogation of genes whose function involves chromatin regulation is likely to affect cells independently of their effect on EWS::FLI1₁ function. Therefore, we expected the RNAi lines shortlisted from the *in-situ* screen to be a mix of bona fide hits that target EWS::FLI1₁ function, and those that affect transcription from 20×(GGAA) μ Sat>YFP through EWS::FLI1-independent effects. The later scenario can arise by two means. The first is through changes to epigenetic stability induced by the loss of chromatin modifier genes (Skora and Spradling, 2010; Lee et al., 2017), which may in turn affect expression from the UAS promoter causing a misexpression of EWS::FLI1₁. Secondly, the RNAi targeted genes may be crucial for salivary gland development, such that their knockdown reduces salivary gland size. While small salivary glands can retain the capacity to maintain transcription from 20×(GGAA) μ Sat>YFP, as is the case for salivary glands expressing ρ EWS::FLI1₁ (see Figure 4.4, on page 47), the *in-situ* comparison of fluorophore levels as compared to healthy controls can be misleading owing to (1) factors that can affect transcription rates in underdeveloped salivary glands and/or (2) the physical coverage of underdeveloped, small salivary glands by surrounding larval tissues. While these factors preclude the classification of RNAi that reduce salivary gland size as bona fide hits, they cannot entirely rule out a potential relevance of the targeted genes to EWS::FLI1 function.

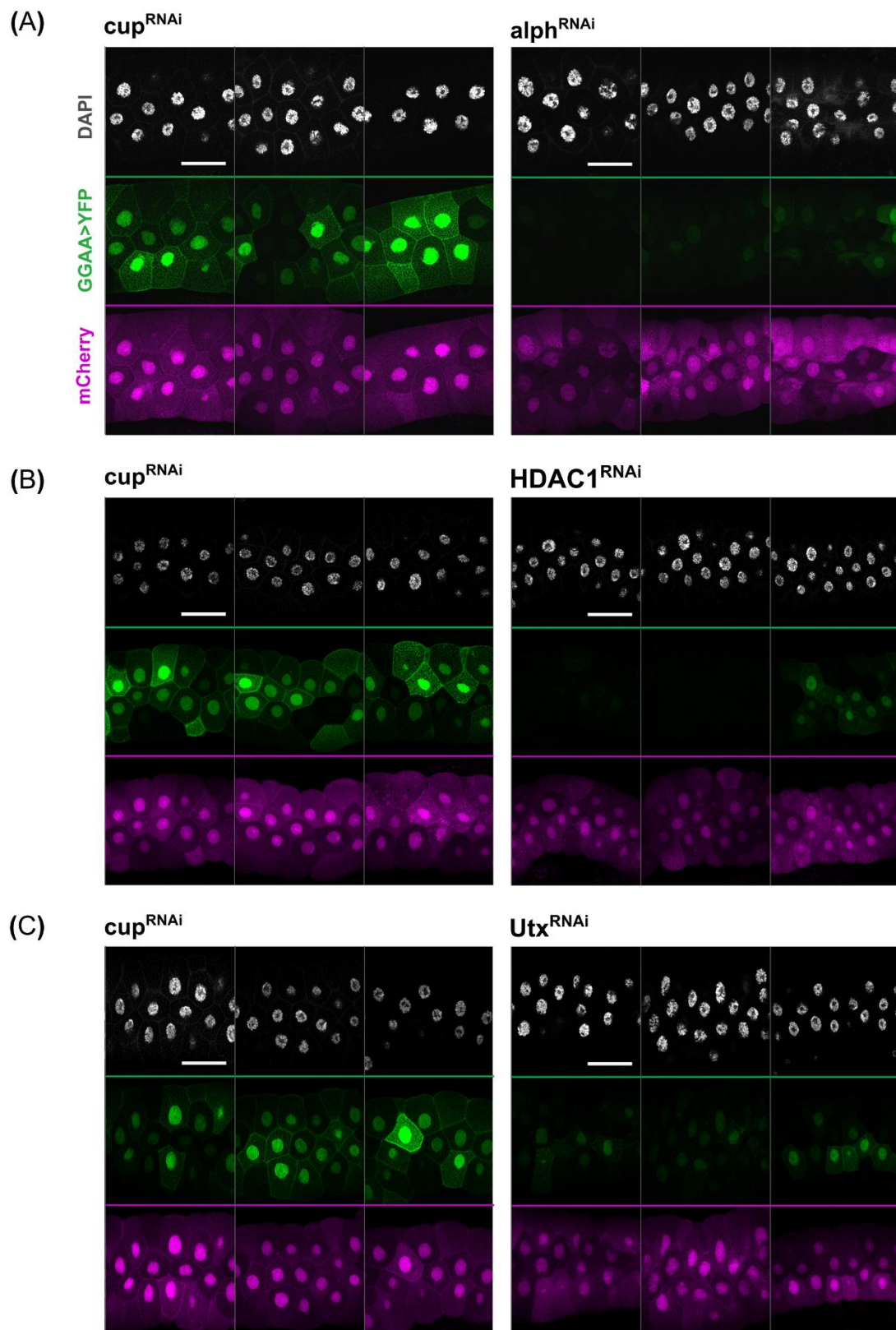
To narrow down on bona fide hits specifically affecting EWS::FLI1-mediated transcription from 20×(GGAA) μ Sat>YFP, I utilized ρ EWS::FLI1₁ that harbours an mCherry fluorophore in the first cistron alongside *EWS::FLI1₁* in the second. This design serves two critical purposes:

Table 4.1: High priority hits from screen for modifiers of EWS::FLI-induced transcription from GGAApSats. The sample averages for YFP and mCherry fluorescence intensities are provided as a ratio of its batch-matched control. Statistical significance of difference between RNAi and control samples (unpaired t-test) are indicated as : NS= not significant, $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***). †RNAi lines 50905 and 60123 both target two gene products Ada2a (FBgn0263738) and Polr2D (FBgn0263757).

Gene	FBID	BDSC ID	mCherry			GGAA>YFP			Salivary gland size
			Ratio of average fluorescence intensities RNAi/control	p-value	significance	Ratio of average fluorescence intensities RNAi/control	p-value	significance	Ratio of average sizes RNAi/control (%)
alph	FBgn0086361	40873	0.88	0.158	NS	0.19	0.002	**	61
HDAC1	FBgn0015805	33725	0.85	0.180	NS	0.31	0.002	**	51
Utx	FBgn0260749	34076	0.87	0.123	NS	0.38	0.009	**	61
Mgtor	FBgn0013756	32941	1.51	0.016	*	0.67	0.011	*	56
Ada2A /Polr2D†	FBgn0263738 /FBgn0263757	50905	1.16	0.121	NS	0.36	0.008	**	36
		60123	1.98	0.000	***	0.64	0.049	*	46

firstly, mCherry levels report for *Gal4-UAS* transcriptional activity, allowing us to distinguish between RNAi lines that genuinely suppressed EWS::FLI1₁ activity and those that simply reduced the *Gal4* driver's function. Secondly, the EWS::FLI1₁ protein levels achieved through this bicistronic construct are inherently lower than those of EWS::FLI1_{1FS}, which is advantageous as these levels do not induce developmental toxicity in the salivary gland. This allows to assess the standalone toxic effect of any given RNAi line, and makes for a cleaner, more EWS::FLI1₁-specific readout. Finally, the effect of each UAS>RNAi over the listed factors (levels of YFP and mCherry, and salivary gland size) was compared to expected levels achieved with a control UAS>RNAi targeting a germline gene, *cup*, that is not expressed in salivary glands (Krause et al., 2022), nor upregulated by any of the EWS::FLI1 transgenes (Molnar et al., 2022, and Appendix B). This ensures that none of the evaluated differences stem from a dilution of Gal4 protein between two UAS promoters.

Unlike with EWS::FLI1_{1FS}, however, the mCherry and YFP levels obtained following *BC*EWS::FLI1₁ expression are too low for clear *in-situ* detection of changes. Therefore, we used confocal microscopy to quantify fluorescence levels in dissected salivary glands. This analysis revealed 6 out of the 63 RNAi lines as high priority hits that significantly reduced YFP



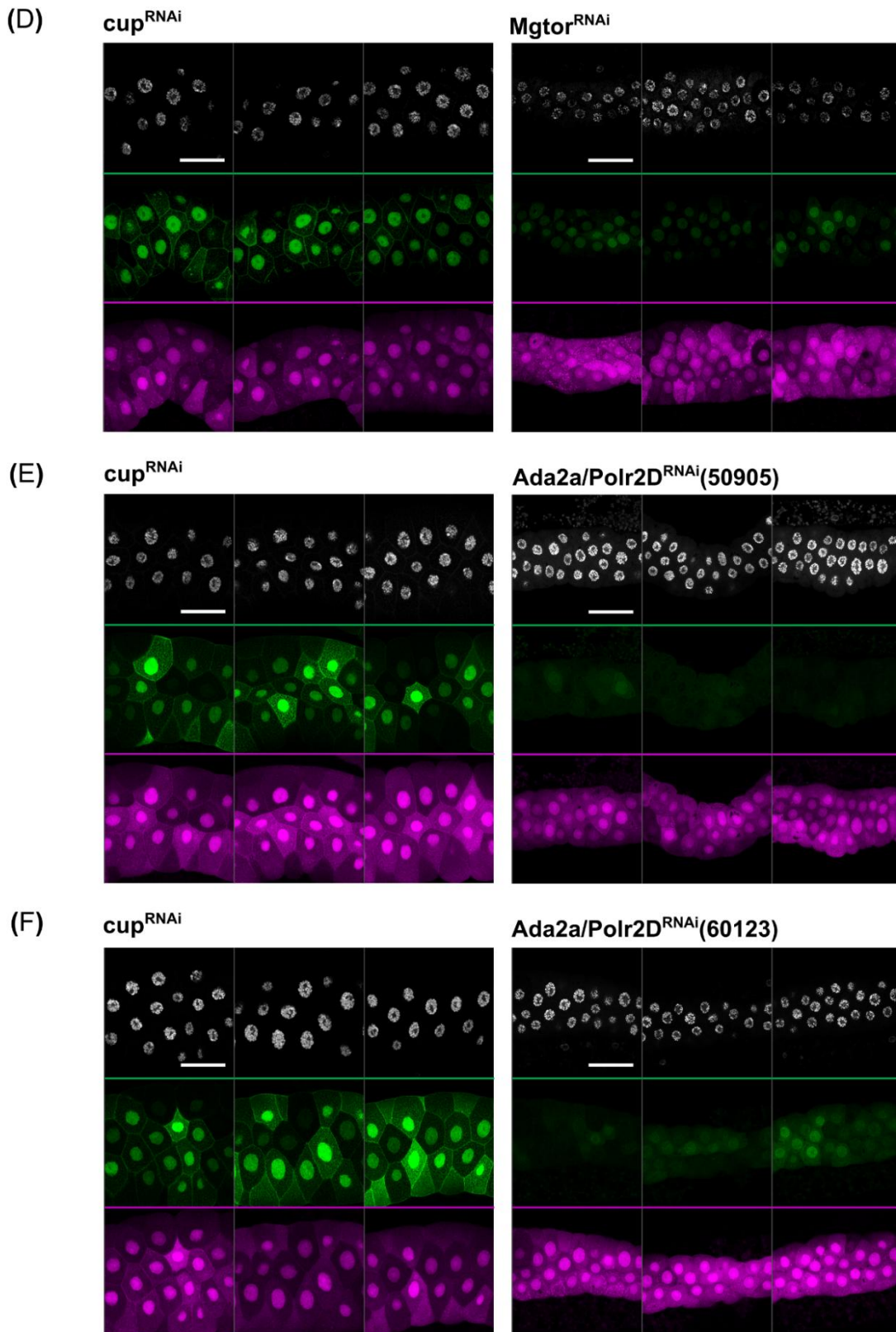


Figure 4.6: Effect of high priority RNAi hits on transcription from UAS and GGAA μ Sats in presence of bCEWS::FLI1₁. Representative salivary glands showing YFP expression from 20 $\times$ (GGAA)>YFP (green) and co-stained with DAPI (grey), from individuals carrying *sal^{EPV}>Gal4*, *bCEWS::FLI1₁*, 20 $\times$ (GGAA) μ Sat>YFP and reach of the indicated UAS>RNAi: (A) *alph^{RNAi}* (B) *HDAC1^{RNAi}* (C) *Utx^{RNAi}* (D) *Mgtor^{RNAi}* and two lines (E-F) targeting three gene products *Ada2a* and *Polr2D*, alongside their respective batch-matched control with the control RNAi targeting *cup*. Scale bar: 100 μ m.

without significantly reducing mCherry levels as compared to control samples expressing *cup^{RNAi}* (Figure 4.6, Table 4.2).

The genes targeted by these high priority suppressors are protein phosphatase *alphet* (*alph*), Histone deacetylase 1 (*HDAC1*), nuclear pore complex component *Megator* (*Mgtor*), histone demethylase *ubiquitously transcribed tetratricopeptide repeat, X Chromosome* (*Utx*), and two RNAi lines (BDSC ID: 50905 and 60123) targeting the same two gene products, *RNA polymerase II subunit D* (*Polr2D*) and transcriptional *Adaptor 2a* (*Ada2a*), both of which are alternative splice variants of a single mRNA transcript.

Importantly, the human orthologs of *alph* and *Utx* promote the transcription of EWS::FLI1-targets regulated through GGAA μ Sats (Grohar et al., 2016; Figuerola-Bou et al., 2023). While the mechanism of involvement has not been investigated for the *alph* ortholog, the *Utx* ortholog KDM6A was found to facilitate the recruitment of BAF complex component BRG1 to EWS::FLI1-bound sites (Figuerola-Bou et al., 2023). While there are no reports implicating the human orthologs of *Mgtor*, *HDAC1*, *Ada2a* and *Polr2D* as regulators of GGAA μ Sat-dependent transcription, they have been identified as dependencies that reduce EwS cell proliferation or viability following CRISPR-Cas9-mediated knock out (Aguirre et al., 2016; Behan et al., 2019). Furthermore, some of *Mgtor* and *HDAC1* have been previously identified by us to physically interact with EWS::FLI1_{1FS}, and their orthologs interact physically with EWS::FLI1 in EwS cells (Li et al., 2012; Selvanathan et al., 2015; Molnar et al., 2022, and unpublished data from González lab). Altogether, these results serve as a proof of principle that the Drosophila EwS model can be reliably used to identify EWS::FLI1 dependencies relevant to human EwS.

Aside from the 6 hits, 41 out of 63 RNAi lines (65%) alter mCherry levels, which is expected given the chromatin-relevant functions of most of the targeted genes, and demonstrates the importance of validation with the bcEWS::FLI1₁ construct. Of these, 39 RNAi lines significantly lower both mCherry levels and YFP levels, reflecting a reduction in expression from the UAS promoter, while 2 RNAi lines significantly increase both mCherry and YFP levels indicating increased expression from the UAS promoter. Finally, the remaining 16 out of 63 RNAi do not significantly reduce YFP levels (Appendix C).

The 2 RNAi lines that significantly increased both mCherry and YFP levels both target Bromodomain-containing BET family protein *female sterile (1) homeotic* (*fs(1)h*) (BDSC ID: 41943 & 41693). The expression of both *fs(1)h^{RNAi}* drastically reduces salivary gland size (down to 30% and 10% of the control, respectively) (Appendix D). Such dramatic reduction in salivary gland size is likely to be perceived as reduced in YFP levels during *in-situ* evaluation under stereomicroscope as mentioned earlier, explaining their selection as hits.

11 out of the 16 RNAi lines that do not significantly reduce YFP levels also dramatically reduce salivary gland size down to less than 40% of control salivary glands (Appendix D), while the remaining 5 RNAi lines do not lower YFP, nor greatly affect salivary gland size. Among these 5 RNAi lines, one targets the P300 ortholog, *nejire* (*nej*) yields mCherry levels averaging 1.5-times its control. This result may reflect a dual effect whereby *nej*^{RNAi} both increases transcription from the UAS promoter, as well as suppresses YFP expression such that the net result is YFP levels similar to that of the control. As transcription from 20×(GGAA) remains comparable to the control, this RNAi was not shortlisted as a high priority hit. The other 4 RNAi lines, targeting histone deacetylase *HDAC3*, a component of the FACT chromatin modifying complex *dre4*, and nucleoporin *Nup50* did not affect mCherry levels.

While the apparent lack of effect of these 4 RNAi lines on bcEWS::FLI1₁ as compared to EWS::FLI1_{1FS}-driven YFP levels may reflect an oncogene dose-dependent function for the targeted genes, this is not the only interpretation. An alternate explanation is that these RNAi are mild suppressors of transcription from UAS, which do not yield significant changes in mCherry fluorescence levels. As previously described, the translation of EWS::FLI1₁ from bcEWS::FLI1₁ mRNA transcripts, which relies on the reinitiation, is inherently less efficient than the cap-dependent translation of EWS::FLI1_{1FS} transcripts (Kozak, 2001). The difference in translation efficiency is what enables achieving lower EWS::FLI1₁ protein levels through the bcEWS::FLI1₁ construct from the same amount of mRNA. Consequently, it is expected that EWS::FLI1_{1FS} protein levels are more sensitive to mild changes in its efficiently translated mRNA transcript levels than EWS::FLI1₁ protein levels are to the same changes in the inefficiently translated bcEWS::FLI1₁ mRNA transcript levels, which would explain the lack of observable RNAi effect in the latter.

The described mild suppression is likely the case for 3 of these 4 RNAi lines: *HDAC3*^{RNAi} (BDSC ID: 64476) and *dre*^{RNAi} (BDSC ID: 34344 & #54855). *HDAC3* and *dre4* were both also targeted by separate RNAi lines (*HDAC3*^{RNAi} BDSC ID: 34778 and *dre4*^{RNAi} BDSC ID: 41677) that did significantly reduce mCherry levels (Appendix C), suggesting that while silencing *HDAC3* and *dre4* does reduce expression from the UAS promoter, the changes induced by the mentioned 3 RNAi lines are too subtle to detect via fluorescence levels.

4.6.1 *alphabet* suppresses EWS::FLI1 driven transcription from GGAA_μSat in a phosphatase-dependent manner

From among the hits identified in the secondary screen, the RNAi targeting *alph* achieved the most significant downregulation of transcription from 20×(GGAA)_μSat>YFP. As a first step towards investigating how *alph*^{RNAi} causes this downregulation, we directly assessed its effect on EWS::FLI1₁ protein levels via western blot (Figure 4.7). This revealed that while the inherent

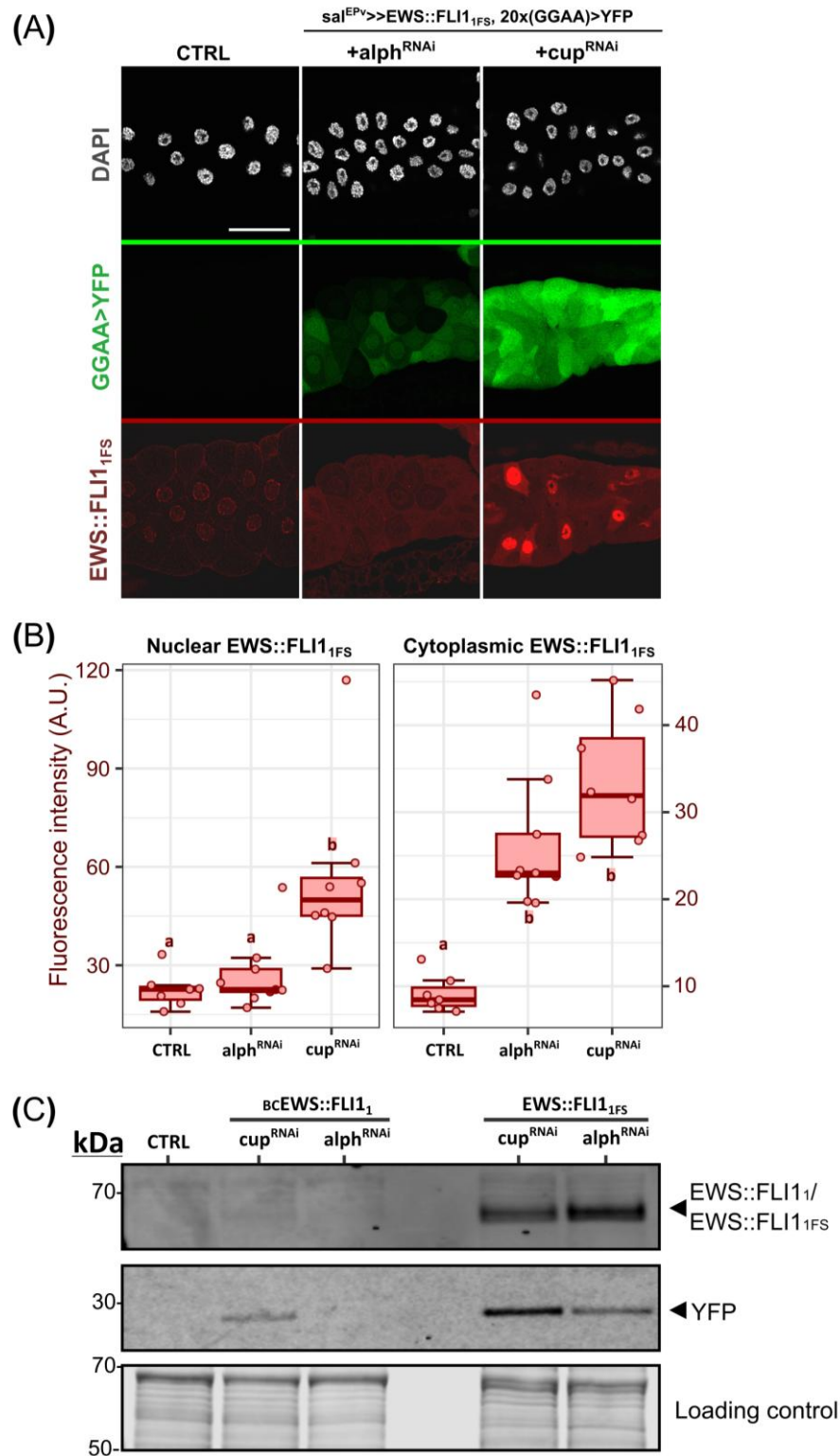


Figure 4.7: Effect of *alph*^{RNAi} EWS::FLI1_{1FS} intracellular localization and protein levels. (A) Representative sections from salivary glands showing YFP expression (green) from 20x(GGAA) μ Sat>YFP and staining for EWS::FLI1_{1FS} (red) Scale bar: 100 μ m **(B)** Quantification of EWS::FLI1_{1FS} fluorescence in nuclei (left) and cytoplasm (right) (arb. units). Letters indicate statistically significant differences between group means (Kruskal–Wallis and Dunn’s test, $p < 0.05$). **(C)** Western blot showing protein levels of EWS::FLI1 and YFP for experimental conditions *sal^{EPV}>Gal4*, *bCEWS::FLI1₁*, 20x(GGAA) μ Sat>YFP/+; *UAS>RNAi*/+ and *sal^{EPV}>Gal4/UAS>EWS::FLI1_{1FS}*; 20x(GGAA)>YFP/+; *UAS>RNAi*/+ with *alph*^{RNAi} and *cup*^{RNAi} as compared to control (CTRL) *sal^{EPV}>Gal4*/+, 20x(GGAA) μ Sat>YFP/+.

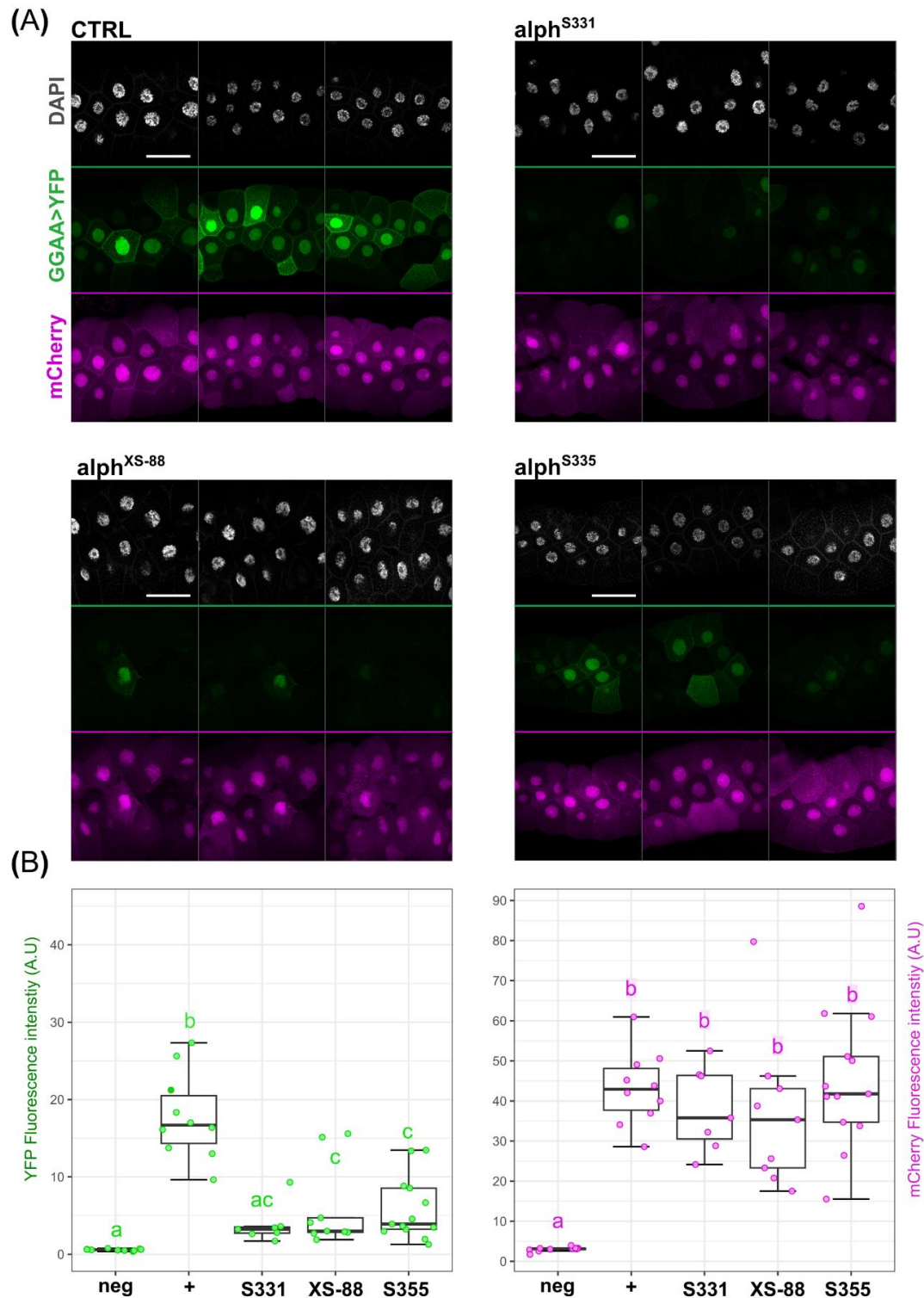


Figure 4.8 α mutant dominant negatively affect GGAA μ Sat activation by Δ C-EWS::FLI1₁, but not EWS::FLI1_{1FS}. Representative salivary glands YFP (green) expression from 20 $\times$ (GGAA) μ Sat>YFP and mCherry (magenta) reflecting expression from the UAS promoter, DAPI (grey) of representative sections in (A) with scale bar: 100 μ m., and their respective quantification in (B). Letters indicate statistically significant differences between group means (Kruskal–Wallis and Dunn’s test, $p < 0.05$). Experimental conditions are CTRL ($sal^{EPV}>Gal4/+$; 20 $\times$ (GGAA) μ Sat>YFP/+), + ($sal^{EPV}>Gal4$, Δ C-EWS::FLI, 20 $\times$ (GGAA) μ Sat>YFP/+), S331 ($sal^{EPV}>Gal4$, Δ C-EWS::FLI, 20 $\times$ (GGAA) μ Sat>YFP/+; $\alpha^{S331}/+$), XS-88 ($sal^{EPV}>Gal4$, Δ C-EWS::FLI, 20 $\times$ (GGAA) μ Sat>YFP/+; $\alpha^{XS-88}/+$), and S355 ($sal^{EPV}>Gal4$, Δ C-EWS::FLI, 20 $\times$ (GGAA) μ Sat>YFP/+; $\alpha^{S355}/+$).

protein levels of BCEWS::FLI1₁ are too low to detect discernible changes, there is no reduction in total EWS::FLI1_{1FS} protein levels in *alph*^{RNAi} samples as compared to controls.

The same western blots were used to reassess YFP levels from the 20×(GGAA) μ Sat>YFP, which show a reduction of almost 50% in YFP protein levels by *alph*^{RNAi} as compared to the control RNAi (Fig. 4-9B). These results demonstrate that the effect of *alph*^{RNAi} is not owing to a reduction of EWS::FLI1₁ translation, which in turn suggests a physical impairment to its function. One hypothesis to explain these observations is reduced EWS::FLI1₁ binding to the chromatin, which can be clearly seen through immunostainings for EWS::FLI1_{1FS} that show intense staining on transcriptionally active regions of the polytene chromosomes (see Figure 1.5 on page 24, Molnar et al., 2022).

To investigate this possibility, I analysed EWS::FLI1_{1FS} distribution in the salivary glands. Under normal conditions, EWS::FLI1_{1FS} signal is highly heterogenous between cells of the same salivary gland, with many cells showing intense fluorescence within their nuclei relative to neighbouring cells. In comparison, *alph*^{RNAi} expressing individuals had far fewer intensely stained nuclei (Figure 4.7-A,B), which suggests that EWS::FLI1_{1FS} binding to chromatin is compromised. Furthermore, the cytoplasmic signal for EWS::FLI1_{1FS} in *alph*^{RNAi} samples is considerably higher than that of salivary glands not expressing the fusion but is not statistically different to that of *cup*^{RNAi}, possibly indicating that EWS::FLI1_{1FS} localization within the cell is not affected by *alph*^{RNAi}.

As *alph* encodes a single-subunit phosphatase (Kamada et al., 2020), we next investigated whether the inhibitory activity of *alph*^{RNAi} could be reproduced by *alph* mutants with abrogated phosphatase activity. For this, we used *alph* mutants that harbour point mutations in their catalytic domains that either completely abolish (*alph*^{S-331}), or severely reduce (*alph*^{XS-88} more than *alph*^{S-355}), their phosphatase activity (Baril and Therrien, 2006). As with *alph*^{RNAi}, each of these mutants was able to reduce BCEWS::FLI1₁-induced YFP levels from 20×(GGAA) μ Sat>YFP and did not significantly reduce mCherry levels (Figure 4.8). These results confirm that *alph*'s activity phosphatase is necessary for EWS::FLI1-dependent neoenhancer formation.

4.7 Insights into EWS::FLI1-driven adult eye phenotypes

The considerable time investment required for the analyses of neoenhancer activity in salivary glands prompted us to explore more scalable and time-efficient alternative EWS::FLI1-induced phenotypes for future screens. Drosophila eye roughness phenotypes are well-established as sensitive readouts for identifying modifiers of human pathogenic genes (Warrick et al., 1998; Bilen and Bonini, 2007; Cukier et al., 2008; Shalaby et al., 2009; Muñoz-Soriano et al., 2016).

The wide range of EWS::FLI1-induced eye phenotypes achieved by combining different eye disc-specific Gal4 drivers with various EWS::FLI1 transgenes makes for a broad phenotypic range, making for excellent readouts for genetic screens.

Elucidating the precise pathways underlying EWS::FLI1-induced eye phenotypes remains an active area of investigation within our group. Within this context, my goal was to investigate whether eye phenotypes could function as proxies for the salivary gland-based readouts. To this end, in parallel with the characterization of their effects in salivary glands, I tested the same 63 RNAi lines for their impact on EWS::FLI1-driven eye roughness. We chose to test RNAi effect on each of the three eye phenotypes induced when expressing either PEWS::FLI1₁ or EWS::FLI1_{1FS} in cells posterior to the eye imaginal disc morphogenetic furrow during third instar larval development via either *sev>Gal4* (*sev>>EWS::FLI1_{1FS}* and *sev>>PEWS::FLI1₁*), or *long-GMR>Gal4* (*GMR>>EWS::FLI1_{1FS}*). The *GMR>>EWS::FLI1_{1FS}* is characterized by extreme roughness and a near complete loss of pigmentation (Molnar et al., 2022), whereas mildest roughness is achieved with *sev>>EWS::FLI1_{1FS}*, which presents with irregularly distributed but well-defined ommatidia. In comparison, *sev>>PEWS::FLI1₁* yields in an intermediate phenotype: poorly developed ommatidia and an overall bumpy appearance that is more evident in the eyes of females that are larger than males.

Utilizing the entire range of phenotypes ensured that any eventual discrepancy between salivary gland and eye roughness modifications would not be due to differences in the extent of ommatidial cells affected by the Gal4 drivers which in turn determines the dynamic range for observing modifications (Freeman, 1996). Specifically, we predicted that the detection of suppressors would be easier in the rougher *GMR>>EWS::FLI1_{1FS}* and *sev>>PEWS::FLI1₁* eyes, while the detection of enhancers would be easier in *sev>>EWS::FLI1_{1FS}* eyes. Indeed, as far as enhancers go, only RNAi lines with no obvious standalone effects were considered as hits.

I found that most of the tested RNAi lines, including the 6 RNAi identified as high priority suppressors of EWS::FLI1₁-driven transcription from GGAA μ Sats, did not affect the eye phenotypes induced by EWS::FLI1₁ (Table 4.2). Exceptions were 5 RNAi lines that enhanced the EWS::FLI1₁-induced eye roughness without notably affecting eye morphology in absence of EWS::FLI1₁ (Figure 4.9). The lack of identified suppressors despite the inclusion of RNAi targeting known interactors of EWS::FLI1 that are necessary for its neomorphic functions, such as RNA Pol II (*Polr2D* and *Polr2C*) and P300 (*nej*) that are core parts of the transcriptional machinery, and components of the BAF and NuRD complexes, suggests that pathways giving rise to eye roughness may not necessarily be relevant to the known oncogenic pathways induced by

Table 4.2: Summary of results from screen for modifiers of EWS::FLI1-induced eye phenotypes. “—”= No effect on indicated phenotype, “↑”= Enhances EWS::FLI1-driven eye phenotype. The sample averages for YFP and mCherry fluorescence intensities are provided as a ratio of its batch-matched control. Statistical significance of difference between RNAi and control samples (unpaired t-test) are indicated as: $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

Gene	FBID	BDSC ID	Eye phenotypes				mCherry			GGAA>YFP			Salivary gland size
			sev>Gal4, UAS-EWS::FLI1 ^{FS}	sev>Gal4, UAS-PEWS::FLI1	longGMR>Gal4, UAS-EWS::FLI1 ^{FS}	Standalone effect of RNAi	Ratio of avg fluorescence intensities RNAi/control	p-value	significance	Ratio of avg fluorescence intensities RNAi/control	p-value	significance	Ratio of average sizes RNAi/control (%)
trr	FBgn0023518	29563	—	↑	↑	—	0.22	0	***	0.02	0	***	77
Spt6	FBgn0028982	32373	—	↑	—	—	0.5	0.005	**	0.08	0.003	**	25
Spt6	FBgn0028982	80476	—	↑	—	—	0.3	0	***	0.21	0.003	**	19
fs(1)h	FBgn0004656	41693	↑	↑	↑	—	1.51	0.04	*	3.21	0.002	**	30
fs(1)h	FBgn0004656	41943	↑	↑	↑	—	1.93	0.03	*	5.78	0.012	*	12

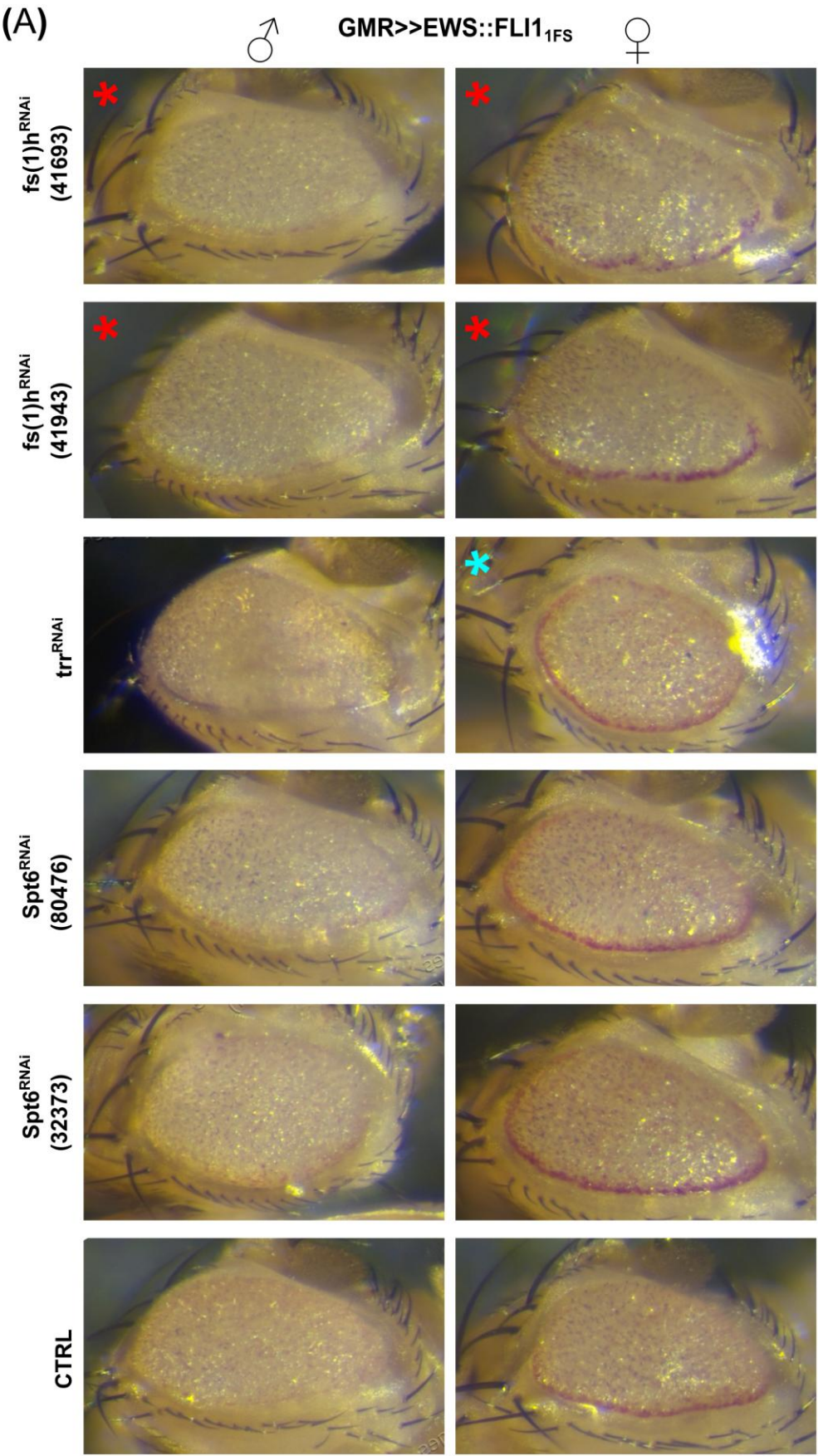
EWS::FLI1 in human EwS. These results indicate that the eye roughness phenotypes cannot serve as proxies for the salivary-gland readouts.

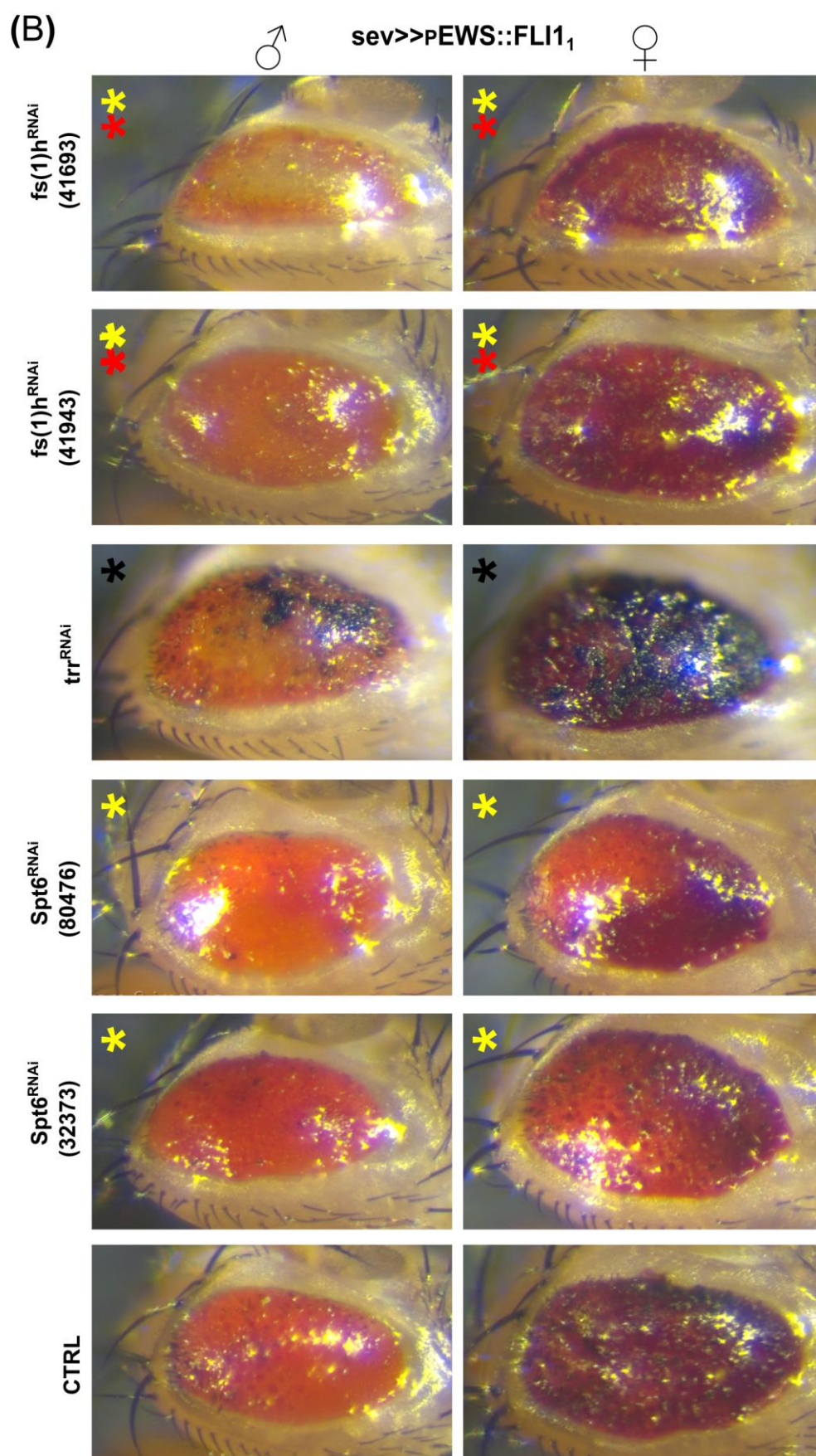
Among the identified enhancers, two RNAi that both target *fs(1)h* consistently enhanced all three EWS::FLI1-driven eye phenotypes. For sev>>PEWS::FLI1₁ and sev>>EWS::FLI1_{1FS} eyes, the *fs(1)h*^{RNAi} induces a “glossy” appearance that is reminiscent to the eye phenotype induced by the *Glazed* gene mutation, which causes a degeneration of corneal nanostructures that normally function to minimize reflection of incoming light (Kryuchkov et al., 2011), along with a reduction in pigmentation. *fs(1)h*^{RNAi} further reduces the already residual red pigmentation of GMR>>EWS::FLI1_{1FS} eyes in females. As mentioned earlier, both *fs(1)h*^{RNAi} increased mCherry and YFP levels in the salivary glands, suggesting that the drastic enhancement of eye roughness is a result of increased EWS::FLI1₁ expression. This result represents an important drawback of eye roughness screens dependent on the Gal4-UAS system: the effect of RNAi over the bipartite expression system itself is not inherently controlled for.

Of the remaining 3 enhancers, two target the *Suppressor of Ty 6* (*Spt6*), which is a transcription elongation factor and regulator of alternative splicing, and one targets methyltransferase *trithorax-related* (*trr*). Both *Spt6*^{RNAi} lines cause an enhancement of only the sev>>PEWS::FLI1₁ eyes, inducing a more “glossy” appearance than control PEWS::FLI1₁

eyes. Finally, *trr*^{RNAi} caused a black necrotic appearance for the sev>>EWS::FLI1₁ eyes and slightly reduced the eye size of females GMR>>EWS::FLI1_{1FS} eyes.

Neither *Spt6*^{RNAi} nor *trr*^{RNAi} enhance sev>>EWS::FLI1_{1FS} eyes, which is the least rough and therefore most amenable to enhancements. Furthermore, the finding that *Spt6*^{RNAi} enhances, rather than suppresses, the eye phenotype is contradictory to the role reported for its human ortholog SUPT6H, whose loss destabilizes EWS::FLI1 mRNA and reduces EWS::FLI1 protein levels (Grohar et al., 2016). Taken together, these results might suggest that the effect of these RNAi lines is not directly specific to EWS::FLI1₁ function but rather arises indirectly as a factor associated with elevated EWS::FLI1₁ expression.





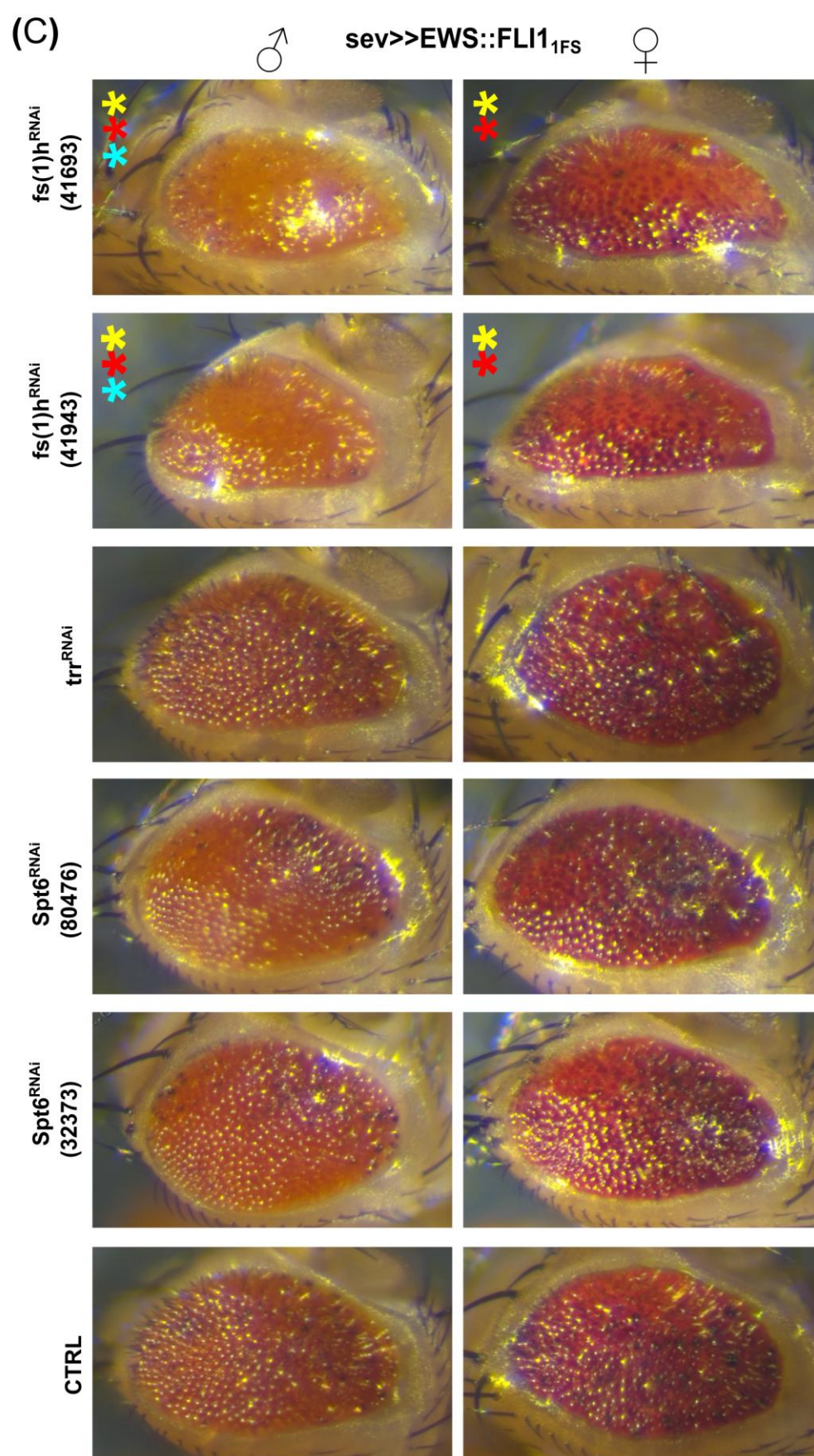


Figure 4.9: RNAi hits that modify EWS::FLI-induced eye phenotypes. Adult eyes for individuals expressing EWS::FLI1_{1FS} or PEWS::FLI1₁ through **(A)** longGMR>Gal4 or **(B-C)** sev>Gal4, and coexpressing the indicated RNAi or control RNAi against cup (CTRL). stars indicate modified phenotypes: increased “glossy” phenotype (yellow), reduced pigmentation (red) reduced eye size (blue), and necrotic spots (black). Scale bars: 0.1 mm.

5. DISCUSSION

Understanding the intricate mechanisms by which EWS::FLI1 activity drives Ewing sarcoma, a notoriously aggressive childhood cancer, remains a critical challenge in paediatric oncology. Our contribution to this effort has been the development and characterization of *Drosophila* models expressing EWS::FLI1, that have been subsequently used to investigate its biology. Leveraging these models, I have been able to (1) characterize the dose-dependent effects of EWS::FLI1 to find differences between GGAA μ Sat-dependent and -independent dysregulation, (2) identify dependencies for EWS::FLI1-mediated neoenhancer formation, and (3) investigate the utility of EWS::FLI1-induced eye roughness phenotypes to investigate EwS-relevant biology.

5.1 FStail tagging as a means to generate animal models for lethal oncogenes

In *Drosophila*, a very clear distinction can be made between toxicity caused by *UAS>EWS::FLI1* in the absence of Gal4, which results in lethality during transgenesis experiments, i.e. the injection of pUAS constructs, and toxicity caused upon Gal4-driven expression which results in lethality in different stages of development determined by the pattern of Gal4 expression. The former can be circumvented by tagging the EWS::FLI1 fusion with the FStail (Molnar et al., 2022). The latter, however, is only partially reduced by the FStail. Here we show that the FStail works by reducing protein levels.

A lack of a model for EwS initiation has precluded identifying the precise origin for EwS (reviewed in section 1.2.9). Within this context, we propose the utility of FStail tagging as a tool to facilitate identifying cell types that are permissive to lower levels EWS::FLI1 expression as a stepping stone to generate transgenic models. Such models will be critical to investigate the origin of EwS, and whether differences in CoO explain the heterogeneity observed in EwS tumours.

Lowering oncogene levels has been reported to improve the modelling of initiation of certain cancer types. For instance, early transgenic murine models for acute promyelotic leukemia (APL) that express the driver fusion oncogene promyelocytic leukaemia (PML)–retinoic acid receptor- α (RAR α) (PML::RAR α) in early myeloid cells only inefficiently induced APL 15%-20% of mice. The penetrance APL was increased up to 90% by lowering PML::RAR α expression levels in the same cells, which was achieved through modifications to the PML::RAR α promoter (Westervelt et al., 2003). This, and other variable penetrance observed using other myeloid-specific promoters suggest that an optimal level for PML::RAR α is necessary for APL development (Welch et al., 2011). Considering these data, it is possible that EWS::FLI1

expression in mice does not yield EwS-like tumours as a factor of suboptimal EWS::FLI1 protein levels rather than low synteny in GGAA μ Sat as has been hypothesized (section 1.2.9).

A similar dose-modulating approach has been used to generate transgenic murine models for rhabdoid tumours by regulating the extent of the driving *SMARCB1* deletion. The tamoxifen-induced ubiquitous deletion of *SMARCB1* during early mouse development causes embryonic or postnatal lethality using standard tamoxifen doses (Han et al., 2016). This lethality was circumvented by using below-standard doses of tamoxifen to induce the deletion, which enabled the identifying the developmental period during which ubiquitous *Smarcb1* deletion induced rhabdoid-like tumours (Han et al., 2016).

Like EwS, the origin of rhabdoid tumours is unknown. The tumour cells from the above-described model were used to investigate tumour origins, which the authors suggest should be various stem cell types based on similarities between their transcriptomic profiles and that of the mouse rhabdoid tumour cells. Combining this model with cell barcoding techniques tagging the hypothetical CoO will ultimately confirm the contribution of each cell type to the final tumour, an approach that could be used upon the development of a transgenic model of EwS initiation.

5.2 EWS::FLI1₁ protein levels and neomorphic functions show a function-specific dose–response relationship

Having confirmed that the FStail reduces EWS::FLI protein levels, we generated new lines that express full-length, unmodified EWS::FLI1₁ from transgenes that were engineered to reduce protein expression to a greater or lesser extent, hence circumventing the need for FStail tagging.

The new EWS::FLI1₁ lines facilitated the generation of a phenotypic series, from which we have learned that the neomorphic effects of EWS::FLI1₁ that we have studied can be categorized in three classes on the basis of how they correlate with EWS::FLI1₁ protein levels. The first includes the assembly of neo-enhancers at GGAA μ Sats that presents a positive linear correlation over a wide range of EWS::FLI1₁ protein levels, from the lowest, (bcEWS::FLI1₁) up to the highest (pEWS::FLI1₁ + Gal80@25 and pEWS::FLI1₁). The second includes massive GGAA μ Sat-independent transcriptional dysregulation including, notably, a significant fraction of orthologs of human Ewing sarcoma signature' genes. Rather counterintuitively, despite the order of magnitude lower level of expressed EWS::FLI1₁, the bcEWS::FLI1₁ transgene is able to significantly dysregulate hundreds of genes albeit with lower fold-changes. Finally, a group of genes upregulated in EWS::FLI1_{1FS} are not upregulated in bcEWS::FLI1₁: they do not follow a linear response but are expressed only if EWS::FLI1 protein levels go above-threshold.

Among the entire group of neural genes, only a few are marginally upregulated in *bcEWS::FLI1*₁ as compared to *EWS::FLI1*_{1FS} and *pEWS::FLI1+Gal80@25*. These results reveal a complex, phenotype-specific correlation between *EWS::FLI1*₁ levels and the expressivity of its phenotypes, that in turn implies fundamental differences in the mechanisms that drive these phenotypes.

Despite a low mutational burden characteristic of paediatric tumours, Ewing sarcoma displays high levels of intratumoral heterogeneity. Several independent findings have suggested that the heterogeneous cellular phenotypes in EwS tumours can be determined by *EWS::FLI1* levels, which may consequently have direct implications in Ewing sarcoma progression (reviewed in 1.2.8). The results of our characterization of the various *EWS::FLI1* transgenes highlight the functional relevance of *EWS::FLI1* expression levels and provide the experimental tools to further investigate in *Drosophila* the molecular pathways affected by the *EWS::FLI* “high” and “low” states observed in human tumours.

5.3 Chromatin-binding genes identified as modifiers of *EWS::FLI1*-mediated transcription from *GGAA μ Sats*

Using the generated transgenic models, we conducted a systematic screen for the role of chromatin genes in *EWS::FLI1* function. Notably, a large fraction (41/63) of the tested chromatin gene-targeting RNAi lines that initially shortlisted as hits for reducing *EWS::FLI1*_{1FS}-driven transcription from *GGAA μ Sat* also lowered transcription from the UAS promoter. It must be reiterated that while these results preclude the classification of these 41 RNAi lines as hits they do not rule out a potential relevance of the targeted genes to *EWS::FLI1* function, which must be pursued through separate experimental setup. A critical implication of this result is that the Gal4/UAS system is sensitive to a substantial number of chromatin proteins. This calls for caution in the interpretation of such screening results, and the necessity to assess the effect on the Gal4/UAS or other utilized expression systems to truly shortlist bona fide hits.

Through the screen identified 6 RNAi lines as high priority hits. The genes targeted by hit RNAi include “chromatin” genes *Mgtor*, *Utx*, *HDAC1*, *Ada2a* and *Polr2D* that are altogether associated with a diverse range of chromatin regulatory mechanisms. Remarkably, the human orthologs of each have been functionally implicated in EwS, validating the potential of the *Drosophila* *EWS::FLI1* transgenic models to identify genes relevant to human EwS.

Of these genes, the mechanism underlying their involvement in transcription from *GGAA μ Sats* has only been investigated for the ortholog of *Utx*. Therefore, the discussion below will focus on the potential mechanisms through which *Mgtor*, *Utx*, and *HDAC1* may be

contributing to EWS::FLI1 function. As Ada2a and Polr2D are simultaneously targeted by a single RNAi, and have distinct functions making it impossible to distinguish which gene's knockdown contributed to the result without further investigation, their potential roles have not been delved into.

Ubiquitously transcribed tetratricopeptide repeat, X Chromosome (Utx)

Utx is the sole ortholog of mammalian Jumanji-C (Jmj-C) family of H3K27-specific lysine demethylases KDM6A/UTX (its closest structural ortholog), KDM6B/Jmjd3 and KDM6C/UTY that demethylate di- and tri-methylated H3K27 (Smith et al., 2008), both of which are marks typically linked to gene repression (Barski et al., 2007; Rosenfeld et al., 2009). KDM6C in particular has residual demethylating activity relative to KDM6A and KDM6B owing to point substitutions of its catalytic domain (Walport et al., 2014), and is thought to function in redundancy to KDM6A (Gažová et al., 2019).

Lacking DNA binding sites, Jmj-C family demethylases are recruited to their targets by other proteins. This recruitment is facilitated by the demethylases' highly conserved tetratricopeptide repeats (TPR), structural motifs that function as protein-protein interaction modules (Kato et al., 2020). In turn, Utx, KDM6A and KDM6B interact with various proteins to promote transcription through mechanisms other than H3K27 demethylation. In both *Drosophila* and mammals, Utx and KDM6A interact with histone acetyltransferase CBP, forming a complex that contains BAF complex component brm in *Drosophila* and its ortholog BRG1 in mouse (also termed SMARCA4) to modulate H3K27 acetylation, a marker of active transcription (Miller et al., 2010; Tie et al., 2012; Xie et al., 2017; Gozdecka et al., 2018). Additionally, Utx promotes the methylation of H3K4 (Herz et al., 2010), likely through the methyltransferase COMPASS-like complex that both KDM6A and KDM6B associate with (Cho et al., 2007; De Santa et al., 2007). Notably, each of these Utx interactors are also known protein partners of EWS::FLI1 that are recruited to GGAA μ Sats (see section 1.2.6.3 Epigenetic reprogramming by EWS::FLI1).

While KDM6C has no reported functional relevance to EwS, a recent pre-print has found both KDM6A and KDM6B to be cofactors of EWS::FLI1, with KDM6A in particular facilitating the recruitment of the BRG1 to EWS::FLI1-bound sites to enable transcriptional activation. (Figuerola-Bou et al., 2023). KDM6A and KDM6B differentially colocalize with EWS::FLI1; while KDM6B occurs on both single motifs and GGAA μ Sats, KDM6A colocalizes preferentially on single GGAA motifs. Accordingly, KDM6A loss only induces the downregulation of EWS::FLI1 targets regulated through a single GGAA motif, while KDM6B knockdown results in the downregulation of GGAA μ Sat-associated target genes (Figuerola-Bou et al., 2023).

Given KDM6A preferentially associates with single GGAA motifs, our finding of *Utx*^{RNAi} suppressing EWS::FLI1-driven transcription from a GGAA μ Sat in *Drosophila* may suggest Utx to functionally recapitulate both KDM6A and KDM6B (Lee et al., 2007). If so, such functional similarity would extend the relevance of Utx to EWS::FLI1-mediated transcription at both GAA μ SATs and single GGAA motifs and, consequently, to both gene up- and down-regulation mediated by EWS::FLI1.

Interestingly, while the abovementioned study found the CRISPR-mediated knockout of KDM6A abrogates proliferation in A673 cells (Figuerola-Bou et al., 2023), such an effect has not been reported in any other whole genome CRISPR-mediated knockout screens investigating dependencies of various EwS cell lines: A673, EW-7, TC-71, TC-32, ES-4, ES8, EW-1, and EW-16 cell lines (Behan et al., 2019; He et al., 2017; Aguirre et al., 2016). These conflicting reports warrant the further investigation of the reason underlying an apparent lack of effect following the loss of a gene that so broadly regulates EWS::FLI1-mediated transcriptional dysregulation.

Histone Deacetylase 1 (HDAC1)

HDAC1 is the sole *Drosophila* ortholog of mammalian deacetylases, HDAC1 and HDAC2 (Lee et al., 2007), two of five mammalian HDACs (in addition to HDAC3, HDAC6 and SIRT1) that have been identified as EwS dependencies. The knockdown of these HDACs or their inhibition using HDAC inhibitors (HDACi) vorinostat (a pan-HDACi) and entinostat (an inhibitor of class 1 HDACs: HDAC1, HDAC2, HDAC3, and HDAC8) abrogates EwS cell proliferation and xenograft tumour growth (Jaboin et al., 2002; Sakimura et al., 2005; Sonnemann et al., 2007; El-Naggar et al., 2019; Ma et al., 2019; García-Domínguez et al., 2021; Schmidt et al., 2021; Ramos et al., 2023). These studies have led to the inclusion of vorinostat and entinostat in combination therapies as part of early phase clinical trials for EwS (NCT04308330/NCT03838042). However, no study has specifically linked HDAC1 activity with transcriptional upregulation from GGAA μ Sats.

HDACs are recruited to their targets through various interacting partners. HDAC association with transcription factors or repressive complexes, such as the NuRD complex that harbours HDAC1 and HDAC2, and the Nuclear receptor co-repressor (NCoR) complex harbouring HDAC3, facilitates the deacetylation of histones to induce transcriptional silencing (Joshi et al., 2013; Park and Kim, 2020). Accordingly, the knockdown of HDAC2 and HDAC3 derepresses EWS::FLI1 down-regulated direct targets *LOX* and *TGFBR2* (Sankar et al., 2014, 2013). Interestingly, HDAC1 does not facilitate the repression of either *LOX* or *TGFBR2* despite also being a core component of the NuRD complex (Lai and Wade, 2011; Sankar et al., 2013). This

would suggest that the mechanism by which HDAC1 function as an EwS-dependency is likely to be independent of its association with repressor complexes.

Alternatively, HDACs can deacetylate non-histone proteins. For instance, recruitment of HDAC2 by the TP53 negative regulator, MDM2, creates ternary complex with MDM2, HDAC1 and TP53 that promotes the deacetylation and subsequent degradation of TP53 (Ito et al., 2002). Similarly, EWS::FLI1 has been shown to physically associate with HDAC1 in EwS cells, inducing the enhanced deacetylation of TP53 (Li et al., 2012), which is also a physical interactor of EWS::FLI1, to prevent cell cycle arrest (Ban et al., 2008; Li et al., 2012; Ban et al., 2014). As our lab has previously identified *Drosophila* HDAC1 to be an interactor of EWS::FLI1_{1FS}, it is likely for it mediate similar functions in *Drosophila*.

Though histone deacetylases are typically associated with transcriptional downregulation, HDAC1's deacetylase activity can promote transcriptional activation, specifically at super-enhancers (SE) in cell lines for leukaemia, rhabdomyosarcoma and breast cancer (Greer et al., 2015; Gryder et al., 2019; Lai et al., 2022). SEs are hyper-active regulatory elements, characterized by enrichment for active histone marks including H3K27Ac, that can be acquired by oncogenes to promote tumour pathogenesis- as is the case for the *MYC* oncogene in rhabdomyosarcoma and breast cancer (Ott et al., 2018; Wang et al., 2019). In breast cancer cells, the deacetylation activity of HDAC1 enables the recruitment of elongation factors to SEs, which promotes *MYC* transcription (Greer et al., 2015; Liu et al., 2022). In rhabdomyosarcoma, the collective inhibition of HDAC1, HDAC2 and HDAC3 via entinostat treatment results in a hyperacetylation of SEs (Gryder et al., 2019). The enhanced accessibility of these hyperacetylated regions disrupts chromatin looping at SEs, diluting transcription supporting interactions to oppose transcription of *MYC* (Gryder et al., 2019). Taken together, it is plausible that the association of EWS::FLI1 with HDAC1 results in the aberrant recruitment of the latter to GGAA μ Sats, where its deacetylase activity optimizes transcriptional through the mechanisms detailed above.

Interestingly, HDAC1 is also recruited by another fusion oncogene, PML::RAR α , to similarly induce TP53 degradation (Insinga et al., 2004). Like EWS::FLI1, PML::RAR α is an aberrant transcription factor that drives APL by driving a widespread transcriptional dysregulation (de Thé and Chen, 2010; Yan et al., 2010; Villiers et al., 2023). PML::RAR α -bound SEs are also co-localized by HDAC1 and p300 (Tan et al., 2021). As such, future studies of HDAC1's role in regulating EWS::FLI1 function may also benefit studies of PML::RAR α biology.

Megator (Mgtor)

Mgtor is a nucleoporin (NUP) that forms part of the nuclear basket of the nuclear pore complex (NPC), a massive non-polymeric complex that is involved in the regulation of trafficking across

the nuclear envelope and of the recruitment chromatin to the nuclear periphery to regulate transcription (Wente and Rout, 2010). Similar to HDAC1, Mgtor also interacts with EWS::FLI1_{1FS} (Molnar et al., 2022), and its human ortholog Translocated Promoter Region (TPR) was similarly identified as a potential EWS::FLI interacting partner via liquid chromatography-mass spectrometry (Selvanathan et al., 2015). Furthermore, large-scale, genome-wide CRISPR-Cas9-mediated knockout screens EwS cell lines have identified TPR loss to abrogate proliferation in each of the EwS cell lines expressing various isoforms of EWS::FLI (EW-7, TC-71, TC-32; ES-4, ES8, EW-1, and EW-16) (Aguirre et al., 2016; Behan et al., 2019). No mechanism has been described for TPR's role in EwS.

The NPC is canonically known for regulating nucleocytoplasmic transport, which TPR plays a direct role in facilitating (Aksenova et al., 2020). However, many studies indicate that Mgtor and its orthologs might also preferentially associate with and promote “open” chromatin states. In humans, for instance, TPR is necessary to maintain heterochromatin-free regions near the NPC, which likely facilitates unimpeded transport (Krull et al., 2010). In *Drosophila* salivary glands, Mgtor binds to chromatin in long, continuous domains that are termed NUP-associated regions (NARs). These Mgtor-bound-NARs are dominated by markers of active transcription, including high occupancy for RNA polymerase II, H4K16ac, as well as males absent on the first (mof), which is a component of the males-specific lethal (MSL) complex that deposits H4K16ac (Vaquerizas et al., 2010). Additionally, Mgtor forms part of an NPC-independent nuclear complex in *Drosophila* spermatocytes, termed the Mad1-containing IntraNuclear Territories (MINT) that is thought to contribute towards establishing or maintaining an “open” chromatin state (Raich et al., 2018). In yeast, Mgtor orthologs Mlp1/2 bind highly transcribed genes, and their binding is proposed to facilitate efficient mRNA export by recruiting these actively chromatin regions to the NPC vicinity (Casolari et al., 2005; García-Benítez et al., 2017). While it is unclear how Mgtor/TPR preferentially associate with and promote open chromatin, their interaction with EWS::FLI1 may contribute transcriptionally active environment required for EWS::FLI1-driven transcription from GGAA μ SATs.

Through their association with chromatin and chromatin modifiers, Mgtor and its orthologs can affect transcription both directions (Vinciguerra et al., 2005; Agarwal et al., 2011; Aksenova et al., 2020; Aleman et al., 2021; Capelson, 2023). Mlp1 in yeast regulates transcription at the NPC by recruiting SUMO protease Ulp1 (Texari et al., 2013). The desumoylation of transcriptional repressor targets of Ulp1, in turn, results in the gene derepression, suggesting a transcriptional activating role for Mlp1. On the other hand, Mlp1 has also been shown to reduce mRNA transcription response to inefficient mRNP assembly (Vinciguerra et al., 2005). In *Drosophila*, Mgtor modulates dosage compensation of male X-chromosome genes; while Mgtor depletion results in widespread up- and downregulation in both males and females,

males present with dramatic upregulation of X genes (Aleman et al., 2021). Altogether, these reports suggest that Mgtor/TPR potentially facilitate both up and downregulation mediated by EWS::FLI1.

5.4 Alphabet's role in EWS::FLI-driven transcription from neoenhancers

The strongest suppression of EWS::FLI1-driven transcription from GGAA μ Sats was achieved by an RNAi line targeting alphabet, the *Drosophila* ortholog of the Ser/Thr protein phosphatases PPM1A and PPM1B. Though not characterized as a “chromatin” gene, *alph*^{RNAi} was included in our screen owing to a prior report showing that RNAi-mediated silencing of human PPM1A in Ewing sarcoma cells reduces the EWS::FLI1-dependent transcriptional upregulation of *NR0B1* in TC-32 EwS cells (Grohar et al., 2016). While this report suggests a role for PPM1A in EWS::FLI1's neoenhancer activating capacity, the mechanism underlying this role has not been investigated.

My data, indicating that alphabet's effect on EWS::FLI1-driven transcription from the GGAA μ Sat neoenhancer is dependent on its phosphatase activity, provides crucial mechanistic insight into its role. The exact substrate(s) of alphabet in this context, and how their dephosphorylation ultimately leads to the observed changes in EWS::FLI1-driven transcription or protein displacement, remain to be elucidated.

Though there are no studies that have performed a systematic characterization of all phosphorylation-regulated domains in EWS::FLI1, phosphorylation of EWS::FLI1 at ser266 has been found to facilitate its transcriptional activity. This ser266 is likely a Protein Kinase C (PKC) phosphorylation site, at which the elimination of phosphorylation by mutation to alanine has been found to reduce EWS::FLI1 DNA binding and transcriptional activity, and results in a nuclear-to-cytoplasmic shift (Olsen and Hinrichs, 2001). Englerin A, an inhibitor of EWS::FLI1-driven upregulation of *NR0B1* and *NKX2.2* was found to deplete phosphorylation at ser266, similarly resulting in reduced DNA binding (Caropreso et al., 2016). These data suggests that the effect of alphabet in facilitating EWS::FLI mediated transcriptional upregulation is not expected if EWS::FLI1 is its direct target through its ser266 dephosphorylation. Thr79 in the EWS N-terminus is another residue shown to be phosphorylated via p38 MAPKs following DNA damage, however the functional relevance of phosphorylation on this residue has not been investigated (Klevernic et al., 2009). Taken together, alph may be dephosphorylating EWS::FLI1 at a non ser266 site, such as Thr79.

PPM1A and PPM1B are broad-spectrum phosphatases that often act in redundancy (Li et al., 2022). Together, they dephosphorylate and subsequently deactivate key kinases (e.g.,

MAPK family members including p38, ERK2, and upstream regulators of JNK) (Hanada et al., 1998; Takekawa et al., 1998; Li et al., 2013; Therrien et al., 2000; Baril and Therrien, 2006; Baril et al., 2009) other signalling molecules (e.g., PPAR γ , and IKK β) (Sun et al., 2009; Khim et al., 2020), and transcription factors (Smad2/3, Pax2) (Lin et al., 2006; Abraham et al., 2015). As such, there is a plethora of potential intermediates whose dephosphorylation-mediated deactivation could be implicated in EWS::FLI1 function. While the substrates of α ph are predicted to be many on account of its homology to PPMs, they have not been systematically investigated and only three are confirmed. These substrates are MAPKKKs Slipper (slpr), Wallenda (Wnd) and TGF β -activated kinase-1 (Tak1), orthologous to human MAP3K9, MAP3K13, and MAP3K7, respectively. The dephosphorylation of these MAPKKKs by α ph downregulates JNK signalling in *Drosophila* (Baril et al., 2009) making them interesting candidates as a starting point for investigating the mechanism of α ph's role in regulating EWS::FLI1-driven transcriptional upregulation.

It is important to note that, despite PPM1A's implication with *NR0B1* upregulation in TC32 EwS cells, CRISPR-Cas9-mediated knockout PPM1A does not affect proliferation or cell viability across various EwS cell lines including TC32 (Aguirre et al., 2016; He et al., 2017; Behan et al., 2019; Tan et al., 2023). As with KDM6A, these observations raise the question of why the loss of a gene, whose function is necessary for EWS::FLI1-mediated transcriptional dysregulation, would not affect EwS cell line proliferation.

The loss of *NR0B1* expression itself affects growth characteristics of only some EwS cells (inhibiting growth in EWS502 and A673, but not TC71 cells, each of which express EWS::FLI1) (Kinsey et al., 2006, 2009; Behan et al., 2019). Therefore, it is entirely possible that genes such as PPM1A and KDM6A should have cell line-specific effects. The critical point is to investigate what distinguishes the cells that are refractory to the loss of *NR0B1* from those that are not.

5.5 EWS::FLI-driven eye roughness

Eye roughness screens have been utilized to conduct landmark studies dissecting signalling and metabolic pathways necessary for eye development, such as the MAPK signalling cascades, whose relevance extends to tumorigenic signalling. Such relevance has been leveraged to conduct screens for modifiers of eye roughness-induced by cancer-related mutations to identify leads that hold potential therapeutic relevance (Avila et al., 2024). For instance, modifier screens for eye roughness induced by the NUP::HOXA9 fusion, a rare fusion that drives AML, have identified several suppressors and enhancers. Among these were two suppressor RNA lines targeting HOX gene co-factors extradenticle (*exd*) and homothorax (*hth*)

whose human orthologs accelerate the onset of HOX-mediated AML (Gavory et al., 2021). A similar example is the eye roughness induced by the BCR::ABL1 fusion which functions as a constitutively active kinase and drives chronic myelogenous leukemia (CML). Drug screens for modifiers of BCR::ABL1-induced eye roughness identified kinase inhibitors dasatinib and ponatinib to rescue the fusion-induced eye roughness (Fogerty et al., 1999; Outa et al., 2020). Given such studies, we expected eye roughness could be similarly utilized to investigate dependencies relevant for EwS.

Instead, our results raise important questions about the extent to which pathways responsible for EWS::FLI1₁-driven eye roughness is relevant to known oncogenic signalling in human EwS. The lack of suppressors identified in our RNAi screen, despite many of the RNAi lines significantly reducing transcription from the UAS promoter in the salivary glands, has two implications. Firstly, this result implies that the effect of EWS::FLI1₁ is likely induced following a relatively short period of EWS::FLI1₁ function, i.e. the period between the initial translation of EWS::FLI1₁, and the degradation mRNA targeted by the RNAi lines that ultimately inhibit transcription from the UAS promoter. Alternative screening tools, such as deficiency or overexpression constructs that do not create such periods, may therefore be better suited to identify suppressors. In support of this conclusion, our group has recently undertaken such screens through which numerous suppressors have been identified.

A relevant suppressor RNAi that targets components directly downstream in the pathway triggered by EWS::FLI1 can be expected to alleviate the eye roughness phenotype. This is because the downstream component's knockdown would precede the delayed reduction of EWS::FLI1 following reduced transcription from the UAS promoter. As such, the lack of identified suppressors may further reflect that genes targeted by these RNAi lines have no effect over the EWS::FLI1-induced pathways that give rise to eye roughness. This leads to the second implication, which concerns the nature of the pathways through which EWS::FLI1₁ induces eye roughness phenotypes. The tested RNAi lines include many that target orthologs of known interactors of EWS::FLI1, such as BAF complex components *mor* and *Snr1* (orthologous to SMARCC1/2 and SMARCB1) necessary for transcriptional activation by EWS::FLI1, *Sce*, whose ortholog, RING1B, is necessary for EWS::FLI1 recruitment to the chromatin (Sánchez-Molina et al., 2020), core transcriptional machinery components *Polr2D*, *Polr2C* and *nej* (orthologous to components of RNA Pol II and P300), spliceosome components *Sf3a1* and *Sf3b5*, *HDAC3* and *HDAC1* (that are components of the corepressor complexes such as NuRD, and whose knockdown derepresses EWS::FLI1-downregulated genes (Sankar et al., 2014, 2013)), and several other genes who have been implicated in mediating EWS::FLI1-dependent transcription from GGAA μ Sats (Grohar et al., 2016). For none of these genes to modify EWS::FLI1-induced eye phenotypes implies that the EWS::FLI1-induced

pathways giving rise to eye roughness may not necessarily be relevant to the known oncogenic pathways induced by EWS::FLI1 in human EwS.

The RNAi against *trr* and *Spt6* only caused evident modifications to *sev>>P*EWS::FLI1₁ but not the *sev>>E*WS::FLI1_{1FS} eyes, which suggests that the observed modifications are not necessarily generated through an enhancement of EWS::FLI1₁-induced pathways. *trr* itself is a regulator of eye tissue growth, with reduced *trr* function inducing cell death in *trr* mutant clones of mosaic adult eyes. Accordingly, the ubiquitous expression of *trr*^{RNAi} in the eye imaginal disc using *ey>Gal4* causes rough eyes (Kanda et al., 2013). Our finding that neither *longGMR>Gal4* nor *sev>Gal4*-driven expression of *trr*^{RNAi} induced roughness on their own suggests that only certain cells are sensitive to apoptosis induced by *trr* loss. The discrepancy in *trr*^{RNAi} effect over *sev>>E*WS::FLI1_{1FS} as compared to *sev>>P*EWS::FLI1₁ eyes may reflect that the cells of the latter are more sensitive to undergoing cell death induced by *trr* loss than those of the former. While *Spt6* has not been investigated within the context of eye development, the depletion of its *SUPT6H* in human cells elevates R-loop formation which leads to increase DNA damage-induced cell death (Nojima et al., 2018; Obara et al., 2020), a function that may be contributing to the increased roughness that we report.

5.6 Future perspectives

Among the different research lines that may derive from this work, I would like to underscore three that are of immediate interest with respect to *alph*'s role in EwS.

The first is to investigate whether *alph* mediates its effect directly through the dephosphorylation of EWS::FLI1 or indirectly through an intermediate suppressor.

The second is to investigate the consequences of *alph* depletion upon the *Drosophila* EwS signature to see whether it can affect EWS::FLI1-induced GGAA_nSat-independent transcriptomic changes.

The third is the need to establish the relevance of *alph* ortholog PPM1A's on growth and migratory phenotypes of EwS cells. Specifically, the lack of effect of CRISPR-mediated PPM1A knockout on proliferation in EwS cell lines despite its role in regulating enabling EWS::FLI1-mediated *NR0B1* upregulation suggests PPM1A might be a cell line-specific vulnerability. This raises three closely related questions that must be addressed: (1) does PPM1A loss affect *NR0B1* expression in EwS cell lines other than TC-32? (2) does the loss of *NR0B1* expression affect growth/proliferation in the same cells where PPM1A loss does not? (3) what distinguishes cell lines whose proliferation is abrogated by the loss of *PPM1A/NR0B1* from those that are refractory to *PPM1A/NR0B1* loss?

6. CONCLUSIONS

- 1) FStail-tagging may be useful in implementing animal models for toxic drivers of disease.
- 2) GGAA μ Sat-independent transcription is a substantial contributor to the EwS transcriptional signature, even at low levels of EWS::FLI1₁ expression.
- 3) Cell toxicity and GGAA μ Sat-dependent transcription present a positive linear correlation with EWS::FLI1₁ protein levels.
- 4) GGAA μ Sat-independent transcription correlates positively, but not linearly, with EWS::FLI1₁ protein levels.
- 5) The transcriptional upregulation of neural genes is a threshold dependent effect that only occurs when EWS::FLI1 protein levels exceed a specific level.
- 6) The EWS::FLI1₁ transgenic Drosophila models can be utilized to identify genes that are relevant to human EwS.
- 7) EWS::FLI1₁-induced phenotypes in the adult eye are not necessarily induced through pathways relevant to the mechanisms that control transcription from GGAA μ Sats.
- 8) Alphet facilitates EWS::FLI1₁-driven neoenhancer formation at GGAA μ Sats in a manner dependent on its phosphatase activity

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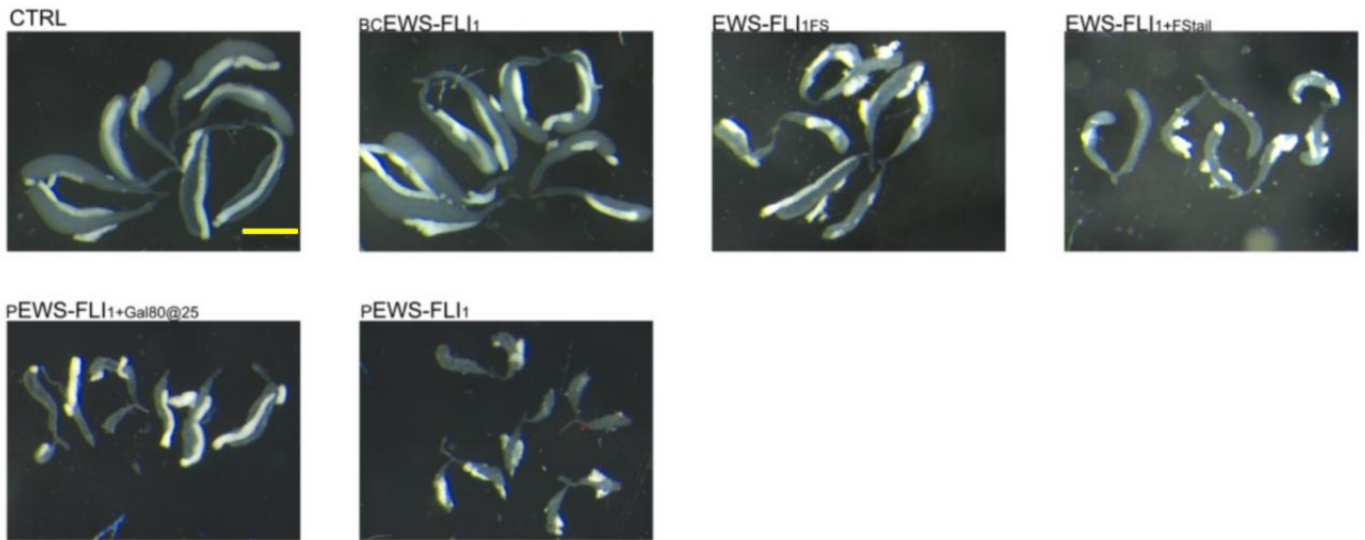
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8. APPENDIX

Appendix A

Appendix A:

A) The toxic effect of each EWS::FLI transgene on salivary gland development. Panels showing representative salivary glands for the following experimental conditions:
 control ($sal^{EPV}>Gal4/+$),
 $BC\text{EWS::FLI1}_1$ ($sal^{EPV}>Gal4/UAS>BC\text{EWS::FLI1}_1; 20\times(GGAA)>YFP/+$),
 EWS::FLI1_{1FS} ($sal^{EPV}>Gal4/UAS>\text{EWS::FLI1}_{1FS}; 20\times(GGAA)>YFP/+$),
 $P\text{EWS::FLI1}$ ($sal^{EPV}>Gal4/UAS>\text{EWS::FLI1}; 20\times(GGAA)>YFP/+$) and
 $P\text{EWS::FLI1}+Gal80@25$ ($sal^{EPV}>Gal4/UAS>\text{EWS::FLI1}; P[tubP>GAL80ts]/20\times(GGAA)>YFP$).
 Scale Bar: 250 a.u.



Appendix B

Appendix B:

- 1) **Excel File: Summary of differential gene expression analysis:** Comparisons of the transcriptomic profiles for each of BCEWS::FLI1₁, EWS::FLI1_{1FS} and PEWS::FLI1₁+Gal80@25C as compared to wild type salivary glands is provided. Each row corresponds to an individual microarray probe. Rejection (rej) values of 1 and -1 denote significantly upregulated or downregulated genes, respectively. Available for download at <https://doi.org/10.6084/m9.figshare.c.7321779>.

Appendix C

Appendix C:

Table – Summary of results from secondary screen for modifiers of EWS::FLI1₁ activity.

The results have been ordered by increasing significance of YFP downregulation. UAS>RNAi lines that significantly reduced YFP levels and do not reduce mCherry levels have been highlighted in yellow. The sample averages for YFP and mCherry fluorescence intensities are provided as a ratio of its respective control. Statistical significance was calculated via unpaired t-test. NS= not significant, $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), “—”= No effect on indicated phenotype, “↑”= Enhances EWS::FLI eye roughness, “X”= Cannot interpret enhancements to EWS::FLI eye roughness due to standalone effect (effect of RNAi with *sev-Gal4* or *longGMR-Gal4*). †RNAi lines 50905 and 60123 target three gene products Ada2a (FBgn0263738) and Polr2D (FBgn0263757).

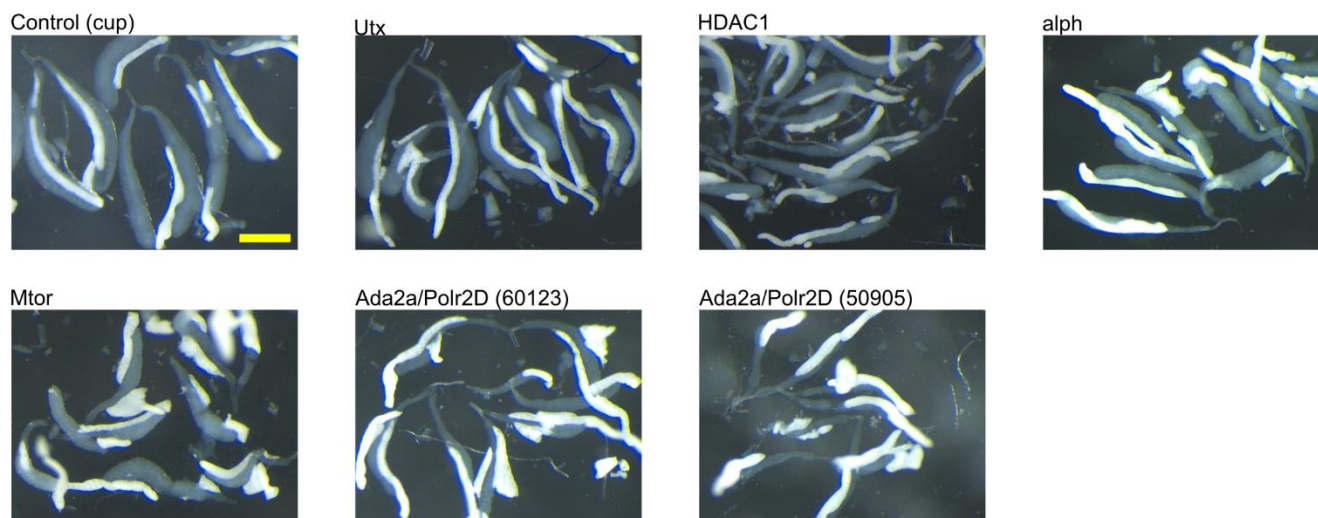
Gene	BDSC ID	FBID	mCherry of BCEWS::FLI1			BCEWS::FLI1-driven 20x(GGAA)YFP			EWS::FLI1 rough eye phenotypes			
			Ratio of average fluorescence intensities RNAi/control	p-value	significance	Ratio of average fluorescence intensities RNAi/control	p-value	significance	sev>Gal4, UAS-EWS::FLI1FS	sev>Gal4, UAS-PEWS::FLI1	longGMR>Gal4, UAS-EWS::FLI1FS	Standalone effect of RNAi if tested
alph	40873	FBgn0086361	0.88	0.158	NS	0.19	0.002	**	—	—	—	
HDAC1	33725	FBgn0015805	0.85	0.180	NS	0.31	0.002	**	—	—	—	
50905 [†]	50905	-	1.16	0.121	NS	0.36	0.008	**	—	—	—	
Utx	34076	FBgn0260749	0.87	0.123	NS	0.38	0.009	**	—	—	—	
Mgtor	32941	FBgn0013756	1.51	0.016	*	0.67	0.011	*	—	—	—	
60123 [†]	60123	-	1.98	0.000	***	0.64	0.049	*	—	—	—	
Nup154	34710	FBgn0021761	0.16	0.000	***	0.06	0.000	***	—	—	—	
Act5C	42651	FBgn0000042	0.30	0.000	***	0.07	0.000	***	X	X	—	↑
dre4	41677	FBgn0002183	0.61	0.004	**	0.07	0.000	***	—	—	—	
trr	29563	FBgn0023518	0.22	0.000	***	0.02	0.000	***	—	↑	↑	—
rept	32415	FBgn0040075	0.32	0.000	***	0.10	0.000	***	—	X	—	↑
tok	66320	FBgn0004885	0.16	0.000	***	0.08	0.000	***	—	—	—	
Bap60	33954	FBgn0025463	0.13	0.000	***	0.04	0.000	***	—	—	—	
Bap60	32503	FBgn0025463	0.27	0.000	***	0.04	0.000	***	—	—	—	
Tbp	55911	FBgn0003687	0.23	0.000	***	0.06	0.000	***	—	—	—	
piwi	34866	FBgn0004872	0.12	0.000	***	0.08	0.000	***	—	—	—	
Ssrp	41719	FBgn0010278	0.36	0.000	***	0.14	0.000	***	—	—	—	
Bap111	26218	FBgn0030093	0.50	0.000	***	0.09	0.000	***	—	—	—	
Sf3b5	34788	FBgn0040534	0.21	0.000	***	0.18	0.000	***	—	—	—	
Su(var)3-9	33401	FBgn0263755	0.19	0.000	***	0.03	0.000	***	X	—	—	↑
uri	65116	FBgn0035025	0.62	0.002	**	0.18	0.001	***	—	—	—	
mbo	77374	FBgn0026207	0.09	0.001	**	0.04	0.002	**	—	—	—	
fs(1)h	41693	FBgn0004656	1.51	0.040	*	3.21	0.002	**	↑	↑	—	—
CG6066	53997	FBgn0039488	0.09	0.000	***	0.05	0.002	**	—	—	—	

APPENDIX C

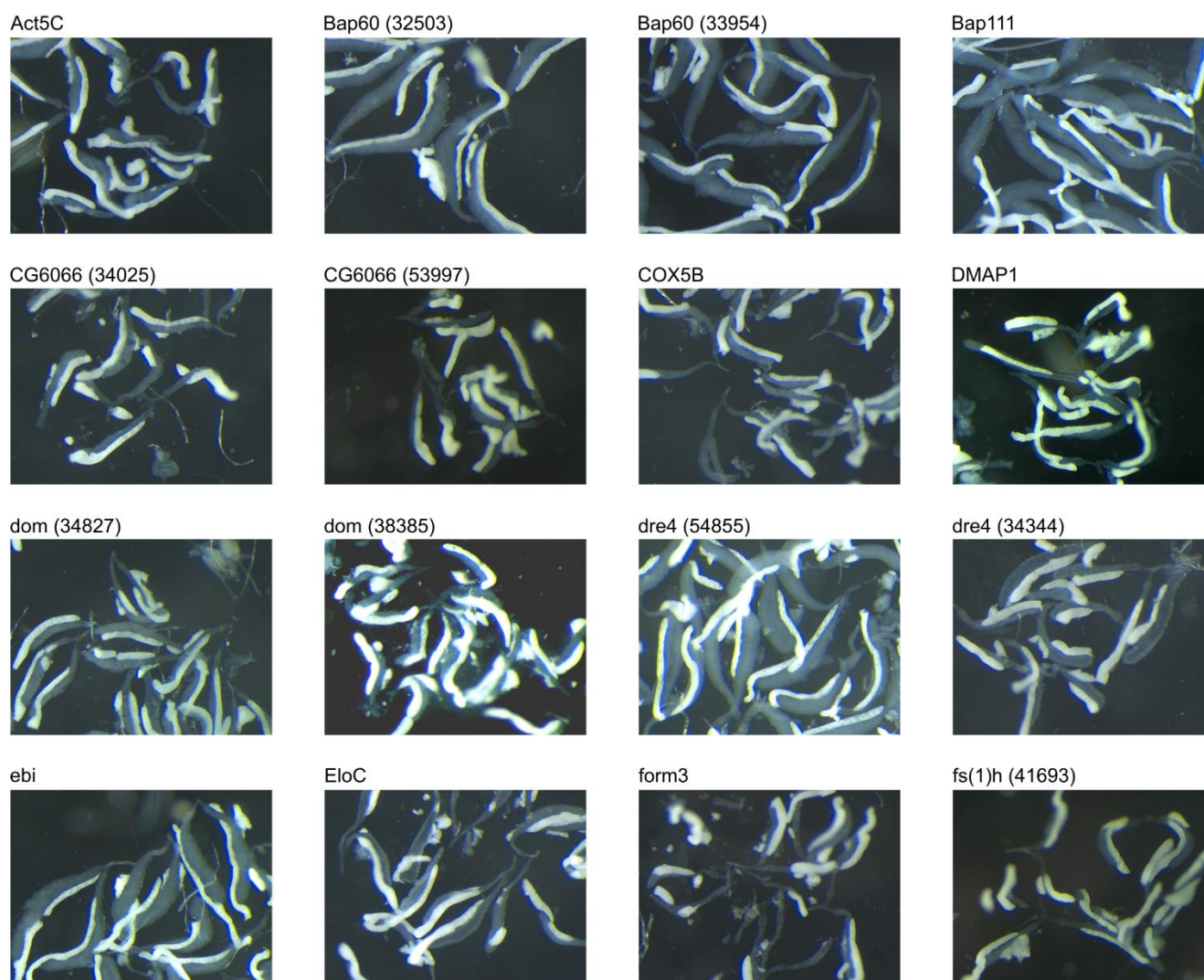
Taf10	62172	FBgn0028398	0.48	0.001	**	0.44	0.002	**	—	—	—	
Sf3b1	33650	FBgn0031266	0.17	0.000	***	0.07	0.002	**	X	X	—	↑
Spt6	32373	FBgn0028982	0.50	0.005	**	0.08	0.003	**	—	↑	—	—
CG6066	34025	FBgn0039488	0.26	0.016	*	0.10	0.003	**	—	—	—	
Sce	67924	FBgn0003330	0.59	0.001	**	0.10	0.003	**	—	—	—	
SmB	64594	FBgn0262601	0.21	0.000	***	0.08	0.003	**	—	—	—	
Polr2C	34567	FBgn0026373	0.18	0.000	***	0.10	0.003	**	—	—	—	
Spt6	80476	FBgn0028982	0.30	0.000	***	0.21	0.003	**	—	↑	—	—
HDAC3	34778	FBgn0025825	0.49	0.000	***	0.31	0.005	**	—	—	—	
Polr2G	57028	FBgn0051155	0.28	0.000	***	0.25	0.005	**	—	—	—	
DMAP1	63666	FBgn0034537	0.25	0.000	***	0.21	0.006	**	—	—	—	
Snr1	67929	FBgn0011715	0.62	0.001	**	0.23	0.006	**	—	—	—	
PolA1	61254	FBgn0259113	0.68	0.011	*	0.22	0.006	**	—	—	—	
mor	34919	FBgn0002783	0.09	0.001	***	0.03	0.008	**	—	—	—	
EloC	34818	FBgn0266711	0.53	0.020	*	0.12	0.011	*	—	—	—	
fs(1)h	41943	FBgn0004656	1.93	0.030	*	5.78	0.012	*	↑	↑	↑	—
ebi	34981	FBgn0263933	0.74	0.004	**	0.52	0.017	*	—	—	—	
Nup93-1	34090	FBgn0027537	0.47	0.000	***	0.54	0.018	*	—	—	—	
Rbbp5	42819	FBgn0036973	0.71	0.012	**	0.49	0.021	*	—	—	—	
Sf3a1	34840	FBgn0266917	0.53	0.005	**	0.38	0.022	*	—	—	—	
form3	32398	FBgn0053556	0.33	0.000	***	0.53	0.023	*	—	—	—	
Sbf	32419	FBgn0025802	0.32	0.000	***	0.33	0.028	*	—	—	—	
COX5B	80383	FBgn0031830	0.44	0.000	***	0.51	0.040	*	—	—	—	
HDAC3	64476	FBgn0025825	0.89	0.211	NS	0.55	0.111	NS	—	—	—	
dom	38385	FBgn0020306	1.35	0.055	NS	1.40	0.145	NS	—	—	—	
Nup50	34580	FBgn0033264	1.15	0.089	NS	1.19	0.146	NS	—	—	—	
Taf1	32421	FBgn0010355	0.77	0.070	NS	1.33	0.147	NS	—	—	—	
dre4	34344	FBgn0002183	0.85	0.122	NS	1.07	0.153	NS	—	—	—	
Taf9	32345	FBgn0000617	0.85	0.122	NS	1.07	0.153	NS	—	—	—	
dre4	54855	FBgn0002183	1.32	0.071	NS	0.78	0.204	NS	—	—	—	
piwi	33724	FBgn0004872	1.25	0.118	NS	0.82	0.236	NS	—	—	—	
Taf2	62168	FBgn0011836	0.80	0.089	NS	1.14	0.303	NS	—	—	—	
Nup62	52927	FBgn0034118	0.55	0.012	*	0.83	0.321	NS	—	—	—	
dom	34827	FBgn0020306	1.11	0.296	NS	0.71	0.334	NS	—	—	—	
Ssrp	51519	FBgn0010278	0.90	0.142	NS	1.10	0.355	NS	—	—	—	
Nipped-A	34849	FBgn0053554	0.75	0.049	*	1.09	0.400	NS	—	—	—	
nej	36682	FBgn0261617	1.49	0.020	*	0.95	0.436	NS	X	—	—	↑
PolA1	53341	FBgn0259113	0.92	0.319	NS	0.98	0.480	NS	—	—	—	
Not1	32836	FBgn0085436	1.05	0.341	NS	1.01	0.493	NS	—	—	—	

Appendix D

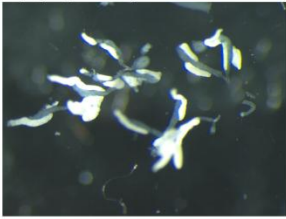
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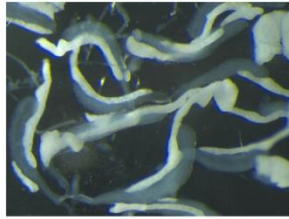
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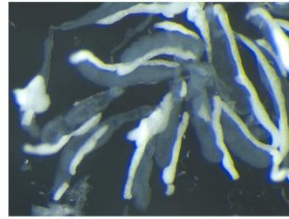
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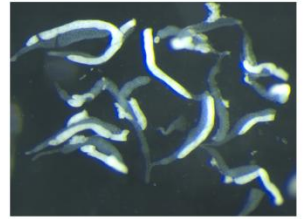
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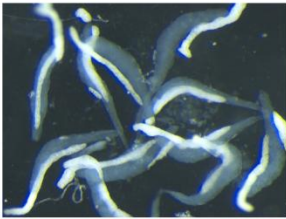
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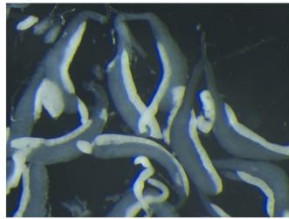
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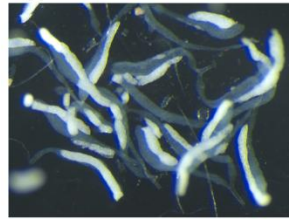
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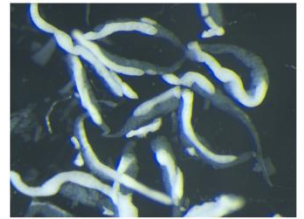
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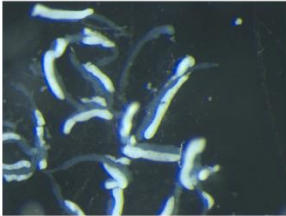
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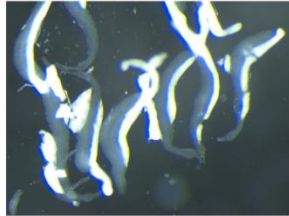
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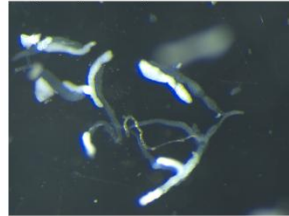
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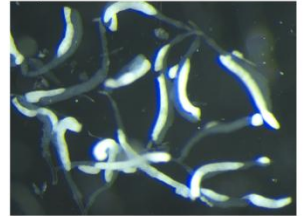
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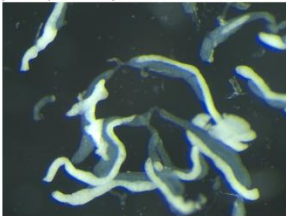
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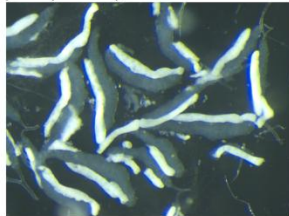
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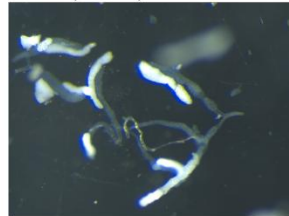
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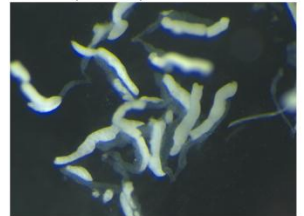
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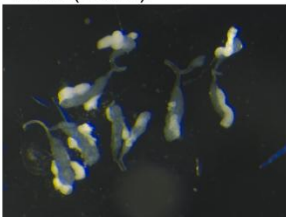
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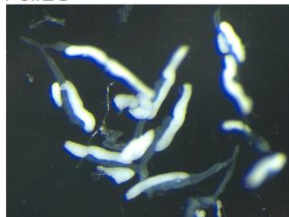
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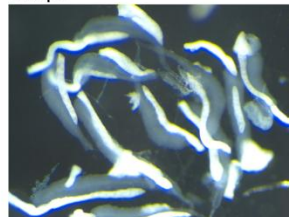
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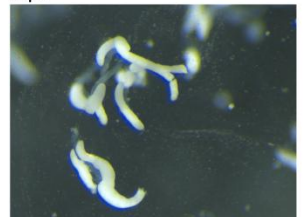
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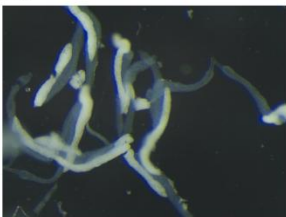
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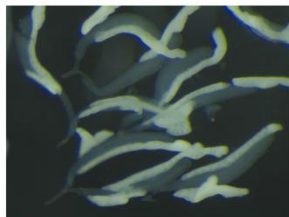
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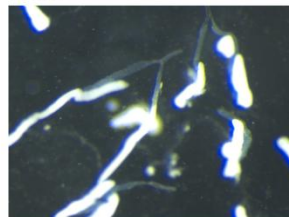
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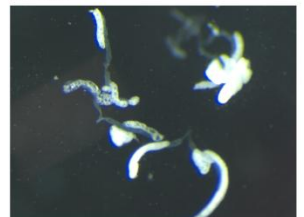
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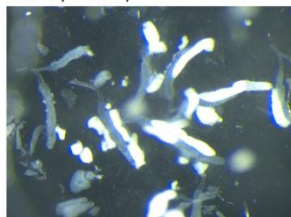
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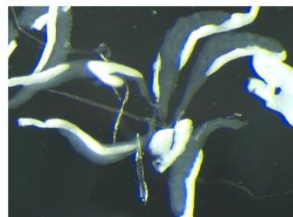
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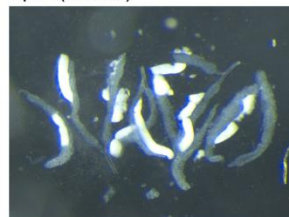
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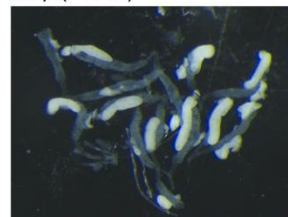
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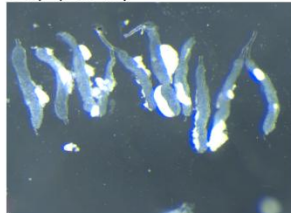
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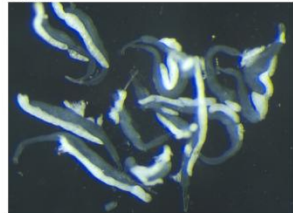
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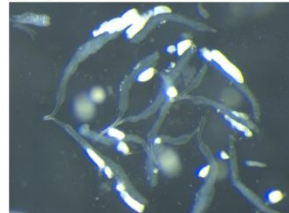
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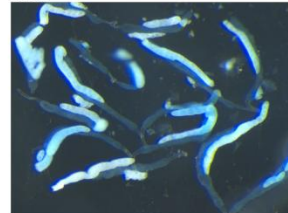
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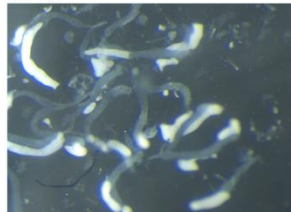
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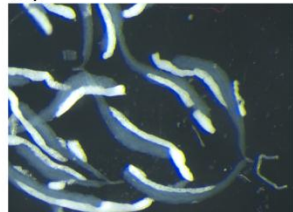
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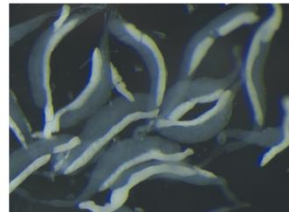
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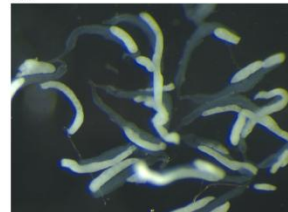
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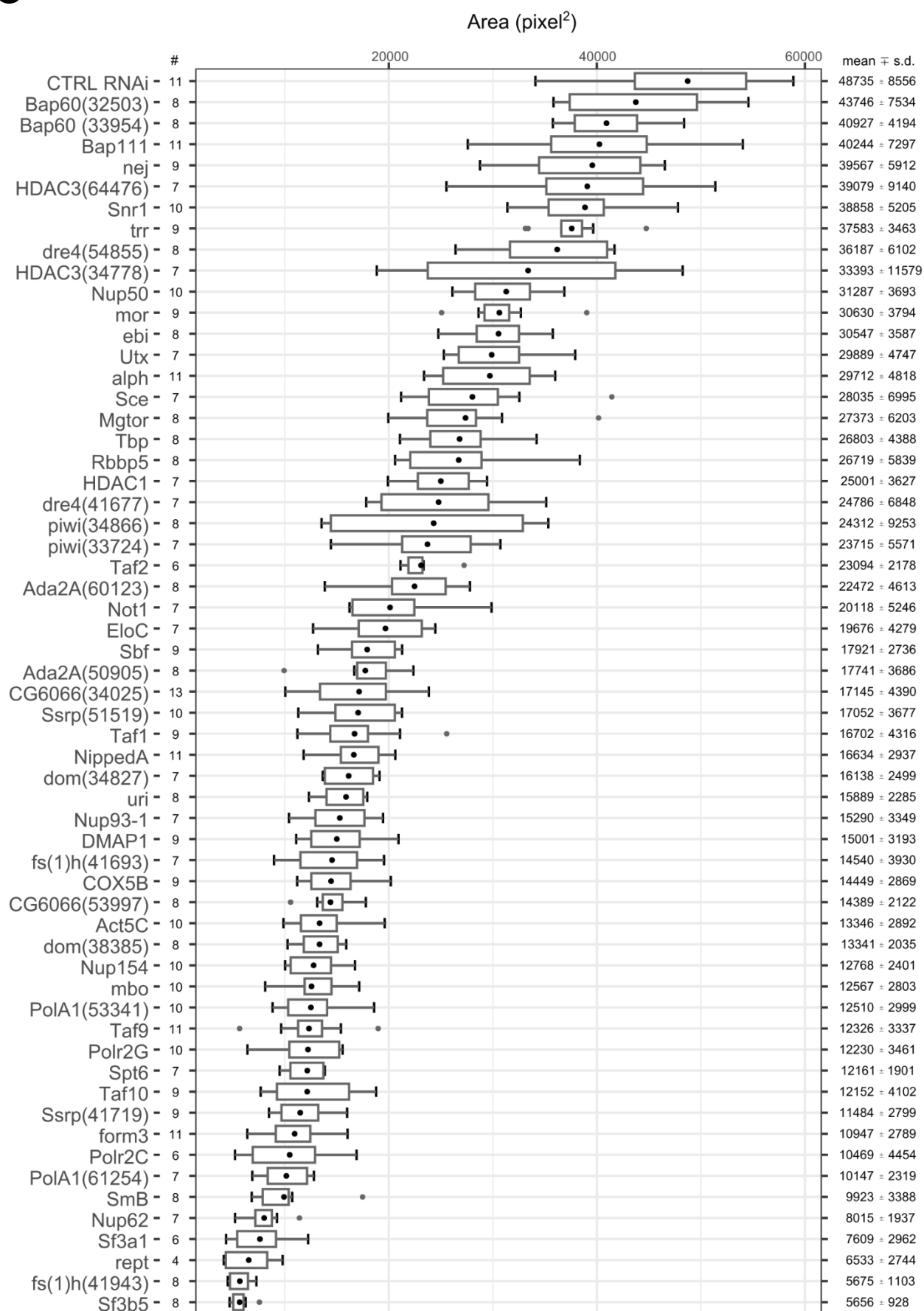
trr



uri



C



Appendix D. The toxic effect of each shortlisted RNAi on salivary gland development. Panels showing representative salivary glands (Representative salivary glands with genotypes *sal^{EPV}>Gal4*, *BC^{EWS}::FLI*, *20×(GGAA) μ Sat>YFP/+; UAS>RNAi/+*) for (A) RNAi lines shortlisted as true suppressors of EWS::FLI-driven transcription from GGAA μ Sats and (B) remaining RNAi lines analysed in the secondary screen. Scale bar in yellow: 250 a.u.(C) Plot of salivary gland sizes (arb. units) ordered by mean size (denoted by black points within the boxplot boxes). The corresponding number of salivary glands measured are shown on the left, and the mean and s.d. (a.u.²) values are shown on the right. BDSC ID numbers have been included within brackets for genes targeted by more than one RNAi lines.

Appendix E

Appendix E: Published Article- Mahnoor S, Molnar C, Velázquez D, Reina J, Llamazares S, Heinen JP, Mora J, Gonzalez C. 2024 Human EWS-FLI protein levels and neomorphic functions show a complex, function-specific dose–response relationship in *Drosophila*. *Open Biol.* 14: 240043. <https://doi.org/10.1098/rsob.240043>



Research



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Human EWS-FLI protein levels and neomorphic functions show a complex, function-specific dose–response relationship in *Drosophila*

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Ewing sarcoma (EwS) is a cancer that arises in the bones and soft tissues, typically driven by the Ewing's sarcoma breakpoint region 1-Friend leukemia virus integration 1 (EWS-FLI) oncogene. Implementation of genetically modified animal models of EwS has proved difficult largely owing to EWS-FLI's high toxicity. The EWS-FLI_{1FS} frameshift variant that circumvents toxicity but is still able to perform key oncogenic functions provided the first study model in *Drosophila*. However, the quest for *Drosophila* lines expressing full-length, unmodified EWS-FLI remained open. Here, we show that EWS-FLI_{1FS}'s lower toxicity is owed to reduced protein levels caused by its frameshifted C-terminal peptide, and report new strategies through which we have generated *Drosophila* lines that express full-length, unmodified EWS-FLI. Using these lines, we have found that the upregulation of transcription from GGAA-microsatellites (GGAA μ Sats) presents a positive linear correlation within a wide range of EWS-FLI protein concentrations. In contrast, rather counterintuitively, GGAA μ Sats-independent transcriptomic dysregulation presents relatively minor differences across the same range, suggesting that GGAA μ Sat-dependent and -independent transcriptional upregulation present different kinetics of response with regards to changing EWS-FLI protein concentration. Our results underpin the functional relevance of varying EWS-FLI expression levels and provide experimental tools to investigate, in *Drosophila*, the effect of the EWS-FLI 'high' and 'low' states that have been reported and are suspected to be important for EwS in humans.

1. Introduction

Ewing sarcoma (EwS) is an aggressive tumour, typically arising from bone and soft tissues, which is reported to be the second most common bone malignancy in children, adolescents and young adults [1–3].

EwS is a paradigm for solid tumour development caused by a single genetic lesion, a fusion between the transactivation domain of any member of the FET (FUS, EWSR1 and TAF15) family of RNA-binding proteins and the DNA-binding domain of one of several E26 transformation specific

(ETS) transcription factors (reviewed in [2]). In more than 80% of patients, the actual proteins involved are EWS RNA-binding protein 1 (EWSR1) and Friend leukaemia integration 1 (FLI1) and the resulting fusion is the EWS-FLI1 oncogene [2,4,5]. About 60% of these fusions have breakpoints at EWSR1 exon 7 and FLI1 exon 6 (i.e. type 1) henceforth referred to as EWS-FLI1 [6–8].

EWS-FLI1 is a pleiotropic oncoprotein with diverse neomorphic functions that include the remodelling of transcriptionally silent micro-satellites made of GGAA repeats (GGAA μ Sats) into active neo enhancers, out competition of ETS transcription factors and forcing aberrant alternative splicing [9–14]. Through these functions, EWS-FLI1 establishes the oncogenic regulatory program that subverts normal cell physiology and drives EwS development (reviewed in [2]).

The overwhelming toxicity of EWS-FLI1 has hampered the development of genetically tractable models of EwS and successful attempts to express it in vertebrates are still rare [15,16].

In *Drosophila melanogaster*, EWS-FLI1 toxicity can be avoided by a frameshift mutant (EWS-FLI1_{FS}) that replaces the 69 amino acid C-terminal tail of EWS-FLI1 with a new 69 amino acid sequence (henceforth referred to as the FStail) [17]. Remarkably, upon expression in *Drosophila*, EWS-FLI1_{FS} interacts with the homologues of EWS-FLI1's human protein partners including subunits of RNA pol II, the BAP/BAF chromatin remodelling complex and others; and causes a massive dysregulation of the transcriptome that affects a fraction of the *Drosophila* homologues of human EwS signature genes [17]. Indeed, such a massive transcriptional dysregulation in *Drosophila* must be largely GGAA μ Sat-independent because, unlike the human genome that contains thousands of GGAA μ Sats of optimal length (i.e. 18 $\times$ to 26 $\times$ (GGAA) [18,19]), the *Drosophila* reference genome (r6.40) contains less than one hundred of which the longest is 9 $\times$ (GGAA). However, as in EwS cells, human EWS-FLI1 can also convert silent GGAA μ Sats into neoenhancers in *Drosophila* as shown with transgenic lines carrying 20 GGAA repeats upstream a heat-shock minimal promoter (HSP) followed by the yellow fluorescent protein (YFP)-coding sequence, (20 $\times$ (GGAA) μ Sat>YFP [17]). Moreover, human EWS-FLI1 is also capable of outcompeting ETS transcription factors and causing aberrant alternative splicing in *Drosophila* [17].

Altogether, these results showed that genetically engineered *Drosophila* strains expressing EWS-FLI1_{FS} may serve as genetically tractable study models to investigate EWS-FLI1 function. However, the question of how the FStail reduces toxicity remained open and so was the quest for a *Drosophila* line capable of expressing the unmodified full-length EWS-FLI1 protein.

Here, we show that EWS-FLI1_{FS}'s reduced toxicity is owed to decreased protein concentration brought about by the FStail. Building on this observation, we have generated new lines carrying transgenes designed to downregulate transcription or translation, hence, avoiding the need for FStail tagging. Using these lines, it is possible to achieve a wide range of full-length, unmodified EWS-FLI1 protein levels that result in a complex phenotypic series. From these series, we have found that activities of EWS-FLI1 like assembly of neo-enhancers at GGAA μ Sats and GGAA μ Sat-independent transcriptomic dysregulation respond differently to varying amounts of EWS-FLI1.

Altogether, our results reveal a complex, phenotype-specific, dose–response relationship between the amount of EWS-FLI1 protein and its neomorphic functions, which in turn implies fundamental differences in the corresponding underlying mechanisms.

2. Results

2.1. The C-terminal peptide of the EWS-FLI1_{FS} variant results in decreased protein levels

The frameshift variant EWS-FLI1_{FS} was discovered as a serendipitous mutant that circumvented embryonic lethality, which was presumably caused by leaky expression from the Upstream Activating Sequence (UAS) of *UAS>EWS-FLI1* (i.e. expression in the absence of any Gal4 driver). Functional assays showed that rather than to the loss of the 69 amino acid C-terminal peptide of EWS-FLI1, the lower toxicity of EWS-FLI1_{FS} was owing to a neomorphic property of the new FStail [17]. We did not know the nature of such a neomorphic property, but a simple hypothesis was that it could be owing to reduced protein expression and/or stability.

To test this hypothesis, we quantified the effect of FStail tagging on YFP. To this end, we generated transgenic animals carrying *UAS>YFP* and *UAS>YFP_{+FStail}*, a fusion of the FStail to the C-terminal end of YFP. By driving *UAS>YFP* and *UAS>YFP_{+FStail}* expression from the *nub>Gal4* driver, we found that both YFP_{+FStail} green fluorescence intensity, as measured by confocal microscopy, as well as YFP_{+FStail} protein levels, as quantified by western blot, are extremely reduced compared with those of unmodified YFP in salivary glands and wing imaginal discs (figure 1a,b).

Because the YFP and YFP_{+FStail} transgenes were inserted at the same genomic site (attp40 [20]), by ϕ C31 integrase-mediated transgenesis [21] and were both expressed by *nub>Gal4*, transcription levels are expected to be comparable. That is unless the FStail-coding sequence affects transcription, which could indeed explain the differences observed at the protein level. To test this hypothesis, we quantified YFP and YFP_{+FStail} transcripts by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) in salivary glands and wing imaginal discs. We found no significant differences in the level of mRNA from each variant in either tissue (figure 1c). Altogether, these results show that the FStail has no significant effect on transcription but affects protein expression and/or stability such that it results in a strong reduction in the concentration of a protein carrying this C-terminal tag. These results substantiate the hypothesis that EWS-FLI1_{FS}'s reduced toxicity is owing to a significant reduction in protein concentration caused by the FStail.

To further substantiate this conclusion, and to get a direct estimate of the effect of the FStail in EWS-FLI1, we generated the EWS-FLI1_{+FStail} transgene, an FStail-tagged version of full-length EWS-FLI1. We found that in stark contrast with unmodified EWS-FLI1, EWS-FLI1_{+FStail} transgenes were obtained at the standard rate of more than five per 200 injected embryos, thus confirming that the sole addition of the FStail can circumvent the toxicity issue of unmodified EWS-FLI1 (figure 2).

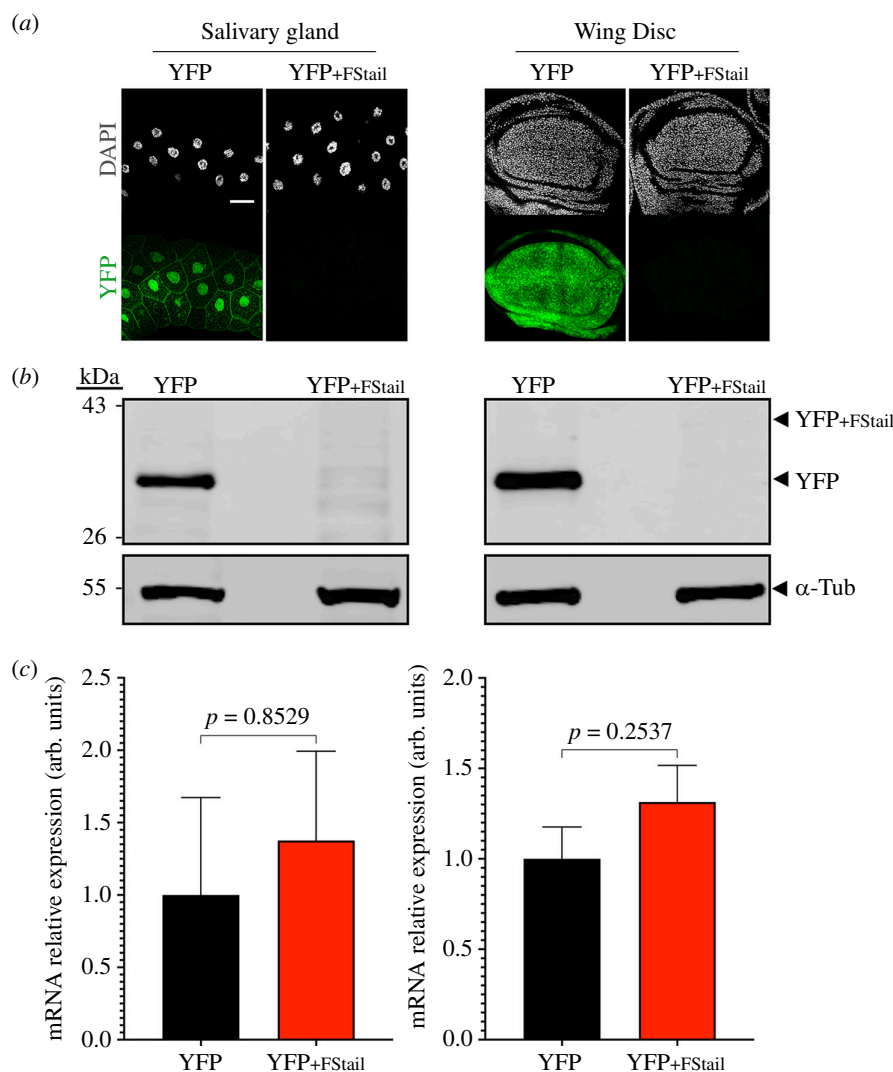


Figure 1. The FStail reduces protein levels without affecting transcription. YFP protein levels observed by fluorescence (a) and western blot (b), and YFP mRNA quantified by RT-qPCR (c) in salivary glands (left) and wing imaginal discs (right) from *nub>Gal4/UAS>YFP*; (YFP) and *nub>Gal4/UAS>YFP_{+FStail}* (YFP_{+FStail}) larvae. Scale bar = 50 μ m.

2.2. Generating *Drosophila* strains carrying unmodified, full-length EWS-FLI transgenes

Having shown that EWS-FLI_{1FS}'s reduced toxicity is owing to a significant reduction in protein concentration caused by the FStail, we decided to test if the same effect could be achieved with transgenes designed to downregulate translation or transcription, so that full-length, untagged EWS-FLI₁ could be expressed. We used two approaches to this aim.

The first approach was to downregulate translation by placing an EWS-FLI₁ coding sequence lacking its own internal ribosome entry site as the secondary open reading frame (ORF) in a bicistronic transcript encoding mCherry as primary ORF. Under these conditions, translation of the secondary ORF is so severely compromised that it has been estimated to be essentially null in the absence of Gal4, hence avoiding potential toxicity issues caused by leaky transcription during transgenesis [22–25].

The second approach was based on P-element-mediated transgenesis [26,27]. Unlike precise targeting to predetermined genomic sites by ϕ C31 integrase [21], P-element transgenes insert nearly randomly in the genome and are, therefore, subjected to unpredictable position effects that may significantly affect transcription. We reasoned that this inherent drawback could be to our advantage because it may be used to select for insertions in genomic regions in which leaky *UAS>EWS-FLI₁* expression is downregulated below toxic levels.

We found that both approaches work well to generate *Drosophila* lines carrying unmodified, full-length EWS-FLI₁ transgenes. ϕ C31 integrase-mediated transgenic lines carrying the bicistronic construct bcEWS-FLI₁ were obtained at the standard rate of more than five per 200 injected embryos, thus showing that this strategy abrogates EWS-FLI₁ embryo toxicity. P-element-mediated transgenesis was much less efficient, producing a single pEWS-FLI transgenic line out of 180 injected embryos (<1%), which is one order of magnitude below the normal rate of P-element-mediated transgenesis (>10%) (figure 2). Such a low rate is not surprising, however, since only the small fraction of P-element insertions subjected to position effects strong enough to abrogate toxic, leaky EWS-FLI₁ expression can be expected to generate viable transgenic animals. DNA sequencing of the new transgenic lines obtained from both approaches confirmed the presence of non-mutated, full-length EWS-FLI₁, as intended, thus validating the two strategies.

		E/T
ϕC31 integrase	EWS-FLI1	200/0
	EWS-FLI1FS	200/>5
	EWS-FLI1+FS _{tail}	200/>5
	bcEWS-FLI1	200/>5
P-element	empty vector	130/20
	pEWS-FLI1	180/1

Figure 2. *Drosophila* transgenic lines expressing full-length human EWS-FLI type 1 protein (EWS-FLI₁). The number of injected embryos ‘E’ and transgenic animals recovered ‘T’ in one round of injection. Transgenesis experiments with full-length EWS-FLI₁, the published frameshift variant (UAS>EWS-FLI_{1FS}), full-length EWS-FLI₁ fused with the C-terminal peptide created by the EWS-FLI_{1FS} frameshift (UAS>EWS-FLI_{1+FS_{tail}}), and a bicistronic construct carrying mCherry followed by full-length EWS-FLI (UAS>bcEWS-FLI₁) were carried out using ϕC31 integrase. The P-element expressing full-length EWS-FLI₁ (UAS>pEWS-FLI₁) was obtained by P-element-mediated transgenesis.

2.3. The collection of full-length EWS-FLI transgenes causes a range of developmental phenotypes

As a first step to characterize the new transgenic lines, we investigated their effect on development upon expression from a variety of Gal4 drivers (figure 3a). We found that as far as the lethality phase, wing disc tumours and cuticular phenotypes in adult flies are concerned, the new variants cause a wide range of expressivity levels.

Top of the range is pEWS-FLI₁ that is lethal when expressed from any of the selected Gal4 drivers, including *longGMR>Gal4* and *sal^{EPv}>Gal4* with which all of the other transgenes are viable. At the bottom of the range is the bcEWS-FLI₁ variant that yields pupal lethality with *da>Gal4*, *Act5C>Gal4* and *48Y>Gal4*, and adult viability with *Mef2>Gal4* and *twi>Gal4*, while the same Gal4 drivers result in embryonic lethality with all other EWS-FLI₁ versions. Moreover, surviving adults carrying bcEWS-FLI₁ and *Mef2>Gal4* or *twi>Gal4* present no noticeable cuticular or lifespan phenotypes. EWS-FLI_{1FS} and EWS-FLI_{1+FS_{tail}} have an intermediate effect, stronger than bcEWS-FLI₁ and weaker than pEWS-FLI₁ (figure 3a). EWS-FLI_{1FS} and EWS-FLI_{1+FS_{tail}} are largely indistinguishable except for the effect on eye roughness and size when driven from *sev>Gal4*, which is stronger in the case of EWS-FLI_{1+FS_{tail}} (figure 3b, row 1).

To ameliorate the strong lethal effect of pEWS-FLI₁, we also generated individuals carrying this construct together with a second transgene, *P[tubP>GAL80^{ts}]*, which expresses the temperature-sensitive allele of the yeast Gal80 protein from the *alphatubulin84B* promoter. Gal80 is an inhibitor of the transcriptional activation domain of Gal4. When expressed in *Drosophila*, Gal80^{ts} is fully functional at 17°C, but it is inactive at 29°C. By raising individuals carrying the pEWS-FLI₁ and *P[tubP>GAL80^{ts}]* transgenes at 25°C we hoped to achieve a partial inactivation of Gal80 function that would in turn partially inhibit Gal4, hence lowering EWS-FLI₁ expression from UAS>pEWS-FLI₁. We refer to this combination as pEWS-FLI₁+Gal80@25.

The stronger effect of pEWS-FLI₁ compared with that of EWS-FLI_{1FS} and EWS-FLI_{1+FS_{tail}} is clearly reflected in their tumorigenic potential in wing imaginal discs. As published [17], EWS-FLI_{1FS} causes wing imaginal disc tumours when driven by *nub>Gal4*, but not by *sal^{EPv}>Gal4*, which is expressed at a later stage in development and over a much smaller area in the wing disc. The same is true for EWS-FLI_{1+FS_{tail}}. In contrast, *sal^{EPv}>Gal4*-driven expression is sufficient for pEWS-FLI₁ to cause wing imaginal disc tumours, and *nub>Gal4*-driven expression of pEWS-FLI₁+Gal80@25 yields even larger tumours in the rare escapers (figure 3b, rows 2 and 3).

2.4. Upregulation of transcription from (GGAA)_nμSat and tissue toxicity present a positive linear correlation with a wide range of EWS-FLI protein concentrations

To further characterize the new full-length EWS-FLI₁ lines, we quantified the efficiency of each transgene at activating transcription from (GGAA)_nμSats, which is one of the hallmarks of the EWS-FLI₁ oncogene and is known to be critical for EwS tumorigenesis [9,18,28,29]. The 20×(GGAA)_nμSat>YFP transgene was used to track (GGAA)_nμSat activation [17]. We chose to carry out these studies on third instar larval salivary glands because they are among the tissues that are more resilient to the detrimental effects of EWS-FLI₁ [17]. EWS-FLI₁ transgenes were expressed from *sal^{EPv}>Gal4* that, like other Gal4 drivers derived from the P{GawB} vector (Flybase FBtp0000352), contains a cryptic larval salivary-gland-specific enhancer element [30].

Using confocal microscopy, we found that the extent of YFP upregulation induced by the collection of EWS-FLI₁ transgenes from the 20×(GGAA)_nμSat>YFP construct spans over a wide range (figure 4a,b). At one extreme, pEWS-FLI₁ and pEWS-FLI₁+Gal80@25 cause the strongest effect (with means ± s.d. of 85 ± 20 arb. units, and 101 ± 26 arb. units, respectively). At the other extreme, bcEWS-FLI₁ induces very low, but measurable levels (10 ± 12 arb. units). EWS-FLI_{1FS} results in intermediate levels of (GGAA)_nμSat transcriptional activation (39 ± 11 arb. units): 386% ($p = 4.4 \times 10^{-2}$) and 46% ($p = 3.6 \times 10^{-2}$) of that caused by bcEWS-FLI₁ and pEWS-FLI₁, respectively. These results show that the extent of YFP activation induced by each transgene can be ordered into a range as follows: control < bcEWS-FLI₁ < EWS-FLI_{1FS} < pEWS-FLI₁+Gal80@25 ≈ pEWS-FLI₁.

(a)

GAL4	Expression	pEWS-FLI1	EWS-FLI1FS	EWS-FLI1+Fstail	bCEWS-FLI1
<i>da>GAL4</i>	Ubiquitous	Embryonic Lethal	Embryonic Lethal	Embryonic Lethal	Pupal Lethal
<i>act5C>Gal4</i>	Ubiquitous	Embryonic Lethal	Embryonic Lethal	Embryonic Lethal	Pupal Lethal
<i>48Y>GAL4</i>	Endoderm	Embryonic Lethal	Embryonic Lethal	Embryonic Lethal	Pupal Lethal
<i>Mef2>GAL4</i>	Mesoderm	Embryonic Lethal	Embryonic Lethal	Embryonic Lethal	Viable
<i>twi>GAL4</i>	Mesoderm	Embryonic Lethal	Embryonic Lethal	Embryonic Lethal	Viable
<i>nub>GAL4</i>	Wing discs / CNS	Embryonic Lethal	Early Pupal Lethal Tumorous wing disc	Early Pupal Lethal Tumorous wing disc	Viable very short lived
<i>ey>GAL4</i>	Eye discs	Larval/Pupal Lethal Headless/eyeless escapers	Pupal Lethal Headless/eyeless escapers	Pupal Lethal Headless/eyeless escapers	Viable
<i>longGMR>GAL4</i>	Eye discs	Larval Lethal	Viable Rough and pigmentless eyes	Viable Rough and pigmentless eyes	Viable
<i>sal^{EPV}>GAL4</i>	Wing discs	Early Pupal Lethal Tumorous wing disc	Viable Abnormal wing	Viable Abnormal wing	Viable
<i>sev>GAL4</i>	Eye discs	Viable Extremely rough eye	Viable Rough eye	Viable Rough eye	Viable

(b)

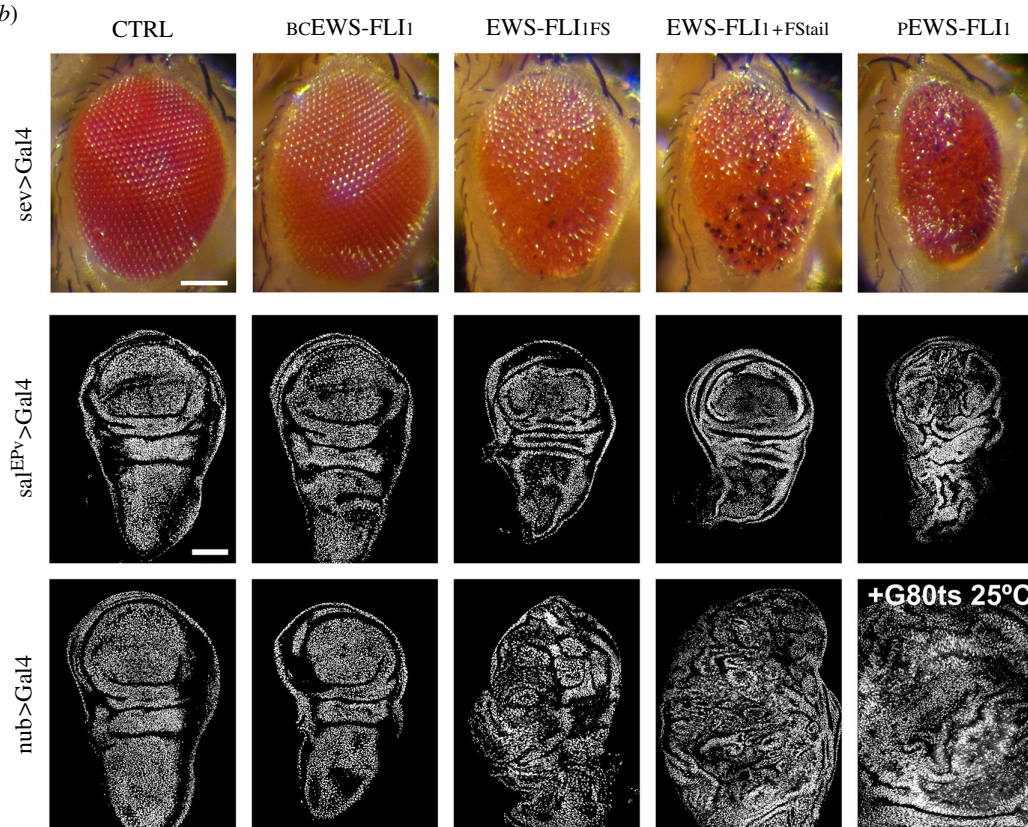


Figure 3. *Drosophila* transgenic lines expressing full-length human EWS-FLI₁ generate a wide range of developmental phenotypes. (a) Summary of developmental phenotypes observed in each EWS-FLI₁ transgenic line, colour coded with red for embryonic lethal, yellow for larval or pupal lethal and cyan for adult viable. (b) Adult eye and third instar wing imaginal discs from individuals expressing the indicated versions of EWS-FLI₁ in ommatidia (*sev>Gal4*), central region of the wing pouch (*sal^{EPV}>Gal4*) or wing pouch and hinge region (*nub>Gal4*). The *nub>Gal4/UAS>EWS-FLI1FS* and *nub>Gal4/UAS>EWS-FLI1+Fstail* wing disc tumours shown are extreme examples to demonstrate the extent of transformation that can be induced by these transgenes; milder phenotypes have been observed. Most *nub>Gal4/UAS>pEWS-FLI1*; *P[tubP>GAL80^{ts}]/+* individuals reared at 25°C die at early stages of development; only two third instar larval escapers that we have observed presented large wing disc tumours (+Gal80 ts 25°C). Scale bars: 0.1 mm or 100 µm for adult eyes and wing discs, respectively.

We next quantified salivary gland sizes, measured as the area of the largest confocal z-section, as a proxy for tissue toxicity (figure 4a,b). We found that salivary gland size is indistinguishable between wild-type and *bCEWS-FLI1* larvae (49k ± 10k px² and 48k ± 9k px², respectively; *p* = 0.9), but is severely compromised by *pEWS-FLI1*, alone or in combination with *P[tubP>GAL80^{ts}]* (13k ± 4k px² and 22k ± 7k px², respectively). Differences between *pEWS-FLI1* and *pEWS-FLI1+Gal80@25* are not significant, although *pEWS-FLI1+Gal80@25* yields a notable number of salivary glands with an intermediary size between that of *pEWS-FLI1* and *EWS-FLI1FS*. As before, *EWS-FLI1FS* results in intermediate levels of salivary gland sizes (30k ± 7k px²) that

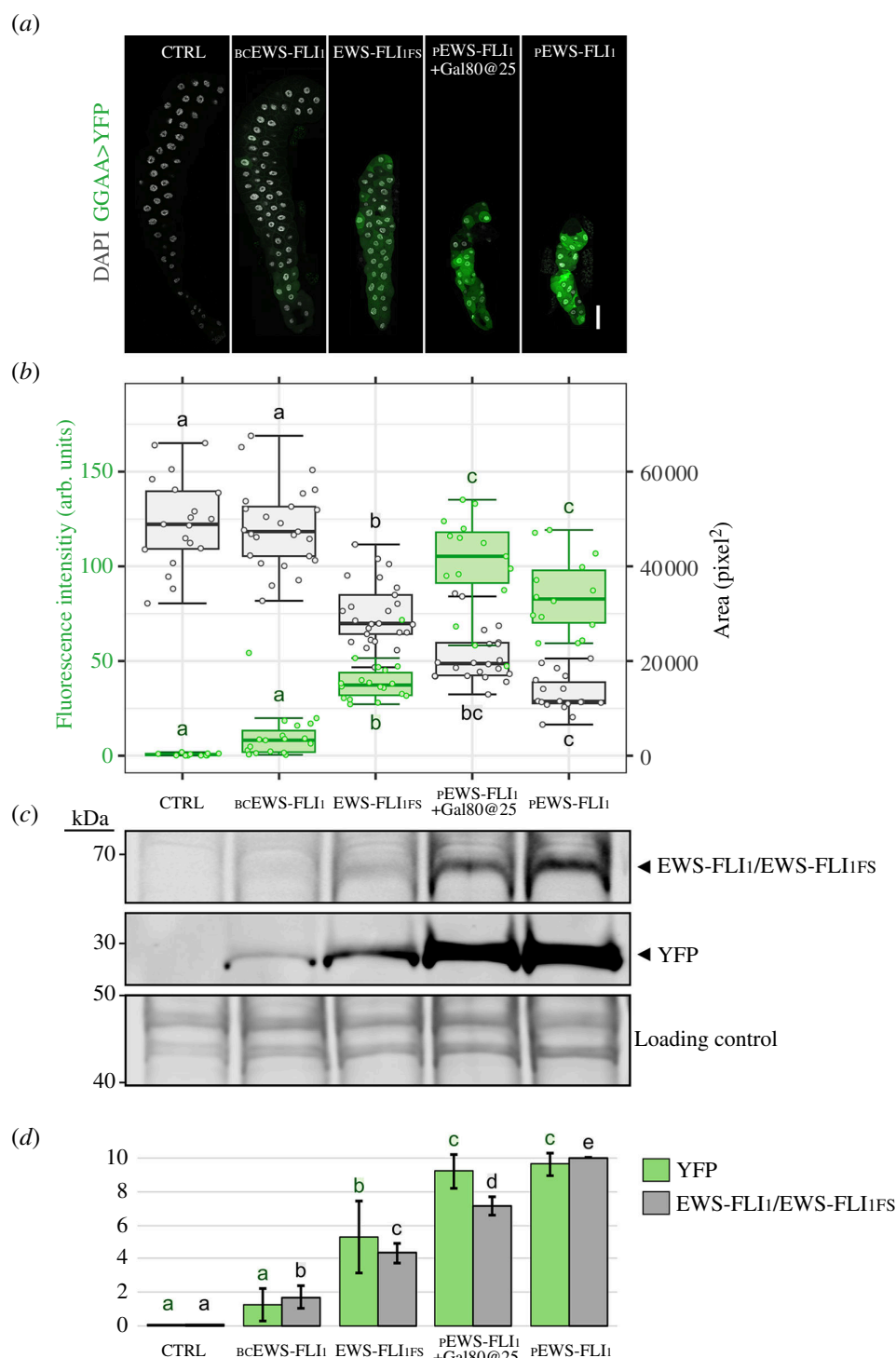


Figure 4. The expressivity of EWS-FLI1-induced phenotypes correlates with protein concentration. (a) Representative salivary glands showing YFP expression from $20\times(GGAA)>YFP$ (green) and co-stained with DAPI (grey). Scale bar: 100 μ m. (b) Quantification of YFP fluorescence (green) and salivary gland size (grey) (arb. units). Letters indicate statistically significant differences between group means (Kruskal–Wallis and Dunn's test, $p < 0.05$). (c) Western blot showing protein levels of EWS-FLI1 and YFP; means $\pm$ s.d. of relative protein band intensities from three replicates are represented in (d). Letters indicate statistically significant differences between group means (ANOVA and Tukey's HSD, $p < 0.05$). Experimental conditions include: control ($sal^{EPV}>Gal4/+$), bcEWS-FLI1 ($sal^{EPV}>Gal4/UAS>bcEWS-FLI1$; $20\times(GGAA)>YFP/+$), EWS-FLI1FS ($sal^{EPV}>Gal4/UAS>EWS-FLI1FS$; $20\times(GGAA)>YFP/+$), pEWS-FLI1 ($sal^{EPV}>Gal4/UAS>EWS-FLI1$; $20\times(GGAA)>YFP/+$) and pEWS-FLI1+Gal80@25 ($sal^{EPV}>Gal4/UAS>EWS-FLI1$; $P[tubP>GAL80^s]/20\times(GGAA)>YFP$).

are approximately 62% ($p = 5.6 \times 10^{-4}$) and 230% ($p = 3.7 \times 10^{-4}$) the size of those from bcEWS-FLI1 and pEWS-FLI1 individuals, respectively. The absence of detrimental effects of bcEWS-FLI1 on salivary glands is interesting considering that it is sufficient for driving transcription from the $20\times(GGAA)\mu Sat>YFP$. These results show that, altogether, the transgenes induce toxicity as follows: control $\approx$ bcEWS-FLI1 $<$ EWS-FLI1FS $<$ pEWS-FLI1+Gal80@25 $\leq$ pEWS-FLI1.

We then carried out western blot to quantify the level EWS-FLI1 protein expressed through each of the EWS-FLI1 transgenes (figure 4c). As compared with EWS-FLI1FS, the levels of EWS-FLI1 protein are about 1.5 and 2.0 times higher in pEWS-FLI1+Gal80@25 and pEWS-FLI1, respectively, thereby validating the approach of raising $P[tubP>GAL80^s]$ -carrying individuals at 25°C to tune down EWS-FLI1 expression from $UAS>pEWS-FLI1$. In contrast, EWS-FLI1 is hardly detectable in bcEWS-FLI1

individuals. Thus, we found that EWS-FLI levels linearly increase along the following range: control < bcEWS-FLI₁ < EWS-FLI₁FS < < pEWS-FLI₁+Gal80@25 < pEWS-FLI₁.

Using the same western blots, we re-assessed the levels of YFP expression from the 20×(GGAA)_μSat>YFP brought about by each of the EWS-FLI₁ transgenes and found them to be largely consistent with those observed by confocal microscopy. This includes the fact that despite approximately 40% higher EWS-FLI₁ levels in pEWS-FLI₁ compared with pEWS-FLI₁+Gal80@25 individuals, YFP expression levels are similar, hence suggesting that, as far as GGAA_μSat transcriptional activation is concerned, EWS-FLI₁ levels are near saturation in pEWS-FLI₁+Gal80@25 individuals.

Altogether, these results demonstrate a tight correlation of EWS-FLI protein levels to both their detrimental effect on salivary gland development and their effect on upregulating transcription from (GGAA)_μSats.

2.5. GGAA_μSat-independent transcriptome dysregulation is not linearly correlated with EWS-FLI protein levels

In spite of its frame-shifted C-terminal tail, EWS-FLI₁FS expression recapitulates in *Drosophila* key oncogenic functions of EWS-FLI₁ like transcriptional dysregulation of hundreds of genes including a fraction of the orthologues of known EWS-FLI's targets in human cells [17]. The question, however, remains open as to whether unmodified full-length EWS-FLI₁ protein could bring about a transcriptomic signature that is closer to that of EwS tumours than the EWS-FLI₁FS's signature.

To address this question, we used Affymetrix microarrays to perform a genome-wide transcriptomic analysis of salivary glands carrying the bcEWS-FLI₁, and pEWS-FLI₁+Gal80@25 transgenes that express very different levels of full-length EWS-FLI₁ (electronic supplementary material, table S1). EWS-FLI₁FS, salivary glands were also included as reference. For each condition, we determined the total number of dysregulated transcripts, quantified the enrichment of two published EwS signatures, and assessed the upregulation of neural genes, a feature that has been reported upon expression of EWS-FLI in human cells [31,32].

We found that although the total number of genes affected in pEWS-FLI₁+Gal80@25 (*n* = 879) and EWS-FLI₁FS (*n* = 849) is larger than in bcEWS-FLI₁ (*n* = 561), all three conditions cause a strong dysregulation of transcription (figure 5a) and the resulting transcriptomes are all highly significantly enriched for the published EWS-FLI₁FS *Drosophila* signature [17] (figure 5b). Moreover, with regards to upregulated genes, approximately one-third of the 404 genes of the Hancock and Lessnick signature [33] were significantly enriched at the top of all three gene set enrichment analysis (GSEA)-ranked *Drosophila* datasets: EWS-FLI₁FS (*n* = 115; FDR *q* = 0.032), bcEWS-FLI₁ (*n* = 117; FDR *q* = 0.004) and pEWS-FLI₁+Gal80@25 (*n* = 125; FDR *q* = 0.030) (figure 5c). Similar results were obtained with the 277 genes of the Kauer *et al.* signature [34]: EWS-FLI₁FS (*n* = 74; FDR *q* = 0.006), bcEWS-FLI₁ (*n* = 82; FDR *q* = 0.002) and pEWS-FLI₁+Gal80@25 (*n* = 101; FDR *q* = 0.02). However, differences between bcEWS-FLI₁ and the other conditions became apparent with regard to the upregulation of genes associated with neural development, a trait of EWS-FLI₁ activity in human cells that is also observed in *Drosophila* salivary glands expressing EWS-FLI₁FS [17]. Gene ontology (GO) biological process 'nervous system' is well represented in both the pEWS-FLI₁+Gal80@25 and the EWS-FLI₁FS transcriptomes (*n* = 121, *p* = 6.8 × 10⁻¹⁸; and *n* = 69, *p* = 8.2 × 10⁻⁵), but is not significantly enriched in the bcEWS-FLI₁ transcriptome with only 13 genes from the same GO being upregulated.

These are remarkable results as far as bcEWS-FLI₁ is concerned, taking into account the extremely low levels of EWS-FLI₁ protein and the lack of toxicity caused by this construct that nonetheless is nearly as good as EWS-FLI₁FS at driving GGAA_μSat-independent transcriptome dysregulation. Altogether, these results show that, unlike GGAA_μSat-dependent transcriptional upregulation, GGAA_μSat-independent transcriptome dysregulation, including ectopic expression of orthologues of human EwS signature genes, is not linearly correlated with EWS-FLI₁ protein levels.

3. Discussion

EWS-FLI cytotoxicity has proven a great hindrance in the development of genetically tractable animal models [15]. In *Drosophila*, a very clear distinction can be made between toxicity caused by UAS>EWS-FLI₁ in the absence of Gal4 and toxicity caused by Gal4-driven expression. The former, which accounts for the extreme difficulty in generating transgenic lines, can be circumvented by tagging the EWS-FLI₁ fusion with the FStail [17]. The latter, however, is only partially reduced by the FStail. Here, we show that the FStail works by reducing protein levels, which in turn suggests that FStail tagging may facilitate the implementation of other animal models expressing EWS-FLI and perhaps other lethal oncogenic fusion proteins.

Building on this observation, we have generated new lines that express full-length, unmodified EWS-FLI₁ from transgenes that were engineered to reduce protein expression to a greater or lesser extent, hence circumventing the need for FStail tagging.

Using the new EWS-FLI₁ lines, it is possible to generate a phenotypic series, from which we have learned that the neomorphic effects brought about by EWS-FLI₁ that we have studied can be categorized in three classes on the basis of how they correlate with EWS-FLI₁ protein levels. The first includes the assembly of neo-enhancers at GGAA_μSats that presents a positive linear correlation over a wide range of EWS-FLI₁ protein levels, from the lowest, (bcEWS-FLI₁) up to the highest (pEWS-FLI₁+Gal80@25 and pEWS-FLI₁). The second includes massive GGAA_μSat-independent transcriptional dysregulation including, notably, a significant fraction of orthologues of human EwS signature' genes. Rather counterintuitively, despite the order of magnitude lower level of expressed EWS-FLI₁, the bcEWS-FLI₁ transgene is able to significantly dysregulate hundreds of genes albeit with lower fold changes. Finally, transcriptional upregulation of neural genes, a landmark of EWS-FLI activity in human cells, is only marginal in bcEWS-FLI₁ compared with EWS-FLI₁FS and pEWS-FLI+Gal80@25. These results reveal a complex, phenotype-specific correlation between EWS-FLI₁ levels and the expressivity of its phenotypes, that, in turn, implies fundamental differences in the mechanisms that drive these phenotypes. In particular, our results suggest that GGAA_μSat-dependent and GGAA_μSat-independent transcriptional upregulation present different kinetics with regard to EWS-FLI protein concentration, opening a new perspective on EWS-FLI-dependent dysregulation of transcription.

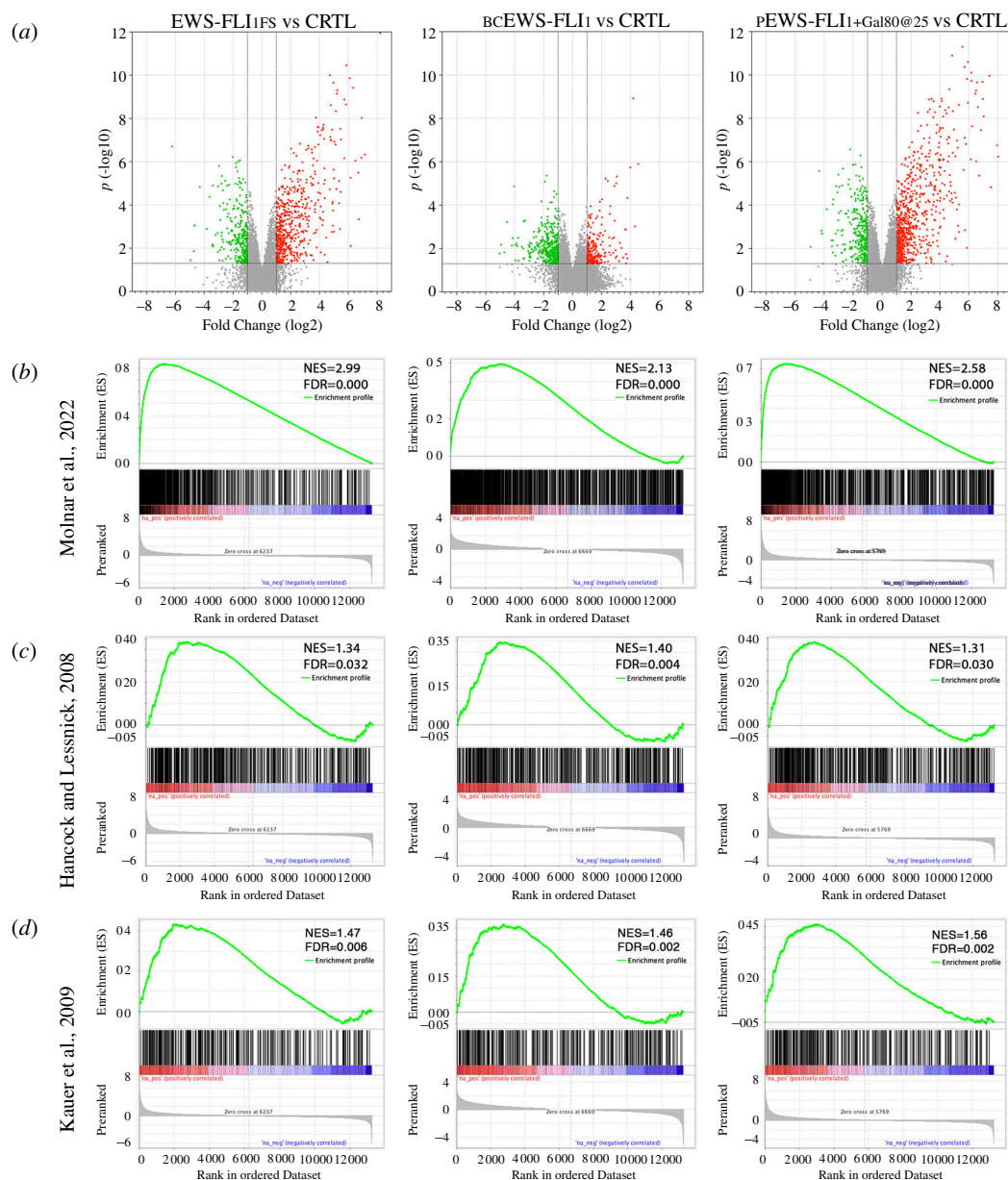


Figure 5. GAApSat-independent transcriptome dysregulation is not linearly correlated with EWS-FLI protein levels. (a) Volcano plots showing changes in gene expression levels in EWS-FLI_{1FS} (*sal^{EPV}>Gal4/UAS>EWS-FLI_{1FS}*), BC-EWS-FLI₁ (*sal^{EPV}>Gal4/UAS>BC-EWS-FLI₁*), and pEWS-FLI₁+Gal80@25 (*sal^{EPV}>Gal4/UAS>EWS-FLI₁; P[tubP>GAL80^{ts}]/+*) compared with control (*sal^{EPV}>Gal4/+*) samples. Red and green dots represent genes that are significantly ($p < 0.05$) up (FC > 2) and downregulated (FC < -2) genes, respectively. (b–d) Gene set enrichment analysis showing the *Drosophila* Molnar et al., 2020 [17] (b) and the human Hancock and Lessnick, 2008 [33] (c) and Kauer et al., 2009 [34] (d) EWS signatures to be significantly enriched (FDR < 0.25) in the ranked datasets of EWS-FLI_{1FS}, BC-EWS-FLI₁ and pEWS-FLI₁+Gal80@25 compared with control.

Despite a low mutational burden characteristic of paediatric tumours, EwS displays high levels of intratumoral heterogeneity largely mediated by alterations to the epigenetic state. Several independent findings have suggested that differences in EWS-FLI levels are a determinant of different cellular phenotypes that may have direct implications for EwS progression [35–43]. Altogether, our results highlight the functional relevance of EWS-FLI expression levels and provide experimental tools to further investigate in *Drosophila* the molecular pathways affected by the EWS-FLI ‘high’ and ‘low’ states observed in human tumours.

4. Material and methods

4.1. Fly stocks

The following fly strains were used in this study: *sal^{EPV}>Gal4* [44], *tub>Gal80^{ts}* (BDSC#: 7018), *UAS>EWS-FLI_{1FS}*, *UAS>EWS-FLI₁+F_{tail}*, *UAS>uORF-EWS-FLI₁* (*UAS>BC-EWS-FLI₁*), *P[UAS>EWS-FLI₁]* (pEWS-FLI₁) and 20×(GGAA)>YFP. All Gal4 lines in figure 3a are described in Flybase. The wild-type strain used was (*w¹¹¹⁸*). All crosses, including controls, were maintained at 25°C.

Table 1. Primers used in this article.

name	sequence
cloning	
El-Cherry-FW	AGGGAATTGGGAATTCATGGCAACTAGC
Cherry-RV	GGCCATGTTATTATGCGGTACCAGAACCTTTG
EWS-FLI-FW	TAATAACATGGCCTCCACCGATTATTCCACGTAC
EWS-FLI-XhoI-RV	TAGAGGTACCCTCGAGTCAGTAGTAGGAGCCCAG
qPCR	
YFP-1-FW	AAGCAGAAGAACGGCATCAA
YFP-1-RV	GGGGGTGTTCTGCTGGTAGT
YFP-2-FW	ATGGTGAGCAAGGGCGAG
YFP-2-RV	TGAACTTGTTGGCCGTTTACGT
RpL32-b-FW	TGTGAATTTTCCTTGTCGCGT
RpL32-b-RV	TGGATAGAGATACATTACGCATA
sequencing	
HSP-FW	GCAACTACTGAAATCTGCCAAG
EWS500-FW	ACCCGATGCAACCACTGA
EWS1100-RV	CAATGCCATGGAAGTGG
SV40	GGCATTCCACCACTGCTCCC

4.2. Genotypes and crossing schemes

For the microarray expression profiling experiment, females *w*; *sal^{EPv}>Gal4* were crossed to males *w¹¹¹⁸*, to males *w*; *UAS>EWS-FLI_{1FS}/CyO* and to males *w*; *UAS>bcEWS-FLI₁/CyO* to obtain *sal^{EPv}>Gal4/+* (CTRL), *sal^{EPv}>Gal4/UAS>EWS-FLI_{1FS}* and *sal^{EPv}>Gal4/UAS>bcEWS-FLI₁* samples. To obtain FL samples, females *w*; *sal^{EPv}>Gal4*; *P[tubP>GAL80^{ts}]* were crossed to males *w¹¹¹⁸*; *P[UAS>EWS-FLI₁]/CyO*, *S*, *Tb*.

For quantification of 20×(GGAA)>YFP fluorescence, EWS-FLI₁ protein levels and salivary gland size, females *w*; *sal^{EPv}>Gal4*; 20×(GGAA)>YFP/TM6B were crossed to males *yw*, *UAS>EWS-FLI_{1FS}/CyO*, to males; *UAS>bcEWS-FLI₁*, and to males *w*; *P[UAS>EWS-FLI₁]/CyO*, *S*, *Tb*, to obtain *sal^{EPv}>Gal4/UAS>EWS-FLI_{1FS}*; 20×(GGAA)μ*Sat*>YFP/+, *sal^{EPv}>Gal4/UAS>bcEWS-FLI₁*; 20×(GGAA)>YFP/+, and *sal^{EPv}>Gal4/P[UAS>EWS-FLI₁]*; 20×(GGAA)>YFP/+, respectively. For pEWS-FLI₁+Gal80@25, females *w*; *sal^{EPv}>Gal4*; *P[tubP>GAL80^{ts}]* were crossed to males *P[UAS>EWS-FLI₁]/CyO*; 20×(GGAA)>YFP/TM6B to obtain *sal^{EPv}>Gal4/P[UAS>EWS-FLI₁]*; 20×(GGAA)>YFP/*P[tubP>GAL80^{ts}]*. All these crosses were maintained at 25°C. Wandering third instar larvae from each cross were dissected, fixed and mounted to visualize the 20×(GGAA)>YFP expression in the salivary glands under the confocal microscope. All the experiments were performed blind. All individuals analysed were between 4 and 6 days old.

4.3. Immunohistochemistry and microscopy

Immunostaining of salivary glands and wing discs was performed as follows: salivary glands and wing discs were dissected in phosphate-buffered saline (PBS), fixed for 30 min in 4% formaldehyde with 0.3% TritonX-100, washed three times in PBS-0.3% Triton X-100 (PBST) for 10 min per wash. DNA was stained with DAPI. Salivary glands and wing discs were mounted in Vectashield (molecular probes). Immunostaining images were acquired with a Leica TCS SP8 scan unit coupled to a microscope and managed by Leica Application Suite X (LAS X) software. The objective used was HC PL APO CS2 40×/1.30 OIL and images were acquired at zoom 1. Fluorophores 405 and 488 were excited with lasers diode and argon, respectively. Image processing was carried out with Fiji. All shown immunofluorescence images correspond to a single Z.

4.4. Quantification and statistical analysis

Quantification of YFP fluorescence levels was carried out using ImageJ to calculate the mean grey values of each region of interest (ROI) in a focal plane per salivary gland acquired with an SP8 Leica confocal image microscope.

Quantification of salivary gland size was performed in Inkscape 1.2.2 using Bezier curves to manually outline individual salivary glands from the distal end till the meeting point of the individual ducts at the proximal end and measure the area of the resulting shape through the visualized path extension.

Both salivary gland size and YFP intensity were represented in boxplots, *p*-values for salivary gland size and GGAAμ*Sat* activation were calculated in R version 4.2.3. by Dunn's test with Holm's method for *p*-value adjustment after rejection of Kruskal–Wallis one-way ANOVA and compact letter display (CLD) was used to represent *p*-values on boxplots. Values of *p* for western blot band intensities were calculated by Tukey's HSD after the rejection of one-way ANOVA.

4.5. Cloning and transgenesis

The pUAS^t P-element EWS-FLI₁ construct was made by cloning the *EcoRI-XhoI* fragment from pUC-GW-Kan EWS-FLI₁ TI that contains the full EWS-FLI₁ ORF in the original pUAS^t vector [45]. The pUAS^t-EWS-FLI₁+FS^tail construct was made by introducing a *BbsI* site into the pUC-GW-Kan EWS-FLI₁ TI. This was achieved by replacing the *BsrGI-XhoI* fragment with a gBlock (IDT) containing the *BbsI* site at the terminus of the ORF. Subsequently, another gBlock (IDT) with the FS sequence was inserted in-frame with EWS-FLI₁ into the *BbsI* and *XhoI* sites. The resulting fragment was subcloned into the *EcoRI* and *XhoI* sites of *pUAS^t-attB* (DGRC Stock 1419 [46]). The pUAS^t-uORF-EWS-FLI₁ construct was made by PCR amplification of the mCherry ORF from the *pLT3-Dam* vector [24] and the EWS-FLI₁ ORF from pUC-GW-Kan EWS-FLI₁ TI. Subsequently, both fragments were fused and cloned by in-fusion (Takara) into *EcoRI* and *XhoI* sites of the *pUAS^t-attB* vector.

All PCR amplifications were performed using KOD polymerase (Merck). Cloning was performed by using In-Fusion Snap Assembly (Takara). Primers were ordered to Sigma. DNA fragments were ordered to IDT.

Transgenic fly lines were generated by BestGene Inc. (φC31 integrase-mediated transgenesis) using the Bloomington stock 24482, and by the Institute for Research in Biomedicine (IRB) Barcelona *Drosophila* Injection Service (P-element-mediated transgenesis) using the *w¹¹¹⁸* line.

All transgenic lines used for experiments were sequenced to confirm that they carry the correct transgene.

gBlock DNA fragments used in this article:

BbsI.FS.*XhoI*: TCAGAAGAAGACCTACTACGAGCTACCATGCCACCAGCAGAAGGTCAACTTCGTGCCACCACATCCGTCTCCATGCCAGTGACGTCCAGCAGCTTCTTTGGCGCTGCTTCCAGTATTGGACGTCCCCGACCGGCGGCATCTACC
CAAACCCAAACGTCCACGCCATCCGAACACCCACGTCCCATCCCATCTGGGCTCTACTACTGACTCGAGTAAGCA

EWS-FLI₁ BbSi-*XhoI*:

GTCCAGCATGTACAAGTACCCGTCCGACATTAGCTACATGCCGAGCTACCATGCCACCAGCAGAAGGT-
CAACTTCGTGCCACCACATCCGTCTCCATGCCAGTGACGTCCAGCAGCTTCTTTGGCGCTGCTTCCAGTATTG-
GACGTCCCCGACCGGCGGCATCTACCCAAACCCAAACGTCCACGCCATCCGAACACCCACGTCCCATCCCATCTGGG
CTCCTACTACGTCTCTCTCGAGTATTAG

4.5.1. Microarray processing

Dissected *Drosophila* salivary glands were collected in 45 µl of a lysis buffer containing 20 mM dithiothreitol (DTT), 10 mM Tris-HCl pH 7.4, 0.5% sodium dodecyl sulfate (SDS) and 0.5 µg µl⁻¹ proteinase K, incubated at 65°C for 15 min and immediately frozen until processing. RNA extraction and cDNA generation was performed at the IRB Barcelona Functional Genomics Core Facility. Briefly, RNA was treated with DNase I and purified using magnetic beads (RNAClean XP, Beckman Coulter). RNA was quantitated with Qubit RNA HS Assay kit (Invitrogen), and RNA integrity was assessed with the Bioanalyzer 2100 RNA Pico assay (Agilent). Twenty-five nanograms of RNA were reverse transcribed and amplified using the whole transcriptome amplification method (WTA2, Sigma Aldrich) with 17 cycles of amplification. cDNA was further purified using a spin column (PureLink Quick PCR Purification Kit, Invitrogen) and quantified using a microvolume spectrophotometer (Nanodrop ND-1000, Thermo-Fisher Scientific).

For microarray processing, 8 µg of cDNA were fragmented and labelled according to the manufacturer's instructions (GeneChip Mapping 250K Nsp Assay Kit, Affymetrix). Array hybridization was performed using the GeneChip Hybridization, Wash and Stain Kit (Applied Biosystems). Briefly, libraries were denatured at 99°C for 2 min before incubation into the *Drosophila* Genome 2.0 arrays (Applied Biosystems). Libraries were hybridized on the arrays for 16 h at 45°C for 60 rpm at GeneChip Hybridization Oven 645 (Affymetrix/ThermoFisher Scientific). Washing and Stain steps were performed using a GeneChip Fluidics Station 450 following the *Drosophila* Genome 2.0 protocol (Affymetrix/ThermoFisher Scientific). Finally, arrays were scanned with a GeneChip Scanner GCS3000 (Affymetrix/ThermoFisher Scientific). The CEL files containing the microarray data were generated with the Command Console software (Affymetrix/ThermoFisher Scientific) and were used for probeset-based gene expression measurements using robust multichip average (RMA) normalization. Results were analyzed using the Transcriptome Analysis Console 4.0 (TAC) software. Genes with an absolute FC of >2 and an *p*<0.05 were considered differentially expressed.

4.5.2. Gene set enrichment analysis

The GSEA pre-ranked algorithm was used to compare the human EWS-FLI signatures from [33,34] to all genes in the *Drosophila* microarrays ranked by average log₂FC. Genesets with an FDR *q*-value of <0.25 were accepted as a significant enrichment. *Drosophila* orthologues of these human signatures were identified using the *Drosophila* RNAi Screening Center Integrative Ortholog Prediction Tool (DIOPT) orthologue mapping online resource [47].

4.5.3. GO analysis

Functional annotation of GO terms was performed using the online tool database and annotation, Visualization and integrated discovery (DAVID) [48]. GOTERM_BIOLOGICAL_PROCESS_4 terms with a *p*<0.05 were accepted as a significant enrichment.

4.5.4. Quantitative real-time PCR

Dissected *Drosophila* larval salivary glands were collected in 45 µl of a lysis buffer containing 20 mM DTT, 10 mM Tris-HCl pH 7.4, 0.5% SDS and 0.5 µg µl⁻¹ proteinase K, incubated at 65°C for 15 min and immediately frozen until processing. RNA extraction and cDNA generation were performed at the IRB Barcelona Functional Genomics Core Facility. Briefly, RNA was treated with DNase I and purified using magnetic beads (RNAClean XP, Beckman Coulter). RNA was quantitated with Qubit RNA HS Assay kit (Invitrogen), and integrity was assessed with the Bioanalyzer 2100 RNA Nano assay (Agilent). Twenty-five nanograms of RNA were reverse transcribed and amplified using the whole transcriptome amplification method (WTA2, Sigma Aldrich) with 17 cycles of amplification. cDNA was further purified using a spin column (PureLink Quick PCR Purification Kit, Invitrogen) and quantified using a microvolume spectrophotometer (Nanodrop ND-1000, Thermo-Fisher Scientific). cDNA yield ranged from 18 to 44 µg. PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) was used for quantitative real-time PCR following manufacturer's instructions. The real-time assays were conducted in a QuantStudio 6 Flex real-time PCR system (Thermo Fisher Scientific) using SYBR green as the detection system and ROX as the reference dye. Two different pairs of primers were designed to amplify YFP cDNA. mRNA levels were assessed from three independent RNA extractions and three technical replicates were performed on each sample. RNA levels were normalized to Ribosomal Protein L32 (*RpL32*) housekeeping gene. The primers used are shown in table 1.

4.6. Western blot

Dissected *Drosophila* larval salivary glands or wing discs were collected in 50 µl of cold PBS. PBS was removed after dissection and the samples were frozen at -20°C. For the detection of YFP_{+FStail} and YFP, eight pairs of salivary glands or 10 wing discs were used per sample from individuals with genotypes *nub>Gal4/UAS-YFP_{+FStail}* and *nub>Gal4/UAS-YFP*, respectively. For detection of EWS-FLI₁, salivary gland pairs were collected from individuals of the genotype *sal^{EPv}>Gal4/P[UAS>EWS-FLI₁]; 20×(GGAA)>YFP/+*, *sal^{EPv}>Gal4/P[UAS>EWS-FLI₁]; 20×(GGAA)>YFP/ P[tubP>GAL80^{ts}]*, *sal^{EPv}>Gal4/UAS>EWS-FLI_{1FS}; 20×(GGAA)>YFP/+*, *sal^{EPv}>Gal4/UAS>bcEWS-FLI₁; 20×(GGAA)µSat>YFP/+*, or *sal^{EPv}>Gal4/+; 20×(GGAA)>YFP/+* and loaded protein quantity was adjusted to be equal to that from 4 *sal^{EPv}>Gal4/+* salivary gland pairs.

Samples were resuspended in 30 µl of 2 × SDS NuPAGE lithium dodecyl sulfate (LDS) sample buffer with 100 mM DTT, lysed via manual grinding and sonication with Bioruptor. Boiled lysates were resolved by 4–12% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Bolt 4–12% Bis-Tris, 1.0 mm, REF: NW04120BOX; Invitrogen), and proteins were transferred to nitrocellulose membranes (0.2-µm pore size, Invitrogen). YFP_{+FStail} and YFP proteins were detected by incubating with anti-GFP primary antibodies overnight at 4°C (mouse anti-GFP) (1:250, REF: 11814460001; Roche, Basel, Switzerland). α-tubulin (Clone Dm1A) protein was detected by incubating with rat anti-TubDM antibody (1:500, REF: T6199; Sigma Aldrich) and was used as the loading control. EWS-FLI₁ proteins were detected by incubating with rabbit anti-FLI primary antibody (1:200, ab133485, Abcam) and mouse anti-EWS (1:500, sc-48404, Santa Cruz Biotechnology) overnight at 4°C. Total protein was detected with REVERT Total Protein Stain Kit (LI-COR) and was used as a loading control. Immunoblots were developed using Alexa Fluor 790 and Alexa Fluor 680 secondary antibodies (1:2500, Jackson ImmunoResearch) or IRDye 800CW (1:2500, LI-COR) with the Odyssey Infrared Imaging System (LI-COR).

Ethics. This work did not require ethical approval from a human subject or animal welfare committee.

Data accessibility. Supporting data have been included in the electronic supplementary material [49].

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. S.M.: formal analysis, investigation, methodology, validation, visualization, writing—original draft; C.M.: formal analysis, investigation, methodology, validation, visualization; D.V.: investigation, methodology, validation, visualization; J.R.: investigation, methodology; S.L.: investigation, methodology; J.P.H.: investigation, methodology; J.M.: conceptualization, resources, supervision; C.G.: conceptualization, funding acquisition, methodology, project administration, resources, supervision, writing—original draft.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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