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Characterization of Hippo downstream effectors in the maintenance of planarian cellular identity

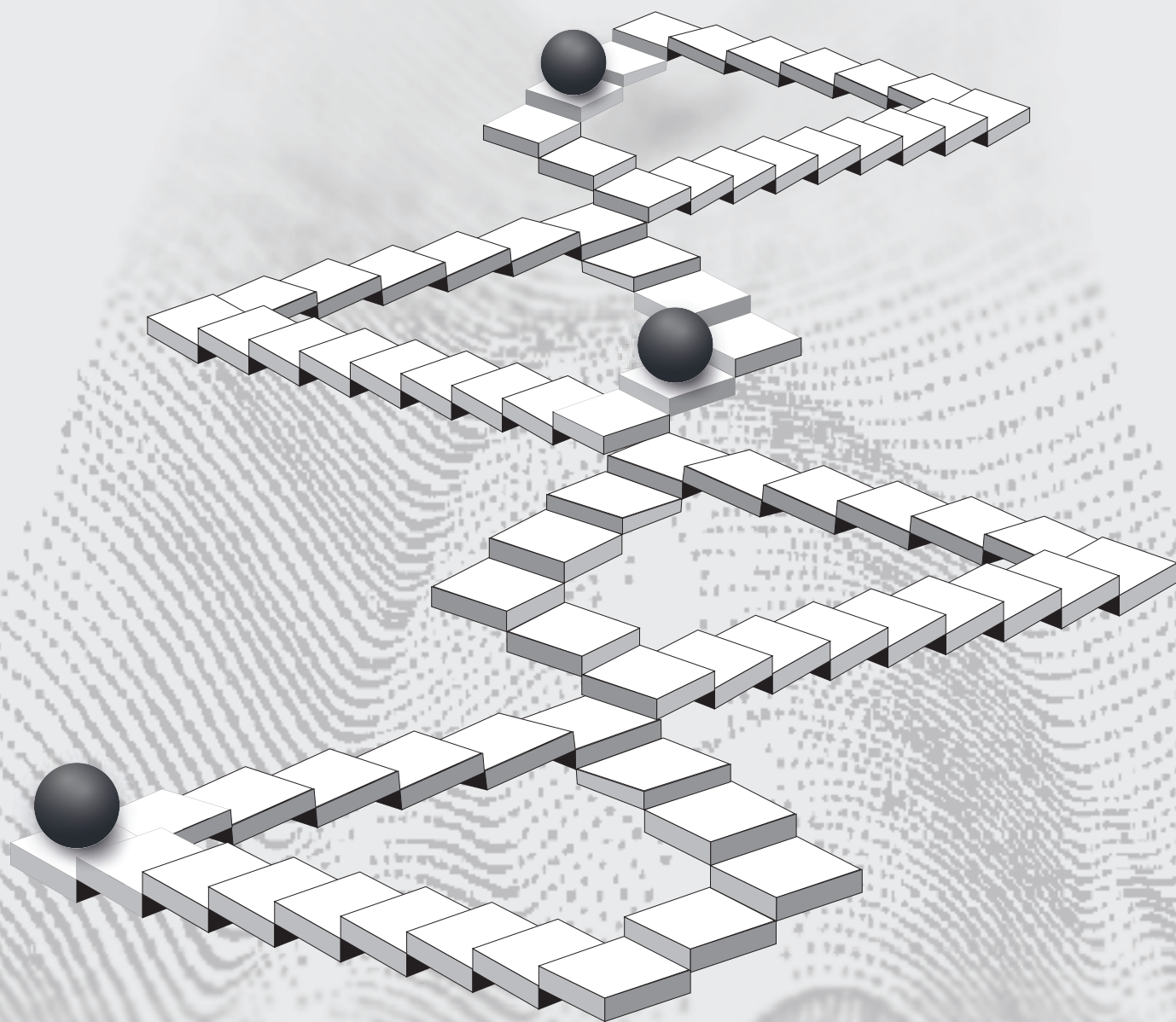
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CHARACTERIZATION OF HIPPO DOWSTREAM EFFECTORS IN THE MAINTENANCE OF PLANARIAN CELLULAR IDENTITY



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**Characterization of Hippo downstream effectors in the maintenance
of planarian cellular identity.**

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“Science and machines can kill each other off or be replaced. Myth, seen in mirrors, incapable of being touched, stays on.”

— Ray Bradbury, The Martian Chronicles

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Abstract:

The study of tissue renewal in adult organisms has widely been viewed as a source to uncover the triggers for the disruption of cell lineages in pathologies such as cancer. One of pathways involved in this process is the Hippo pathway. The Hippo pathway acts as a mechanosensor of extracellular physical forces. These inputs lead to the control of organ growth through the regulation of cellular fate. However, the exact mechanisms and biological processes that underly this maintenance of cellular fate remain elusive and require further characterization. One of this biological processes is cellular senescence. Cellular senescence has been linked with the onset of tumoral transformation and other age-associated disorders similar to dysregulations of the Hippo pathway. Other processes that share similarities with Hippo in regards to tumorigenesis include the regulation of cell cycle progression, cytoskeleton maintenance and transcriptional regulation. Nonetheless, the connection between the Hippo pathway and these biological processes have been poorly characterized so far. To better understand these mechanisms we use planarians (*Schmidtea mediterranea*). These are ageless flatworms able to regenerate any body part and constantly resize according to food availability.

The aim of this thesis revolves around highlighting the importance of cellular senescence, transcriptomic and cytoskeletal regulation and cell cycle progression downstream of the Hippo pathway to properly maintain cellular differentiation *in vivo*. In order to assess the role of these biological processes we use a RNAi screening approach by inhibiting putative effectors of the pathway representative of these biological functions.

All in all, we found that cellular senescence predates and contributes to the loss of cellular differentiation. Moreover senescence rescues are able to prevent such phenotype. Furthermore we show that defects in maintenance of the cytoskeletal architecture impair proper metaphase progression of the cell cycle and induce dedifferentiation. We also detect that deregulation in transcription also impairs the progression of the cell cycle and is associated with loss of cellular identity. Finally we also correlate defects in the progression of the cell cycle with proliferation and alterations in growth and degrowth dynamics according to the nutrient status of the planarian.

In conclusion we affirm that the biological processes of cellular senescence, cytoskeletal maintenance, transcriptional regulation and cell cycle progression are linked with the Hippo molecular pathway and their physiological regulation are key for maintaining the cellular fate.

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GENERAL INTRODUCTION

PREFACE: TISSUE SELF-RENEWAL MAINTENANCE

Tissues self-renewal and homeostatic maintenance are biological processes accomplished thanks to cell proliferation and the establishment of the different cellular identities. The fine tuning of these processes are also key in order to avoid pathologies in the adult and maintain the tissues. Often, the alteration of the pathways involved in these processes lead to an increase of organ size coupled with overgrowths. However, the exact mechanisms and molecular pathways that underly these processes remain elusive and require further characterization. [1] Furthermore, the study of these mechanisms has been regarded as a tool to better understand pathologies that involve the disruption of cellular identity such as cancer. For instance, the accumulation of senescent cells has been regarded as one of the biological processes linked with the onset of tumoral transformation and other age-associated disorders.[2] Nonetheless, the connection between the aforementioned molecular pathways and the triggers of tissue dystrophies remains poorly studied. To better understand these connection, the experimental model used is the freshwater flatworm *Schmidtea mediterranea*, an organism that constantly resize and renew their tissues according to food availability. [3]

CHAPTER 1: PLANARIAN AS A MODEL SYSTEM

Schmidtea mediterranea is a fresh water flat worm also known as planaria from the Dugesiidae family, Tricladida order, Platyhelminthes phylum of the Protostomia clade [4]. Freshwater planarians can be found all over the world, with the exception of Antarctica and some islands. They live in a great variety of freshwater bodies, including rivers, streams, ponds, lakes, springs, caves, and also in artificial water reservoirs and canals. Freshwater planarians need the continuity of water bodies to survive since they cannot tolerate desiccation [5]. The genus *Schmidtea* is found endemic to the Western Palearctic region. *S. mediterranea* appears scattered across the western Mediterranean islands and is also found in two localities on the coasts of Catalonia (Spain) and one in continental Tunisia.[6] This species is viewed as a model organism generally used in regeneration and developmental studies [7].

NEOBLASTS IN PLANARIAN REGENERATION AND HOMEOSTASIS

Planarians can regenerate an entire organism from any fragment of their body by rebuilding lost tissue with the correct pattern. On the other hand, they are also constantly resizing according to food availability and temperature through a process that involves re-patterning of existing tissues [7–9].

The high regenerative capability of these animals resides on their population of adult stem cells, called neoblasts. Neoblasts are somatic (also called adult) stem cells present throughout the planarian mesenchyme and absent in the pharynx and in front of the eyes. They represent around 25-30% of the total animal cell population. Nowadays, neoblasts are collectively considered pluripotent stem cells. Nonetheless, the exact number and precise localization remains unknown. Pluripotency is viewed as a transient state that leads to a majority of multipotent progeny cells. These different multipotent cells later give rise to the different tissues[10]. They are irradiation sensitive cells with a unique transcriptomic profile that allow them to be detected by in-situ hybridization with transcripts against *smad-piwi* [11] and *smad-h2b* [12]. They are small cells (5-8 μm) with a high nucleus:cytoplasm ratio [10]. This feature together with their X-ray sensitivity and their ability to rapidly expel the fluorescent DNA-binding dye Hoechst 33342 allow them (X1) to be separated and quantifiable by Fluorescence-activated cell sorting from progenitors (X2) and differentiated cells (Xins) [13]. Moreover, they present indefinite telomerase activity allowing them to maintain their telomere longitude during regeneration and fission reproduction providing the animal its alleged immortality[14].

Previous studies have revealed that the population of neoblasts appears heterogeneous with pluripotent stem cells called cNeoblasts together with lineage-committed progenitors

segregated between gamma (γ)-class, zeta (ζ)-class and nu (ν)-class[15]. In which γ -class is related with gut lineage; ζ -class with epidermal lineage and ν -class with the neural one [13, 16]. A representation of the model can be seen in Figure 1. As active cycling cells, neoblasts can be detected by immunohistochemistry with anti-PH3 antibody [17] or through BrdU incorporation assays [18].

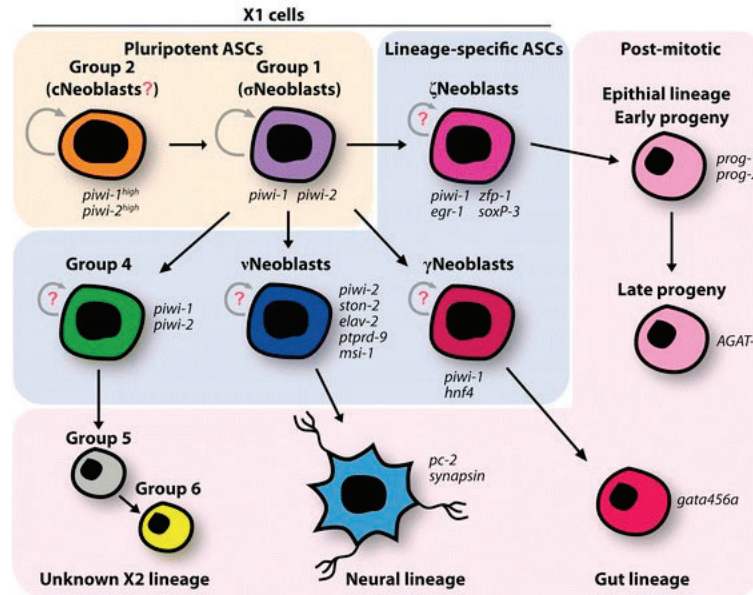


Figure 1: Model of planarian stem cell hierarchies. It is based on single cell RNA-seq (scRNA-seq) and Waterfall/pseudotime analyses. cNeoblasts are hypothesized to give rise to the σ Neoblasts both with pluripotent capabilities depicted on the first tier. These give rise to ζ , γ , ν , and Group 4 neoblasts represented at the middle tier. This model argues that each of these neoblasts subclasses give rise to tissue specific cell lineages depicted on the third tier such as epithelium for ζ Neoblasts, gut for γ Neoblasts, and neurons for ν Neoblasts. Question marks depict the cell types with unknown ability to self-renew. Modified from [13].

After any amputation, neoblasts respond to the injury and proliferate, forming unpigmented new tissue at the stump region named blastema [19]. Two mitotic peaks are observed during this process. The first one occurs around 6h after the injury and is general, as it takes place across the entire animal. The second one appears around 48h after injury and is local, as it occurs specifically in the stumped area [17]. In the case of head regeneration, the process is completed after 14 days.

Silencing of several signaling pathways such as the JNK leads to the formation of smaller blastemas since it modulates the expression of wound response genes together with triggering an early apoptotic response post amputation and attenuating the onset of stem cell mitosis for a coordinated regeneration response[20].

During homeostasis, neoblasts are the source of the constant cell turnover required to maintain the body size. They produce the new cells that migrate and undergo multiple determination steps, intercalate in their target tissue and differentiate in the proper cell type. Changes in the body size are achieved through variations in the balance between cell proliferation and cell death. Under starvation conditions animal size reduction correlates with an increase of cell death whereas mitotic quota stays basal. Conditions revert upon nutrient availability: a decrease of cell death and mitotic increase occurs[21].

PLANARIAN ANATOMY

Planarians are triploblastic animals around 3 to 8 mm in length that share with vertebrates a bilateral symmetry and well-defined dorsoventral, mediolateral and anteroposterior axes [22–24]. They are also acoelomates with an enveloping tissue across organs called parenchyma.

S. mediterranea presents a sexual strain that through cross reproduction generates capsules with multiple embryos named cocoons, and an asexual strain which accomplishes reproduction through fission processes, generating a genetically homogenous population [7]. The later strain has been used for the development of this project.

They are characterized by an anterior cephalic region with two eyes connected by an optic chiasm [25]. They also present a bilobed cerebral ganglion dorsally located. This ganglion constitutes the animal nervous system together with two ventral nerve cords. These cords progress up to the posterior region of the animal from which a nervous plexus derives. This nervous plexus is responsible for the innervation of the musculature, pharynx and gut. It can be labeled for instance by Immunostaining using an antibody against SYNAPSIN (3C11)[26]. Several types of neurons have been described in the planarian brain. These include: serotonergic [27], dopaminergic [28], glutamatergic [29], octopaminergic [30], GABAergic [31], cholinergic [32] and chemoreceptors [33]. Dopaminergic neurons can be detected using markers for *tyrosine hydroxylase* (*smed-th*) since it is the rate limiting enzyme in dopamine synthesis. On the other hand, chemoreceptors can be detected with *cintillo* a member of the DEG/ENaC superfamily of sodium channels. Its name means "head ribbon" in Spanish [34, 35]. On the other hand, neural committed neoblasts and early progeny can be visualized by markers against the transcription factor Collier/Olfactory-1/Early B-cell factor (*smed-coe*) [27] and the homeobox transcription factor *smed-nkx2.1* [36] among others. The LIM homeobox transcription factor *smed-lhx1/5-1* has also been characterized in planarians as required for the maintenance and regeneration of the serotonergic lineage [37, 38].

The digestive system is constituted by a blind intestine ramified into three main branches that converge into a pharynx located in the center of the animal. This pharynx is located ventral to the muscular mouth used for food ingestion[39] It also presents an excretory system composed by protonephridia based on a dorsal network of channels with specialized flame cells with constant beating flagella.[40]

The muscular system extends throughout the whole planarian body and shapes it. It is composed by three fiber layers: circular, diagonal and longitudinal, from outside to inside. It acts as a plexus connecting the dorsal and ventral part of the animal and shapes organs such as the pharynx and the gut [41]. It is a major secretor of extracellular matrix (ECM) together with other elements that compose the matrisome of the animal [27].

The animal is also covered by a monolayer of epithelial tissue. It is composed by differentiated non-ciliated and multi-ciliated cells on top of a basal lamina [42]. Locomotion is accomplished mainly by the action of cilia located in the ventral region. They also present secretory structures in the apical part called rhabdites that connect with several secretory cells present on the parenchyma [43]. These are responsible for the mucus secretions that act as a protector layer for the animal. A summary of the planarian anatomic structures can be observed at Figure 2.

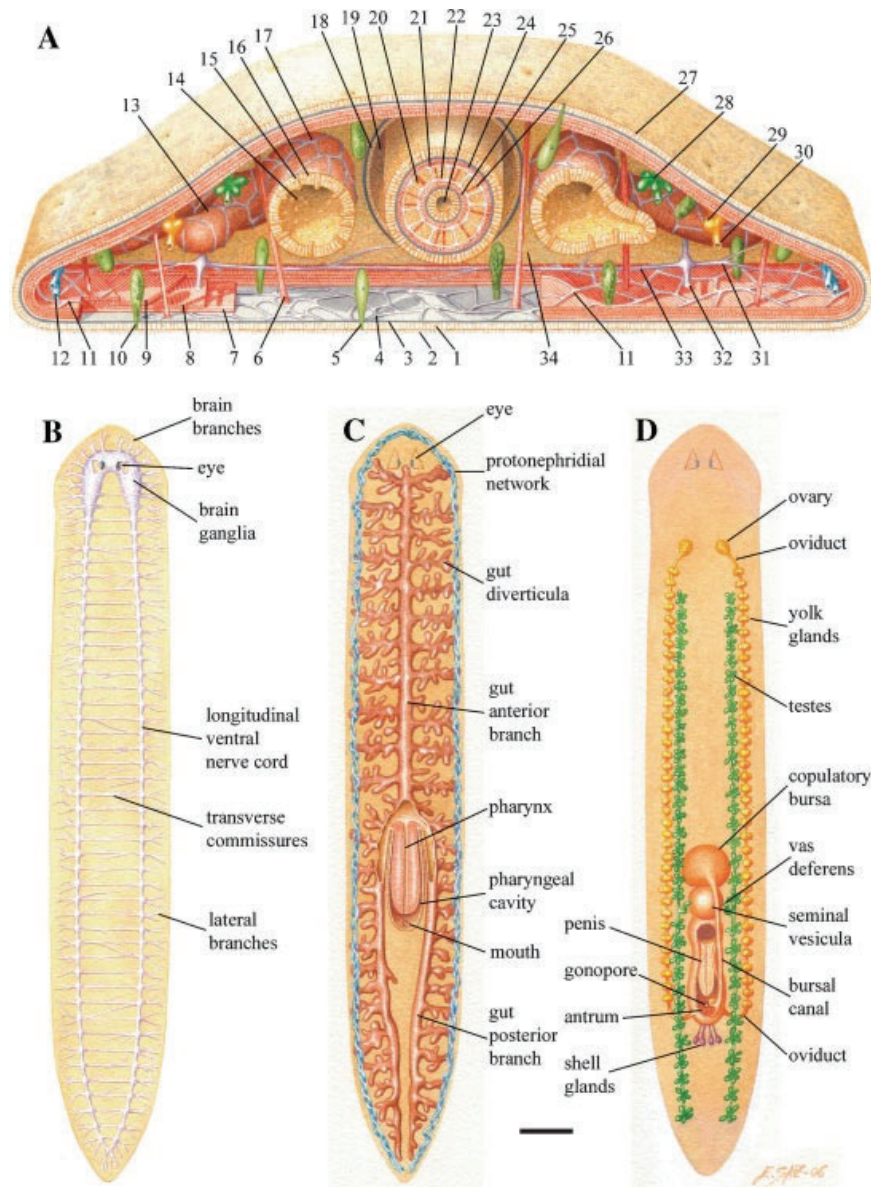


Figure 2: Diagram of the major organ systems in the freshwater planarian *Schmidtea mediterranea*. A. Transverse section of the animal. The most remarkable features include the three muscular layers, the pharynx and its cavity at the medial part of the animal and the epidermal monolayer with their cilia projections. 1, ventral epithelial cells; 2, cilia; 3, basal membrane; 4, subepidermal nerve plexus; 5, gland cells; 6, dorso-ventral muscles; 7, circular muscles; 8, longitudinal muscles; 9, diagonal muscles; 10, rhabdite cells; 11, submuscular nerve plexus; 12, protonephridial network; 13, gut diverticula; 14, gut lumen; 15, goblet cells; 16, gastrodermal cells; 17, gastrodermal plexus; 18, pharynx cavity wall; 19, pharynx cavity; 20, pharynx parenchyma; 21, pharynx outer nerve net; 22, pharynx inner nerve net; 23, pharynx lumen; 24, pharynx outer musculature; 25, pharynx inner musculature; 26, pharynx radial musculature; 27, dorsal epithelial cells; 28, testis; 29, yolk glands; 30, oviduct; 31, lateral nerves; 32, longitudinal ventral nerve cord; 33, transverse commissures; 34, parenchyma. B. Representation of the central nervous system characterized by a bilobed brain ganglia together with the two ventral nerve cords and their projections C: Depictions of the digestive system characterized by a blind ramified gut with the pharynx at the center and the excretory system composed of a dorsal protonephridia network. D: Representation of the hermaphrodite reproductive system of the sexual strain. Scale bar: 1mm. Obtained from [7].

The epidermal lineage has been well characterized in planarians. The epidermal committed neoblasts called ζ -neoblasts lose their proliferative capabilities once they start expressing *smed-nb32* and migrate towards the epidermis. Afterwards the sequential expression of *smed-agat/smed-zpuf/smed-vim* correlates with the appearance of differentiated epidermal cells localized in the sub-epidermal or epidermal layer. Finally *smed-vim+* (*vimentin*) cells terminally differentiate to the different polarized epidermal cell types [44, 45].

CHAPTER 2: HIPPO PATHWAY AND DISEASE

This chapter will consist in a summarized description of the Hippo pathway as well as its evolutionary significance together with the regulators, implications in disease and in the planarian model necessary to contextualize the results obtained in the lab.

THE HIPPO PATHWAY

The Hippo pathway is a cell-cell communication pathway initially described as a regulator of organ size through cell death and proliferation regulation in response to the environment. Its inhibition lead to the appearance of overgrowths in *Drosophila* [46] and an increase of organ size coupled with overgrowths in mammals [47]. Nonetheless, recent studies indicate a wider variety of functions of the pathway besides growth control[48], placing it as a pivotal pathway in the regulation of cell fate and maintenance of pluripotency [49]. It has also been directly linked with tumorigenesis in animal models and in humans [50].

The Hippo pathway consists of a highly-conserved kinase cascade which modulates the phosphorylation and nuclear localization of Yorkie, a nuclear effector responsible for the transcription of multiple genes. At its core, Hippo (Hpo) (Mst1/2 in mammals) interacts with the adaptor Salvador (Sav) and phosphorylates Warts (Wts) (Lats 1/2 in mammals) which mainly phosphorylates on S168 the main nuclear effector of the pathway named Yorkie (Yki) (YAP/TAZ in mammals) and leads to its degradation. Unphosphorylated Yki on the contrary is able to enter the nucleus and interact with several DNA binding factors to induce the transcription of its targets. For instance, Scalloped (Sd) which was the first described DNA-binding partner of Yki, characterized in *Drosophila* wing discs [51, 52].

Environmental signals that impact on cell-cell contact, as tight junctions or cell adhesions, are the main inputs that trigger the activation of the pathway. The subcellular localization and activity of YAP/TAZ are tightly regulated by cell substrate rigidity and topography, actin cytoskeleton remodeling, and by specific regimens of cell stretching [53, 54]. Therefore, YAP/TAZ act as mechanosensors and mechanotransducers able to regulate cellular proliferation, differentiation and death thorough environmental signals[55]. Some upstream regulators described in *Drosophila* act at this level, triggering the inhibition of the pathway core kinase cassette. The main ones include the Expanded-Merlin-Kibra (Ex-Mer-Kbr) complex or the signals regulated by the transmembrane Fat protocadherin [56]. Downstream of the kinase cassette, Yki can interact with Sd, or with other DNA binding transcription factors in a tissue-specific manner, triggering a transcriptional specific response. For example, Yki interacting with Homothorax (Hth) and Teashirt (Tsh) in the progenitor cells of *Drosophila* eye imaginal discs leads to the expression of the microRNA *bantam*[57], while the Yki-Sd complex activates the expression of *diap1* (*Drosophila* inhibitor of apoptosis) and *CTGF* (Connective Tissue Growth Factor) [58]. These evidences reveal a high complexity in both the Hippo pathway trigger and response elements (Figure 3).

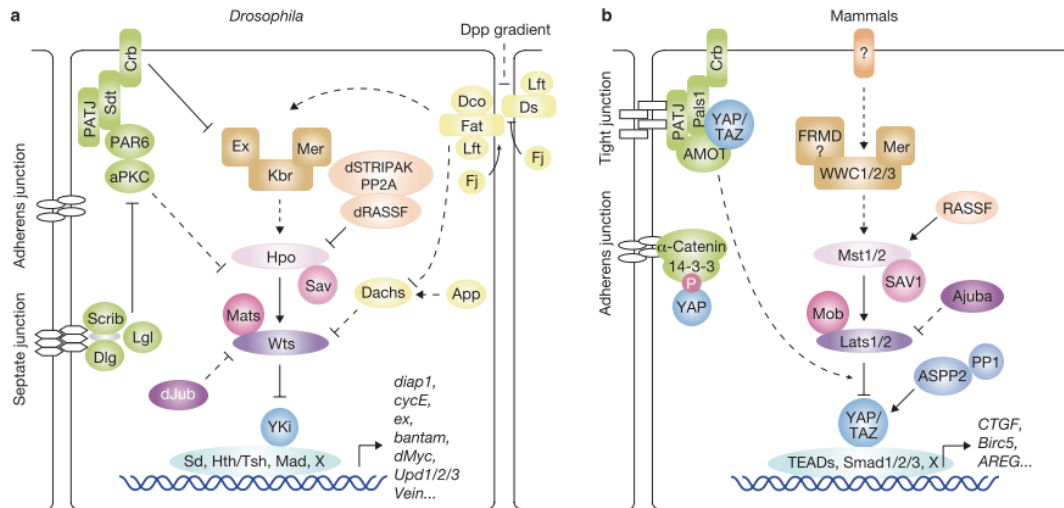


Figure 3: Hippo pathway components. Representation of the key components of the pathway in *Drosophila* and mammals. Obtained from [52].

EVOLUTIONARY PRESPECTIVE

The Hippo pathway presents its core elements in a wide array of species from premetazoan to mammals. Mats, Hippo or Warts appear in unicellular species such as *Tetrahymena thermophila* (earlier eukaryotes), *Acanthamoeba castellanii* (Amoebozoa & Dictyostelia) and *Fonticula alba* (Holomycota) respectively [59]. This is in contrast to *Capsaspora owczarzaki* (Holozoa) which presents an intact Hippo pathway[60] (Figure 4). Nonetheless, these three pathway components retain their intrinsic activities presenting a conserved function in human cells with conserved key domains and capacity of integration into the Hippo regulatory network. These evidences suggest that the pathway could originate from these ancestor components.

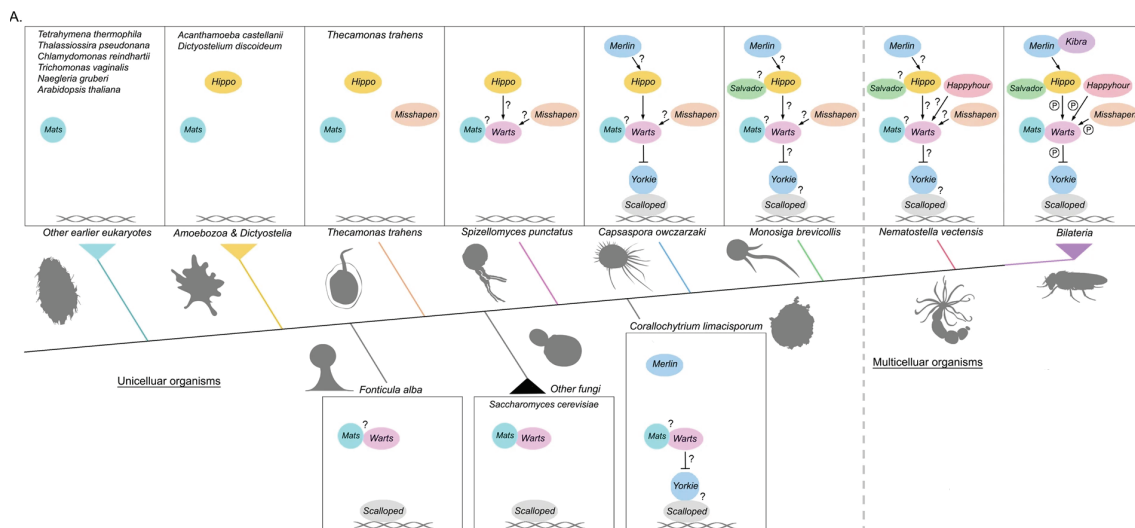


Figure 4: Schematic illustration of the Hippo pathway evolution from unicellular organisms to Bilateria. The color of lines was matched with the firstly emerged Hippo pathway component in the indicated species. The presumed complex formation and regulation were indicated by a question mark. Obtained from [59].

On the other hand, the number of paralogous genes that are present in the mammalian pathways are absent in the *Drosophila* one. This can be attributed to a whole genomic duplication in fishes where the paralogues were firstly identified. This is because the majority of these paralogs have been later found conserved in amphibians and mammals. An specific

example of this process is the emergence of TAZ as a duplication of the original Yorkie giving its redundant role in the mammalian Hippo pathway [59].

HIPPO REGULATORS AND APICAL-BASAL POLARITY

It has been described that many upstream regulators of the Hippo pathway are components of the adherens junctions (AJ), tight junctions (TJ) or members of the apical-basal complexes. These elements are key to maintain adherence and the differentiated state among epidermal cells. They divide these cells into an apical domain and a basolateral one thanks to the establishment of a mesh of actin-myosin filaments. These filaments connect the junctional complexes together and form a cell cortex under the inner face of the plasma membrane [61]. Specifically, *lg2* has been described as a modulator of the Par complex. This is an upstream inhibitor complex of Hippo. It acts through the stabilization of the apical complex preventing the interaction of the aPKC with the core kinases cascade of the Hippo pathway [61, 62].

Moreover, it presents two distinctive roles that have been hypothesized to be key in the aforementioned maintenance of cell polarity:

The first one is related to the ability of Lgl family of proteins to regulate polarized exocytosis as demonstrated by yeast models [63, 64]. This finely tuned mechanism allows the transport of specific secreted and transmembrane macromolecules to the cell surface in order to maintain the molecular identities of the apical, lateral and basal membrane domains. The specific mechanism is thought to be through the interaction with t-SNARE proteins (target soluble N-ethylmaleimide attachment protein receptor) found in plasma membranes and other components of the exocyst complex or by regulating the fusion of post-Golgi transport vesicles to target membranes [63] (Figure 5).

The second role involves the Lgl family of proteins being part of the cytoskeleton through their interactions with the heavy-chain of the non-muscular myosin II (NMII). This is an actin-based motor protein essential for proper cell adhesion, migration and polarity.

Generally, unphosphorylated Lgl is localized at the basolateral domain where it restricts activity of the nonmuscle myosin II isoform A (NMII-A) and inhibits the assembly of actin-myosin II cytoskeleton. On the contrary, phosphorylated Lgl appears at the apical domain dissociated from the cytoskeleton, unable to influence the actin-myosin II cytoskeleton [65, 66]. This allows the proper differentiation of the two cellular poles (Figure 5).

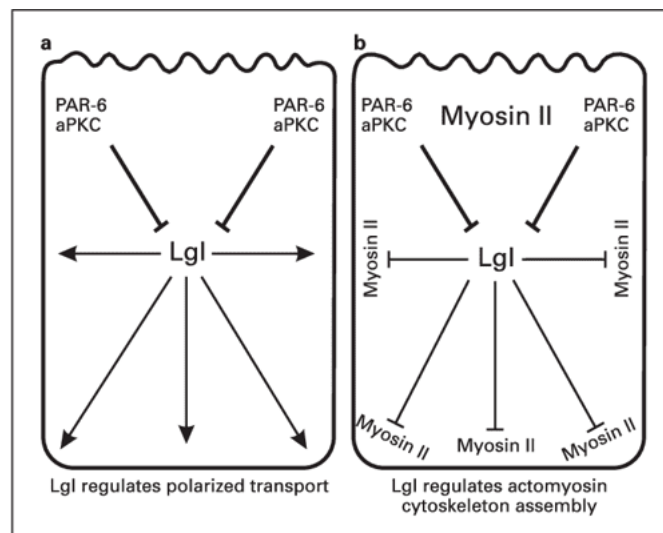


Figure 5: Two models of Lgl function in the maintenance of cell polarity. **a.** Lgl as a regulator of polarized transport. Phosphorylation by aPKC inactivates Lgl at the apical membrane domain and directs its transport activities toward the basolateral membrane. **b.** Lgl as a regulator of actomyosin cytoskeleton. Nonphosphorylated, active Lgl at the basolateral

membrane domain negatively regulates the assembly of the actin-myosin II cytoskeleton, which is formed at the apical membrane where Lgl is inactivated by aPKC. Asymmetric assembly of the actomyosin cytoskeleton helps maintain cell polarity. Obtained from [67].

Defects in both functions lead to a loss in the cellular apical-basal polarity. This attribute has most commonly been associated with the epidermal-mesenchymal transition. This phenomenon that plays a key role in development in features such as the establishment of the mesoderm during gastrulation or in the emigration of the neural crest from the neural tube [68, 69] can also be ectopically reactivated in diseases such as cancer [70]. During tumor progression, this process can allow cells to acquire a gain of stem-like properties that correlate with a more malignant phenotype and poor prognosis. For example, Snail1 has been characterized as one of the main stemness inducers among the plethora of transcription factors that play a role in epithelial-mesenchymal transition (EMT) (others include Zeb, Twist, and Prx families) of mesenchymal cells of different origins such as embryonic cells, healthy adult cells or cells with a pathological origin [71].

ROLE OF HIPPO IN CANCER

The role of *hippo* itself in cancer appears to be more ambiguous since it is reported to present dual roles of a tumor suppressor and an oncogene [72, 73]. The hypothesis involved with this duality of functions are diverse and extensive. This is in part due to the fact that the effectors YAP and TAZ bind to various transcription factors depending to the cellular context. Also, Hippo itself presents a crosstalk from and to other pathways. Finally, mutations to upstream regulators of the pathway themselves often lead to several different outcomes [74, 75]. One example of tumor suppression occurs in mouse intestine regeneration where loss of YAP leads to WNT hypersensitivity resulting in stem cell expansion and hyperplasia [76]. On the other hand, an example of oncogenic process indicates that the nuclearization of YAP/TAZ in differentiated tissues often leads to increase of genomic instability, EMT together with increased migration, metastasis and increased resistance to anoikis (apoptosis induced by the lack of cell attachment) [77, 78]. Nonetheless it must be remarked that the majority of these de-regulations occur through epigenetic silencing mechanisms. This is the reason why seldom mutations associated with core Hippo pathway genes have been characterized in human cancers. [79]

ROLE OF HIPPO IN PLANARIANS

The first study of the Hippo pathway on Platyhelminthes was performed in *Macrosomum lignano* and revealed the functional conservation of the pathway between flatworms and mammals. The results showed that Hippo silencing leads to the regeneration of bigger structures together with an increase in proliferation and overgrowths formation in homeostasis. On the other hand, upon *yki* silencing a decrease on proliferation was observed [80].

Studies in *Schmidtea mediterranea* revealed a blotted phenotype upon *yki* silencing due to defects in the excretory system coupled with an unexpected increase in cell proliferative rates [81]. Nonetheless a more recent study performed in our lab regarding *hippo* silencing in the same species revealed an increase of mitosis coupled with an increase of Yki protein levels in the nucleus, suggesting that in fact the role of Hippo signal in growth control is also conserved in *S. mediterranea*. The blotted phenotype after *yki* inhibition could be caused by a specific role of Yki in the excretory system, which masks its role in growth.

Furthermore, *hippo* silencing in planarians also resulted in the decrease of apoptosis and the development of overgrowths and unpigmented regions (Figure 6A). Despite this shift on cellular turnover mechanisms, body size and cell number were maintained. This result was explained by the observation that mitotic cells were accumulated in M phase, due to defects on the ensemble of the mitotic spindle. Finally, in the unpigmented regions and overgrowths, markers of pluripotency and of terminally differentiated cells were co-expressing aberrantly, pointing to a loss of identity commitment, a feature shared with tumor progression (Figure 6B).

Moreover, it was found that *hippo* inhibition in planarians depleted of cycling cells (or neoblasts) may lead to the dedifferentiation of postmitotic cells. Specifically, under sublethal irradiation doses (17,5 Gy) *hippo* (RNAi) animals increased the number of *smedwi*⁺ cells compared to the sublethal irradiated animals. Importantly, anti-H3P immunostaining indicated there were no differences in the number of mitotic cells between the sublethal irradiated/*hippo* (RNAi) planarians and the sublethal controls. This indicated that the increase of *smedwi*⁺ cells was not attributed to an increased proliferation but rather due to the dedifferentiation of postmitotic cells that regain the expression of stem-cell markers.

Thus, in *S.mediterranea* *hippo* inhibition leads to overgrowths and unpigmented regions which are composed by undifferentiated cells. Three observations could account for this phenotype: the arrest of cells in M phase, the decrease of apoptosis (which impedes the elimination of unhealthy cells) and the inability of differentiated cells to maintain their differentiated state. All these features are known to be present in any tumoral process (Figure 6 B).

To further analyze the cellular and molecular nature of the pathway, transcriptomic analysis of *hippo* silenced animals after one, three and four weeks of inhibition were performed and compared with respective controls in order to identify putative target genes of the pathway that appear differentially up or downregulated [82].

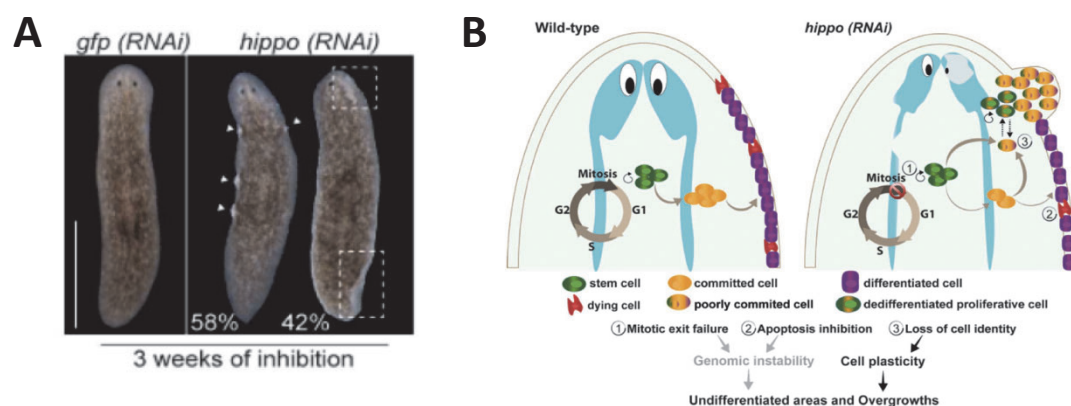


Figure 6: Hippo pathway in planarians. A. *hippo* RNAi phenotype in planarians. White arrowheads indicate overgrowths and white discontinuous boxes unpigmented regions. B. Model of the Hippo pathway role in planarians. Modified from [82].

CHAPTER 3: SENESCENCE

Cellular senescence is a complex concept that involves many different biological functions that are still in the process of being properly characterized. Therefore, this chapter will be focused on the characteristics that are able to contribute to the discussion according to the results found in planarians.

According to the International Cell Senescence Association (ICSA) cellular senescence is defined as a cell state triggered either by stress or some specific physiological processes characterized by a prolonged and generally irreversible cell-cycle arrest, secretory features, macromolecular damage and altered metabolism. These features can often be inter-dependent [83].

CELL CYCLE ARREST

The cell cycle arrest that occurs in cellular senescence implies the cessation of cell division. It is characterized by profound chromatin changes, and tumor-suppressor activation [84–88]. These features may result in genomic instability that leads to chronification, resulting in chromosomal instability, DNA damage, loss of cell identity and failure of the cell death

programs activation (apoptosis) [89–92]. This type of arrest differs from quiescence for the fact that the latter is temporary with proliferation being reinstated afterwards. On the other hand, this kind of arrest is also different from terminal differentiation since in this case cells acquire specific functions triggered by different pathways from the ones involved in senescence. Moreover, senescent cells acquire a new and distinctive phenotype different from the ones that identify differentiated cells. It must be remarked though, that in specific circumstances, especially during tumorigenesis, senescent cells can reenter the cell-cycle and trigger dedifferentiation [93, 94].

Senescent cell cycle arrest is mainly regulated through the tumor suppressor pathways of retinoblastoma (pRB) family and p53 proteins [95]. pRB1 is phosphorylated by cyclin dependent kinases (CDKs; CDK4, CDK6, CDK2). This phosphorylation leads to the repression of the transcription factor E2F family activity, which are required for the progression of cell cycle. This occurs in senescent cells thanks to the persistent activation of pRB [96, 97].

SENESCENT ASSOCIATED SECRETORY PHENOTYPE

Senescent cells secrete a wide variety of factors globally included in the term senescent associated secretory phenotype (SASP). Some of the most definitory molecules of the SASP include cytokines, chemokines and matrix metalloproteases. It mediates many of the senescence effects. Especially, it mediates the spread of senescence in an autocrine and paracrine manner [98, 99]. The composition of the SASP varies wildly across cell types, the original stimulus and the endurance of the senescent phenotype[100]. Thus, it is important to properly characterize it in each biological context. Specially because it will unveil novel and context-specific secretory signatures tied to different types of senescent progressions.

TELOMERE SHORTENING

One of the most defining characteristics of cellular senescence in regards to damage is telomere shortening consequence of serial passages of DNA replication due to the DNA-end replication problem [101]. Telomeres are evolutionary conserved repetitive sequences of DNA found as loops at chromosomal ends and stabilized by the Shelterin protein complex. This makes the region inaccessible by the DNA damage response (DDR) and the double-strand DNA break (DSB) repair pathways [102].

Telomere shortening culminates in the destabilization of the DNA loops, uncapping of the telomeres resulting in telomere dysfunction-induced foci (TIFs) and the activation of DDR causing the cell cycle arrest through *p53* as mentioned before. This can also be replicated by disrupting genes involved in telomere maintenance [103].

Senescence can also be induced through persistent DNA damage caused by several genotoxic agents such as irradiation (ionizing and UV).

LYSOSOME ALTERATIONS

Lysosomes have been reported to increase in number and size in senescent cells [104]. They also accumulate defects leading to a change in their pH to more basic. This change is specifically related to senescence-associated beta-galactosidase (SA- β -gal) activity. Nonetheless, although the SA- β -gal is prominent in senescent cells it has been reported to be not required nor determinant for a cell to become senescent [105]. Accumulations of aggresomes in these defective lysosomes, especially of lipofuscin also stimulate expression of the anti-apoptotic factors of the Bcl-2 family, providing the cell with apoptosis resistance. Apoptosis resistance is also one of the key features of senescent cells [106].

NUCLEAR CHANGES

Downregulation of Lamin B1 is also one of the main attributes linked to cellular senescence. Lamin B1 is a major component of the nuclear lamina [107, 108]. Its downregulation leads to a reduction of the nuclear integrity coupled with a special rearrangement of H3K9me3 giving rise to senescence-associated chromatin structures[109]. Interestingly, these epigenetic

modifications appear to be stimulus and context dependent. For instance, oncogene induced senescence leads to the loss in local connectivity of regions rich with H3K9m3 and Lamin B1 which in turn perturbs long-range chromatin interactions [110]. On the other hand, replicative senescence shows loss of long-range and gain of short-range chromosomal interactions [111].

Downregulation of Lamin B1 coupled with the reduction of nuclear integrity also facilitates the SASP excretion by contributing to the formation of cytoplasmic chromatin fragments (CCF) [112].

A summary of all the previously mentioned markers is depicted in Figure 7.

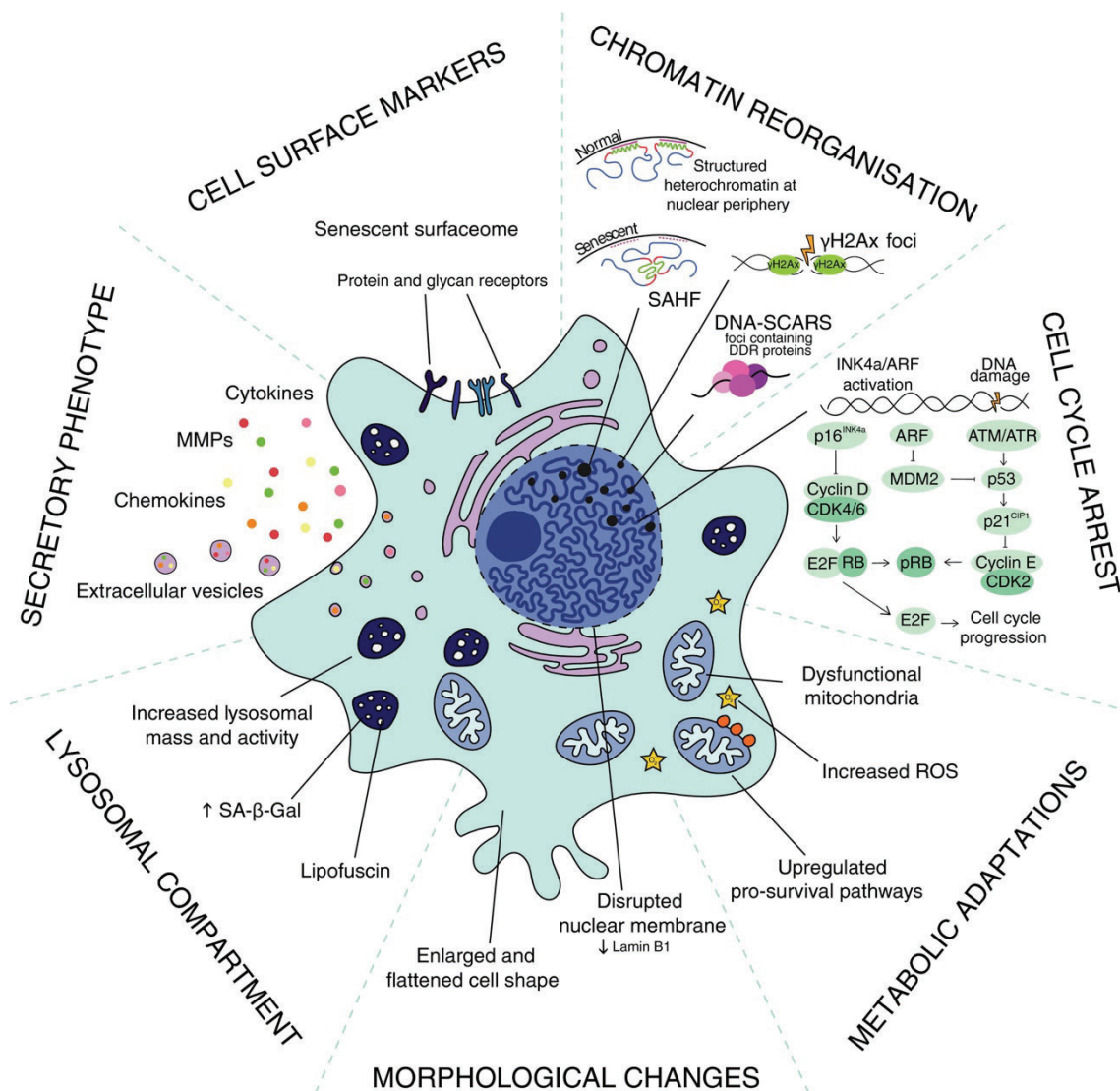


Figure 7: General hallmarks of cellular senescence. The ones discussed in this chapter include: the regulators of cell cycle arrest *p53* and *pRb*, components of the senescence associated secretory phenotype (SASP), DNA scars, senescence-associated beta-galactosidase activity (SA-β-gal), upregulation of pro-survival pathways in the form of apoptosis resistance and downregulation of Lamin B1 and disruption of the nuclear membrane. Obtained from [113].

SENESCENCE IN DEVELOPMENT

Senescence appears at various sites at specific time windows during development across multiple evolutionary distant organisms. It presents similarities with stress-induced

senescence which leads to a permanent cell cycle arrest but also presents distinctive features [114].

These features could potentially be categorized in three branches: organ regression, cellular balance and tissue growth.

In the first category we have the example of the regression of the mesonephros in mammals and birds also known as the pronephros in axolotl and *Xenopus*. This structure acts as a temporary kidney for the embryo to be replaced at later stages of development and which parts are reused to generate other structures in the adult. Inhibition of senescence in the mesonephros in mice has revealed defects in the establishment of tissues originated from such structure [115].

In the second category we find the senescence present in the mouse endolymphatic sac that could modulate the balance of cell populations by causing arrest and posterior clearance of certain subpopulations in comparison to others [115].

In the third category, we find the presence of senescent cells at the apical ectodermic ridge where they could act as a signaling center regulating limb patterning and growth [116].

All and all, these differences between developmental senescence and stress-induced senescence suggest that the latter could be adapted from the first one as a novel mechanism to offer protection against stress conditions [114].

SENESCENCE IN REGENERATION

Senescence is also present in regenerative processes in a wide range of organisms. As mentioned in development it also plays timed specific roles with different outcomes depending on the model organism and the tissue affected.

There are examples of a transient tissue-damaged induced senescence promoting regeneration in models such as in zebrafish fin regeneration [117] and in salamander limb regeneration [118]. In these cases the incomplete elimination of senescent cells could lead to fibrotic tissue and block tissue formation [119].

Moreover, induced cell reprogramming towards pluripotency in mice through the expression of the Yamanaka factors (Oct4, Sox2, Klf4 and c-Myc) is enhanced in tissues with an accumulation of senescent cells [119].

A general consensus of the pathways involved in these processes as well as the specific SASP factors that could be involved in each of them has yet to be reached. Nonetheless, it is generally agreed that the mechanisms should involve mechanisms of proliferation, dedifferentiation, matrix remodeling or immune system activation respectively [120].

Another aspect of senescent cells in regeneration occurs more commonly in mammalian organ regeneration such as in the liver or the kidneys [121, 122]. In these contexts, senescent cell accumulation results in regeneration impairment most likely due to the accumulation of non-proliferative cells and deregulation of the SASP with pro-fibrogenic factors that promote tissue fibrosis. Nonetheless, once more, understanding the fine differences between such senescent footprints will be the key to properly understand the different mechanisms of tissue regeneration across species [120].

SENESCENCE IN CANCER

Senescence also presents a dual role in tumorigenic transformations. Traditionally, stress-induced senescence has been viewed together with apoptosis as a tumor suppressor mechanism that prevents cell cycle progression and thus tumor formation. Therefore disabling senescence is an step required for cancer cells to grow [2]. However, it is described that the SASP exuding from senescent cells present a paracrine effect which carry a cocktail of pro-

tumorigenic factors as well including pro-inflammatory cytokines, growth factors and matrix-remodeling enzymes aiding in the proliferation, metastasis and migration of tumorigenic cells.

Overall, further characterization of the different roles of each type of senescent cells present in the tumor microenvironment as well as the timeframe of their activation will help elucidate the triggers for tumoral transformation as well as the development of new targeted therapies [123].

ROLE OF SENOLYTICS AND SENOMORPHICS

Senolytics are broadly used as treatments to eliminate senescent cells since they present a senescent-specific cell killing activity[124]. Among these compounds Navitoclax (ABT-263) specifically inhibits the Bcl-2 antiapoptotic family of proteins in order to compensate the decrease of apoptosis linked with cellular senescence [125].

Senomorphics on the other hand, suppress senescence by suppressing SASP expression. In the case of Rapamycin this is achieved through the decrease activation of Stat3 pathway [126].

SENESCENCE IN PLANARIANS

In planarians it has been previously reported that in post-irradiation conditions an accumulation of senescent cells occurs [127]. However, the lack of further evidences still rise concerns of the actual evidences of senescence existing as a physiological mechanism in planarians. One of the main concerns involve their lack of conservation of several components of DDR and cell cycle checkpoints components. This suggest that as a whole, DNA damage response mechanisms as well as the cell cycle checkpoints could have drifted as a collective over the course of evolution. As an specific example, it has been reported that DNA damage response after irradiation downstream of ATM highlights a loss of *p53* function in driving apoptosis in planarians [128].

CHAPTER 4: CELLULAR DEDIFFERENTIATION

Cellular differentiation refers to the process that a cell undergoes from the asymmetric stem cell division until it achieves its terminal identity. During this path, the cell gains progressive commitment towards its final identity thanks to a dynamic combination of specific transcription factors that allow the activation of the appropriate genetic programs in order to obtain a specific differentiated cell from each lineage[129].

In order to maintain proper tissue functions, the identity of their constituent cells must be stable. This stabilization is maintained thanks to the establishment of specific epigenetic programs (through histone modifications and specific enhancer conformations) that allow the activation of a fixed genetic program key for the cellular identity stability [130]. Nonetheless, such programs can be altered and trigger a loss of cell identity. Thus, cell dedifferentiation refers to events in which differentiated or committed cells acquire characteristics of less mature cells [131]. During this process, the telomeres are reset and start to elongate, although the exact dynamics and lengths in different tissues remain still to be elucidated [132, 133]. A clear example of this process *in vitro* is the production of iPSC cells from terminally differentiated cells through the overexpression of a specific combination of transcription factors, the Yamanaka factors mentioned in the previous chapter[134]. However, an even more striking example of this production of stem cells has been characterized *in vitro* just by altering the Hippo pathway. In this case, transient expression of YAP/TAZ was sufficient to induce dedifferentiation of a range of differentiated cells into somatic stem cells of the same tissue [49]. It has also been reported that ectopic activation of YAP in adult mice promote the dedifferentiation of hepatocytes [135]. On the other hand, halting YAP overexpression induces the rapid reappearance of differentiated cells in the intestinal crypts [76]. These evidences go in line with the *hippo* RNAi planarian model introduced in chapter 3 [82].

Nonetheless, dedifferentiation is a mechanism that also occurs naturally. Specifically, after tissue loss or damage in some species. As indicated in the previous chapter, the limb regeneration in urodele amphibians is achieved through this mechanism. After amputation, the cells adjacent to the wound dedifferentiate and form a blastema of undifferentiated cells that proliferate and later redifferentiate in order to regenerate the tissues of the lost limb [136].

This concept is difficult to visualize in the classical model of the Waddington landscape. In this model sequential developmental fate decisions induce an adult stem cell (ASC) to gain a defined identity according to descending cell lineages [137]. Nonetheless, a new model has recently been proposed, the so called “wobbling Penrose landscape”, which is not as strongly influenced by the vertebrate model of stem cells and encompasses better the diversity of stemness existing across metazoans. In this model, cell totipotency follows the logic of a Penrose staircase, an “Escherian loop” of stemness that juxtaposes the implicit gravitational force represented in the Waddington landscape, refuting the idea that a stem cell begins its journey at the top of a hill. Thus, at any point, the stem cell can initiate cascades of cellular phenotypes at different hierarchies of potency. These cascades may give rise to different lineages but with the ability to turn back at any point in ascending trajectories of potency. This means different forms of cellular dedifferentiation, for example. All these paths depict a dynamic or rather “wobbling” landscape. This representation therefore is opposed to the inevitable progressive loss of stem cell potency [138]. The visual representation model can be seen in Figure 8.

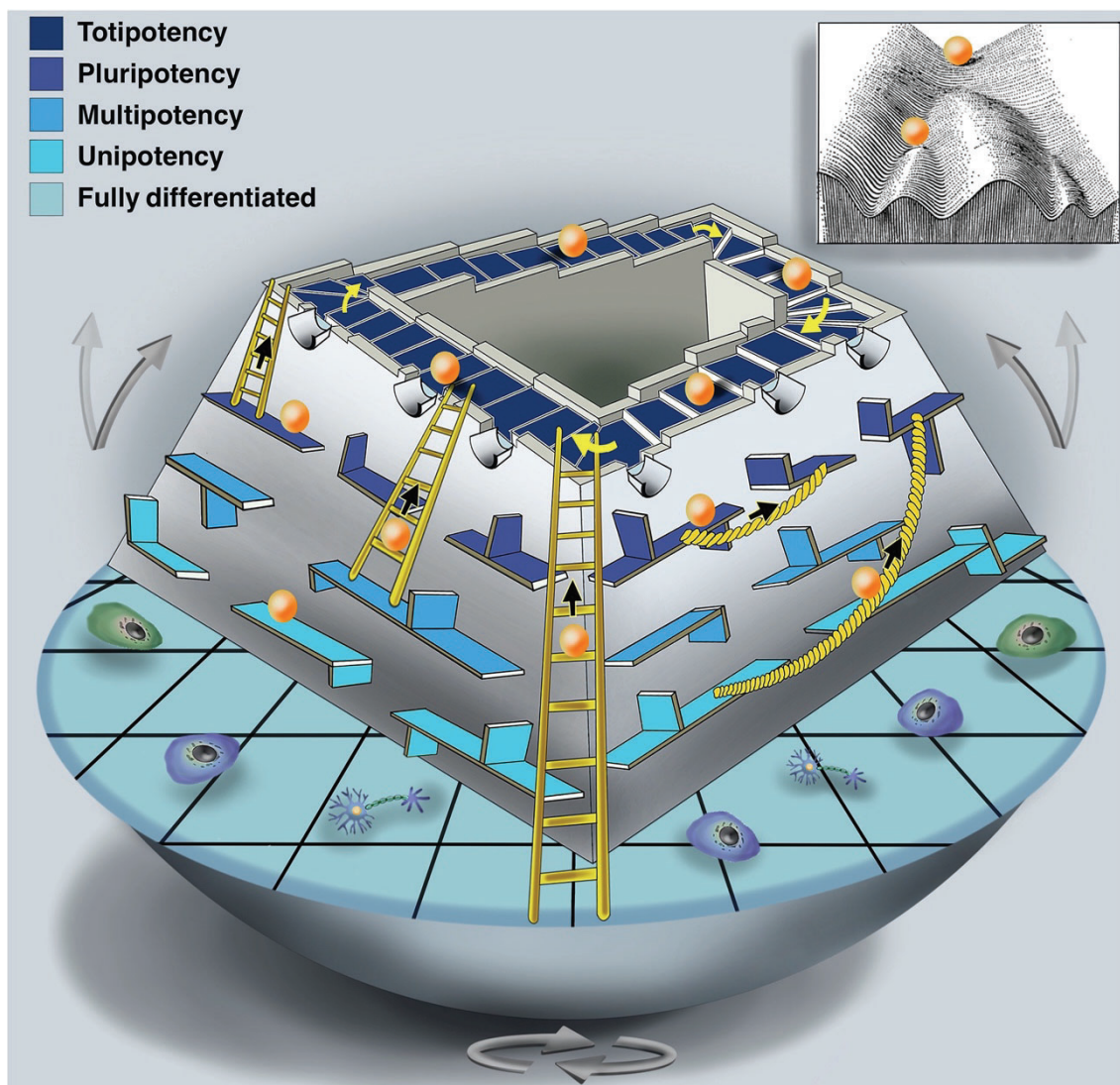


Figure 8: A graphical visualization of the ‘wobbling Penrose landscape’ metaphor. The dark blue stairs represent the Penrose staircase of stemness in which the stem cell depicted in orange make turns in a continuous loop. At any step, represented by funnels, the cell can descend stemness echelons depicted as gray downhill walls and remain at that level or further descend to a differentiated state depicted in the lighter blue tones depicted in the upper legend. The Escherian movement attributed to the cell allow a continuous passage to any stemness passage through dedifferentiation processes for example, depicted by the ropes or back to totipotency depicted by the ladders. It opposes the hilly Waddingtonian landscape depicted in the upper insert. Obtained from[138].

CHAPTER 5: CELL CYCLE

The cell cycle is the universal process of cell division to generate identical daughter cells achieved through accurate genome duplication followed by proper division. The whole process takes 24h for human cells cultured *in vitro*. The cycle is divided in four sequential phases: G1, S, G2, M. During the S-phase (S for DNA *synthesis*) the genomic DNA is accurately duplicated. This phase takes roughly half of the cycle. Together with the *gap phases* G1 and G2 it makes up the interphase of the cycle that takes up to 95% (23 hours) of the cycle. This is followed by the M-phase (for *mitosis*) which is the shortest phase of the cycle (1 hour) that segregates the DNA copies and divide the nucleolus and cytoplasm (cytokinesis) culminating in the formation of the two daughter cells. It must be remarked that the duration of the cell cycle phases is widely variable between different species and cell types. The budding yeast for example progresses through the 4 phases in just 1,5 hours and in the *Xenopus* fertilized egg (during early development) just in under 30 minutes due to the lack of G1 and G2 phases [139, 140].

G1 and G2 are required for the appropriate cell and organelle growth but moreover, they provide check-points in order to ensure cell cycle sustainability before entering the S and M phases respectively. In yeast for example, the lack of nutrition leads to a cell cycle arrest at G1 until conditions improve and the cell-cycle is postponed [141]. This effect has been connected with the energy requirements and other ATP intermediates necessary for DNA duplication. This has also been connected with the enhancement of mitochondria fusion. During the G1/S transition mitochondria form hyperpolarized tubes prior to mitosis. This hyperpolarization enhances catabolic capabilities to overcome the energetic requirements to bypass this stage of the cell cycle[142]. Thus, G phase may be very variable in length. When cells remain arrested for very long periods of time they enter a quiescence stage of the cycle, called G0[140]. Examples of G2 arrest also occur in *Caenorhabditis elegans* larvae under starving conditions [141]. An ideogram of the 4 phases can be seen at Figure 9.

Mistakes produced during the DNA duplication, chromosome separation or insufficient growth during the cell cycle need to be detected and consequently the cycle must be blocked until the situation is resolved. Therefore, in the majority of eukaryotic cells exist three main checkpoints of cell cycle progression: the restriction point at late G1, at the G2/M transition and at the spindle-assembly metaphase to anaphase transition during M-phase. The core regulation of these checkpoints is mediated by the *cyclin dependent kinases* (Cdks) which are activated by their binding to their respective paired cyclins. Cyclins, are proteins that undergo processes of synthesis and degradation according to each phase of the cycle thus regulating the whole process. In vertebrates 4 Cdk proteins exist; one for each cell cycle stage [143]. In yeast, on the contrary, one Cdk binds to all cyclins.[139]

- 1) Cyclin E is highest in late G1 and binds to Cdk2 protein and activates the G1-S transition.
- 2) Cyclin A is highest during S phase until mitosis and binds to Cdk2 and Cdk1 to promote chromosome duplication.
- 3) Cyclin B is highest at the G2/M transition and goes down at mid-mitosis. It binds to Cdk1 to enter the M phase.
- 4) Finally, Cyclin D synthesis is initiated at early G1 phase. It interacts with Cdk4 and Cdk6 and allows the progression through the restriction point.

An ideogram of the 4 phases and their regulations can be seen at Figure 9.

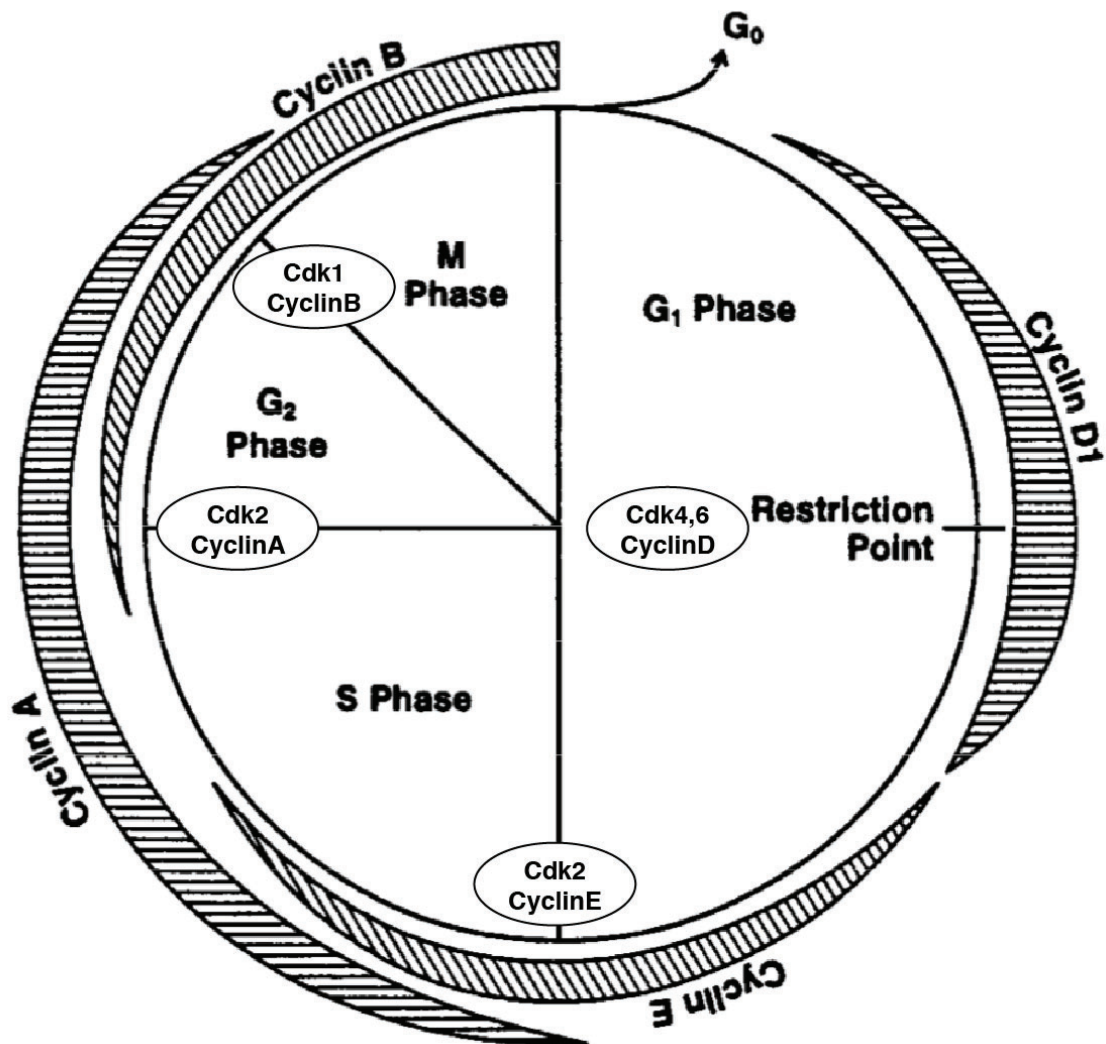


Figure 9: Cell cycle progression and checkpoints. The cell cycle consists of four phases (G₁, S, G₂ and M) regulated by different Cyclin-Cdk complexes. Cyclin levels change depending of the phase of the cycle and are represented by the thickness of the crescents. The restriction point at G₁ marks the decision to enter a new cycle or remain quiescent (G₀). Obtained from [143].

In planarians, there are some peculiarities that suggest that some differences exist in their cell cycle progression. One of the most sticking ones is the lack of conservation of MAD1 and MAD2, two near-universally conserved proteins responsible of the spindle-assembly checkpoint (SAC), guarding cells from aneuploidies by arresting the cycle as long as at least on chromosome remains unattached to the mitotic spindle. Other components of the SAC complex have also been lost in planarians. Arrests in the M checkpoint occur therefore MAD1/2 independently. [144]

In planarians Rb has conserved the ancestral role in cell division and cell death. Nonetheless flatworms as a phylum have undergone a loss of an activating E2F homolog. Flatworms have also lost a *cyclinE* homolog, a critical regulator of Rb activity in other systems. Its canonical binding partner though, CDK2 still exists in planarians but has very low expression based on RNA-seq data suggesting it may no longer function in cell cycle [145].

Planarians also present only a single homolog of the vertebrate *p53* family with both tumor-suppressor (vertebrate *p53*) and self-renewal (vertebrate *p63*) properties. This goes in line with the hypothesis of an ancestral molecule that presented the properties of *p53*, *p63* and *p73* in vertebrates and was further split amongst its paralogs. Furthermore, so far, no homolog of *p21* has been found in planarians thus making impossible to test if the *smad-p53* functions through *p21* to trigger cell cycle arrest after stress is inflicted [146]. This evidences go in line with the previous chapter where it was mentioned the lack of apoptotic induction of *p53* post irradiation stress [128].

Overall these peculiarities of the planarian cell cycle open the question of how they affect their proliferation capabilities and which evidences one must gather to better understand how the cell cycle checkpoint mechanisms work in this particular system. Therefore, this thesis benefits from the information the assays in cell cycle progression may provide in the Hippo pathway context. This is due to the important implications the cycle holds in relation to the proliferation and loss of cell identity previously discussed.

CHAPTER 6: GROWTH AND mTOR PATHWAY

Cell cycle entry and progression in order to trigger proliferation described in the previous chapter lead to continuous decrease in cell size with every division. Therefore, cell growth is essential to allow this continuous cell turnover. This growth requires large amounts of the novo synthesized macromolecules (proteins, lipids and nucleic acids). This synthesis requires large amounts of energy consumption. Thus, cells are able to monitor the amount of nutrients and energy reserves they possess before entering the cycle. One key example is the ability of the *Drosophila* ovaries to adjust their division rate according to nutrient availability and reduce the production of eggs in times of scarcity [147]. One of the key pathways being described to play a role in this context is the TOR pathway since it monitors growth and proliferation in response changes in nutrient availability [148].

The TOR pathway is an evolutionary conserved molecular pathway with TOR as its core element [149]. TOR (target of Rapamycin) is a serine/threonine kinase that monitors signals from nutrients like amino acids and growth factors like insulin and insulin-like growth factor (IGF). One of their main downstream target effectors is the synthesis of proteins for appropriate growth. These include cell-cycling-driving proteins such as cyclins described in the previous chapter [150]. Thus, it has been hypothesized that the checkpoint at late G1 in which cells happen to enter a new cell cycle or not in order to trigger proliferation could be mediated by TOR. This evidence is gathered from the fact that inhibition of TOR signaling arrests cells at late G1. This could be a way for the cell to determine if they have sufficient nutrients and energy available before committing to proliferation. This could be similar to the checkpoint described in yeasts [151]. The proposed mechanism suggests that under nutrient and energy rich conditions, TOR is active and increases protein synthesis. This leads to an increase of Cyclin E levels which promotes the progression of the late G1 phase of the cycle as previously described [150]. Thus, TOR ends up promoting the progression of the cycle in a nutrient and energy dependent way.

THE MTOR PATHWAY

TOR appears in two different complexes, mTORC1 and mTORC2. In mTORC1 it interacts with a regulatory associated protein of TOR (raptor) and its role is mainly focused in protein translation. mTORC2 on the other hand presents the rapamycin-independent companion of TOR (rictor) and its role is mainly focused in actin cytoskeleton organization [149].

mTORC1 receive diverse upstream stimuli: mainly by extracellular growth factors or through amino acids. These growth factors act via tyrosine kinase receptors (RTKs) which signals are propagated to mTORC1 via PI3K/Akt or RAS/Erk signaling cascades [149, 152, 153]. Akt positively modulates mTORC1 in two different ways: through phosphorylating and inactivating the tuberous sclerosis proteins 1 and 2 complex (TSC1/2) (a negative regulator of mTORC1) and through phosphorylation and dissociating from mTORC1 the TORC1 inhibitory factor

proline-rich Akt substrate of 40 kDa (PRAS40). It is worth mentioning that a negative regulator of this PI3K-Akt axis is the phosphatase and tensin homologue (PTEN) which acts as a tumor suppressor that appears mutated in many cancers [154].

mTORC1 presents many effectors as well. The most characteristic ones involve translation regulation. This role is done by the ribosomal protein S6 kinases (S6Ks) and also by the eukaryotic initiation factor 4E(eiF4E)-binding proteins (4E-BPs). The later ones repress translation initiation by inhibiting the translation initiation factor eiF4E. Thus, TORC1 activation through phosphorylation leads to inactivation of 4E-BPs [149].

Moreover, it has been seen that Rapamycin (inhibitor of mTOR) is able to suppress DNA double strand break repair through the recruitment impairment of BRCA1 and Rad51 to DNA repair foci [155]. Similar effects have been associated with oncogenic forms of Akt that lead to the cytoplasmatic retention of BRCA1 and Rad51 independently of phosphorylation. This is in spite of the positive role of Akt in non-oncogenic cells [156].

GROWTH IN PLANARIANS AND MTOR

As mentioned in the planarian chapter, planarians constantly regulate their body size according to food availability. Thus, upon feeding, planarians grow. A depiction of this process can be visualized in Figure 10. It has been described that planarians present a mitotic peak after 1 day of feeding and progressively decrease their proliferative ratio until they reach a plateau by day 5 [157, 158]. In parallel, it has been observed that during the first days post-feeding cell death decreases to a minimum allowing the cell balance to shift towards an accumulation in numbers [159].

So far, the insulin/mTOR signaling pathway has been the only one described in planarians to be able to control their body size. Inhibition of insulin-like peptides or mTOR leads to a lack of proliferation and growth impairment [160–162]. Moreover, a novel negative regulator of mTOR named SMG-1 has also been described in planarians. It's downregulation has been associated with an hyperproliferative response that leads to the formation of lethal ectopic overgrowths [161]. The tumor suppressor PTEN has also been described to present a similar phenotype in planarians upon downregulation leading to overgrowths formation [163].

Strikingly, a de novo and taxonomically restricted family of genes identified as *blitzschnell* (*bls*) was also described to play a role in growth and cell proliferation in planarians. Their silencing lead to faster regeneration and an increase of cell number during homeostasis. In feeding conditions this increase in cell number leads to an increase in body size whereas in starving conditions it leads to a decrease of cell size and the formation of overgrowths. Interestingly, its expression is downregulated after feeding. Moreover, there are hints that suggest *bls* genes are integrated within the insulin/Akt/mTOR network. For instance, *bls* could be acting as an inhibitor of the mTOR pathway since upon *bls*2/3/5 inhibition *mTOR* and *Akt* appear upregulated and vice versa.

Overall, these phenotypes in planarians are the representation of the impairment of the tumor suppressor function and the associated defects in the progression of the cell cycle. This is explored in the previous chapter with the phenotype presented upon the inhibition of the *p53* homolog [146].

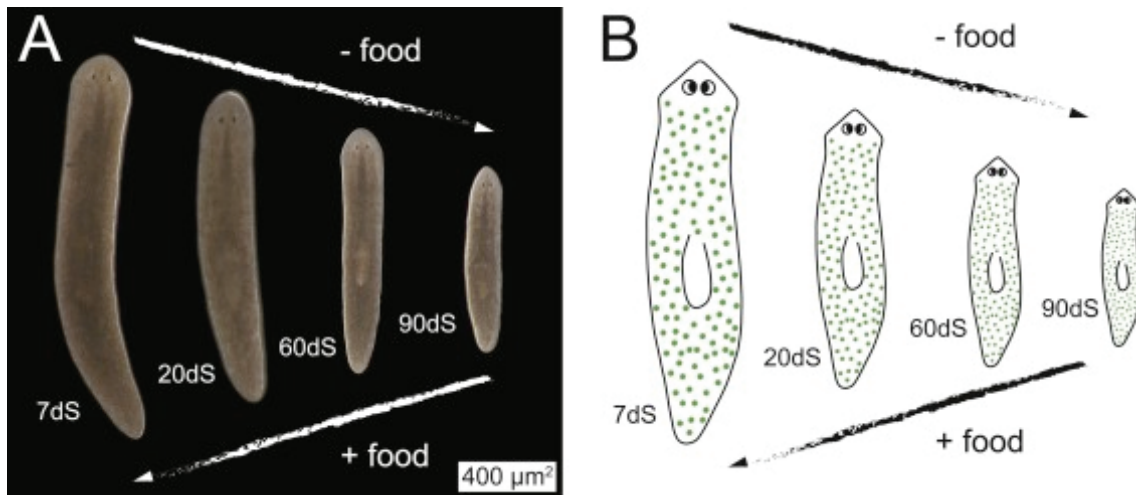


Figure 10: Planarian dynamics of growth and degrowth. A. Animal size comparison during a process of starvation from 7 to 90 days. Lack of food leads to a planarian degrow whereas food intake allows them to grow back again. B. Ideogram representing the mitotic neoblasts during a process of starvation. Despite being less in number the more their decrease in size, the relative proportion is maintained. Obtained from[164].

OBJECTIVES:

General Objective

The general objective of this thesis has been to understand the molecular mechanisms required for tissue renewal, maintenance of cellular identities and growth regulation. I have focused in the characterization the Hippo signaling pathway by studying differentially expressed genes (DEGs) in *hippo* RNAi planarians compared to controls.

Specific Objectives

- 1. To reanalyze a previous RNA-seq experiment to find DEG that function in cellular senescence in other model systems.**
 - 1.1. To functionally characterize those genes by RNAi experiments to see whether they are able to phenocopy *hippo* RNAi.
 - 1.2. To establish markers to detect senescence in planarians
 - 1.3. To investigate whether the downregulation of those genes is able to modulate senescence.
 - 1.4. To test senolytic drugs in planarians and investigate whether they could rescue the phenotype of *hippo* RNAi.
- 2. To reanalyze a previous RNA-seq experiment to find the most prevalent GO Ontology Terms that could mediate the Hippo-mediated tissue renewal and investigate their function.**
 - 2.1. To identify representative genes of each GO Ontology Term and study their function by RNAi to see whether they are able to phenocopy *hippo* RNAi
 - 2.2. To functionally characterize by RNAi and with the appropriate tools the implication of several candidates in the GO Ontology Terms-related function.
- 3. To define the role of *ccdc175*, a putative effector of *hippo*, in tissue renewal linking cellular senescence and cellular differentiation.**
 - 3.1. To pursue the identification of putative promoters of *ccdc175* to better characterize its tissue specification.
- 4. To study the function of *mitofusin2 (mfn2)* by RNAi experiments in planarians in two different contexts: feeding and starvation.**

RESULTS:

CELLULAR SENESENCE MEDIATES THE FORMATION OF OVERGROWTHS AFTER HIPPO SILENCING IN PLANARIANS

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The accumulation of senescent cells in old organisms, or upon chronic damage, contributes to chronic inflammation and age-associated disorders, including cancer. On the other hand, the Hippo signaling pathway is a key hub in the control of cellular renewal and is found systematically deregulated in tumoral processes. However, the relationship between the Hippo signaling, cellular senescence and tumoral transformation has been poorly studied.

Planarians (*Schmidtea mediterranea*) are ageless flatworms that endow a continuous tissue renewal and have the ability to regenerate. It has been demonstrated that Hippo inhibition in planarians promotes cellular dedifferentiation and the formation of overgrowths. In this study we characterize the function of two downstream *hippo* target genes related with cellular senescence (*syne1* and *trrap*). Functional characterization of their RNAi inhibition revealed a decrease of apoptosis and the presence of overgrowths, phenocopying *hippo* inhibition. Interestingly, the appearance of senescent cells before the formation of the overgrowths in those RNAi animals, as well as in *hippo* RNAis, was demonstrated by using different markers: accumulation of SA- β -Gal, a decrease of *Imnb1* expression levels and increase of *p53* and *pRb*. Moreover, a partial rescue of the phenotype was observed when *hippo* RNAi animals were treated either with the senolytic drug Navitoclax or the senomorphic drug Rapamycin.

Our results suggest that in planarians cellular senescence contributes to the onset of tumoral transformation observed when Hippo is downregulated.

INTRODUCTION

Cellular senescence implies the cessation of cell division. This is caused by several external stressors. Failure in relieving the stress and/or repairing the damage results in genomic instability and chronification, which leads to DNA damage, loss of cell identity and failure in the activation of cell death programs (apoptosis). Senescence is characterized by tumor-suppressor activation, profound chromatin changes and secretome alterations [1–5]. Moreover, accumulation of senescent cells contributes to age-dependent chronic inflammation and age-associated disorders. [6–9]

On the other hand, regeneration and tissue renewal are essential homeostatic processes in adult organisms that require a strict control since its misregulation may contribute to diseases, such as cancer. Due to the tight relation found between the physiological processes responsible for organ growth and the pathological ones linked to tumor formation [10, 11], the Hippo pathway and its effectors have gained the interest of the scientific community in regards to their role on cancer progression.

The Hippo pathway is a cell-cell communication pathway initially described as a regulator of organ size through cell death and proliferation in response to the environment, which inhibition leads to the appearance of overgrowths in *Drosophila* [12] and an increase of organ size coupled with overgrowths in mammals [13]. Recent studies indicate a wider variety of functions of the pathway besides growth control, placing it as a pivotal pathway in the regulation of cell fate and maintenance of pluripotency [14]. It has also been directly linked with tumorigenesis in animal models and in humans [15]. These evidences reveal a high complexity in the Hippo pathway response elements. However, the relationship between the Hippo signaling, cellular senescence and tumoral transformation has been poorly studied.

Recently, the Hippo pathway has been characterized in planarians as a hub in regulating cell renewal through the control of cell death, differentiation and proliferation in response to cell-cell contact [16]. *Schmidtea mediterranea* is an emerging model in biomedicine broadly used in regeneration and stem cell studies. Planarians can regenerate an entire organism from any fragment of their body by rebuilding lost tissue with the correct pattern. On the other hand, they are also constantly resizing according to food availability through a process that involves re-patterning of existing tissues [17]. *smed-hippo* (*hippo* from now on) silencing in planarians results in the decrease of apoptosis and the development of overgrowths and unpigmented regions formed by cells with a loss of identity, a feature shared with tumor progression in mammals. It has been reported that cellular senescence plays a role in multiple biological processes from tissue repair to organismal ageing [18–23].

Here we study two genes whose expression is modulated by the Hippo pathway in planarians and are related to cellular senescence in liver tumorigenic cell lines Hep3B and Huh7 and in hepatocellular carcinoma cells : *syne1* and *trrap* [24–26]. We adapt senescent assays to planarians to detect it upon RNAi inhibition of *nesprin-1*, *smed-syne1* (*syne1* from now on), *transformation/transcription domain associated protein*, *smed-trrap* (*trrap* from now on) and *hippo*. We also present a senolytic and a senomorphic treatment as tools to rescue senescence-associated phenotypes in planarians [27–29]. These drugs prevent the formation of the characteristic overgrowths of *hippo* RNAi. All in all, we describe the role of *hippo* regulating cellular senescence and maintaining cellular identity in planarians.

RESULTS

***hippo* putative effectors *syne1* and *trrap* phenocopy *hippo* RNAi**

We re-analyzed a previously published RNA-seq experiment on *hippo* RNAi planarians at 1, 3 and 4 weeks of inhibition compared to controls and focused at the 1 week RNAi timepoint to

check for early response transcripts that could be linked with the initiation of the *hippo* RNAi phenotype but still are kept downregulated at the later timepoints of 3 and 4 weeks (Figure 1 A, A')[16]. At this early stage, *syne1* and *trrap* were revealed as two of the few downregulated transcripts with a significant Log2 Fold Change. (Figure 1B). Both of them stayed downregulated at 3 and 4 weeks as well (Figure 1C). *gfp* is used as a control because it is a gene not present in planarians and its inhibition does not affect its transcriptome.

We found *syne1* and *trrap* as interesting candidates, as these represent novel putative downstream effectors of the Hippo pathway in any organism and they have been previously linked to senescence in other model systems. Syne1 is known to act as the link between the actin cytoskeleton and the nucleus and has been related with the maintenance of nuclear shape through cytoskeleton interactions. Its role has been mainly described in tumorigenic cell lines. It was shown to be involved in the transduction of mechanic signals and chromatin status. Defects in its expression cause nuclear deformations that lead to cellular senescence [24]. On the other hand, Trrap is an adapter protein found in complexes with histone acetyltransferase activity (HAT) that plays an important role in the DNA Double-strand break (DSB) repair mechanism. Moreover, *trrap* has been described as an oncogene in several tumorigenic contexts. Specifically, in hepatocellular carcinoma cells it has been reported that depletion of *trrap* induces G2/M arrest and senescence in a p53- and p21-independent manner[25, 26].

Our results showed that *hippo* RNAi phenocopy in *syne1* and *trrap* RNAi animals regarding the presence of overgrowths and/or unpigmented regions after 3 weeks of inhibition in a significant number of planarians (Figure 1D). To determine if the phenotype was caused by an early imbalance of cellular populations due to a decrease of apoptosis as previously described in *hippo* RNAi [16], we performed a Caspase-3 activity assay [30] after 2 weeks of RNAi. We confirmed that RNAi of either *syne1* or *trrap* presented a decrease of apoptotic activity matching *hippo* RNAi phenotype (Figure 1E). As negative control, other candidate effectors unrelated to senescence and with diverse expression patterns (*ly1 antibody reactive*, *smed-lyar*; from now on *lyar*, *serine/arginine repetitive matrix 2*, *smed-srrm2*; from now on *srrm2* and *sco-spondin*, *smed-sspo*; from now on *sspo*) (Figure S1B, S1C) were also tested and presented a lower penetrance of the unpigmented and overgrowth phenotype (Figure S1A) although also presented a decrease in apoptosis (Figure S1D). A summary of the biological function attributed to each candidate can be seen at Table S1 and a representation of their conserved domains between planarians and humans are depicted in Figure S1E.

***hippo*, *syne1* and *trrap* downregulation positively correlate with senescence markers**

In order to characterize the possible role of the Hippo pathway in regulating senescence we first established senescence markers in planarians. These include the transcript quantification of the senescent markers *smed-p53* (*p53* from now on) , *smed-pRb* (*pRb* from now on) and *smed-lmn1* (*lmn1* from now on) and the improvement of a previously published senescence-associated beta-galactosidase (SA- β -gal) activity assay in whole-mount planarians [31]. We validated *lmn1* as a senescent marker by testing its expression in a post-irradiation context [31] in which senescence is induced. Downregulation of Lamin B1 coded by the gene *lmn1* is one of the main attributes linked to cellular senescence since Lamin B1 is a major component of the nuclear lamina [32, 33]. It is responsible for keeping the nuclear lamina integrity as well as the chromatin conformation of the nuclei [34].

We validated *lmn1* level as a senescent marker by testing its expression in a post-irradiation context [31] in which senescence is induced. We could corroborate by whole mount in-situ hybridization (WISH) that the level of *lmn1* decreased progressively each day post irradiation until the seventh day of post irradiation (Figure S2A). *lmn1* expression analysis by WISH and qPCR revealed that the expression decreased in *syne1*, *trrap* and *hippo* RNAi animals

after 1 week of inhibition (Figure S2B). Second, the levels of *p53* and *pRb* appeared significantly upregulated in all 3 downregulations (Figure 2A). Finally, we stained for SA- β -gal activity at the same conditions. SA- β -gal activity was detected in *hippo*, *syne1* and *trrap* RNAi animals after 1 week of inhibition before the emergence of the phenotype. The accumulation of positive cells was mostly detected at the periphery of the animal close to the dorsoventral border (Figure 2B).

The senolytic drug Navitoclax and the macrolide compound Rapamycin rescue the *hippo* RNAi phenotype

To better understand the importance of senescence in the *hippo* RNAi phenotype, rescue experiments were conducted co-injecting dsRNA of *hippo* together with the drugs Navitoclax (16 nM) or Rapamycin (40 nM) for a period of 3 consecutive weeks. The objective was to detect how important is the role of cellular senescence in *hippo* RNAi animals for the formation of unpigmented regions and overgrowths. We thought that it could also help to elucidate how much of the phenotype is due to senescence. Whereas Navitoclax acts by activating the apoptotic pathway, Rapamycin is not a senolytic strictly speaking and removes senescent cells through the inhibition of the mTOR pathway activating autophagy [35, 36]) (Figure 3A, A'). The Navitoclax concentrations were extrapolated from senolytic xerograph assays performed on mice [27] whereas the Rapamycin concentration was adapted from a previous experiment on homeostatic planarians. This specific Rapamycin concentration was used to ensure that the mTOR pathway was being effectively inactivated in these animals [29].

No phenotype was observed in the co-injections of *gfp* dsRNA together with either Rapamycin or Navitoclax compared to the *gfp* controls co-injected with DMSO. However, injection of dsRNA of *hippo* together with Rapamycin revealed a rescue phenotype of up to 40% of the animals (10 out of 15) compared to animals injected with *hippo* RNAi and DMSO (5 out of 15 animals). In the case of the Navitoclax, the rescue percentage was 60% (11 out of 15 animals) compared to animals injected with *hippo* RNAi and DMSO (2 out of 15 animals) (Figure 3B). We consider as a rescue the animals that present no visible phenotype after the injections compared to their respective controls. On the other hand, animals that present unpigmented areas across their bodies or overgrowths protruding from the sides of the animal are considered to present the phenotype. Representatives of each category for each condition are summarized in Figure B'. The contingency tables of significance of each condition are found in Figure 3B''.

In order to measure the senolytic effect of Navitoclax through the activation of apoptosis we next checked the apoptotic activity in *hippo* RNAi planarians rescued with Navitoclax. We observed higher levels of caspase-3 activity when compared to the *hippo* RNAi animals co-injected with DMSO (Figure 3C) suggesting that a rescue is occurring through an activation of apoptotic activity.

As a readout of the effect of rapamycin, the number of cells positive for phospho-histone H3 (H3P⁺) [37] was analyzed. We expected a decrease in mitosis when treating *hippo* RNAi planarians with Rapamycin compared with untreated *hippo* RNAi planarians. A significantly decrease in the case of *hippo* RNAi animals treated with Rapamycin compared to untreated *hippo* RNAi animals was observed (Figure 3D). We also detected the previously published [29] effect of Rapamycin in control planarians as a slight decrease of H3P⁺ cells in rapamycin treated *gfp* controls compared to untreated controls (Figure 3D) [29]. This results suggests that Rapamycin compensates the increase of H3P⁺ cells observed after *hippo* RNAi.

DISCUSSION

The results reported here suggest that *hippo* leads to the formation of overgrowths through a prior accumulation of senescent signals (Figure 1). RNA-seq experiments on *hippo* RNAi animals have shown two conserved genes which have been previously related to senescence. Among them we found *syne1* and *trrap*, whose RNAi phenocopies the one of *hippo*. *syne1* and *trrap* have been described to present a prevalent role in preventing senescence *in vitro* and in the mouse model respectively (TableS1) [24, 25]. We also show that their silencing leads to a decrease in apoptotic activity 1 week prior the emergence of the overgrowths. This suggests that a decrease in apoptotic activity is a prerequisite for this phenotype.

More importantly though, we have been able to quantify at a transcriptomic level some bona fide senescent markers (*p53*, *pRb* and *Imnb1*) which change their expression levels accordingly at early stages of *hippo* inhibition as well as in the case of the inhibition of senescence candidates *syne1* and *trrap*. It has been seen in other model organisms such as in mice and that the transcriptomic levels of *p53* and *pRb* increase and the levels of *Imnb1* decrease upon cellular senescence[38]. This suggests that a peak of senescence is a prerequisite for this phenotype [39, 40]. Nonetheless, it must be remarked that proteomic levels of expression of these markers and phosphorylation activation of p53 and pRb provide more direct evidence of cellular senescence than the transcript levels [41, 42]. We have also performed SA- β -gal staining upon *hippo*, *syne1* and *trrap* inhibition and detected an accumulation of cellular senescence aggregates in all three cases. Senescence-associated beta-galactosidase (SA- β -gal) activity refers to the beta-galactosidase activity associated with lysosomes that present a more basic pH. This basicity has often been associated with an accumulation of defects related with cellular senescence[43]. Nonetheless we still require a method to empirically quantify such accumulations of SA- β -gal positive cells and elucidate the type of cell populations positive for the staining. Taken together, these preliminary evidences open the door to expand the field of senescence in planarians in other contexts beyond what we know nowadays in the post irradiation context [31] and encourage us to adapt more accurate techniques to the model.

Finally, the senomorphic and senolytic rescue experiments we performed with Rapamycin and Navitoclax after *hippo* RNAi gave us some evidences of the necessity of the accumulation of senescent cells in order to trigger cellular dedifferentiation. Nonetheless there are other experiments we think that are required to further emphasize such evidences. One example would be to check by SA- β -gal staining if the positive aggregates found in *hippo* RNAi are prevented in both co-injection assays of Rapamycin or Navitoclax combined with *hippo* RNAi. Moreover the transcriptomic levels of the senescent markers *p53*, *pRb* and *Imnb1* should also be assessed in both drug rescues by qPCR. We expect that all these results would point to a decrease in all the senescence markers after the rescues in comparison to the *hippo* RNAi conditions. However until we have this data we cannot discard that the rescue might be caused due to other side effects caused by the drugs. Considering the important role that autophagy play in the suppression of cellular senescence and that it can be triggered specially by Rapamycin both in *in vivo* and *in vitro* contexts [44][45], it is possible that rapamycin treatment in *hippo* RNAi animals overactivate autophagy mechanisms and thus reduce the number of senescent cells leading to a reduction of overgrowths and unpigmented regions. However, further experiments are necessary to demonstrate the prevention of the senescent phenotype in planarian cells by Rapamycin. The same case can be argued for Navitoclax, a potent pro-apoptotic pharmaceutical that has already been tested in mammals in the context of tumor recession [20, 21, 27].

It shall be remarked that in planarians the phenotypical rescue that such drugs provide drastically differ between one and the other. Lethality appears much higher after the Navitoclax rescue treatments in comparison to Rapamycin. This could be due to the fact that Navitoclax

is intrinsically more toxic for planarians at the current used concentration. Therefore more replicates at lower concentrations of Navitoclax would be interesting to test in order to corroborate this hypothesis. Nonetheless, this also provides a notion of the intrinsic complexity that cellular senescence and its pathological ramifications entail [20, 46]. Not only that but also emphasizes the importance of the molecular targets that one must bear in mind when designing senolytic therapies since the implications of each treatment may present drastically different effects. Nowadays each of them only treats a small aspect of the senescence universe. The use of other specific senolytic drugs such as Dasatinib and Quercetin [47, 48] would further emphasize the role that cellular senescence play in *hippo* RNAi planarians to trigger the formation of overgrowths.

To sum up, we show for the first time the role that cellular senescence could have in planarians in order to trigger the rise of overgrowths and cellular dedifferentiation attributed to the knockdown of the Hippo pathway. We know the dual role senescence has in tumorigenic transformation includes a tumor suppressor mechanism to prevent cell cycle progression as well as a paracrine tumor inducing effect aiding in the proliferation, metastasis and migration of tumorigenic cells [49, 50]. Similarly the role of *hippo* itself in cancer appears to be ambiguous as well since it is reported to present both roles tumor suppressor and oncogene depending on the context and interactions with other pathways [51, 52]. At the end, despite their similarities, further investigations are required to connect further Hippo, senescence and their potential common role in tumoral transformation.

Our results indicate that cellular senescence act as one of the earliest drivers for the cellular dedifferentiation reported upon the knockdown of the pathway [14, 16]. Interestingly, there are still no evidences to indicate that such trigger for dedifferentiation occur in other model organisms. We also adapted preexisting senescence assays from other model organisms to study senescence in planarians [28, 29, 31]. Finally, we also open the door to use planarians as a novel model system for antitumorigenic senolytic treatments keeping in mind the limitations that still exist in the field and the side effects these components might have. We think that planarians provide an excellent in vivo landscape to test these peculiarities and other novel treatments thanks to their high plasticity and the versatility of experimental designs they provide in the senescent field.

METHODS

Planarian culture

The planarians used in this study are the asexual clonal strain of *S. mediterranea* BCN-10 biotype and were maintained as previously described [53] in planarian artificial medium (PAM) water [54]. Animals were fed twice per week with liver, and those used in starvation experiments were starved for 1 week.

Transcriptomic analysis

Transcriptomic analysis of previously published data [16] were performed on the Raw sequencing data in FASTQ format as well as the transcript abundances found in the NCBI Gene Expression Omnibus (GEO) [55] accessible using GEO Series accession number GSE95130.

Sequence analyses

A fragment of *Smed-sspo* was identified from [56]. The other candidate genes were identified from the genome [57] and a RNAi fragments were amplified using specific primers (Table S3). Sequence identity comparison was carried out using the pairwise alignment tool in Jalview suite v2.11[58]. Sequence comparison against the GenBank database was performed using the NCBI BLAST network server (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). Potential orthologs were searched for using tBLASTx where possible to allow a certain level

of tolerance in case of a high degree of divergence. Domain characterization of each transcript was carried out using Scan prosite [59].

RNA interference analysis dsRNA

dsRNA was synthesized by *in vitro* transcription (Roche) and microinjection performed, as previously described [60], following the standard protocol of 3 × 32 nl injection of dsRNA for 3 consecutive days. To achieve stronger inhibition, we performed the same protocol over 3 consecutive weeks in starved planarians. Control animals were injected with dsRNA for green fluorescent protein (GFP), a gene not present in planarians.

Drugs treatments

Rapamycin ([HY-10219](#)) and Navitoclax ([HY-10087](#)) were obtained from Med Chem Express (MCE) and were freshly diluted to 40 nM and 10 nM, in planarian water before daily prepharyngeal injections, respectively. Three pulses of 32 nL were injected per each per worm. Injections were always scheduled 2–3 hours before respective dsRNA injections [27–29].

Caspase 3 activity assay

At the end of the second week of RNAi inhibition, protein extraction was performed. BioRad protein reagent was used to obtain protein concentration of the cell lysates. Fluorometric analysis of caspase-3 activity was performed as described previously [30]. Fluorescence was measured in a luminescence spectrophotometer (Perkin- Elmer LS-50) using Fluostar Optima microplate fluorescence reader (BMG Labtech), applying the following settings: excitation, 380 nm; emission, 440 nm. 20 mg of protein extract was used to determine enzyme activity, incubating for 2 h at 37 °C with 20 µM caspase-3 substrate Ac-DEVD-AMC or 2 ml from a stock of 1 mg/ml for a final volume of 150 µl. 3 biological replicates with three technical replicates per biological replicate were analyzed per condition. Caspase-3 inhibitor Z-DEVD-FMK was used as a control.

Quantitative real-time PCR

Total RNA was extracted from a pool of five planarians per condition using TRIzol reagent (Invitrogen). cDNA was synthesized as previously described [30]. Expression levels were normalized to that of the housekeeping gene *ura4*. All experiments were performed using three biological per condition and three technical replicates per biological replicate. The design of specific primers corresponding to *lmnb1*, and the use of previously published ones for *p53* and *pRb* [61] allowed to check their levels of expression after the RNAi experiments. All primers used in this study are shown in TableS2.

Whole-mount *in situ* hybridization

Probes were synthesized *in vitro* using SP6 or T7 polymerase and DIG- or FITC- modified (Roche) from previously cloned fragments using primers found in TableS1 as previously described [62]. For colorimetric whole-mount *in situ* hybridization, animals were sacrificed using 5% N-acetyl-L-cysteine (NAC), fixed with 4% formaldehyde and permeabilized with Reduction Solution (50 mM DTT, 1% NP-40, 0.5% SDS in 1× PBS). The fixative and whole-mount *in situ* hybridization protocol used has been previously described [63].

Immunohistochemistry

Whole-mount immunohistochemistry was performed as previously described [64]. Animals were killed with 2% HCl and fixed with 4% formaldehyde. The primary antibody used was

rabbit anti-phospho-histone H3 (Ser10) (D2C8) (PH3) (1:500; Cell Signaling Technology; 9701). The secondary antibody used was Alexa 568-conjugated goat anti-rabbit (1:1000; Molecular Probes; A-11011). Nuclei were stained with DAPI (1:5000; Sigma-Aldrich).

Single-Cell Sequencing Data

hippo, *syne1*, *trrap*, *lyar*, *srrm2*, and *sspo* gene expression profiles (dd_Smed_v6_4384_0, dd_Smed_v6_15904_0, dd_Smed_v6_4555_0, dd_Smed_v6_5281_0, dd_Smed_v6_3805_0 and dd_Smed_v6_3183_0 respectively) were obtained from the planaria single-cell database hosted by the Rajewsky lab at the Berlin Institute for Medical Systems Biology of the Max Delbrück Center, Berlin [65].

SA- β -galactosidase staining

The β -galactosidase activity was analyzed adapting the protocols described previously [50,51] [66]. Planarians were fixed with 2% formaldehyde + 0.2% glutaraldehyde for 5 min, washed twice with PBS and stained with the staining solution containing: 20 mg/ml of X-gal (Roche;10703729001) in dimethylformamide, 0.2 M citric acid/sodium phosphate buffer pH=6, 100 mM potassium ferrocyanide, 5 M sodium chloride and 1 M magnesium chloride. Planarians were incubated 1h and 40 min at 37 °C.

Imaging

In vivo images were taken with sCMEX-3 High End Digital Microscope Camera DC.3000s (Visual Inspection Technology). Whole mount *in situ* hybridization and immunostaining samples were observed with a *Leica* stereoscope and images were taken with *Progress C3 Jenoptik* camera. A *Leica* MZ16F microscope (Leica Microsystems) was used to observe the SA- β -galactosidase aggregates in the samples and obtain images. Images were treated using Image J and Photoshop CS6 software. Quantifications of H3P were done manually using the tool cell counter in Image J and normalized by animal area determined with the measurement tool.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 6. Two-sided Student's *t*-tests ($\alpha=0.05$) were performed to compare the means of two populations. Comparisons of more than 2 populations were done with ANOVA test. Previous confirmation of normality and homogeneity was performed with the Shapiro–Wilk test and Bartlett test respectively. To determine the p-value accumulated for each pairwise comparison the Tukey HSD test was used. Fisher.test from the R package was used to compare more than two phenotypic variants between two populations. Results were plotted using GraphPad Prism 6. To compare different populations, we used box plots depicting the median, the 25th and 75th percentiles (box), and all included data points (colored dots). Whiskers extend to the largest and the smallest data point. To visualize the percentage phenotype in each population we used the Stacked Bars plots. Each phenotype was assigned a distinct color.

REFERENCES

1. Campisi J, D'Adda Di Fagagna F (2007) Cellular senescence: When bad things happen to good cells. *Nature Reviews Molecular Cell Biology* 8:729–740. <https://doi.org/10.1038/nrm2233>
2. Campisi J (2013) Aging, cellular senescence, and cancer. *Annual Review of Physiology* 75:685–705. <https://doi.org/10.1146/annurev-physiol-030212-183653>
3. Da Silva-álvarez S, Collado M (2016) Cellular Senescence. *Encyclopedia of Cell*

- Biology 3:511–517. <https://doi.org/10.1016/B978-0-12-394447-4.30066-9>
4. Rodriguez-Brenes IA, Wodarz D, Komarova NL (2015) Quantifying replicative senescence as a tumor suppressor pathway and a target for cancer therapy. *Scientific Reports* 5:1–13. <https://doi.org/10.1038/srep17660>
 5. Kumari R, Jat P (2021) Mechanisms of Cellular Senescence: Cell Cycle Arrest and Senescence Associated Secretory Phenotype. *Frontiers in Cell and Developmental Biology* 9:1–24. <https://doi.org/10.3389/fcell.2021.645593>
 6. Debacq-chainiaux F, Ameer R Ben, Bauwens E, et al (2016) Cellular Ageing and Replicative Senescence. Springer International Publishing, Cham
 7. Yousefzadeh M, Henpita C, Vyas R, et al (2021) DNA damage—how and why we age? *eLife* 10:1–17. <https://doi.org/10.7554/eLife.62852>
 8. Olan I, Narita M (2022) Senescence: An Identity Crisis Originating from Deep Within the Nucleus. *Annual Review of Cell and Developmental Biology* 38:1–21. <https://doi.org/10.1146/annurev-cellbio-120420-013537>
 9. Rapp YG, Ransbotyn V, Grafi G (2015) Senescence meets dedifferentiation. *Plants* 4:356–368. <https://doi.org/10.3390/plants4030356>
 10. Halder G, Johnson RL (2011) Hippo signaling: growth control and beyond. *Development* 138:9–22. <https://doi.org/10.1242/dev.045500>
 11. Zhao B, Tumaneng K, Guan K-L (2011) The Hippo pathway in organ size control, tissue regeneration and stem cell self-renewal. *Nature Cell Biology* 13:877–883. <https://doi.org/10.1038/ncb2303>
 12. Xu T, Wang W, Zhang S, et al (1995) Identifying tumor suppressors in genetic mosaics: the *Drosophila* *lats* gene encodes a putative protein kinase. *Development* 121:1053–63. <https://doi.org/10.1101/gad.3.9.1273>
 13. Lu L, Li Y, Kim SM, et al (2010) Hippo signaling is a potent in vivo growth and tumor suppressor pathway in the mammalian liver. *Proceedings of the National Academy of Sciences* 107:1437–1442. <https://doi.org/10.1073/pnas.0911427107>
 14. Panciera T, Azzolin L, Fujimura A, et al (2016) Induction of Expandable Tissue-Specific Stem/Progenitor Cells through Transient Expression of YAP/TAZ. *Cell Stem Cell* 19:725–737. <https://doi.org/10.1016/j.stem.2016.08.009>
 15. Yin MX, Zhang L (2014) Hippo signaling in epithelial stem cells. *Acta Biochimica et Biophysica Sinica* 47:39–45. <https://doi.org/10.1093/abbs/gmu111>
 16. de Sousa N, Rodríguez-Esteban G, Rojo-Laguna JI, et al (2018) Hippo signaling controls cell cycle and restricts cell plasticity in planarians. *PLoS Biology* 16:1–28. <https://doi.org/10.1371/journal.pbio.2002399>
 17. Saló E (2006) The power of regeneration and the stem-cell kingdom: Freshwater planarians (Platyhelminthes). *BioEssays* 28:546–559. <https://doi.org/10.1002/bies.20416>
 18. Yun MH (2018) Cellular senescence in tissue repair: Every cloud has a silver lining. *International Journal of Developmental Biology* 62:591–604. <https://doi.org/10.1387/ijdb.180081my>
 19. Antelo-Iglesias L, Picallos-Rabina P, Estévez-Souto V, et al (2021) The role of cellular senescence in tissue repair and regeneration. *Mechanisms of Ageing and Development* 198:111528. <https://doi.org/10.1016/j.mad.2021.111528>

20. McHugh D, Gil J (2018) Senescence and aging: Causes, consequences, and therapeutic avenues. *Journal of Cell Biology* 217:65–77. <https://doi.org/10.1083/jcb.201708092>
21. Di Micco R, Krizhanovsky V, Baker D, d'Adda di Fagagna F (2021) Cellular senescence in ageing: from mechanisms to therapeutic opportunities. *Nature Reviews Molecular Cell Biology* 22:75–95. <https://doi.org/10.1038/s41580-020-00314-w>
22. Yu Q, Walters HE, Pasquini G, et al (2023) Cellular senescence promotes progenitor cell expansion during axolotl limb regeneration. *Developmental Cell* 58:2416–2427.e7. <https://doi.org/10.1016/j.devcel.2023.09.009>
23. Salinas-Saavedra M, Febrimarsa, Krasovec G, et al (2023) Senescence-induced cellular reprogramming drives cnidarian whole-body regeneration. *Cell Reports* 42:112687. <https://doi.org/10.1016/j.celrep.2023.112687>
24. Sur-Erdem I, Hussain MS, Asif M, et al (2020) Nesprin-1 impact on tumorigenic cell phenotypes. *Molecular Biology Reports* 47:921–934. <https://doi.org/10.1007/s11033-019-05184-w>
25. Kwan S, Sheel A, Song C, et al (2020) Depletion of TRRAP Induces p53-Independent Senescence in Liver Cancer by Down-Regulating Mitotic Genes. *Hepatology* 71:275–290. <https://doi.org/10.1002/hep.30807>
26. Murr R, Vaissière T, Sawan C, et al (2007) Orchestration of chromatin-based processes: Mind the TRRAP. *Oncogene* 26:5358–5372. <https://doi.org/10.1038/sj.onc.1210605>
27. Chen J, Jin S, Abraham V, et al (2011) The Bcl-2/Bcl-X L/Bcl-w inhibitor, navitoclax, enhances the activity of chemotherapeutic agents in vitro and in vivo. *Molecular Cancer Therapeutics* 10:2340–2349. <https://doi.org/10.1158/1535-7163.MCT-11-0415>
28. Oviedo NJ, Pearson BJ, Levin M, Alvarado AS (2008) Planarian PTEN homologs regulate stem cells and regeneration through TOR signaling. *DMM Disease Models and Mechanisms* 1:131–143. <https://doi.org/10.1242/dmm.000117>
29. González-Estévez C, Felix DA, Smith MD, et al (2012) SMG-1 and mTORC1 Act Antagonistically to Regulate Response to Injury and Growth in Planarians. *PLoS Genetics* 8:e1002619. <https://doi.org/10.1371/journal.pgen.1002619>
30. González-Estévez C, Felix DA, Aboobaker AA, Saló E (2007) Gtdap-1 promotes autophagy and is required for planarian remodeling during regeneration and starvation. *Proceedings of the National Academy of Sciences of the United States of America* 104:13373–13378. <https://doi.org/10.1073/pnas.0703588104>
31. Perrigue PM, Najbauer J, Jozwiak AA, et al (2015) Planarians as a model of aging to study the interaction between stem cells and senescent cells in vivo. *Pathobiology of Aging & Age-related Diseases* 5:30052. <https://doi.org/10.3402/pba.v5.30052>
32. Freund A, Laberge RM, Demaria M, Campisi J (2012) Lamin B1 loss is a senescence-associated biomarker. *Molecular Biology of the Cell* 23:2066–2075. <https://doi.org/10.1091/mbc.E11-10-0884>
33. Shimi T, Butin-Israeli V, Adam SA, et al (2011) The role of nuclear lamin B1 in cell proliferation and senescence. *Genes and Development* 25:2579–2593. <https://doi.org/10.1101/gad.179515.111>
34. Chandra T, Ewels PA, Schoenfelder S, et al (2015) Global reorganization of the nuclear landscape in senescent cells. *Cell Reports* 10:471–483. <https://doi.org/10.1016/j.celrep.2014.12.055>

35. Zhu Y, Tchkonina T, Fuhrmann-Stroissnigg H, et al (2016) Identification of a novel senolytic agent, navitoclax, targeting the Bcl-2 family of anti-apoptotic factors. *Aging Cell* 15:428–435. <https://doi.org/10.1111/ace.12445>
36. Tai H, Wang Z, Gong H, et al (2017) Autophagy impairment with lysosomal and mitochondrial dysfunction is an important characteristic of oxidative stress-induced senescence. *Autophagy* 13:99–113. <https://doi.org/10.1080/15548627.2016.1247143>
37. Hendzel MJ, Wei Y, Mancini MA, et al (1997) Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. *Chromosoma* 106:348–360. <https://doi.org/10.1007/s004120050256>
38. González-Gualda E, Baker AG, Fruk L, Muñoz-Espín D (2021) A guide to assessing cellular senescence in vitro and in vivo. *The FEBS Journal* 288:56–80. <https://doi.org/10.1111/febs.15570>
39. Ito T, Igaki T (2016) Dissecting cellular senescence and SASP in *Drosophila*. *Inflammation and Regeneration* 36:1–8. <https://doi.org/10.1186/s41232-016-0031-4>
40. Schmitt CA, Wang B, Demaria M (2022) Senescence and cancer — role and therapeutic opportunities. *Nature Reviews Clinical Oncology* 19:619–636. <https://doi.org/10.1038/s41571-022-00668-4>
41. Rodier F, Muñoz DP, Teachenor R, et al (2011) DNA-SCARS: Distinct nuclear structures that sustain damage-induced senescence growth arrest and inflammatory cytokine secretion. *Journal of Cell Science* 124:68–81. <https://doi.org/10.1242/jcs.071340>
42. Ben-Porath I, Weinberg RA (2005) The signals and pathways activating cellular senescence. *International Journal of Biochemistry and Cell Biology* 37:961–976. <https://doi.org/10.1016/j.biocel.2004.10.013>
43. Robbins E, Levine EM, Eagle H (1970) Morphologic changes accompanying senescence of cultured human diploid cells. 148–162
44. Wang R, Yu Z, Sunchu B, et al (2017) Rapamycin inhibits the secretory phenotype of senescent cells by a Nrf2-independent mechanism. *Aging Cell* 16:564–574. <https://doi.org/10.1111/ace.12587>
45. Sasaki N, Itakura Y, Toyoda M (2020) Rapamycin promotes endothelial-mesenchymal transition during stress-induced premature senescence through the activation of autophagy. *Cell Communication and Signaling* 18:1–14. <https://doi.org/10.1186/s12964-020-00533-w>
46. Van Deursen JM (2014) The role of senescent cells in ageing. *Nature* 509:439–446. <https://doi.org/10.1038/nature13193>
47. Hickson LTJ, Langhi Prata LGP, Bobart SA, et al (2019) Senolytics decrease senescent cells in humans: Preliminary report from a clinical trial of Dasatinib plus Quercetin in individuals with diabetic kidney disease. *EBioMedicine* 47:446–456. <https://doi.org/10.1016/j.ebiom.2019.08.069>
48. Justice JN, Nambiar AM, Tchkonina T, et al (2019) Senolytics in idiopathic pulmonary fibrosis: Results from a first-in-human, open-label, pilot study. *EBioMedicine* 40:554–563. <https://doi.org/10.1016/j.ebiom.2018.12.052>
49. Nardella C, Clohessy JG, Alimonti A, Pandolfi PP (2011) Pro-senescence therapy for cancer treatment. *Nature Reviews Cancer* 11:503–511. <https://doi.org/10.1038/nrc3057>

50. Da Silva-Álvarez S, Collado M (2021) The Jekyll and Hyde of Senescence in Cancer: TIMP1 Controls the Switch from Tumor-Controlling to Tumor-Promoting Senescence. *Cancer Cell* 39:13–15. <https://doi.org/10.1016/j.ccell.2020.12.013>
51. Zhao B, Lei QY, Guan KL (2008) The Hippo-YAP pathway: new connections between regulation of organ size and cancer. *Current Opinion in Cell Biology* 20:638–646. <https://doi.org/10.1016/j.ceb.2008.10.001>
52. Moroishi T, Hansen CG, Guan KL (2015) The emerging roles of YAP and TAZ in cancer. *Nature Reviews Cancer* 15:73–79. <https://doi.org/10.1038/nrc3876>
53. Fernández-Taboada E, Moritz S, Zeuschner D, et al (2010) Smed-SmB, a member of the LSM protein superfamily, is essential for chromatoid body organization and planarian stem cell proliferation. *Development* 137:1583–1583. <https://doi.org/10.1242/dev.051847>
54. Cebria F, Cebrià F, Newmark PA (2005) Planarian homologs of netrin and netrin receptor are required for proper regeneration of the central nervous system and the maintenance of nervous system architecture. *Development* 132:3691–3703. <https://doi.org/10.1242/dev.01941>
55. Barrett T, Wilhite SE, Ledoux P, et al (2012) NCBI GEO: archive for functional genomics data sets—update. *Nucleic Acids Research* 41:D991–D995. <https://doi.org/10.1093/nar/gks1193>
56. Cote LE, Simental E, Reddien PW (2019) Muscle functions as a connective tissue and source of extracellular matrix in planarians. *Nature Communications* 10:1592. <https://doi.org/10.1038/s41467-019-09539-6>
57. Grohme MA, Schloissnig S, Rozanski A, et al (2018) The genome of *Schmidtea mediterranea* and the evolution of core cellular mechanisms. *Nature* 554:56–61. <https://doi.org/10.1038/nature25473>
58. Waterhouse AM, Procter JB, Martin DMA, et al (2009) Jalview Version 2-A multiple sequence alignment editor and analysis workbench. *Bioinformatics* 25:1189–1191. <https://doi.org/10.1093/bioinformatics/btp033>
59. de Castro E, Sigrist CJA, Gattiker A, et al (2006) ScanProsite: Detection of PROSITE signature matches and ProRule-associated functional and structural residues in proteins. *Nucleic Acids Research* 34:362–365. <https://doi.org/10.1093/nar/gkl124>
60. Sánchez Alvarado A, Newmark PA (1999) Double-stranded RNA specifically disrupts gene expression during planarian regeneration. *Proceedings of the National Academy of Sciences of the United States of America* 96:5049–54. <https://doi.org/10.1073/PNAS.96.9.5049>
61. Peiris TH, Ramirez D, Barghouth PG, et al (2016) Regional signals in the planarian body guide stem cell fate in the presence of genomic instability. *Development (Cambridge)* 143:1697–1709. <https://doi.org/10.1242/dev.131318>
62. Pascual-Carreras E, Marin-Barba M, Herrera-Úbeda C, et al (2020) Planarian cell number depends on Blitzschnell, a novel gene family that balances cell proliferation and cell death. *Development* 147:. <https://doi.org/10.1242/dev.184044>
63. Currie KW, Brown DDDR, Zhu S, et al (2016) HOX gene complement and expression in the planarian *Schmidtea mediterranea*. *EvoDevo* 7:2663–2670. <https://doi.org/10.1186/s13227-016-0044-8>
64. Ross KG, Omuro KC, Taylor MR, et al (2015) Novel monoclonal antibodies to study tissue regeneration in planarians. *BMC Developmental Biology* 15:2.

<https://doi.org/10.1186/s12861-014-0050-9>

65. Plass M, Solana J, Wolf FA, et al (2018) Cell type atlas and lineage tree of a whole complex animal by single-cell transcriptomics. *Science* 360:1–17. <https://doi.org/10.1126/science.aag1723>
66. Dimri GP, Lee X, Basile G, et al (1995) A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proceedings of the National Academy of Sciences of the United States of America* 92:9363–9367. <https://doi.org/10.1073/pnas.92.20.9363>

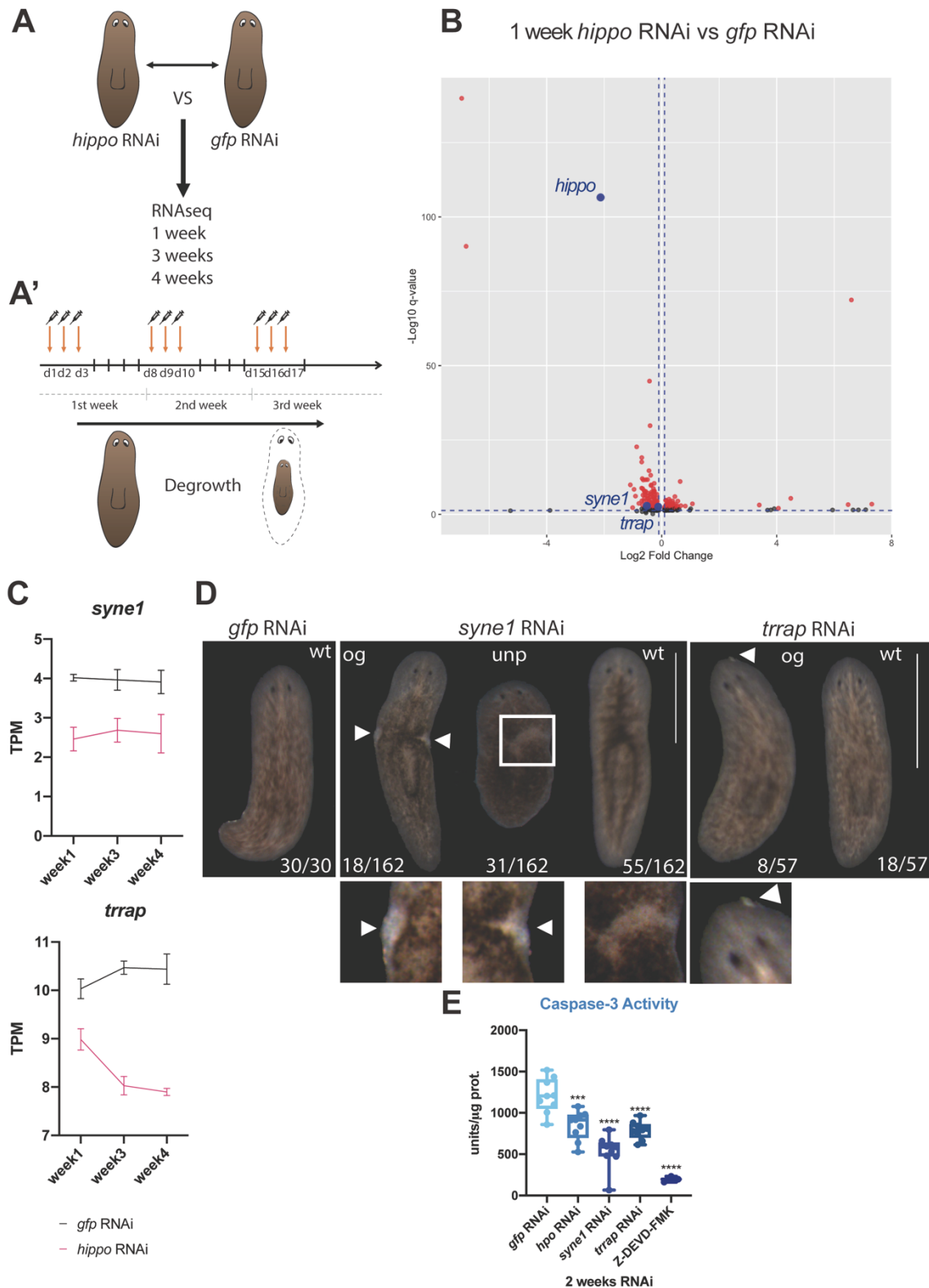


Figure 1: RNA-seq experiments on *hippo* RNAi planarians find *syne1* and *trrap* as putative effectors of *hippo*. (A) Cartoon depicting the experimental design of the *hippo* RNAi RNA-seq [16]. (A') Schematic illustrating the RNAi experimental procedure of the putative *hippo* effectors. (B) Volcano plot of differentially expressed genes between one week of *hippo* RNAi inhibition vs *gfp* RNAi controls. Differentially expressed genes are defined using edgeR with raw counts at 0,1 Log2FoldChange and filtered with a False Discovery Rate cutoff of 1% and

a q-value of 0,05. (C) Graph showing the transcripts per kilobase million (TPM) of *syne1* and *trrap* in the RNA-seq of *hippo* RNAi animals (pink) and controls (*gfp* RNAi, black). (D) Stereomicroscopic image of live *gfp*, *syne1* and *trrap* RNAi planarians depicting overgrowths and unpigmented regions ($n \geq 30$) at 3 weeks after RNAi treatment. White arrows indicate overgrowths, the white box a zoom frame of an unpigmented region. (E) Quantification of caspase-3 enzymatic activity in *hippo*, *trrap*, *syne1* and *gfp* RNAi animals after 2 weeks of injections. Z-DEVD-FMK corresponds to the negative control. Data is represented as mean $\pm$ s.e.m * $p < 0.05$; *** $p < 0.001$ was analyzed with ANOVA test. Scale bars: 1mm. RNAi, RNA interference; *gfp*, green fluorescent protein; og, overgrowth; unp, unpigmented regions; wt, wild type.

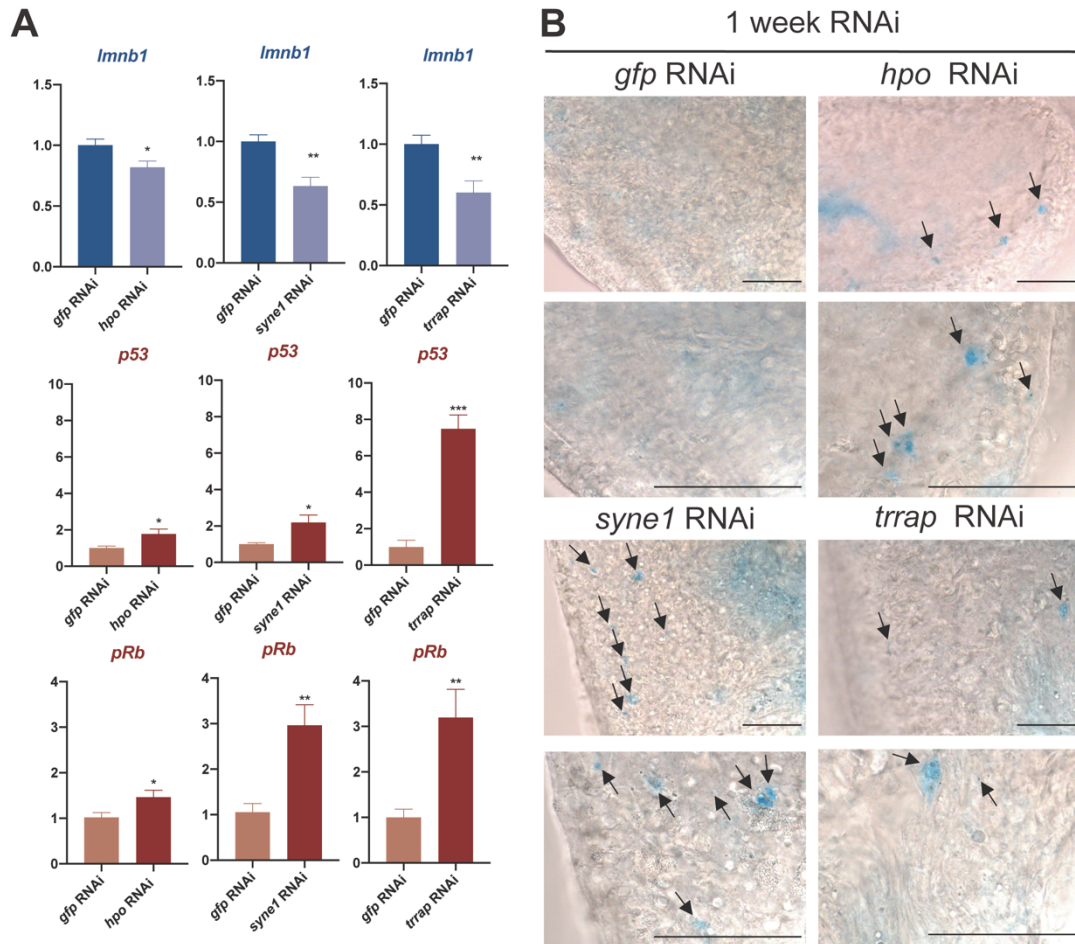


Figure 2: *hippo*, *syne1* and *trrap* downregulation correlate with positive senescence markers. (A) qPCR quantification of *lmnb1*, *p53* and *pRb* expression after 1 week of *hippo*, *trrap* and *syne1* RNAi respectively. In all the panels, relative expression is plotted as $2^{-\Delta\Delta CT}$ values. Data are analyzed by Student t test (indicar tipus de t-test) and represented as mean $\pm$ s.e.m * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$. (B) SA-β-gal activity staining in *gfp*, *hpo*, *syne1* and *trrap* RNAi animals. Black arrows point to positive staining for SA-β-gal activity. Scale bar 50 μm. All experiments were performed in planarians subjected to 1 week of RNAi. RNAi, RNA interference; *gfp*, green fluorescent protein; *hpo*, *hippo*.

function from the R package. (C) Quantification of caspase-3 enzymatic activity in *hippo*, and *gfp* RNAi animals treated with Navitoclax or DMSO after 3 weeks of injections. Z-DEVD-FMK corresponds to the negative control. Data is represented as mean $\pm$ s.e.m * $p < 0.05$; *** $p < 0.001$ was analyzed with ANOVA test. Scale bar 200 μ m. RNAi, RNA interference; *gfp*, green fluorescent protein; *hpo*, *hippo*; Rap, rapamycin; Nav, navitoclax; wt, wild type; unp; unpigmented regions; og, overgrowth.(D) Quantification of mitotic cells (H3P+) normalized by area after rescue experiments of *hpo* and *gfp* RNAi (control) animals with Rapamycin or DMSO as drug control. The experiments were performed in planarians subjected to 3 weeks of injections (n $\geq$ 5). Data is represented as mean $\pm$ s.e.m * $p < 0.05$; *** $p < 0.001$ was analyzed with ANOVA test. *gfp*, green fluorescent protein; *hpo*, *hippo*; ns, non-significant.

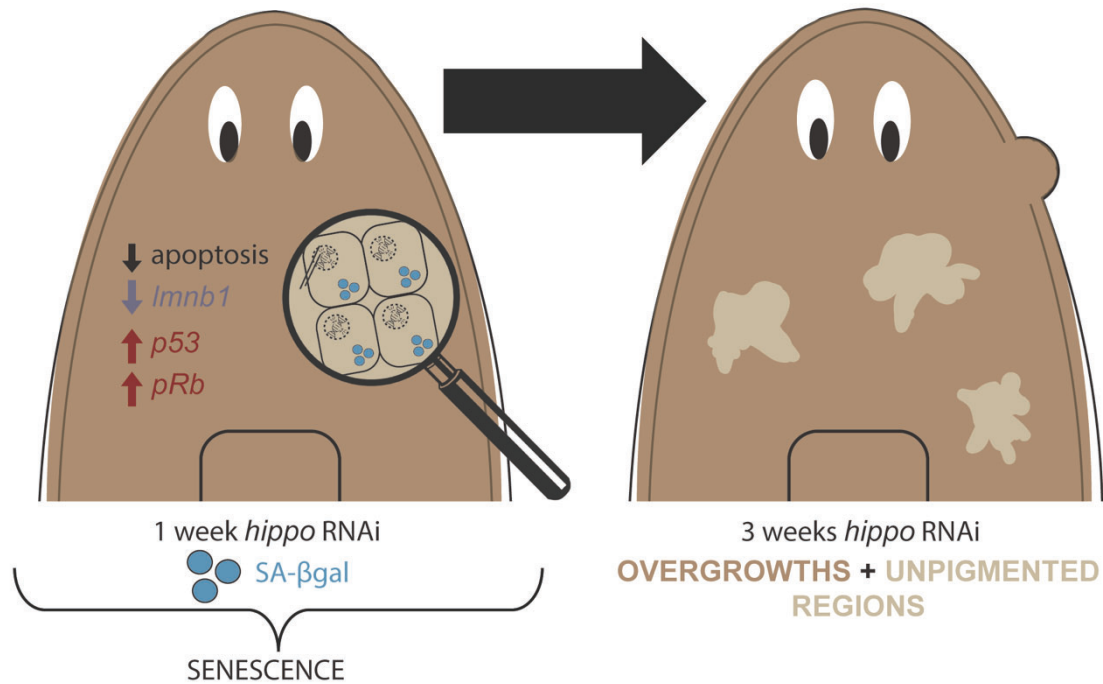


Figure 4: Model representing *hpo*-induced onset of senescence in planarians. In an early stage of *hippo* RNAi inhibition planarians accumulate a myriad of senescence markers such as a transcriptomic increase of *p53* and *pRb* and a decrease of *lmnb1* coupled with a decrease of apoptotic activity and an accumulation of SA-β-gal . This accumulation of senescence cells is later required for the emergence of the overgrowths and unpigmented regions after 3 weeks of *hippo* RNAi inhibition.

SUPPORTING INFORMATION

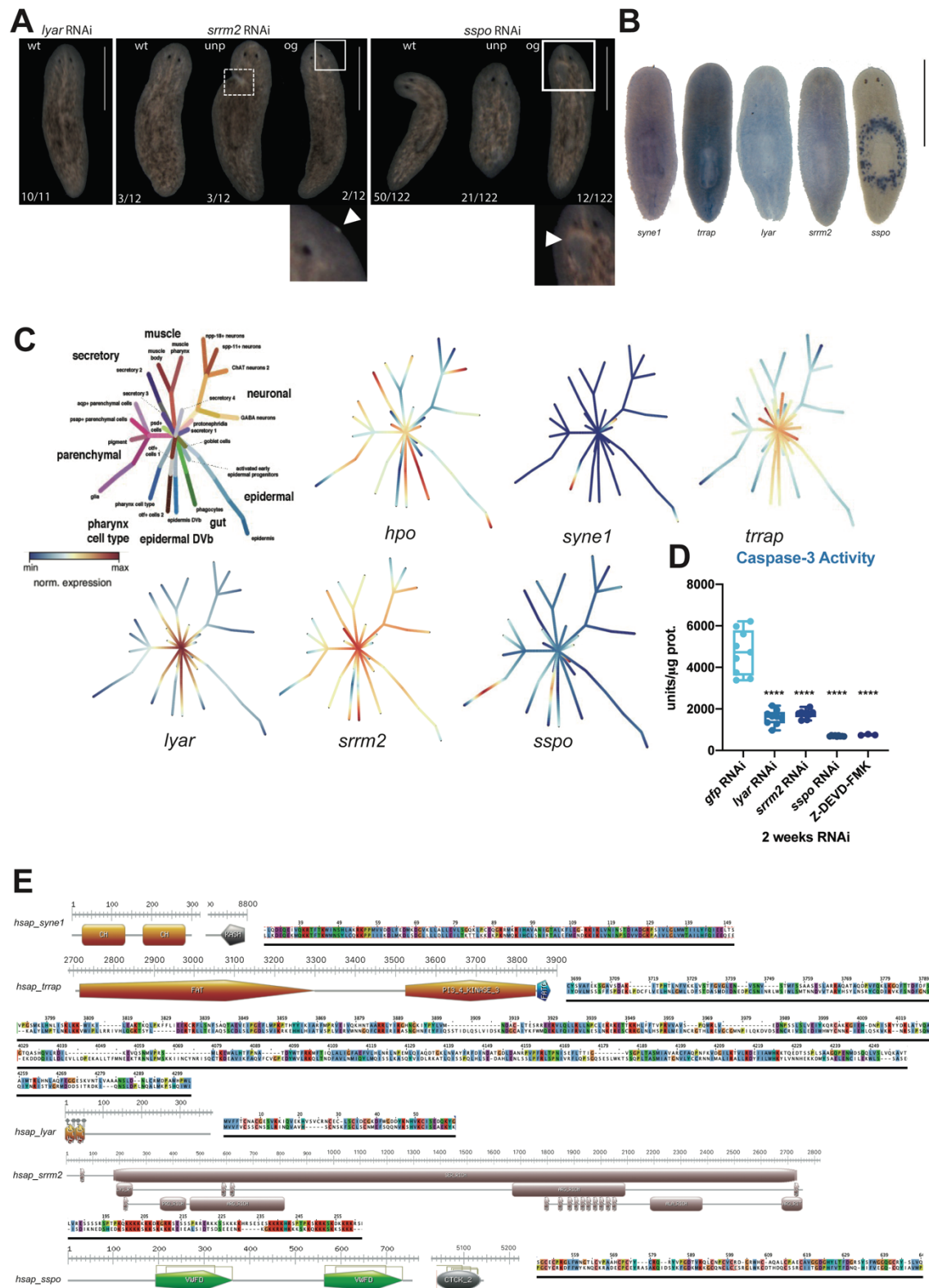


Figure S1: RNA-seq analysis of hippo RNAi animals finds several putative effectors which show an expression pattern and RNAi phenotype similar to that of *hippo* (A) Stereomicroscopic image of live *lyar*, *ssrm2* and *sspo* RNAi planarians depicting overgrowths and unpigmented regions (n ≥ 11). White arrows indicate overgrowths, the white box a zoom

frame and the white discontinuous box mark an unpigmented region. (B) Whole-mount *in situ* hybridization analysis of the expression of *syne1*, *trrap*, *lyar*, *srrm2* and *sspo* in wt animals. (C) Schematic representation of single-cell transcriptomic expression of *hippo*, *syne1*, *trrap*, *lyar*, *srrm2* and *sspo* genes (according to [65]). (D) Quantification of caspase-3 enzymatic activity in *gfp*, *lyar*, *srrm2*, *sspo* and RNAi animals after 2 weeks of injections (n=9). Z-DEVD-FMK corresponds to the negative control (n=3). Data is represented as mean $\pm$ s.e.m *p < 0.05; ***p < 0.001 was analyzed with ANOVA test. (D) Domain architectures of *syne1*, *trrap*, *lyar*, *srrm2* and *sspo* conserved between planarians and human annotated using ScanProsite [59]. Alignment displayed with Jalview. Scale bars: 1mm. *gfp*, green fluorescent protein; *hpo*, *hippo*; wt, wild type; unp; unpigmented regions; og, overgrowth.

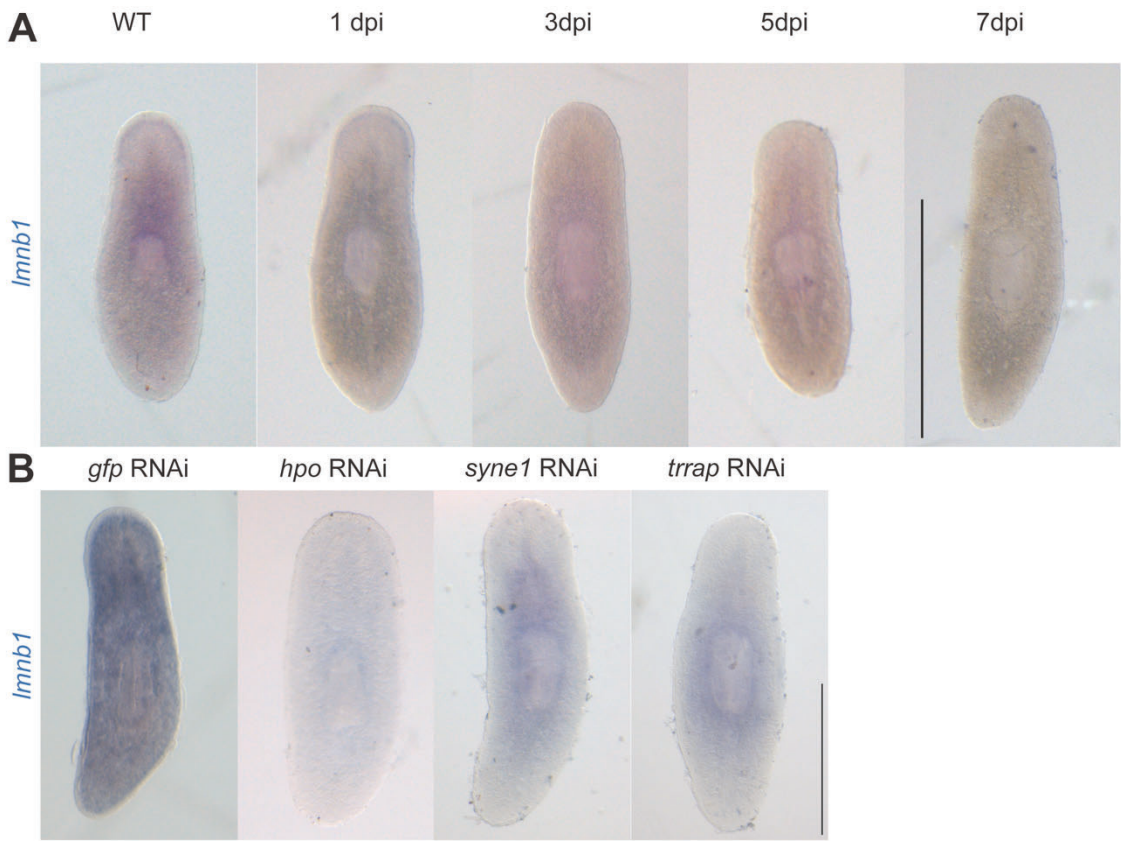


Figure S2: *Imnb1* acts as a downregulated marker of cellular senescence (A) Whole-mount *in situ* hybridization analysis of the expression of *Imnb1* at wildtype conditions and after 1, 3, 5 and 7days post-irradiation with X-ray at 60 Gy. (B) Whole-mount *in situ* hybridization analysis of the expression of *Imnb1* in *gfp*, *hpo*, *syne1* and *trrap*, and RNAi animals after 1 week of injections. Scale bars: 1mm. *gfp*, green fluorescent protein; *hpo*, *hippo*; wt, wild type; dpi, days post-irradiation.

<i>syne1</i>	Link between the actin cytoskeleton and the nucleus. Involved in the transduction of mechanic signals and chromatin status.
<i>trrap</i>	Adapter protein found in complexes with histone acetyltransferase activity (HAT) leading to transcription activation. It also plays a role in DSB repair.
<i>lyar</i>	Plays a role in the maintenance of the appropriate processing of 47S/45S pre-rRNA and in transcription regulation.
<i>srrm2</i>	Required for pre-mRNA splicing as component of the spliceosome.
<i>sspo</i>	Involved in the modulation of neuronal aggregation.May be involved in developmental events during the formation of the central nervous system.

Table S1: Summary of the biological function of the candidate genes.

	transcriptome id	forward	Reverse	forward	reverse
<i>syne1</i>	dd_Smed_v6_15904_0	TTCTCCAGAAAAAGCCCAAG	TCGCCTTCTTTCATGGTTTT	CGGAAATAAACGGTGGCTTA	GCATCTTGTAGCCAGCTTCC
<i>trrap</i>	dd_Smed_v6_4555_0	GCTTTTTAGTCCCGATGAG	TCGTTGCTGAATTTCACTCG	TCGAGCATTGCAATCAAAG	ACGTATTGGGATGCCTTCAG
<i>lyar</i>	dd_Smed_v6_5281_0	TGAAGGAAAGCTCGGAAGAA	TCGGGATTTCTTCAACTTCCTC	TCCGTCACGAAAAAGATCAA	TCGGGATTTCTTCAACTTCCTC
<i>srrm2</i>	dd_Smed_v6_3805_0	GGTGGTAGGATTGTCGGAAA	TCTGCATCAGAATCGTGCTT	CCTCCGATACAAAAGGTCA	TCTGCATCAGAATCGTGCTT
<i>sspo</i>	dd_Smed_v6_3183_0	GCAAAGAGAAGCCGGTATTG	CGTTTCTTTTCTCCGCATC	CGAGCACAAGTTCAGAATC	CACGAATTGCATTGACATC
<i>lmnb1</i>	dd_Smed_v6_850_0_2			AAGGCTGAACAGAGGGAACA	GCAACGCTAATATTACCCGAG

Table S2: Table depicting the primers used for RNAi and probe synthesis of each candidate gene together with its transcript id.

	qPCR	
	forward	reverse
<i>p53</i>	TGGTTGCATTGCATCGGAACA	GGCCCAGCAATATATTACTTCGGC
<i>pRb</i>	CCACGAGATCCTCCAAATGT	CGTGTGAACATTGGTTTTGC
<i>lmnb1</i>	GGGAAATACGGTCTCGTGTG	CAAATTTCCAGAGCAGTCG

Table S3: Table depicting the primers used for qPCR analysis.

HIPPO PATHWAY CONTROLS TISSUE RENEWAL THROUGH CYTOSKELETON MAINTENANCE AND TRANSCRIPTIONAL REGULATION IN PLANARIANS

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The Hippo pathway acts through mechanosensing extracellular physical forces and transduce them to nuclear specific transcriptional programs. Its inhibition leads to an increase of organ size coupled with overgrowths. However, its role in the abnormal growth of cells remains elusive. Recent studies place it as pivotal in the regulation of cell fate and specially the maintenance of the differentiated state. To better understand this connection, we study its function in planarians, organisms that constantly resize and renew their tissues according to food availability.

Planarian *hippo* downregulation leads to defects in stem cell cycle progression and in the maintenance of the differentiated fate driving the formation of overgrowths. How this is regulated is currently unknown. We analyzed an RNA-seq on *hippo* RNAi planarians and found two main biological functions significantly downregulated. In the present work we characterize two representative candidate genes for each of these functions: *syne1* and *med17*. RNAi for either of these genes showed altered cell cycle progression and signs of cellular dedifferentiation phenocopying *hippo* downregulation. However, while *syne1* RNAi planarians were not able to maintain cell polarity, *med17* RNAi planarians displayed problems in mitosis, DNA replication and transcription.

All and all, these evidences highlight the importance of transcriptomic and cytoskeletal regulation downstream of *hippo* to properly maintain cellular differentiation *in vivo*. They also put planarians on the spot as a model to understand the fine tune of cell renewal in adult organisms.

INTRODUCTION

Tissue renewal and growth are tightly regulated homeostatic processes which misregulation may lead to cancer. Due to this connection between development and pathological tumor formation [1, 2], the Hippo pathway and its effectors have gained the interest of the scientific community in regards to their role on cancer progression.

The Hippo pathway is a key regulator of growth through mechanosensing extracellular physical forces and transducing them to nuclear specific transcriptional programs. Its inhibition leads to the appearance of overgrowths in *Drosophila* [3, 4] and an increase of organ size coupled with overgrowths in mammals [5, 6]. Nonetheless, recent studies indicate a wider variety of functions of the pathway besides growth control, placing it as a pivotal pathway in the regulation of cell fate and maintenance of pluripotency [7–9]. It has also been directly linked with tumorigenesis in animal models and in humans [10–12]. These evidences reveal a high complexity in the Hippo pathway response elements.

One of the key functions described to be involved in the upstream regulators of the pathway relates to the establishment of apical-basal polarity during cellular differentiation [13]. Specifically, *lgl2* (*lethal Giant Larvae Homolog 2*) has been described as a modulator of the upstream inhibitor complex of Hippo named PAR through the stabilization of the apical complex [14]. Moreover, the loss of apical-basal polarity has also been denoted to be one of the first steps towards epidermal-mesenchymal transition, a key process of tumor metastasis and gain of malignancy directly linked to loss of cellular identity [15, 16]. Such loss is also one of the key direct observable features of the disruption of the pathway [14, 17].

In spite of some direct effectors of the pathway being already described in the literature such as *diap1*, *bantam* [18] which transcriptional signatures directly relate with the formation of overgrowths and dedifferentiation upon misregulation, the global frame and scope the pathway reaches downstream remains to be elucidated.

Recently, the Hippo pathway has been characterized in planarians as a hub in regulating cell renewal through the control of cell death, differentiation and proliferation in response to cell-cell contact [19]. *Schmidtea mediterranea* is a freshwater flatworm also known as planaria (Platyhelminthes) from the Dugesidae family and Tricladida order. *Schmidtea mediterranea* is a model organism generally used in regeneration and developmental studies. Planarians can regenerate an entire organism from any fragment of their body by rebuilding lost tissue with the correct pattern. This high regenerative capability resides on their population of adult stem cells, called neoblasts. Neoblasts are somatic pluripotent (also called adult) stem cells present throughout the planarian mesenchyme and absent in the pharynx and in front of the eyes. They represent around 25-30% of the total animal cell population. [20]. They are also capable of rescaling according to food availability [20, 21].

Smed-hippo (*hippo* from now on) silencing in planarians results in the accumulation of cycling cells and the development of overgrowths and unpigmented regions composed by cells with a loss of identity commitment, a phenotype shared with tumor progression [15, 19].

In this study, we survey the function of several putative *hippo* target genes and focus in representatives of prominent cellular functions that we found associated with the deregulation of the Hippo pathway in planarians. These functions are alterations in microtubule processes and DNA replication and transcription. We focused in one representative for each function: *nesprin-1*, *smed-syne1* (*syne1* from now on), and *mediator complex subunit 17*, *smed-med17* (*med17* from now on).

Both *syne1* and *med17* were found systemically downregulated in a RNA-seq performed after 1, 3 and 4 weeks of *hippo* RNAi inhibition compared to *gfp* RNAi control animals [19]. *Syne1* has been related with the maintenance of nuclear shape through cytoskeleton interactions. Its role has been mainly described in tumorigenic cell lines (among them the liver cell lines Hep3B and Huh7) [22] and in the *syne1* knockout (KO) mouse model. In the first case Nesprin-1 (aka *syne1*) deficiency was associated with an altered nuclear membrane structure and nuclear morphology. In the case of the KO mice, defects in the anchoring of the muscular nuclei at the neuromuscular junction as well as in the spacing of the extra synaptic nuclei along the muscular fiber were shown [23]. On the other hand, *med17* has been described as a subunit of the Mediator complex. Mediator plays a key role in the modulation of transcription activity. The role of *med17* has been characterized in yeast. It is described as a structural hub key for the stabilization of the other subunits of the Mediator complex.[24] Moreover, enzymatic experiments with TATA-containing DNA templates and HeLa cell extracts indicated that the recruitment of Med17 to the preinitiation transcription complex together with the rest of subunits occur in a stepwise assembly model dependent on the presence of general initiation factors.[25]

All and all, here we show that RNAi inhibition of *syne1* and *med17* phenocopy the dedifferentiation and overgrowths found in *hippo* RNAi. Our data indicate that *syne1* could trigger this phenotype through disruptions in the cytoskeleton, whereas *med17* RNAi would cause it through the deregulation of RNAPolIII activity.

RESULTS

***hippo* RNAi transcriptomic analysis reveal a connection between transcription regulation, cytoskeleton stability and the emergence of the overgrowth phenotype**

We reanalyzed a previously published RNA-seq for *hippo* RNAi [19] to find putative effectors systemically downregulated after 1, 3 or 4 weeks of *hippo* silencing in *Schmidtea mediterranea*. To better understand the gene expression dynamics, we used TCseq [26] to check temporal patterns in the differentially expressed genes. By clustering the different dynamics in 9 clusters (Figure S1A,B) we were able to identify different groups of genes that were downregulated (c1 and c5) or upregulated (c7) in all tree timepoints. We also observed clusters with fluctuating dynamics (c3, c4, c6, c8 and c9). In order to classify the putative functions affected in each cluster a GO enrichment analysis was performed. Clusters of mostly upregulated dynamics (c2,c3,c4,c9) were related with catabolism and proteolysis. Clusters of downregulated tendencies on the other hand were involved in microtubule dynamics (clusters 1 and 5) and transcription regulation (c1) as well as those involved in M phase regulation (c5). Finally, clusters with fluctuating dynamics presented functions related with telomere maintenance (c3 and c8). We found interesting the GO associated to clusters with downregulated genes. To investigate further, we performed RNAi assays for representative candidates of clusters 1 and 5. These candidates were: *microtubule associated protein rp/eb family Member 1*, *smed-mapre1* (*mapre1* from now on); *supt6h interacting protein*, *smed-iws1* (*iws1* from now on); *cell division cycle 25*, *smed-stg* (*stg* from now on) and *tankyrase*, *smed-tnks* (*tnks* from now on). Mapre1 (aka Eb1) is a plus-end tracking protein (+TIP) that binds to the plus-end of microtubules and regulates the dynamics of the microtubule cytoskeleton and is involved in mitotic spindle positioning [27]. In planarians, it appears highly expressed in neural and epidermal progenitors [28]. Iws1 is a transcription factor which plays a key role in defining the composition of the RNA polymerase II (RNAPII) elongation complex and in modulating the production of mature mRNA transcripts [29]. In planarians, it appears highly expressed in *otf*⁺ neurons. Stg (aka *cdc25*) acts as a dosage-dependent inducer in mitotic control. It is a tyrosine protein phosphatase required for progression of the cell cycle [30]. In planarians it is highly expressed in glia cells as well as several differentiated neurons including *otf*⁺, *npp-18*⁺ and GABA. Finally, *tnks* is a Poly-ADP-ribosyltransferase involved in various processes such as Wnt signaling pathway, telomere length and vesicle trafficking. In planarians it appears highly expressed in epidermal,

muscular, neural and glia progenitors as well as in GABA and Chat neurons, the pharynx and in goblet cells[31]. Results indicated a phenocopy of the *hippo* RNAi phenotype in all 4 candidates, with the presence of unpigmented regions and overgrowths in percentages superior to 20% for each phenotype per RNAi. Specifically, 13 out of 13 animals presented unpigmented regions in *mapre1* RNAi animals followed by *iwis1* RNAi animals that presented 3 out of 15 animals with unpigmented regions or overgrowths and 9 out of 15 that presented both phenotypes. *Stg* RNAi animals present 4 out of 15 animals with unpigmented regions and 6 out of 15 present both unpigmented regions and overgrowths. Finally *tnks* RNAi animals present 4 out of 15 animals with unpigmented regions, 1 with overgrowths and 6 out of 15 animals present both unpigmented regions and overgrowths (Figure S2A,B).

Among other systematically downregulated transcripts after *hippo* RNAi inhibition we selected *syne1* and *med17* due to the strong penetrance of the *hippo* phenotype in the RNAi animals and also because they share the relevant biological functions associated with the *hippo* RNA-seq that we saw altered in the downregulated clusters of the previously mentioned TCseq analysis. *Syne1* acts as a link between the actin cytoskeleton and the nucleus and is responsible for its proper shape maintenance [22]. Upon inhibition the connection between the actin cytoskeleton and the nucleus is lost and mechanotransduction and mechanosensation are heavily impaired [23]. Coincidentally, such features have been strongly connected with the deregulation of Hippo in recent reports [26, 27]. In planarians it appears highly expressed in the pharynx and protonephridia tissue as represented in the previous chapter. On the other hand, *med17* acts as a component of the Mediator complex which plays a key role maintaining the epigenetic landscape for a proper RNAPolIII transcription [24, 25]. This is one of the few subunits that have been described to be evolutionary conserved in planarians and across the Spiralia clade [28]. Moreover, its inhibition in planarians has also led to regeneration defects upon amputation similar to the inhibition of other subunits [29, 30]. It also appears highly expressed in the neoblasts and progenitors of almost all the planarian tissues and in some differentiated cells such as pigment cells, secretory and some differentiated neurons (Figure 1B).

***syne1* and *med17* RNAi animals present overgrowths and unpigmented regions together with disruptions in the nervous system**

We performed RNAi experiment for *hippo* and *gfp*, injecting planarians for 3 weeks. Results showed that *hippo* RNAi phenocopy *syne1* and *med17* RNAi with the presence of overgrowths and/or unpigmented regions after 3 weeks of inhibition (22 out of 66 animals in *med17* RNAi planarians and 49 out of 162 *syne1* RNAi animals)(Figure 1D). Due to the strong penetrance of the phenotype we decided to keep these candidates as representatives to study more in depth the role of microtubule dynamics and transcription regulation in the *hippo* RNAi phenotype.

Planarian nervous system is constituted by a bilobed cerebral ganglion dorsally located together with two ventral nerve cords. These progress up to the posterior region of the animal from which the peripheral nerve plexuses derive. Immunostaining using an antibody against SYNAPSIN (3C11) allows visualization of planarian nervous system [32]. In both *syne1* and *med17* RNAi animals synapsin staining depicts disrupted regions of the nerve cords. Specifically we see 9 out of 20 *med17* RNAi animals and 8 out of 16 *syne1* RNAi planarians with disruptions in the nerve cords compared to no animal in *gfp* RNAi controls (Figure 1E) . To investigate the number of mitoses following RNAi injections, we used an antibody that recognizes Histone H3 phosphorylated at serine 10 (anti-H3P). This mark is present from the onset of mitosis to the telophase [33]. In the case of *syne1* RNAi animals the immunostaining reveals an increase of H3P+ cells, as described in *hippo* RNAi animals [19] (Figure 1F). On the other hand, no significant changes were observed in *med17* RNAi animals when quantifying H3P+ cells.

Overall, we detect the presence of overgrowths and unpigmented regions in *med17* and *syne1* RNAi animals, as previously reported in *hippo* RNAi conditions [19]. Nonetheless we only see a significant accumulation of mitotic cells mimicking *hippo* RNAi phenotype in the case of *syne1* RNAi animals and not in the case of *med17* RNAi. These results go in line with the altered biological functions reported previously from the TCseq reinforcing the important roles that microtubule dynamics and transcription regulation play in the onset of the *hippo* RNAi phenotype.

***syne1* and *med17* are required for the correct progression through the cell cycle**

Previous studies have reported defects in cell cycle progression after impairment of the Hippo pathway [34]. Since we have seen accumulations of H3P+ cells in *syne1* RNAi animals as observed in *hippo* RNAi animals [19] we analyzed whether an impairment of the cell cycle could be occurring, as described in *hippo* RNAi planarians. To test possible differences across the progression of the cell cycle we treated cells with 5-bromoethynyl- 20-deoxyuridine (BrdU) and analyzed the proportion of cells in M and S phase 16 h later in *syne1*, *med17* and *hippo* RNAi animals and the respective controls (Figure S3A). We detected similar number of BrdU+ cells in all RNAi conditions when compared to controls, indicating that a similar number of cells are entering the cell cycle during the period of BrdU administration (Figure 2A and S3B). Double staining with anti-H3P revealed a significant accumulation of H3P+BrdU+ cells in all RNAi conditions and an accumulation of H3P+BrdU- cells only in *hippo* and *syne1* RNAi animals (Figure 2A', 2A'', 2A''', S3B). This indicates that in *hippo* and *syne1* RNAi animals, cells cannot complete the M phase, as we detect a higher number of cells stuck in M phase during the 16h BrdU pulse. This result corroborates previous results analyzing *hippo* RNAi animals [19], and demonstrates that *syne1* RNAi produces the same alteration of the cell cycle. In *med17* RNAi animals we don't detect the accumulation of H3P+BrdU- cells during the pulse suggesting that cells advance correctly through the M phase and therefore cannot properly surpass the G2 phase (Figure 2A).

Next, we sought to determine whether *syne1* RNAi affects mitotic fidelity. We focused in *syne1* since that was the candidate that showed defects in the M phase transition of the cell cycle from the BrdU experiments. For that, we inspected mitotic defects in dissociated cells of *syne1* RNAi animals by double immunostaining with anti- α -tubulin and anti-H3P antibodies [35]. Results indicated an accumulation of cells in prophase compared with the controls coupled with high number of defective anaphases and metaphases (Figure 2B). Almost half of the prophase cells presented abnormal microtubule distribution and spindle poles in *syne1* RNAi planarians. Similarly, almost half of the metaphasic cells presented chromosomal misalignments as well as microtubule defects. Our results indicate that *syne1* is required for proper chromosome segregation during mitosis as its inhibition leads to the accumulation of cells in M phase. This result is in accordance with the previously published *hippo* RNAi phenotype [19] which supports the idea of *syne1* as a positive downstream effector of *hippo*.

***syne1* and *med17* are required for the maintenance of neural differentiation**

Dopaminergic neurons can be detected using markers for *tyrosine hydroxylase* (*smed-th*, *th from now on*) since it is the rate limiting enzyme in dopamine synthesis. Its pattern of expression appear at the inner arches of the dorsal bilobed cerebral ganglions of planarians [36] Quantification of dopaminergic neurons in *syne1* and *med17* RNAi animals compared to controls reveal a significant decrease in the number of dopaminergic cells as well as a decrease in dopaminergic population density in the ganglions (Figure 3A). This goes in line with the defects in the peripheral nerve plexuses previously reported in *hippo* RNAi animals.[19] Moreover we also see an accumulation of neural committed neoblasts. Neural committed neoblasts can be visualized by whole-mount in situ hybridization (WISH) with the markers *Collier/Olfactory-1/Early B-cell factor* (*smed-coe*, *coe from now on*) [37] and the homeobox transcription factor *smed-nkx2.1*(*nkx2.1 from now on*) [38] when each of the

markers are coexpressed with the neoblast marker *piwi*. *Piwi+/coe+* and *piwi+/nkx2.1+* cells in *syne1*, *med17* and *hippo* RNAi increase in number compared to *gfp* RNAi controls (Figure 3B, 3B'). Moreover, in these conditions, the density of cells co-expressing both markers appear to increase and concentrate in the region between the base of the dorsal cerebral ganglions and the pharynx of the planarian, in the medial region flanked by the two ventral nerve cords. These results suggest that neural committed neoblast accumulate close to the nerve cords and point towards defects in the maintenance of the differentiation process.

Next, we sought to investigate whether the accumulation of committed neoblasts was not only restricted to the neural lineage but more widespread to different lineages. For that we performed fluorescence activated cell sorting (FACS) analysis. This technique combines the irradiation sensibility of neoblasts with cell cycle analysis using the DNA dye Hoechst. It allows the segregation of 3 cell populations named X1, X2 and X-insensitive [39]. X1 present high levels of Hoechst and is lost upon 24 of post-lethal-irradiation. Therefore, it is referred as the stem cell gate. The X2 population appears as a Hoechst side population with low Hoechst levels. It presents an enrichment of progenitor markers and is referred to as the progenitor population. Finally, the X-insensitive population is irradiation-insensitive and is composed of mature cells encompassing most tissues of the planarian [40]. Our results revealed that in *syne1* and *med17* RNAi animals the progeny (X2 population) was 4.3 and 1.5-fold larger than the *gfp* RNAi controls, respectively. Similarly, *hippo* RNAi animals also presented a 2.43 and 1.8-fold increase in their progeny population (X2 population) when compared to *gfp* RNAi animals (Figure S43A,B).

In line with these results, we decided to quantify the global length of the telomeres in those planarians. Planarians are known to present indefinite telomerase activity allowing them to maintain their telomere longitude during regeneration providing the animal its alleged immortality [41]. Moreover telomere elongation has been linked with cellular dedifferentiation processes in other organisms, although the exact dynamics and lengths in different tissues remain still to be elucidated [42, 43]. To that aim we perform a telomere qPCR (TelqPCR). This technique determines the ratio of telomere (T) repeat copy number to a single-copy (S) gene copy number (called T/S ratio) in experimental samples as compared with a reference DNA sample. Our results indicate that in *syne1*, *med17* and *hippo* RNAi animals telomeres are longer than in the *gfp* RNAi control planarians (Figure 3C).

All in all, the fact that we detect elongated telomeres and the increase of the progeny population in *hippo*, *syne1* and *med17* RNAi animals observed by FACS indicate that the accumulation of progenitors could be caused by mechanisms of cellular dedifferentiation as previously reported in *hippo* RNAi planarians[19].

***syne1* and *med17* are required for the maintenance of epidermal differentiation**

To further explore the idea that there where defects occurring in the process of cellular differentiation we decided to check by FISH if we see alterations in the pattern of expression of markers associated with the differentiation of the epidermal tissue. Quantification of cells stained for the epidermal marker vimentin (*smed-vim*, *vim* from now on) [44] together with the stem cell marker *piwi* (*smedwi-1*) revealed an increase in *smedwi-1+/vim+* cells in *syne1*, *med17* and *hippo* RNAi animals versus controls (Figure 4A). This increase is also present in *hippo* RNAi animals and suggests a failure of the epidermal cells to progress appropriately through the differentiation process.

***hippo* and *syne1* are required for the maintenance of the epidermal cell polarity**

Loss of the epidermal cell polarity leads to epidermal mesenchymal transition coupled with a loss of cell identity in diseases such as cancer [45]. In order to check if the Hippo pathway maintains the differentiated fate through controlling cell polarity, we decided to analyze the structure of the cytoskeleton in planarians with deregulated Hippo pathway. To that avail, we characterized the role of *Igl2* in planarians as a positive control for these experiments since it

plays a major role in epidermal apical-basal polarity establishment [16]. *Igl2* has been described as a modulator of the Par complex. This is an upstream inhibitor complex of Hippo. It acts through the stabilization of the apical complex preventing the interaction of the aPKC with the core kinases cascade of the Hippo pathway [14, 46]. *Igl2* is present in neoblasts in planarians (Figure S5B,C). Its RNAi in planarians phenocopies *hippo* RNAi (Figure S5A), since it regulates proliferation (Figure S5D) and causes an impairment of neural tissue differentiation (Figure S5F).

In order to check for more direct evidences of morphological changes of the cytoskeleton that could be directly affecting the *hippo* phenotype we decided to check by DAPI staining the nuclear distribution and morphology of the epidermal monolayer. This is due to the fact that disruptions in the actin cytoskeleton assemble with the adherens junctions and the nuclei often lead to loss of nuclear circularity. This nuclear deformations have been correlated with the onset of diseases such as cancer, progeria and muscular dystrophies [47]. RNAi of *hippo*, *syne1* and *Igl2* lead to a decrease in nuclear circularity without changes in surface area. (Figure 5A, 5A' and 5A'') These results match with the ones reported in Huh7 cells (a human liver cancer cell line) with a mutation in *SYNE1* to reduce Nesprin-1 expression [22]. A disruption of the epidermal monolayer can also be detected in all three cases through an increase of the deviation of the nuclear density quantifications (Figure 5A and 5A''').

Furthermore, in order to assess the apical-polarity defects of the epidermis we used β -catenin-2 [19] and actin immunostaining. β -catenin-2 is a component of the adherens junctions that labels the boundary between the apical and basal domain. Adherens junctions divide the cell into an apical domain and a basolateral one thanks to the establishment of a mesh of actin-myosin filaments [14]. In control planarians, β -catenin-2 labels the membranes of epidermal cells, while in *Igl2*, *syne1* and *hippo* RNAi animals the signal appears delocalized, suggesting a delocalization of adherens junctions (Figure 5B). Transposed epidermal projections of the immunostaining showed that while in controls β -catenin-2 signal is located above the nuclear layer, in *Igl2*, *syne1* and *hippo* RNAi animals β -catenin-2 staining falls below the nuclear position. (Figure 5B') This is quantified by measuring the distance between the adherens junctions boundary and the basal lamina of the epidermal monolayer. The results indicated a significant shortening of the distance between them in the cases of *Igl2*, *syne1* and *hippo* RNAi (Figure 5B'') when compared to controls. We also detected disruptions in the actin cytoskeleton in *Igl2* RNAi animals (Figure S5E) as well as in the transposed projections of *Igl2*, *syne1* and *hippo* RNAi animals. 3D reconstructions also showed the disruption of the epidermal monolayer and nuclei deformations together with the disruption of the β -catenin-2 apical position (Figure 5B').

Altogether, this data demonstrate a mislocalization of adherens junctions in *Igl2*, *syne1* and *hippo* RNAi animals, supporting the role of the Hippo signaling in maintaining the apical-basal polarity of the epidermal cells.

Mediator activity enhancement phenocopy and aggravate *hippo* RNAi phenotype

In order to check if transcriptional regulation is an important role of the Hippo pathway in modulating the differentiated cellular state, we tested a specific drug (Cdk8/19 inhibitor) that disturbs transcription through altering the activity of the mediator complex [48]. The idea stems from the fact that the mediator complex play a key role in maintaining the proper expression of embryonic stem cell program by forming super-enhancer structures in the genome in cooperation with master transcription factors (Oct4, Sox2 and Nanog) [49]. The Cdk8/19 inhibitor acts mechanistically blocking the aforementioned kinases removing the ability to repress the mediator complex at the enhancer locations in the genome. This has been reported to lead an overall increase in the recruitment of RNAP II and the stabilization of a more naïve transcriptional program. Specifically, the in vitro experiments that tested the Cdk8/19i drug indicated a shift in the expression of primed to naïve markers in mouse and human induced pluripotent stem cells. At the end the drug was aiding the reconfiguration of

enhancers in these cells during the reprogramming to facilitate the process of cellular dedifferentiation [48, 50]. Thus, we tested whether administration of the Cdk8/19 inhibitor in planarians could phenocopy the *hippo* RNAi phenotype. We tested several concentrations and we found that at 3,2 nM administration the drug induces the appearance of overgrowths and unpigmented areas (Figure 6B) similar to the ones of *hippo*, *syne1* and *med17* RNAi planarians.

To check whether both mechanisms, transcriptional regulation and Hippo signaling, could be functioning in a synergic manner, we injected low doses of the Cdk8/19 inhibitor together with *gfp* dsRNA, which did not produce any phenotype. However, the results show that co-injections of the drug with *hippo* RNAi produced an aggravation of the *hippo* RNAi phenotype (Fig 6C, C'). The percentage of animals presenting unpigmented regions and overgrowths in the drug+RNAi condition was significantly higher than with only the drug or *hippo* dsRNA alone (Figure 6B,B').

All in all, we demonstrate that overactivation of the mediator complex promotes an enhancement of the phenotype observed after *hippo* RNAi inhibition. This result further strengthens the connection between transcriptional activity deregulation and the presence of overgrowths and unpigmented regions in planarians tied to the dedifferentiation process.

DISCUSSION

The Hippo signaling pathway has been found deregulated in a myriad of tumoral processes. However, its role in the abnormal growth of cells remains elusive. Initially, the Hippo signaling pathway was shown essential for the control of cell proliferation and growth. Nowadays, it is seen as a complex mechanism that controls cellular behavior in response to the physical environment. In a previous study, *hippo* inhibition by RNAi was characterized in planarians [19]. Those results indicated cellular dedifferentiation and the formation of overgrowths, which correlated with a decrease in cell death, an arrest in mitosis and the increase in cell plasticity. In this work, we have reanalyzed by dynamic clustering the *hippo* RNAi transcriptome and observed a downregulation of genes involved in microtubule maintenance and transcriptomic regulation as has been suggested in other models [51–53].

RNAi functional assays of representative candidates of these functions phenocopied *hippo* RNAi with the presence of overgrowths. Some of the most extreme RNAi phenotypes were related to two quite unknown candidates representing each of the previously mentioned biological function: *syne1* and *med17*. The RNAi animals of both candidates presented disruptions of the nervous system. This type of phenotype has been associated previously with cellular dedifferentiation after *hippo* RNAi inhibition[19]. Nonetheless a dilemma in that case was and still remains of the true mechanism of this process. This is because in the planarian context the loss of proper markers of differentiated tissues can be still attributed to defects in the process of commitment and differentiation of the progenitor cells and not necessarily a complete dedifferentiation process so radical as the reprogramming we see in vitro. On other organisms the role of these genes has been defined to be key in the stem cell response. In the case of *syne1* it has been proven in mesenchymal stem cells (MSC) to have a key role in nuclear strain transfer from the cytoskeleton. This role is key for the activation of mechanoactive signaling pathways such as ERK signaling that are essential for a proper differentiation. On the other hand in vivo experiments in zebrafish of several subunits of the Mediator complex reveal that many of them appear essential for the correct differentiation of the nervous system as well as for maintaining the stem cell population.[54–56] Both evidences reinforce the functional relation between *hippo* and both candidates as downstream effectors.

We have shown that *syne1* RNAi animals present accumulations of H3P positive cells just like in the context of *hippo* RNAi. These evidences combined with our BrdU labeling experiments revealed that in *syne1* RNAi animals, cells are having trouble completing the M

phase and we can detect more cells stuck in that phase from the 16h BrdU pulse and before as previously described in *hippo* RNAi animals [19]. On the other hand, in *med17* RNAi animals we do not detect the accumulation before the pulse suggesting that cells are passing correctly through the M phase and therefore are having trouble surpassing the G2 phase. This could stem from the fact that *med17*, as a subunit of the Mediator complex, is required for the active transcription of the elements that compose the cell cycle impairing cells to surpass the G2 phase where transcription is most active [57]. On the other hand, *syne1* being a connector of the cytoskeleton and the nuclei plays a more prominent role in the assemble of the mitotic spindle during the mitosis.[58] This could be the reason why it leads to M phase arrests upon inhibition in planarians.

Following this train of thought, if we focus in *syne1* RNAi animals we detect cells that present an improper chromosome configuration and mitotic spindle assemble similar to the ones found in *hippo* RNAi planarians [19]. There are further evidences that link central elements of the Hippo pathway with key mitotic phases in model organisms including yeast and fruit flies and mammals. Specifically, the Hippo kinases homologs in yeast have been connected with M phase exit of the cell cycle triggering cytokinesis. In *Drosophila*, Hippo also showed notable spindle defects upon downregulation leading to deregulations of cell cycle exit. And finally in mammalian cells (HeLa) Mst2 depletion caused chromosome misalignment [59–62]. All these evidences in other organisms reinforce our results that show that planarian Hippo pathway regulates the cytoskeletal configuration and the proper progression of the cell cycle and this happens though the regulation of *syne1*. I will be interesting to test whether *syne1* function regulating the cytoskeleton is conserved in other model systems.

In agreement with the former results and the loss of differentiated neural tissue in *hippo*, *syne1* and *med17* RNAi planarians we detect a significant decrease of dopaminergic neurons coupled with a significant increase of neural committed neoblast populations. We also detect an increase of *smedwi-1+/vim+* cells in comparison to controls indicating a loss of the hierarchical steps in the establishment of the epidermal fate. Also, we have shown by FACS that an accumulation of progenitors occur after down-regulating *hippo*, *syne1* or *med17*. That together with the telomeric elongation that we observe in the three RNAi phenotypes have shown to be evidences of dedifferentiation in other models. This has been seen in vivo through reprogramed mice by TRF1 upregulation as well as from in vitro derived human iPSC cells [42, 43]. In parallel, in vitro assays overexpressing YAP have detected similar results of cellular dedifferentiation. It has been reported that in vitro YAP/TAZ expression turns differentiated mammary gland cells into mammary stem cells, differentiated fetal neurons into multipotent neural stem cells and Pancreatic exocrine cells into progenitors. Moreover, YAP/TAZ hyperactivation in vivo is also able to reprogram somatic cells in organs such as in the liver. In this context YAP overexpression transdifferentiate mature hepatocytes into ductal cells bearing characteristics of hepatic progenitors [7, 63]. These results suggest that differentiated cells are unable to maintain the differentiation state and go back to be neoblast progenitors which accumulate and form overgrowths. Nonetheless, our results cannot rule out the possibility that cellular differentiation might be occurring in a defective manner triggering the expression of lineage markers at the wrong times.

We see evidences of cellular misorganization. These evidences include the loss of nuclear circularity in both *hippo* and *syne1* RNAi animals as it has been previously detected in human cells in vitro. These results reported that in Huh7 cells (a human liver cancer cell line) with a mutation in SYNE1 that reduces Nesprin-1 expression nuclear circularity is impaired. Nesprin-1 has also been reported to play a role in promoting nuclear localization of YAP through the induction of nuclear deformation in cultured cells [22, 64]. Moreover, the loss of epidermal apical basal polarity seen in both conditions mimics the one of *lgl2*. *Lgl2* has been described as a modulator of the Par complex. This is an upstream inhibitor complex of Hippo. It acts through the stabilization of the apical complex impeding the interaction of the aPKC with the

core kinases cascade of the Hippo pathway. Its role is the maintenance of the polarity through the stabilization of the apical complex as has been broadly described in other organisms. Two distinctive roles have been hypothesized to be key in the aforementioned maintenance of cell polarity. The first one is related to the ability of Lgl family of proteins to regulate polarized exocytosis as demonstrated by yeast models [65, 66]. This finely tuned mechanisms allow the transport of specific secreted and transmembrane macromolecules to the cell surface in order to maintain the molecular identities of the apical, lateral and basal membrane domains. The second one described in *Drosophila* describe the Lgl family of proteins as being part of the cytoskeleton through their interactions with the heavy-chain of non-muscular myosin II. This is an actin-based motor protein essential for proper cell adhesion, migration and polarity [16, 67, 68]. All these evidences suggest that the *lgl* family of proteins and more specifically *lgl2* acts as an upstream modulator of the Hippo pathway and also play a key role in maintaining the apical-basal epidermal cell polarity. Moreover the loss of epidermal cell polarity we see in *lgl2* RNAi animals is also mimicked by *hippo* and *syne1* RNAi planarians further reinforcing the connection between cellular polarization and the maintenance of the differentiated state thanks to a proper maintenance of the cellular cytoarchitecture.

We observe that part of the *hippo* RNAi phenotype is due to disturbing global transcription through altering the mediator complex. In our planarian context we altered the activity of this complex by injecting a Cdk8/19 inhibitor in the animal. The Cdk8/19 inhibitor acts mechanistically blocking the aforementioned kinases removing the ability to repress the mediator complex at the enhancer locations in the genome. This has been reported to lead an overall increase in the recruitment of RNAP II and the stabilization of a more naïve transcriptional program. In our case, either through *med17* RNAi or through the Cdk8/19i drug we are able to generate overgrowths and unpigmented regions. A previous study of *smed-med14* indicated that an alteration of the transcription program could be responsible of the loss of cell identity in planarians. It also depicted that the inhibition of other subunits such as *med17*, which have a much prominent role in the conformation of the whole complex, was able to trigger such effects [24]. Moreover the in vitro experiments that tested the Cdk8/19i drug revealed a prominent shift from primed to naïve features in mouse and human pluripotent cells [48, 50]. All these evidences together with our in vivo results state that Hippo could be modulating the epigenetic landscape essential for proper cellular differentiation affecting the super-enhancer regulation of genes through the activity of the Mediator complex [49, 69]. It still is important to remark that this regulation that we perceive in planarians is of an indirect nature. At the end of the day the changes we see in the candidate effectors we study originate from transcript downregulations found downstream of *hippo* RNAi inhibition. We therefore think that the molecular pathway connecting them with *hippo* remains unknown and require of further studies.

All in all, we conclude that Hippo could be maintaining the cell differentiated state through two distinctive mechanisms: the stability of the cytoskeleton and the regulation of transcriptional programs.

METHODS

Planarian culture

The planarians used in this study are the asexual clonal strain of *S. mediterranea* BCN-10 biotype and were maintained as previously described[70] in planarian artificial medium (PAM) water[71]. Animals were fed twice per week with liver, and those used in starvation experiments were starved for 1 week.

TCseq Transcriptomic analysis

Transcriptomic analysis were performed on the Raw sequencing data in FASTQ format as well as the transcript abundances found in the NCBI Gene Expression Omnibus (GEO) [72] accessible using GEO Series accession number GSE95130.

TCseq [26] was used to determine clusters of gene behavior based on their expression patterns over the analyzed time points. The z-score transformed log2 fold changes between *hippo*-RNAi samples and control samples were used as input, and the algorithm “cmeans” was selected for computing the cluster scores. Clusters were assigned to genes by introducing a cut-off of 0.8 to the product of the maximum cluster score (as reported by the cmeans algorithm) and the standard deviation of all scores.

Sequence analyses

Candidate genes were identified from the genome [73] and RNAi fragments were amplified using specific primers:

hippo RNAi

F: GAATCAGCTGCTCAGAGCCC

R: CTCGGCTTGCAAGTCTGAATC

med17 RNAi

F: GCTGAATGGAATTACTTTGGA

R: CCACCATTTTCTGGAAATAATG

mapre1 RNAi

F: CAATTGACAGTTTAATGAAAGAACG

R: ATCATCAGGAGGTCCGAATC

iws1 RNAi

F: ACGACAATGAAAGTTTAACTGGG

R: TGATAGACGACCACATGAAGG

stg RNAi

F: AGAGTTTGTGTTACCGTGC

R: TCATTTGGTCCTGGGATTCCT

tnks RNAi

F: CCAACCAATCAAGGATACCG

R: CGAATTCCATTTGTCCGTTT

lgf2 RNAi

F: TTATTGGACACGGTGATGGA

R:CCATGTTGCGATGATAATCG

Sequence identity comparison was carried out using the pairwise alignment tool in Jalview suite v2.11[74]. Sequence comparison against the GenBank database was performed using

the NCBI BLAST network server (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). Potential orthologs were searched for using tBLASTx where possible to allow a certain level of tolerance in case of a high degree of divergence. Domain characterization of each transcript was carried out using Scan prosite[75].

Single-Cell Sequencing Data

syne1, *med17*, *mapre1*, *iws1*, *stg*, *tko* and *lgl2* gene expression profiles (dd_Smed_v6_15904_0, dd_Smed_v6_9246_0, dd_Smed_v6_63370_0, dd_Smed_v6_8811_0, dd_Smed_v6_7858_0, dd_Smed_v6_8547_0 and dd_Smed_v6_7127_0 respectively) were obtained from the planaria single-cell database hosted by the Rajewsky lab at the Berlin Institute for Medical Systems Biology of the Max Delbrück Center, Berlin [76].

RNA interference analysis dsRNA

dsRNA was synthesized by in vitro transcription (Roche) and microinjection performed, as previously described [77], following the standard protocol of 3 × 32 nl injection of dsRNA for 3 consecutive days. To achieve stronger inhibition, we performed the same protocol over 3 consecutive weeks in starved planarians. Control animals were injected with dsRNA for green fluorescent protein (GFP), a gene not present in planarians.

Immunofluorescence

Whole-mount immunofluorescence was performed as previously described [78]. Animals were killed with 2% HCl and fixed with 4% formaldehyde. The primary antibody used were: mouse anti-SYNAPSIN (3C11) (anti-SYNORF-1, 1:50; Hybridoma Bank); rabbit anti-phospho-histone H3 (Ser10) (D2C8) (PH3) (1:500; Cell Signaling Technology; 9701) rabbit anti-β-catenin-2 (anti-Bcat2) (1:2,000) [79]; anti-actin JLA20 (1:2; Developmental Studies Hybridoma Bank). The secondary antibodies used were Alexa 568-conjugated goat anti-rabbit (1:1000; Molecular Probes; A-11011) and Alexa 488-conjugated goat anti-mouse (1:400; Molecular Probes; A-11001). Nuclei were stained with DAPI (1:5000; Sigma-Aldrich).

Whole-mount BrdU staining

Whole-mount BrdU staining was performed as previously described [80].

Immunofluorescence in dissociated cells

Immunohistochemistry in dissociated cells was performed as previously described [19]

Whole-mount *in situ* hybridization

Probes were synthesized *in vitro* using SP6 or T7 polymerase and DIG- or FITC- modified (Roche) from previously cloned fragments using primers found in TableS1. RNA probes were purified by ethanol precipitation and the addition of 7.5 M ammonium acetate. For colorimetric whole-mount *in situ* hybridization, animals were sacrificed using 5% N-acetyl-L-cysteine (NAC), fixed with 4% formaldehyde and permeabilized with Reduction Solution (50 mM DTT, 1% NP-40, 0.5% SDS in 1× PBS). For fluorescence whole-mount double *in situ* hybridization, animals were sacrificed using 5% N-acetyl-L-cysteine (NAC), fixed with 4% formaldehyde and permeabilized with Reduction Solution (50 mM DTT, 1% NP-40, 0.5% SDS in 1× PBS). The fixative and whole-mount *in situ* hybridization protocol used has been previously described [81] or whole-mount fluorescence *in situ* hybridization, animals were sacrificed with 7.5% NAC and fixed with 4% formaldehyde. Fluorescence *in situ* hybridization was carried out as described previously [82]. For double fluorescence *in situ* hybridization (dFISH) an azide step

(150 mM sodium azide for 45 min at room temperature) was added. Nuclei were counter stained with DAPI (1:5000; Sigma).

Drugs treatment

Cdk8/19i drug (ETP-47799) was obtained from Manuel Serrano's lab (IRB) [50]. Concentrated stocks were freshly diluted in planarian water at 2,9 nM (for the RNAi co-injection) and 3,2 nM (for the drug solo experiment) before daily prepharyngeal injections (three pulses of 32 nL each per worm). Injections were always scheduled 2–3 hours before respective dsRNA injections when applied.

Telomere quantitative PCR (telomere qPCR)

Telomere quantitative PCR was performed as previously described [83] in Supplemental Experimental Procedures.

Flow cytometry

Planarians were dissociated as previously described and sorted using a Becton Dickinson FACSaria without calcein [84]. Three biological triplicates of at least 40,000 live-cell events were processed for each replicate in supplementary material Fig. S3.

Imaging

In vivo images were taken with sCMEX-3 High End Digital Microscope Camera DC.3000s (Visual Inspection Technology) in a Zeiss Stemi SV 6 binocular loupe. Whole mount Brightfield colorimetric *in situ* hybridization and immunostaining samples were observed with a *Leica* stereoscope and images were taken with *Progress C3 Jenoptik* camera (Jena, TH, Germany). A Zeiss LSM 880 confocal microscope (Zeiss, Oberkochen, Germany) was used to obtain confocal images of whole-mount fluorescent *in situ* hybridization samples, BrdU assays and epidermal immunostainings. Z-stacks of immunohistochemistry on dissociated cells were acquired with a Leica Thunder microscope (Carl Zeiss, Jena).

Quantification of H3P+ cells were done using freely available ImageJ software [85] with the analyze particle function and normalized by animal area determined with the measurement tool. To build 3D reconstructions, confocal sections were deconvolved using Huygens Deconvolution Software (Scientific Volume Imaging) and processed using Imaris Software (Bitplane). Fiji/ImageJ was also used to show representative confocal stacks for each experimental condition.

Colocalization quantification was performed using the equivalent areas using the “ROI-manager” tool in Fiji. Nuclear area in BrdU experiments was measured using the “threshold” tool and mask conversion for all samples. Quantification of BrdU+ cells and single-cell populations were carried using the analyze particle function.

The circularity index and mean nuclear area of cells was measured using at least 146 nuclei per animal following the same process.

The nuclear density Nuclear stained (DAPI) images were transformed into a mask using the “threshold” tool and converted to a mask. The mask was used to obtain the nuclear signal with the analyze particle function.

To determine mean distance between the apical pole and to the base of epidermal cells, the distance between the adherent junctions (bcat2) to the basal lamina of the cell was measured.

Measurements were taken in three different regions of the same section and the mean value was calculated.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 6. Comparisons of more than 2 populations were done with ANOVA test($\alpha=0.05$). Previous confirmation of normality and homogeneity of data was performed with Shapiro–Wilk test and Bartlett test respectively. In the case of the phenotypical distribution experiments, the p-value accumulated for each pairwise comparison was determined using the Tukey HSD test. Fisher.test from the R package was used to compare more than two phenotypic variants between two populations.

REFERENCES

1. Nieto MA, Sargent MG, Wilkinson DG, Cooke J (1994) Control of cell behavior during vertebrate development by Slug, a zinc finger gene. *Science* 264:835–839. <https://doi.org/10.1126/science.7513443>
2. Fazilaty H, Rago L, Kass Youssef K, et al (2019) A gene regulatory network to control EMT programs in development and disease. *Nature Communications* 10:. <https://doi.org/10.1038/s41467-019-13091-8>
3. Justice RW, Zilian O, Woods DF, et al (1995) The Drosophila tumor suppressor gene warts encodes a homolog of human myotonic dystrophy kinase and is required for the control of cell shape and proliferation. *Genes and Development* 9:534–546. <https://doi.org/10.1101/gad.9.5.534>
4. Harvey KF, Pflieger CM, Hariharan IK (2003) The Drosophila Mst ortholog, hippo, restricts growth and cell proliferation and promotes apoptosis. *Cell* 114:457–467. [https://doi.org/10.1016/S0092-8674\(03\)00557-9](https://doi.org/10.1016/S0092-8674(03)00557-9)
5. Dong J, Feldmann G, Huang J, et al (2007) Elucidation of a Universal Size-Control Mechanism in Drosophila and Mammals. *Cell* 130:1120–1133. <https://doi.org/10.1016/j.cell.2007.07.019>
6. Lei Q-Y, Zhang H, Zhao B, et al (2008) TAZ Promotes Cell Proliferation and Epithelial-Mesenchymal Transition and Is Inhibited by the Hippo Pathway. *Molecular and Cellular Biology* 28:2426–2436. <https://doi.org/10.1128/mcb.01874-07>
7. Panciera T, Azzolin L, Fujimura A, et al (2016) Induction of Expandable Tissue-Specific Stem/Progenitor Cells through Transient Expression of YAP/TAZ. *Cell Stem Cell* 19:725–737. <https://doi.org/10.1016/j.stem.2016.08.009>
8. Sarikaya DP, Extavour CG (2015) The Hippo Pathway Regulates Homeostatic Growth of Stem Cell Niche Precursors in the Drosophila Ovary. *PLoS Genetics* 11:1–28. <https://doi.org/10.1371/journal.pgen.1004962>
9. Voltres A, Hevia CF, Engel-Pizcueta C, et al (2019) Yap/Taz-TEAD activity links mechanical cues to progenitor cell behavior during zebrafish hindbrain segmentation. *Development (Cambridge, England)* 146:. <https://doi.org/10.1242/dev.176735>
10. Yin MX, Zhang L (2014) Hippo signaling in epithelial stem cells. *Acta Biochimica et Biophysica Sinica* 47:39–45. <https://doi.org/10.1093/abbs/gmu111>
11. Sharif AAD, Hergovich A (2018) The NDR/LATS protein kinases in immunology and cancer biology. *Seminars in Cancer Biology* 48:104–114. <https://doi.org/10.1016/j.semcancer.2017.04.010>
12. Sung V, Luo W, Qian D, et al (2003) The Ste20 kinase MST4 plays a role in prostate cancer progression. *Cancer Research* 63:3356–3363

13. Zhao B, Tumaneng K, Guan K-L (2011) The Hippo pathway in organ size control, tissue regeneration and stem cell self-renewal. *Nature Cell Biology* 13:877–883. <https://doi.org/10.1038/ncb2303>
14. Martin-Belmonte F, Perez-Moreno M (2012) Epithelial cell polarity, stem cells and cancer. *Nature Reviews Cancer* 12:23–38. <https://doi.org/10.1038/nrc3169>
15. Roten A Van, Barakat AZ, Wouters A, et al (2018) A carcinogenic trigger to study the function of tumor suppressor genes in *Schmidtea mediterranea*. *DMM Disease Models and Mechanisms* 11:. <https://doi.org/10.1242/dmm.032573>
16. Vasioukhin V (2006) Lethal Giant Puzzle of Lgl. *Developmental Neuroscience* 28:13–24. <https://doi.org/10.1159/000090749>
17. Sousa-Ortega A, Vázquez-Marín J, Sanabria-Reinoso E, et al (2023) A Yap-dependent mechanoregulatory program sustains cell migration for embryo axis assembly. *Nature Communications* 14:. <https://doi.org/10.1038/s41467-023-38482-w>
18. Peng HW, Slattey M, Mann RS (2009) Transcription factor choice in the Hippo signaling pathway: Homothorax and yorkie regulation of the microRNA bantam in the progenitor domain of the *Drosophila* eye imaginal disc. *Genes and Development* 23:2307–2319. <https://doi.org/10.1101/gad.1820009>
19. de Sousa N, Rodríguez-Esteban G, Rojo-Laguna JI, et al (2018) Hippo signaling controls cell cycle and restricts cell plasticity in planarians. *PLoS Biology* 16:1–28. <https://doi.org/10.1371/journal.pbio.2002399>
20. Reddien PW, Alvarado AS (2004) Fundamentals of Planarian Regeneration. *Annual Review of Cell and Developmental Biology* 20:725–757. <https://doi.org/10.1146/annurev.cellbio.20.010403.095114>
21. Saló E (2006) The power of regeneration and the stem-cell kingdom: Freshwater planarians (Platyhelminthes). *BioEssays* 28:546–559. <https://doi.org/10.1002/bies.20416>
22. Sur-Erdem I, Hussain MS, Asif M, et al (2020) Nesprin-1 impact on tumorigenic cell phenotypes. *Molecular Biology Reports* 47:921–934. <https://doi.org/10.1007/s11033-019-05184-w>
23. Razafsky D, Hodzic D (2015) Nuclear envelope: Positioning nuclei and organizing synapses. *Current Opinion in Cell Biology* 34:84–93. <https://doi.org/10.1016/j.ceb.2015.06.001>
24. Harper TM, Taatjes DJ (2018) The complex structure and function of Mediator. *Journal of Biological Chemistry* 293:13778–13785. <https://doi.org/10.1074/jbc.R117.794438>
25. Hwa JB, Yun KK, Roeder RG (2006) Human mediator enhances basal transcription by facilitating recruitment of transcription factor IIB during preinitiation complex assembly. *Journal of Biological Chemistry* 281:15172–15181. <https://doi.org/10.1074/jbc.M601983200>
26. Mengjun W (2023) TCseq: Time course sequencing data analysis. R package version 1.8.0
27. Askham JM, Vaughan KT, Goodson H V, Morrison EE (2002) Evidence that an interaction between EB1 and p150(Glued) is required for the formation and maintenance of a radial microtubule array anchored at the centrosome. *Molecular biology of the cell* 13:3627–45. <https://doi.org/10.1091/mbc.e02-01-0061>
28. Rozanski A, Moon HK, Brandl H, et al (2019) PlanMine 3.0 - improvements to a mineable resource of flatworm biology and biodiversity. *Nucleic Acids Research* 47:D812–D820. <https://doi.org/10.1093/nar/gky1070>
29. Liu Z, Zhou Z, Chen G, Bao S (2007) A putative transcriptional elongation factor hlws1

- is essential for mammalian cell proliferation. *Biochemical and biophysical research communications* 353:47–53. <https://doi.org/10.1016/j.bbrc.2006.11.133>
30. Timofeev O, Cizmecioglu O, Settele F, et al (2010) Cdc25 phosphatases are required for timely assembly of CDK1-cyclin B at the G2/M transition. *The Journal of biological chemistry* 285:16978–90. <https://doi.org/10.1074/jbc.M109.096552>
 31. Cook BD, Dynek JN, Chang W, et al (2002) Role for the related poly(ADP-Ribose) polymerases tankyrase 1 and 2 at human telomeres. *Molecular and cellular biology* 22:332–42. <https://doi.org/10.1128/MCB.22.1.332-342.2002>
 32. Fraguas S, Barberán S, Ibarra B, et al (2012) Regeneration of neuronal cell types in *Schmidtea mediterranea*: An immunohistochemical and expression study. *International Journal of Developmental Biology* 56:143–153. <https://doi.org/10.1387/ijdb.113428sf>
 33. Hendzel MJ, Wei Y, Mancini MA, et al (1997) Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. *Chromosoma* 106:348–360. <https://doi.org/10.1007/s004120050256>
 34. Zhang L, Yang Y, Xie L, et al (2024) JCAD deficiency delayed liver regenerative repair through the Hippo–YAP signalling pathway. *Clinical and Translational Medicine* 14:. <https://doi.org/10.1002/ctm2.1630>
 35. Gutiérrez-Gutiérrez Ó, Felix DA, Salvetti A, et al (2021) Regeneration in starved planarians depends on TRiC/CCT subunits modulating the unfolded protein response. *EMBO reports* 22:e52905. <https://doi.org/10.15252/embr.202152905>
 36. Nishimura K, Kitamura Y, Agata K (2008) [Molecular mechanism of brain regeneration and reconstruction of dopaminergic neural network in planarians]. *Brain and nerve = Shinkei kenkyu no shinpo* 60:307–17
 37. Cote LE, Simental E, Reddien PW (2019) Muscle functions as a connective tissue and source of extracellular matrix in planarians. *Nature Communications* 10:1592. <https://doi.org/10.1038/s41467-019-09539-6>
 38. Currie KW, Molinaro AM, Pearson BJ (2016) Neuronal sources of hedgehog modulate neurogenesis in the adult planarian brain. *eLife* 5:1–18. <https://doi.org/10.7554/eLife.19735>
 39. Molinaro AM, Pearson BJ (2016) In silico lineage tracing through single cell transcriptomics identifies a neural stem cell population in planarians. *Genome Biology* 17:1–17. <https://doi.org/10.1186/s13059-016-0937-9>
 40. Molinaro AM, Pearson BJ (2018) Myths vs. FACS: What do we know about planarian stem cell lineages? *International Journal of Developmental Biology* 62:527–535. <https://doi.org/10.1387/ijdb.180066bp>
 41. Tan TCJ, Rahman R, Jaber-Hijazi F, et al (2012) Telomere maintenance and telomerase activity are differentially regulated in asexual and sexual worms. *Proceedings of the National Academy of Sciences of the United States of America* 109:4209–4214. <https://doi.org/10.1073/pnas.1118885109>
 42. Marión RM, López de Silanes I, Mosteiro L, et al (2017) Common Telomere Changes during In Vivo Reprogramming and Early Stages of Tumorigenesis. *Stem Cell Reports* 8:460–475. <https://doi.org/10.1016/j.stemcr.2017.01.001>
 43. Puri D, Wagner W (2023) Epigenetic rejuvenation by partial reprogramming. *BioEssays* 45:1–8. <https://doi.org/10.1002/bies.202200208>
 44. Tu KC, Cheng LC, Vu HTK, et al (2015) Egr-5 is a post-mitotic regulator of planarian epidermal differentiation. *eLife* 4:1–27. <https://doi.org/10.7554/eLife.10501>
 45. Nieto MA, Huang RYYJ, Jackson RAA, Thiery JPP (2016) Emt: 2016. *Cell* 166:21–45.

<https://doi.org/10.1016/j.cell.2016.06.028>

46. Grzeschik NA, Parsons LM, Allott ML, et al (2010) Lgl, aPKC, and Crumbs Regulate the Salvador/Warts/Hippo Pathway through Two Distinct Mechanisms. *Current Biology* 20:573–581. <https://doi.org/10.1016/j.cub.2010.01.055>
47. Khatau SB, Hale CM, Stewart-Hutchinson PJ, et al (2009) A perinuclear actin cap regulates nuclear shape. *Proceedings of the National Academy of Sciences of the United States of America* 106:19017–19022. <https://doi.org/10.1073/pnas.0908686106>
48. Lynch CJ, Bernad R, Calvo I, Serrano M (2020) Manipulating the Mediator complex to induce naïve pluripotency. *Experimental Cell Research* 395:. <https://doi.org/10.1016/j.yexcr.2020.112215>
49. Whyte WA, Orlando DA, Hnisz D, et al (2013) Master transcription factors and mediator establish super-enhancers at key cell identity genes. *Cell* 153:307–319. <https://doi.org/10.1016/j.cell.2013.03.035>
50. Nóbrega-pereira S, Calvo I, Blanco-aparicio C, et al (2020) Stem cells Stabilising naïve pluripotency Targeting DNA repair for cancer therapy Phase separation amplifies transcription inhibition of CDK8 / 19 Mediator kinases. 22:
51. Hu L, Li H, Zi M, et al (2022) Why Senescent Cells Are Resistant to Apoptosis: An Insight for Senolytic Development. *Frontiers in Cell and Developmental Biology* 10:1–17. <https://doi.org/10.3389/fcell.2022.822816>
52. Hillmer RE, Link BA (2019) The Roles of Hippo Signaling Transducers Yap and Taz in Chromatin Remodeling. *Cells* 8:502. <https://doi.org/10.3390/cells8050502>
53. Galli GG, Carrara M, Yuan WC, et al (2015) YAP Drives Growth by Controlling Transcriptional Pause Release from Dynamic Enhancers. *Molecular Cell* 60:328–337. <https://doi.org/10.1016/j.molcel.2015.09.001>
54. Driscoll TP, Cosgrove BD, Heo SJ, et al (2015) Cytoskeletal to Nuclear Strain Transfer Regulates YAP Signaling in Mesenchymal Stem Cells. *Biophysical Journal* 108:2783–2793. <https://doi.org/10.1016/j.bpj.2015.05.010>
55. Wang X, Yang N, Uno E, et al (2006) A subunit of the mediator complex regulates vertebrate neuronal development. *Proceedings of the National Academy of Sciences of the United States of America* 103:17284–17289. <https://doi.org/10.1073/pnas.0605414103>
56. Burrows JTA, Pearson BJ, Scott IC (2015) An in vivo requirement for the mediator subunit Med14 in the maintenance of stem cell populations. *Stem Cell Reports* 4:670–684. <https://doi.org/10.1016/j.stemcr.2015.02.006>
57. Agrawal R, Singh A, Giri J, et al (2023) MEDIATOR SUBUNIT17 is required for transcriptional optimization of root system architecture in Arabidopsis. *Plant physiology* 192:1548–1568. <https://doi.org/10.1093/plphys/kiad129>
58. Fan J, Beck KA (2004) A role for the spectrin superfamily member syne-1 and kinesin II in cytokinesis. *Journal of Cell Science* 117:619–629. <https://doi.org/10.1242/jcs.00892>
59. Xiao Y, Dong J (2021) The hippo signaling pathway in cancer: A cell cycle perspective. *Cancers* 13:. <https://doi.org/10.3390/cancers13246214>
60. Weiss EL (2012) Mitotic exit and separation of mother and daughter cells. *Genetics* 192:1165–1202. <https://doi.org/10.1534/genetics.112.145516>
61. Bettencourt-Dias M, Giet R, Sinka R, et al (2004) Genome-wide survey of protein kinases required for cell cycle progression. *Nature* 432:980–987. <https://doi.org/10.1038/nature03160>
62. Chiba S, Ikeda M, Katsunuma K, et al (2009) MST2- and Furry-Mediated Activation of

- NDR1 Kinase Is Critical for Precise Alignment of Mitotic Chromosomes. *Current Biology* 19:675–681. <https://doi.org/10.1016/j.cub.2009.02.054>
63. Moya IM, Halder G (2018) Hippo–YAP/TAZ signalling in organ regeneration and regenerative medicine. *Nature Reviews Molecular Cell Biology*
 64. Chang YC, Wu JW, Wang CW, Jang ACC (2020) Hippo Signaling-Mediated Mechanotransduction in Cell Movement and Cancer Metastasis. *Frontiers in Molecular Biosciences* 6:1–7. <https://doi.org/10.3389/fmolb.2019.00157>
 65. Lehman K, Rossi G, Adamo JE, Brennwald P (1999) Yeast Homologues of Tomosyn and lethal giant larvae Function in Exocytosis and Are Associated with the Plasma Membrane SNARE , Sec9. *Journal of Cell Biology* 146:125–140
 66. Zhang X, Wang P, Gangar A, et al (2005) Lethal giant larvae proteins interact with the exocyst complex and are involved in polarized exocytosis. *Journal of Cell Biology* 170:273–283. <https://doi.org/10.1083/jcb.200502055>
 67. Parsons L, Grzeschik N, Richardson H (2014) Igl Regulates the Hippo Pathway Independently of Fat/Dachs, Kibra/Expanded/Merlin and dRASSF/dSTRIPAK. *Cancers* 6:879–896. <https://doi.org/10.3390/cancers6020879>
 68. Cao F, Miao Y, Xu K, Liu P (2015) Lethal (2) giant larvae: An indispensable regulator of cell polarity and cancer development. *International Journal of Biological Sciences* 11:380–389. <https://doi.org/10.7150/ijbs.11243>
 69. Thandapani P (2019) Super-enhancers in cancer. *Pharmacology and Therapeutics* 199:129–138. <https://doi.org/10.1016/j.pharmthera.2019.02.014>
 70. Fernández-Taboada E, Moritz S, Zeuschner D, et al (2010) Smed-SmB, a member of the LSM protein superfamily, is essential for chromatoid body organization and planarian stem cell proliferation. *Development* 137:1583–1583. <https://doi.org/10.1242/dev.051847>
 71. Cebria F, Cebrià F, Newmark PA (2005) Planarian homologs of netrin and netrin receptor are required for proper regeneration of the central nervous system and the maintenance of nervous system architecture. *Development* 132:3691–3703. <https://doi.org/10.1242/dev.01941>
 72. Barrett T, Wilhite SE, Ledoux P, et al (2012) NCBI GEO: archive for functional genomics data sets—update. *Nucleic Acids Research* 41:D991–D995. <https://doi.org/10.1093/nar/gks1193>
 73. Grohme MA, Schloissnig S, Rozanski A, et al (2018) The genome of *Schmidtea mediterranea* and the evolution of core cellular mechanisms. *Nature* 554:56–61. <https://doi.org/10.1038/nature25473>
 74. Waterhouse AM, Procter JB, Martin DMA, et al (2009) Jalview Version 2-A multiple sequence alignment editor and analysis workbench. *Bioinformatics* 25:1189–1191. <https://doi.org/10.1093/bioinformatics/btp033>
 75. de Castro E, Sigrist CJA, Gattiker A, et al (2006) ScanProsite: Detection of PROSITE signature matches and ProRule-associated functional and structural residues in proteins. *Nucleic Acids Research* 34:362–365. <https://doi.org/10.1093/nar/gkl124>
 76. Plass M, Solana J, Wolf FA, et al (2018) Cell type atlas and lineage tree of a whole complex animal by single-cell transcriptomics. *Science* 360:1–17. <https://doi.org/10.1126/science.aaq1723>
 77. Sánchez Alvarado A, Newmark PA (1999) Double-stranded RNA specifically disrupts gene expression during planarian regeneration. *Proceedings of the National Academy of Sciences of the United States of America* 96:5049–54. <https://doi.org/10.1073/PNAS.96.9.5049>

78. Ross KG, Omuro KC, Taylor MR, et al (2015) Novel monoclonal antibodies to study tissue regeneration in planarians. *BMC Developmental Biology* 15:2. <https://doi.org/10.1186/s12861-014-0050-9>
79. Chai G, Ma C, Bao K, et al (2010) Complete functional segregation of planarian β -catenin-1 and -2 in mediating Wnt signaling and cell adhesion. *Journal of Biological Chemistry* 285:24120–24130. <https://doi.org/10.1074/jbc.M110.113662>
80. Lin AYT, Pearson BJ (2017) Yorkie is required to restrict the injury responses in planarians. *PLoS Genetics* 13:1–30. <https://doi.org/10.1371/journal.pgen.1006874>
81. Currie KW, Brown DRR, Zhu S, et al (2016) HOX gene complement and expression in the planarian *Schmidtea mediterranea*. *EvoDevo* 7:2663–2670. <https://doi.org/10.1186/s13227-016-0044-8>
82. King RS, Newmark PA (2013) In situ hybridization protocol for enhanced detection of gene expression in the planarian *Schmidtea mediterranea*. *BMC developmental biology* 13:8. <https://doi.org/10.1186/1471-213X-13-8>
83. Iglesias M, Felix DA, Gutiérrez-Gutiérrez Ó, et al (2019) Downregulation of mTOR Signaling Increases Stem Cell Population Telomere Length during Starvation of Immortal Planarians. *Stem Cell Reports* 13:405–418. <https://doi.org/10.1016/j.stemcr.2019.06.005>
84. Reddien PW, Oviedo NJ, Jennings JR, et al (2005) Developmental biology: SMEDWI-2 is a PIWI-like protein that regulates planarian stem cells. *Science* 310:1327–1330. <https://doi.org/10.1126/science.1116110>
85. Schindelin J, Arganda-Carreras I, Frise E, et al (2012) Fiji: An open-source platform for biological-image analysis. *Nature Methods* 9:676–682. <https://doi.org/10.1038/nmeth.2019>

overgrowths and unpigmented regions. White boxes highlight unpigmented regions and arrowheads overgrowths. Scale bar: 1mm. Four phenotypes could be observed: wild type (wt); unpigmented regions (unp); overgrowth (og); and dead. (n ≥ 30). (E) Anti-SYNAPSIN (3C11) immunostaining of an unpigmented region in *gfp*, *syne1* and *med17* (RNAi) animals (n ≥ 7). Scale bar: 1mm (F) Quantification of mitotic cells (H3P+) normalized by area after rescue experiments of *gfp* (control), *hpo*, *med17* and *syne1* RNAi animals. The experiments were performed in planarians subjected to 3 weeks of injections (n ≥ 10). Data is represented as Box & whiskers min to max. Showing all points. *p < 0.05; ***p < 0.001; ****p < 0.0001 and was analyzed with ANOVA test.

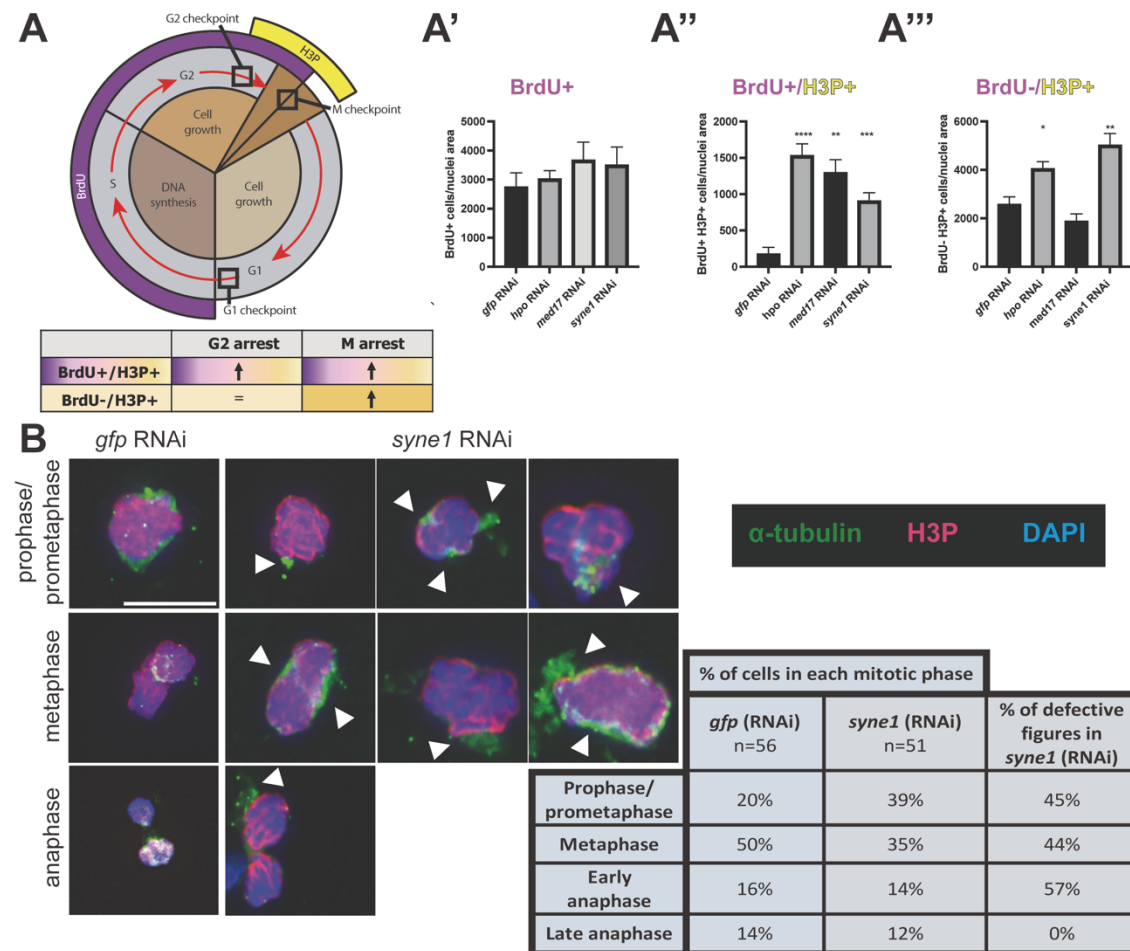


Figure 2: *syne1* and *med17* are required for the progression of the cell cycle at different phases. (A) Schematic and table showing the accumulation of BrdU and H3P during the cell cycle in planarians and the levels correlating to arrests in G2 and M phases. (A') Quantification of BrdU+ cells in *hpo*, *med17*, *syne1* (RNAi) animals and corresponding controls. (A'') Quantification of double-positive BrdU+/H3P+ cells in *hpo*, *med17*, *syne1* (RNAi) animals and corresponding controls. (A''') Quantification of BrdU-/H3P+ cells in *hpo*, *med17*, *syne1* (RNAi) animals and corresponding controls. Experiments were performed after a 16-h BrdU pulse. All graphs correspond to animals subjected to *hippo* RNAi for 3 weeks (n ≥ 7). Error bars represent standard error. Data were analyzed by ANOVA test. **p < 0.05; ***p < 0.001, ****p < 0.0001. (B) α-tubulin arrangement in *syne1*(RNAi) and control cells, as revealed by double immunostaining with anti-α-tubulin and anti-H3P. Nuclei are stained with DAPI. The percentage of each mitotic phase and of defective figures found in *syne1* (RNAi) cells is shown in the table. White arrowheads indicate disorganized arrays of spindle microtubules and mislocalized poles. Scale bar: 10 μm.

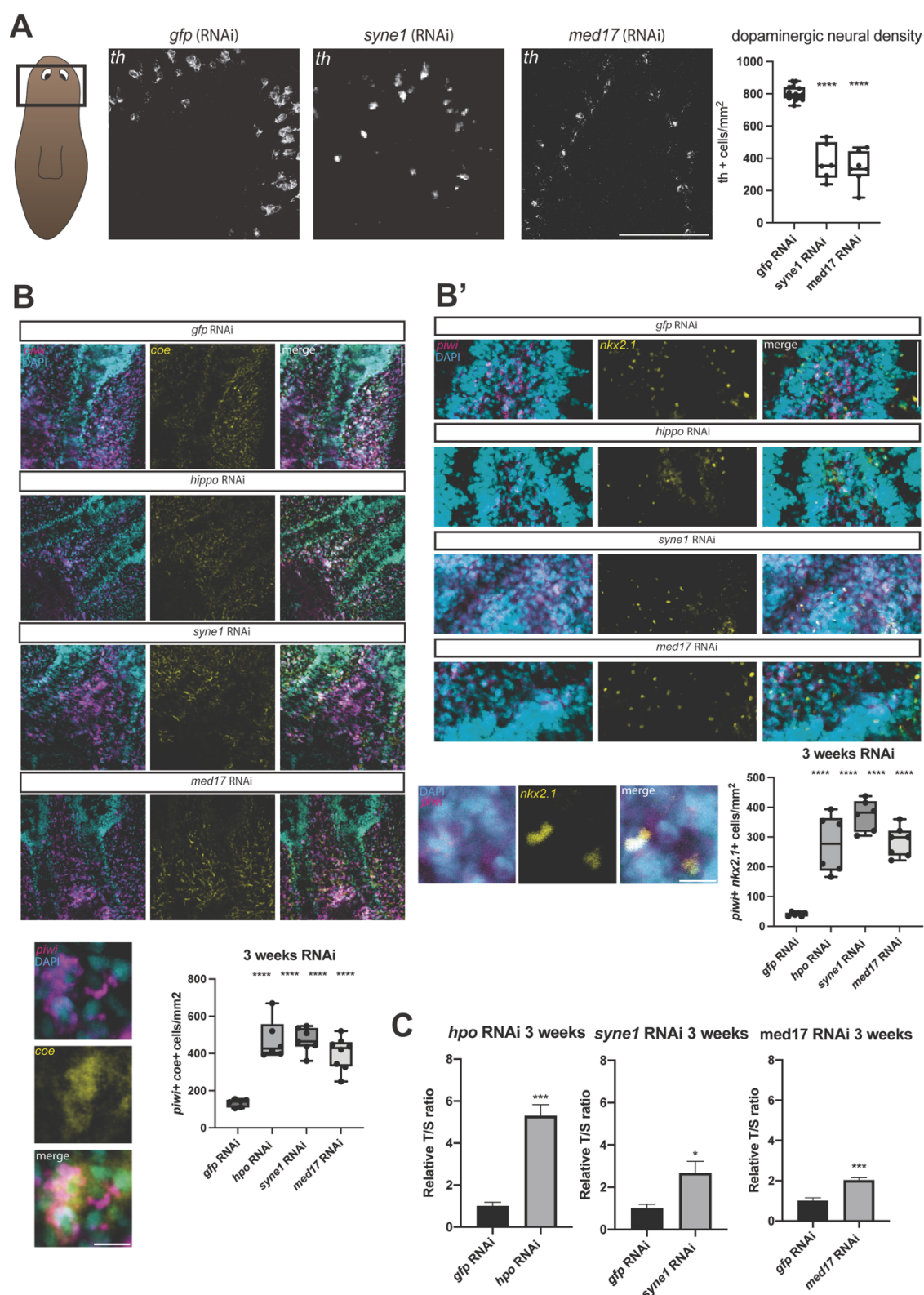


Figure 3: *syne1* and *med17* are required for the maintenance of neural differentiation. (A) FISH for *th*⁺ in control, *syne1* (RNAi) and *med17* (RNAi) animals injected for 3 weeks. The corresponding quantification is shown ($n \geq 6$). The total number of *th*⁺ cells was normalized with respect to the head area (from the anterior tip to the pharynx). Error bars represent standard error. Data were analyzed by ANOVA test. **** $p < 0.0001$. Scale bar: 100 μ m. (B, B') Double FISH with markers of different neural cell progenitors: *smedwi-1/coe*, *smedwi-1/nkx2.1* and corresponding quantification ($n \geq 6$). Representative magnifications of either colocalization can

be seen. Data is represented as Box & whiskers min to max. Showing all points. Data were analyzed by ANOVA test. **** $p < 0.0001$. Scale bars: 100 μm and 10 μm . (C) Telomere quantitative PCR (qPCR) comparing control and *hpo*, *syne1* and *med17* RNAi planarians respectively. qPCR was performed on genomic DNA for 3 biological replicates (5 planarians per replicate) per condition. The qPCR determines the ratio of telomere (T) repeat copy number to a single-copy (S) gene (genomic DNA from dd_Smed_v6_2426_0_1) copy number (T/S ratio) compared with a reference DNA sample (*gfp* RNAi) (two-tailed Student's t-test, * $p < 0.05$; ** $p < 0.001$). Error bars represent standard error.

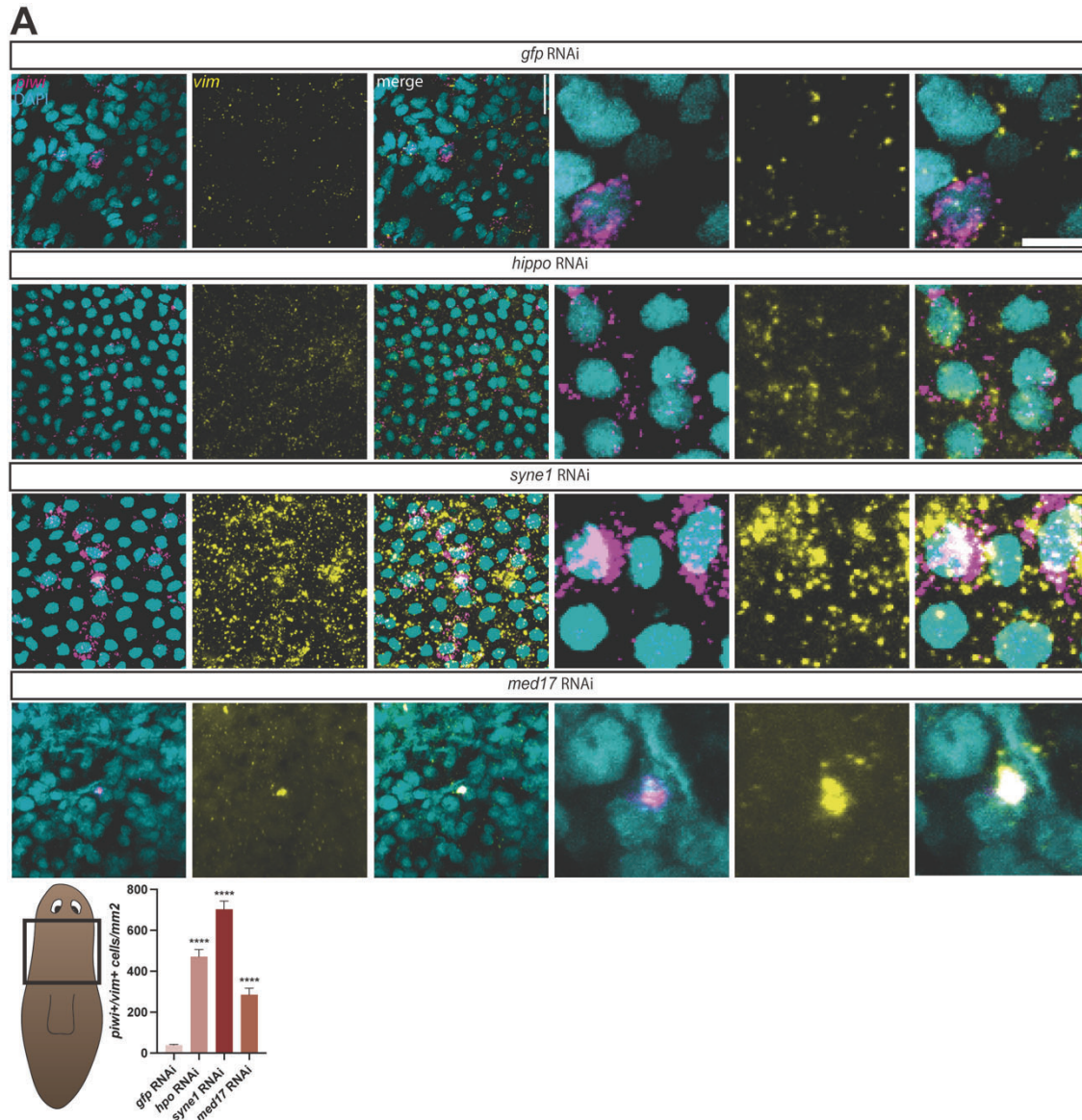


Figure 4: *syne1* and *med17* are required for the maintenance of epidermal differentiation. (A) Double FISH with the neoblast marker *smedwi-1* (*piwi*) and epidermal cell marker *vimentin* (*vim*) and corresponding quantification. Data is represented as mean $\pm$ s.e.m **** $p < 0.0001$. Data was analyzed with ANOVA test ($n \geq 7$). Scale bars: 20 μm and 10 μm .

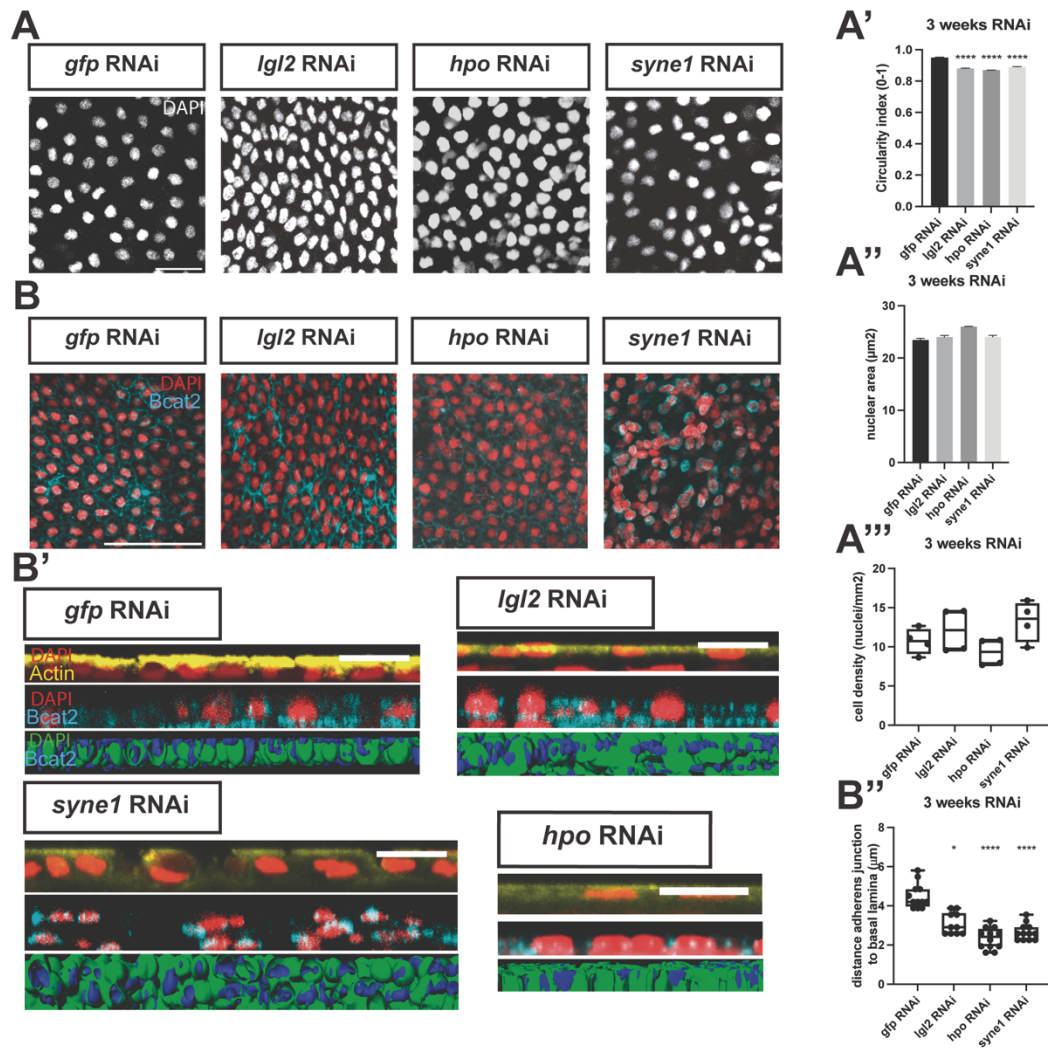


Figure 5: *hippo* and *syne1* are required for the maintenance of the epidermal cell polarity. (A) . DAPI staining of epithelial cells of the pre-pharyngeal region. Scale bar: 20 μm (A') Quantification of the mean circularity index of the nuclei. Data was analyzed with ANOVA test (n≥6). Data is represented as mean ± s.e.m (A'') Quantification of the mean nuclear cell area. Data was analyzed with ANOVA test (n≥6). Data is represented as mean±s.e.m (A''') Quantification of the mean epidermal cell density. Data was analyzed with ANOVA test (n=4). Data is represented as Box & whiskers min to max. Showing all points. (B) Immunostaining of the epithelia with anti-bcat2 antibody (adherens junctions) and DAPI. Scale bar: 50 μm (B') anti-Bcat2 and anti-Actin immunostaining and DAPI of *gfp*, *lgl2*, *hpo* and *syne1* RNAi planarians. The x-z confocal projections are shown together with the IMARIS reconstructions of the anti-Bcat2 and DAPI immunostainings depicting the position of the adherens junctions in the epidermis. Scale bar: 10 μm (B'') Quantification of distance between the adherens junctions and the basal lamina. Data was analyzed with ANOVA test (n≥10). Data is represented as Box & whiskers min to max. Showing all points.

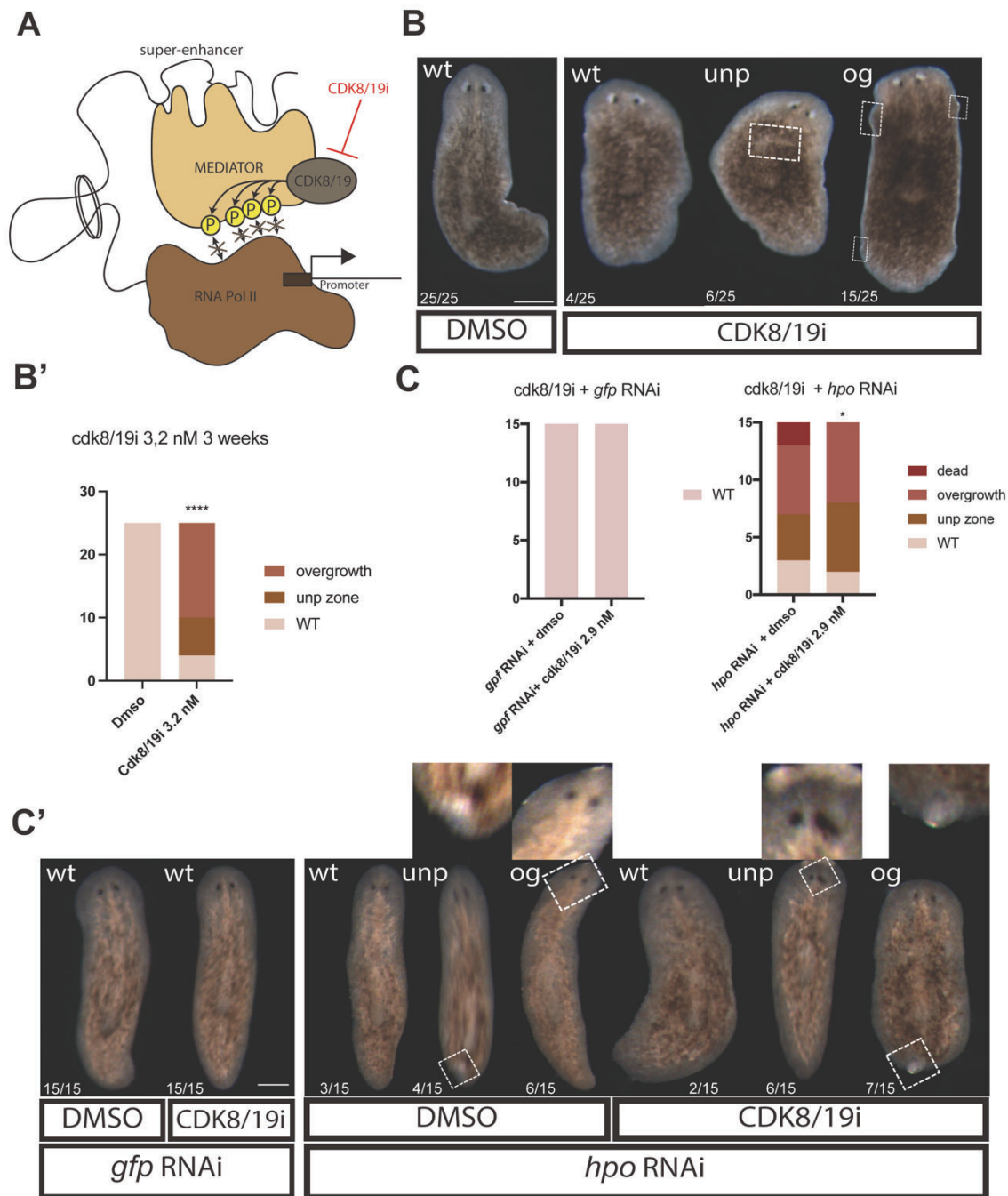


Figure 6: Mediator activity enhancement phenocopy and aggravate *hippo* RNAi phenotype (A) Ideogram depicting the role of the Cdk8/19i drug in the overactivation of the mediator complex. Adapted from [48]. (B) Stereomicroscopic image of live planarians treated with Cdk8/19i compared to DMSO controls. White discontinuous boxes highlight overgrowths and unpigmented regions. Three phenotypes could be observed: wild type (wt); unpigmented regions (unp) and overgrowth (og). (B') The graph indicates percentage quantification of the 3 phenotypes. Data was analyzed with Fisher test ($n=25$). (C) Graphs of live *hippo* RNAi planarians treated with Cdk8/19i. *gfp* RNAi is used as the negative RNAi control and the DMSO present in the drug solution as the drug negative control. Four phenotypes could be observed: wild type (wt); unpigmented regions (unp); overgrowth (og); and dead. The graph show percentage quantification of the 4 phenotypes. Data was analyzed with Fisher test ($n=15$). (C') Stereomicroscopic image of the 4 phenotypes. White discontinuous boxes highlight overgrowths and unpigmented regions.

A

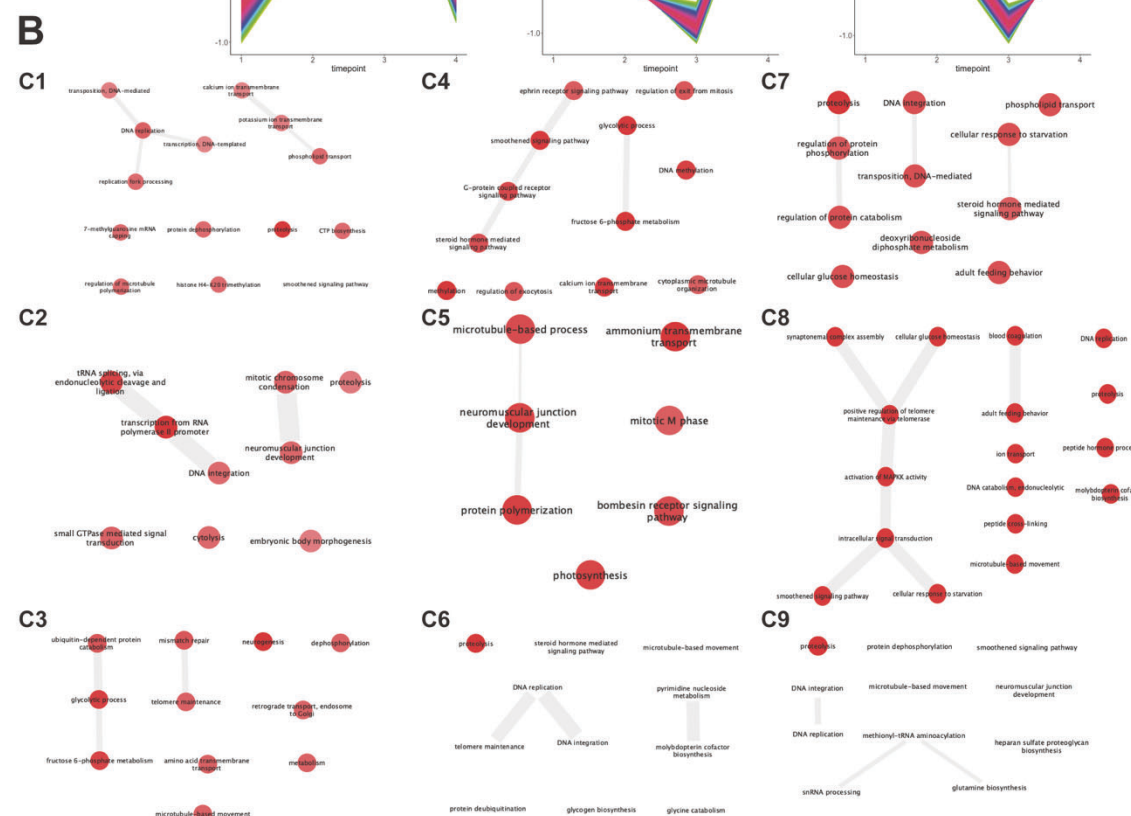
Hippo signaling pathway: MST1/2 inhibits LATS1/2, which inhibits YAP/TAZ. YAP/TAZ promotes transcription of target genes.

RNAseq experiment: *hippo* RNAi vs *gfp* RNAi. Time points: 1 week, 3 weeks, 4 weeks.

B

Gene expression heatmaps showing z-score (logFC) over time (timepoint 1-4) for different gene clusters. The color scale indicates the z-score (from -1.0 to 1.0). The number of genes in each cluster is indicated in the top right corner of each plot.

- Cluster 1: 72 genes
- Cluster 2: 47 genes
- Cluster 3: 118 genes
- Cluster 4: 31 genes
- Cluster 5: 22 genes
- Cluster 6: 56 genes
- Cluster 7: 93 genes
- Cluster 8: 85 genes
- Cluster 9: 118 genes



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knockdown animals and negative slopes indicated genes reducing expression in knockdown animals. The number of genes belonging to each cluster is indicated. (B) Gene Ontology term enrichment of differentially expressed genes in different clusters visualized by ReviGO. The size of the circles denotes the number of genes; circle color indicates the p-value of each term. Highly similar GO terms are linked in the graph.

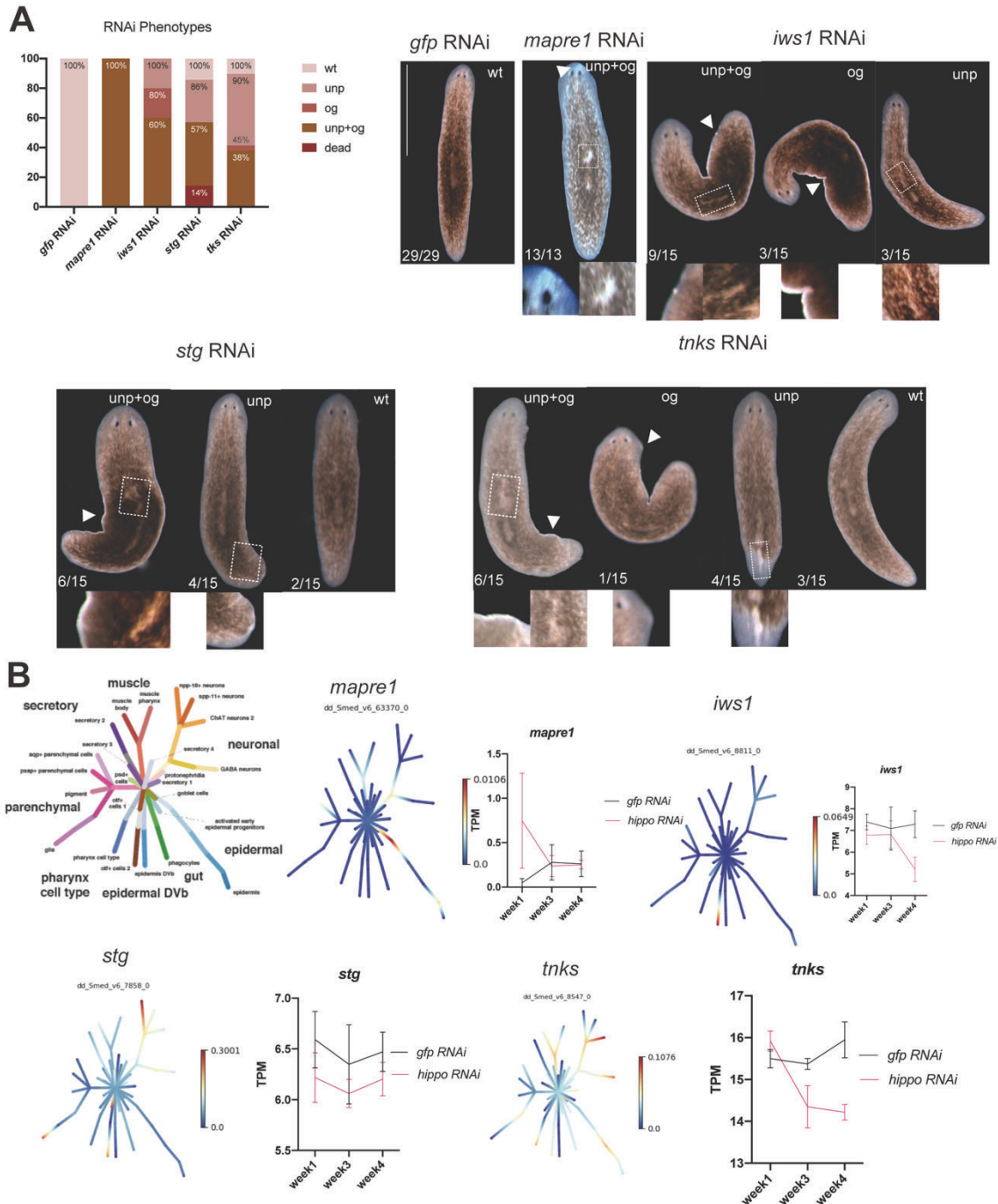


Figure S2: : Putative effectors from downregulated clusters involved in microtubule dynamics and transcription regulation phenocopy *hippo* RNAi. (A) Stereomicroscopic image of live RNAi planarians depicting overgrowths and unpigmented regions. Magnifications highlight overgrowths and unpigmented regions. Five phenotypes could be observed: wild type (wt); unpigmented regions (unp); overgrowth (og); unpigmented regions (unp) + overgrowth (og) and dead. Graphs show percentage quantification of the 4 phenotypes (n ≥ 15). (B) Schematic representation of single-cell transcriptomic expression (according to [76]) together with graph showing the transcripts per kilobase million (TPM) of *mapre1*, *iws1*, *stg*

and *tnks* in the RNA-seq of *hippo* (RNAi) animals (pink) and controls (*gfp* RNAi, black). Scale bar 0.5 mm.

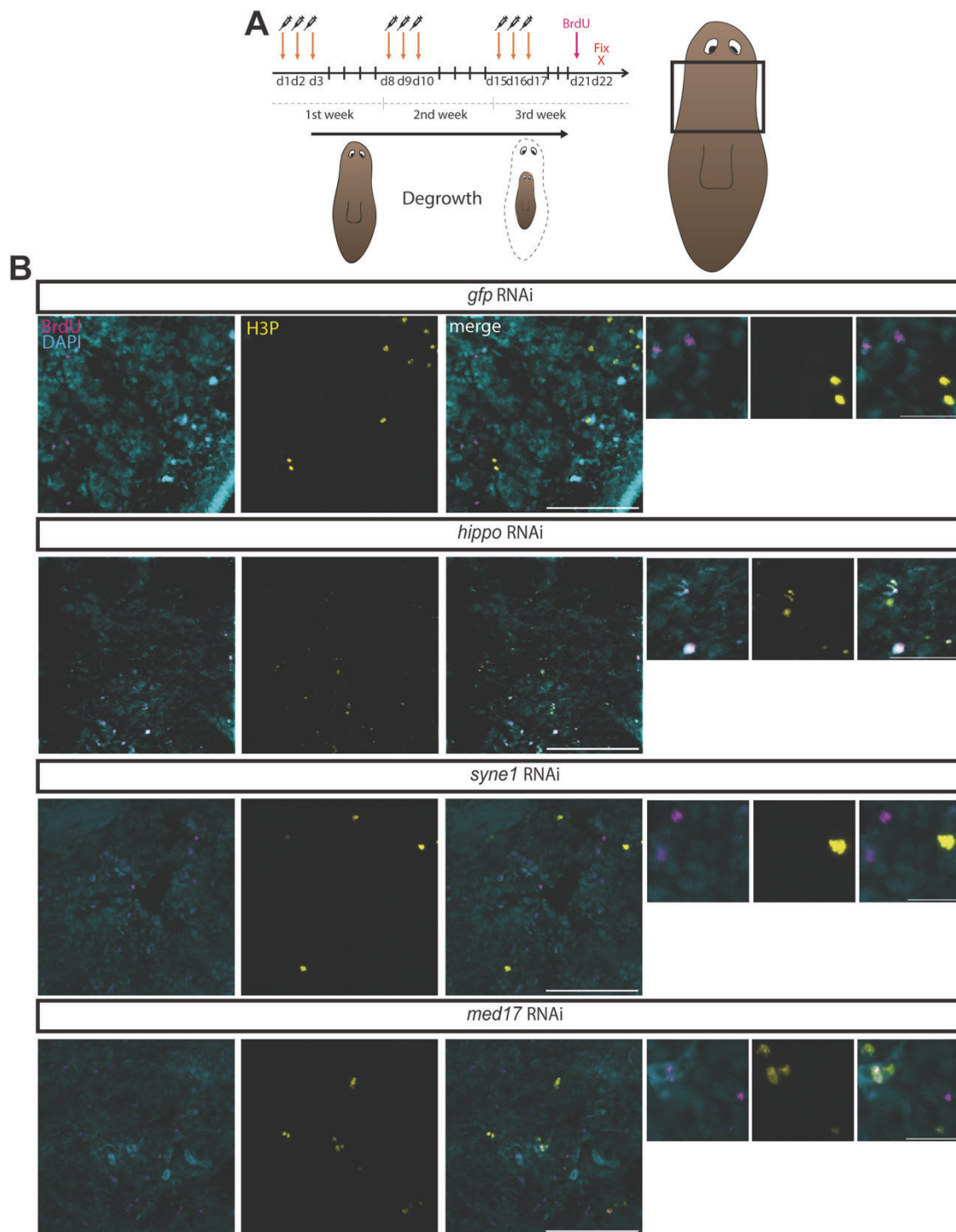


Figure S3: *med17* is essential for S/G2 transition and *hippo* and *syne1* are essential for G2/M transition in planarians. (A) Cartoon illustrating the BrdU pulse procedure. Animals were starved for 1 week, injected with *hippo* dsRNA for 3 weeks, and then injected with BrdU and fixed 16 h later. (B) BrdU labeling combined with immunostaining with anti-H3P antibody in the pre-pharynx region in controls and in planarians subjected to *hippo*, *syne1* and *med17* RNAi for 3 weeks. Scale bars: 100 μ m and 25 μ m.

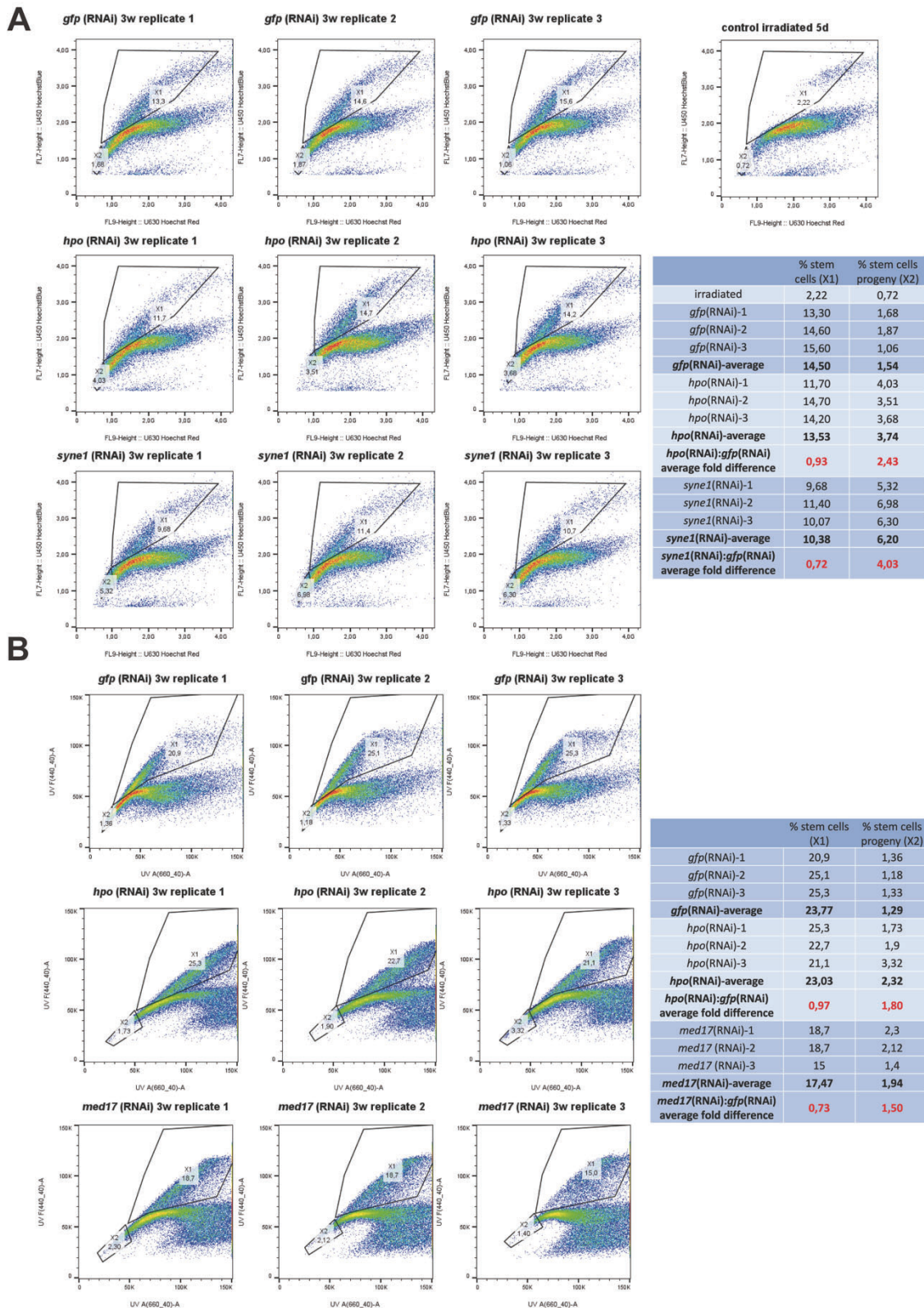


Figure S4: Flow cytometry analysis demonstrates an expanded progenitor cell population in *hippo*, *syne1* and *med17* (RNAi) animals. (A) A flow cytometry profile of animals irradiated with 60 Gy. 5 days prior. Black polygons indicate the gates for stem cells (X1) and for stem cell progeny (X2) coupled with profiles of animals at 3 weeks of *gfp*, *hpo* and *syne1* RNAi using the gating profile of the irradiated sample. The percent enrichment of stem and progenitor populations in each RNAi sample are depicted in table format. Averaged

triplicate enrichment percentages are **bolded**. Both *hpo* and *syne1* RNAi show a fold enrichment in progenitor populations compared to control (*gfp* RNAi) (red bold text). (B) Flow cytometry profiles of animals at 3 weeks of *gfp*, *hpo* and *med17* RNAi. The percent enrichment of stem and progenitor populations in each RNAi sample are depicted in table format. Averaged triplicate enrichment percentages are **bolded**. Both *hpo* and *med17* RNAi show a fold enrichment in progenitor populations compared to control (*gfp* RNAi) (red bold text).

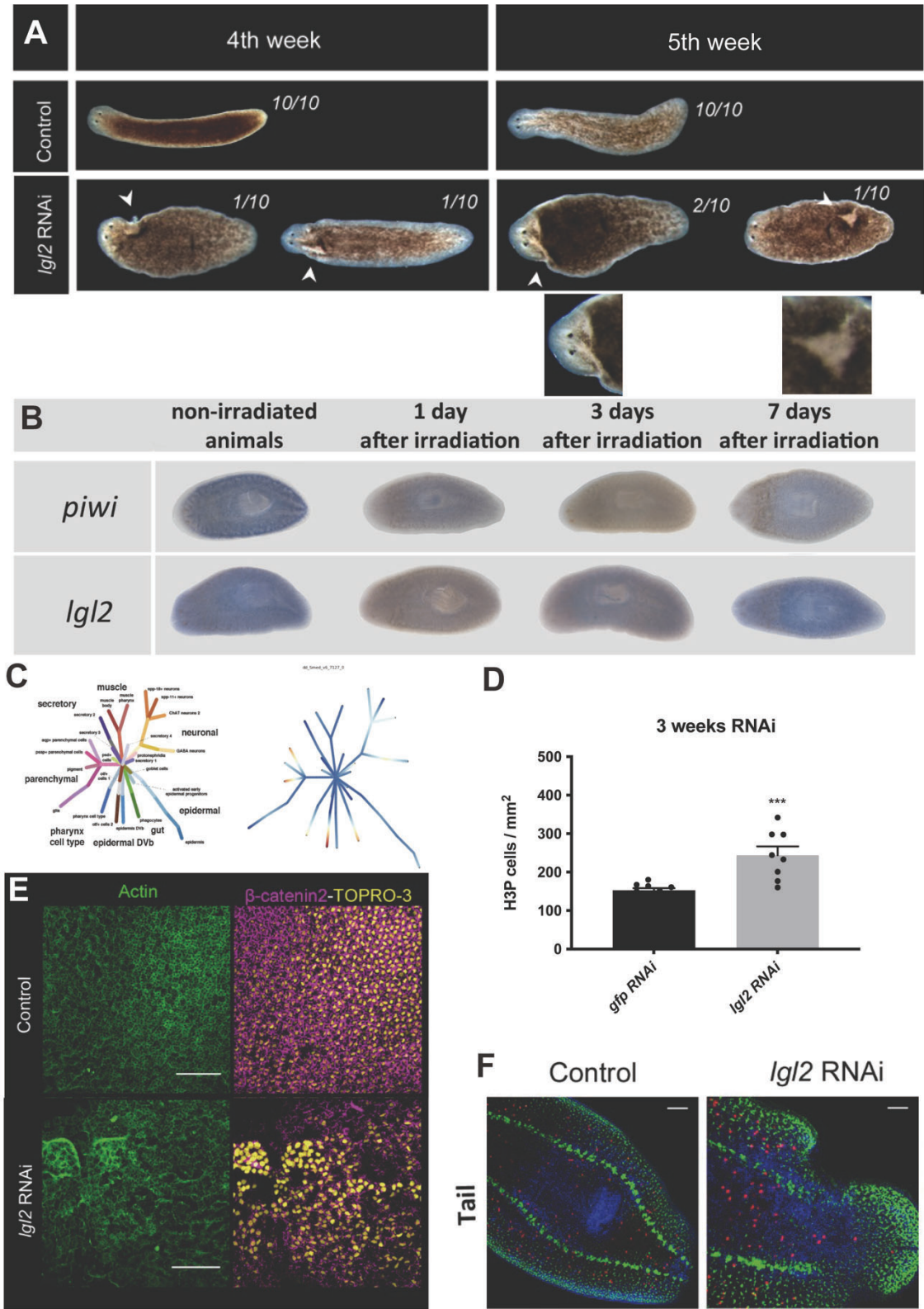


Figure S5: Functional characterization of *lgl2* in planarians. (A) In vivo phenotypes of *lgl2* RNAi after 4 weeks and 4 weeks and 4 days of injection respectively. Ratios of all animals are showed (small white ratios). White arrowheads indicate overgrowths or depigmented regions. (B) Expression pattern of *lgl2* and *piwi*, one, three and seven days after gamma-irradiation. (C) Schematic representation of single-cell transcriptomic expression of *lgl2* (*dd_Smed_v6_7127_0*) (according to [76]). (D) Quantification of mitotic cells (H3P+) normalized by area after rescue experiments of *gfp* (control) and *lgl2* RNAi animals. The experiments was performed in planarians subjected to 3 weeks of injections ($n \geq 8$). Data is represented as mean $\pm$ s.e.m *** $p < 0.001$ and was analyzed with two-tailed Student's t-test. (E) Disruption of actin and β -catenin2 structures in *lgl2* RNAi animals compared to controls after 4 weeks of RNAi inhibition. Immunostaining with anti-actin (green) and anti- β -catenin2 antibodies (magenta) and Topro-3 (yellow). Scale bar: 50 μ m. (F) Deficiencies of nervous system homeostasis in *lgl2* RNAi animals vs control after 4 weeks of RNAi inhibition. Immunostaining with anti-SYNAPSIN (3C11) (green) and anti-H3P antibodies (red) and TOPRO-3 (blue). Scale bar: 100 μ m.

CCDC175 REGULATES THE RENEWAL OF THE NEURAL TISSUE IN PLANARIANS

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Tissue renewal in adult animals has widely been a field of study for a better understanding of the onset of cell lineage disruptive pathologies such as cancer. However, the role of many genes involved in the abnormal growth of cells remains elusive. Recent studies have placed *hippo* as pivotal in the regulation of cell fate and specially the maintenance of the differentiated state. To better understand similar phenotypes of poorly studied candidate genes, we test their function in planarians, organisms that constantly resize and renew their tissues according to food availability. Here we focus in their nervous system since is one of the tissues that presented more cellular identity defects upon *hippo* inhibition.

We identified in planarians the homolog of vertebrate *ccdc175*, a coiled-coil protein which function is unknown. Here we describe the *in vivo* role of *ccdc175* in tissue differentiation. We demonstrate that *ccdc175* is required in planarians for neural tissue maintenance through the regulation of the cell cycle and senescence preventing cellular dedifferentiation. Furthermore, we find that *ccdc175* has a potential promoter for *lhx1/5-1*, a TF and nervous marker previously described in planarians, reinforcing its role in neural differentiation.

All in all, we present an evolutionary conserved coiled-coil gene involved in the maintenance of cellular balance through the regulation of the cell cycle and cellular senescence with a prominent role in the differentiation of the nervous system.

INTRODUCTION

Tissue renewal is a tightly regulated process in adult organisms. However, deregulations in it often lead to abnormal growth. Defects in tissue renewal leads to alterations in the regulation of cell fate and specially the maintenance of the differentiated state. In extreme cases eventually culminate in diseases such as cancer.

Freshwater planarians show high tissue turnover and regeneration capabilities and are ideal model organisms to study the evolutionary functional conservation of tumor suppressor genes. Such evidences have been found in the case of *p53* with animals presenting defects in the control of proliferation, self-renewal and lineage specification[1]. Similar phenotypes have been characterized in the study of the JNK pathway [2], the Hippo pathway [3, 4] and the insulin/Akt/mTOR signaling network [5–7] in planarians, reinforcing the validity of the model as a tool to better understand proper tissue maintenance and cellular differentiation *in vivo*.

hippo silencing in planarians results in the accumulation of cells in M phase and the development of overgrowths and unpigmented regions composed by cells with loss of identity commitment, a phenotype shared with tumor progression [3, 8]. It has been reported that cellular senescence plays a role in multiple biological processes from tissue repair to organismal ageing. This also include pathological developments such as inflammation and cancer. As such it is plausible to think that a connection may exist between cellular senescence and loss of cellular identity in the development of the planarian overgrowth and unpigmented phenotype [9–14].

Cellular senescence implies the cessation of cell division. This is caused by several external stressors. Failure in relieving the stress and/or repairing the damage results in genomic instability and chronification, which leads to DNA damage, loss of cell identity and failure in the activation of cell death programs (apoptosis). Senescence is characterized by tumor-suppressor activation, profound chromatin changes and secretome alterations [15–19] Accumulation of senescent cells contributes to age-dependent chronic inflammation and age-associated disorders. [20–23]

We focus on the nervous system of the planarian. A tissue previously described to present defects in cellular differentiation in the *hippo* RNAi phenotype. Planarian nervous system is composed by a bilobed cerebral ganglion dorsally located connected to two ventral nerve cords. These cords progress up to the posterior region of the animal from which a nerve plexus derives [24]. Several types of neurons have been described in the planarian brain. In this work we focused in dopaminergic cells and chemoreceptors [25].

Here we described for the first time the *in vivo* role of *Coiled-Coil Domain Containing 175* (*ccdc175*) in nervous tissue differentiation. *Ccdc175* is a coiled-coil domain protein conserved across metazoans found positively selected in a long-lived bat species [26] with a protein-protein interaction with *p53* [27]. We find that *ccdc175* is systematically downregulated after *hippo* RNAi inhibition. Moreover, its inhibition leads to the formation of overgrowths in planarians similar to the reported *hippo* RNAi phenotype[3]. We demonstrate that *smcd-ccdc175* (*ccdc175* from now on) is required in planarians for the progression of the cell cycle and apoptosis. *ccdc175* is expressed in the nervous system and we show that it is essential for the proper differentiation of the nerve cords. We find that *ccdc175* presents a putative promoter for the planarian LIM-homeobox 1/5-1 (*smcd-lhx1/5-1*; *lhx1/5-1* from now on) , a transcription factor that specifies nervous tissue in planarians, which supports its role in neural differentiation.

RESULTS

***ccdc175* RNAi planarians present overgrowths together with disruptions in the nervous system**

ccdc175 is an uncharacterized highly evolutionary conserved coiled-coil protein (Figure S2A) which appears downregulated in the *hippo* RNAi transcriptome after 1, 3 and 4 weeks of inhibition when compared to controls (Figure 1A). Single-cell data revealed *ccdc175* to be highly expressed in cholinergic and GABAergic neurons as well as in the epidermis and in the pharynx (Figure 1B). To study its function we injected dsRNA of *ccdc175* in planarians and analyzed possible changes in morphology and of the nervous system. Planarian nervous system is constituted by a bilobed cerebral ganglion dorsally located together with two ventral nerve cords. These cords progress up to the posterior region of the animal from which a nervous plexus derives. Immunostaining using an antibody against SYNAPSIN (3C11) allows the visualization of planarian nervous system [24]. Sustained inhibition by RNAi for 3 weeks (Figure 1C), leads to emergence of overgrowths in almost 40% of the injected animals similar to the *hippo* RNAi phenotype described in planarians. Moreover, we also observed the formation of disrupted nerve cords in 4 out of 6 injected animals (Figure 1D).

***ccdc175* is required for the progression of the cell cycle and apoptosis**

In order to see if the overgrowth phenotype is related to a proliferation imbalance, we decided to check the mitotic levels in these animals. To investigate the number of mitoses following RNAi injections, we used an antibody that recognizes Histone H3 phosphorylated at serine 10 (anti-H3P). This mark is present from the onset of mitosis to telophase [28]. *ccdc175* RNAi reveals an increase of H3P positive cells after 3 weeks of inhibition when compared to the *gfp* RNAi controls (Figure 1E). Moreover, to further check if the phenotype resembled the one described in the case of *hippo* RNAi we decided to check the apoptotic activity of the *ccdc175* RNAi planarians as well. Apoptosis activity is measured through a fluorometric analysis of caspase-3 activity of planarian cell lysates by a luminescence spectrophotometer [29]. The results indicated that *ccdc175* RNAi planarians presented a decrease in apoptotic activity after two weeks of RNAi inhibition before the emergence of the overgrowth phenotype (Figure 1F).

Overall, these results show an imbalance in cell renewal in planarians upon *ccdc175* RNAi inhibition. More interestingly, they phenocopy *hippo* RNAi animals with the formation of overgrowths together with an accumulation of H3P+ cells and with the decrease of apoptotic activity 1 week prior to the emergence of the phenotype.

***ccdc175* RNAi leads to increased cellular senescence and shortening of the telomeres**

In order to see if the decrease in apoptosis was correlated with an increase of cellular senescence, we decided to check for the cellular senescent markers we established in planarians in paper 1 of this doctoral thesis (page 41). These include the transcript quantification of the senescent markers *p53*, *pRb* and *Imnb1*, and the improvement of the SA- β -gal staining in wholemount planarians [36]. Downregulation of Lamin B1 coded by the gene *Imnb1* is one of the main attributes linked to cellular senescence since Lamin B1 is a major component of the nuclear lamina [30, 31]. It is responsible for keeping the nuclear lamina integrity as well as the chromatin conformation of the nuclei [32]. *p53* and *pRb* on the other hand are regarded as senescent markers since they are activated and upregulated in order to be the main triggers of the cell cycle arrest associated with cellular senescence [33–35]. SA- β -gal activity was seen increased in *ccdc175* RNAi animals after 1 week of inhibition before the emergence of the phenotype. The accumulation of positive cells was mostly detected at the periphery of the animal close to the dorsoventral border (Figure 2A). This was coupled with the transcriptomic increases of *p53* and *pRb* and decrease both by qPCR and in situ of *Imnb1* (Figure 2C and S1E).

Finally, we decided to measure the telomere length in this context in order to see if they presented changes linked with cellular senescence. This is due to the fact that telomere

shortening has been described to be a signature for cellular senescence [34]. Planarians are known to present infinite telomerase activity allowing them to maintain their telomere length during regeneration providing the animal its alleged immortality [37]. To that aim we perform a telomere qPCR (TelqPCR). This technique determines the ratio of telomere (T) repeat copy number to a single-copy (S) gene copy number (called T/S ratio) in experimental samples as compared with a reference DNA sample. The results indicated that a shortening of the telomeres occurred after just 1 week of *ccdc175* RNAi inhibition followed by an elongation at three weeks compared to the *gfp* RNAi controls (Figure 2B). This result indicates that after the peak of senescence that occurs after 1 week of *ccdc175* inhibition, it follows cellular dedifferentiation that is associated to the elongation of the telomeres [38, 39].

Overall, after *ccdc175* RNAi we observe an accumulation of senescence markers and an earlier shortening of the telomeres before the rise of the phenotype, followed by a later elongation of the telomeres coinciding with the emergence of the overgrowths and the signs of cellular dedifferentiation.

***ccdc175* is required for the differentiation of the epidermal and nervous system**

To further explore the potential loss of cellular differentiation, we decided to explore potential defects in the progression of the cell cycle by BrdU incorporation assays combined with H3P+ immunolabeling in order to label cells from the G2 entry through the onset of mitosis up to the telophase [28] (Figure S1B). The results indicated an accumulation of BrdU+/H3P+ cells after a 16h of BrdU induction (Figure 2D') with no significant accumulation of cells entering the cell cycle either in total BrdU+ cells or BrdU-/H3P+ cells (Figure 2D, 2D''). This showed that in *ccdc175* RNAi animals, cells cannot complete the G2 phase prior to enter mitosis.

To further explore this idea, we decided to check by in situ colocalization possible alterations in the pattern of expression of the markers associated with differentiation of the epidermal tissue. We analyzed *smad-agat* (*agat* from now on) [40] as a epidermal terminally committed progenitor marker and the neoblast marker *smadwi-1* (*piwi* from now on) [41]. The results show an increase of the cells coexpressing both markers (*agat*+/ *piwi*+) cells upon 3 weeks of *ccdc175* inhibition. The colocalizations quantified were localized in the anterior portion of the animal between the pharynx and the eyes (Figure 2E).

Moreover, we also characterized the epidermis morphology in *ccdc175* RNAi animals. Planarians epidermis is composed of one monolayer of cells. We observed an increase of the nuclear density of the epidermal monolayer upon *ccdc175* RNAi (Figure S1C'). This increase of nuclear density leads to a loss of nuclear circularity (Figure S1C''), without an increase of nuclear surface (Figure S1C, S1C''). These results indicate nuclear deformations without changes in size.

Furthermore, in order to assess the apical-polarity defects of the epidermis we used the β -catenin-2 [3] and actin immunostaining. (Figure S1D, S1D'). β -catenin-2 is a component of the adherens junctions that labels the boundary between the apical and basal domain. Adherens junctions divide the cell into an apical domain and a basolateral one thanks to the establishment of a mesh of actin-myosin filaments [42]. Closer inspection of the apical basal polarity reveals a compaction of the epidermal layer in the *ccdc175* RNAi condition in the transposed image projections. Moreover, when we quantify the distance between the basal lamina and the adherens junction we saw a mild significant increase in height of a micron without changes in polarity alluding to a stretch of the tissue consequence of the increased cellular density. This means that the accumulation of cells in the epidermal monolayer is causing an increase in the tissues height without changes in polarity.

Both of these results indicate defects in epidermal cell differentiation in planarians *ccdc175* RNAi coupled with an accumulation of cells in the epidermal layer.

In order to characterize more in depth the disruptions in the nervous system in *ccdc175* RNAi animals we decided to characterize some neural populations and neural progenitors. We saw

that *ccdc175* RNAi lead to a decrease of dopaminergic neurons and chemoreceptors (Figure 4A). Dopaminergic neurons can be detected using markers for *tyrosine hydroxylase* (*smed-th*, *th* from now on) since it is the rate limiting enzyme in dopamine synthesis. On the other hand, chemoreceptors can be detected with *smed-cintillo* (*cintillo* from now on) a membre of the DEG/ENaC superfamily of sodium channels meaning "head ribbon" in Spanish [43, 44]. Following these evidences, we decided to check for an accumulation of neural committed neoblasts in this context. Neural committed neoblasts can be visualized by markers against the transcription factor Collier/Olfactory-1/Early B-cell factor (*smed-coe*; *coe* from now on) [45] and the homeobox transcription factor *smed-nkx2.1* (*nkx2.1* from now on) [46] when each of the markers are coexpressed with the neoblast marker *piwi*. *Piwi*+/*coe*+ and *piwi*+/*nkx2.1*+ cells in *ccdc175* RNAi animals increase in number compared to *gfp* RNAi controls. (Figure 4B, 4C).

All and all, these experimental evidences depict an impairment of neural differentiation occurring after *ccdc175* RNAi.

A *Lhx1/5-1* conserved motif in the *ccdc175* promotor region, suggest that *lhx1/5-1* could be an upstream transcriptional regulator of *ccdc175*

To further understand the potential role of *ccdc175* in neural differentiation we searched for transcription factors (TF) previously been related with neural differentiation in planarians that could have conserved binding domains in the regulatory region of *ccdc175*. To that avail we found *lhx1/5-1* as a potential candidate. *Lhx1/5-1* is a member of the *lhx* family of transcriptions factors that has been linked with the differentiation of the nervous system during the development of multiple model organisms [47, 48] and in the planarian nervous system regeneration as well [49]. The conserved promoter binding domain of *lhx1/5-1* together with it homology score against the human *lhx1* can be seen in Figure 3A together with the distance to the transcription starting site (TSS) of the planarian *ccdc175* coding gene. It is also worth mentioning that single cell analysis and quantification of the cells co-expressing *lhx1/5-1* and *ccdc175* revealed a collinearity in the coexpression of these markers across several tissue lineages (Figure 3B). Finally, we observed the conservation *in silico* of the *lhx1* or *lhx5* human domain in the active promoter region of *ccdc175* across several metazoan species. These include the species of *Mus musculus*, *Danio Rerio*, *Xenopus tropicalis* and *Hydra vulgaris*. A table of the conserved promoter region of *ccdc175* of several metazoans coupled with the presence of *lhx1/lhx5* motifs can be seen in Table S3. Furthermore, a tree that indicates the *lhx* family of genes appears conserved across all metazoans can be seen in Figure S2B with all the planarian homologs of the *lhx* family. To sum up, the fact that we see a conserved binding motif of the human *lhx1* in the promotor region of the planarian gene *ccdc175* coupled with the lineage collinearities of coexpression of both transcripts across tissues strongly suggest that *lhx1/5-1* could bind in the promoter region of *ccdc175* in planarians. Moreover the fact that we see the conservation of the motif in the promoter region of *ccdc175* in other metazoans further reinforce this promoter connection. In parallel, experimental data revealed that after 1 week of *lhx1/5-1* RNAi a downregulation of *ccdc175* transcriptomic levels occur. This was as seen both by qPCR (Figure 3A) and in situ (Figure 3B).

All and all, these *in silico* evidences imply the role of *lhx1/5-1* as an upstream transcriptional regulator of *ccdc175*.

DISCUSSION

In human disorders *ccdc175* has been related with cone-rod dystrophy heterogeny [50]. Moreover it also has been suggested to present a putative role in longevity in some bat spices[26]. However, its function remains unknown. In this study, we have for the first time functionally characterized *ccdc175* in vivo. *ccdc175* is a highly conserved coiled-coil protein which inhibition leads to defects in neural tissue renewal in planarians such as the phenotypes characterized upon the inhibition of the Hippo pathway in the same organisms. These defects that lead to the formation of overgrowths and the disruption of the nerve cords are linked to

accumulation of mitotic cells and decrease of apoptotic activity. Such imbalance has been described already after *hippo* inhibition in planarians and the similarities bear a stronger meaning after the fact that the *ccdc175* transcript appear downregulated after the downregulation of *hippo* on those animals[3].

SA- β -gal staining and the quantification of senescent markers (*p53*, *pRb* and *Imnb1*) revealed an early peak of senescence before the rise of the overgrowth phenotype. We also observed shortening of telomeres. It remains to be characterized which are the specific cells that are presenting the telomeric shortening as well as the type of cells that are experimenting the accumulation of senescent markers. These experiments require further development of molecular technics in planarians such as double staining of telomeric in situ hybridization or SPiDER- β Gal with different cellular markers. Nonetheless they would help elucidate in more detail the biological function of *ccdc175*.

The phenomenon of a peak of cellular senescence has been described in other contexts of cellular imbalance, mainly in cancer [51, 52] where the peak in senescence leads to alterations in the regulation of the cell cycle that may lead to uncontrollable growth [53, 54] or loss of cell identity [8, 38, 39]. Such defects are visible as well in *ccdc175* RNAi planarians, since we observed the accumulation of cells in G2 phase. This is also reinforced with an elongation of the telomeres at this stage. At the end these effects lead to an accumulation of cells with an identity crisis in the form of dysplastic tissue responsible of the visible phenotype.

In order to unveil further connections between *ccdc175* and cellular differentiation we analyzed the promotor region of the gene and found a promising TF related with tissue differentiation. We selected as *lhx1/5-1* as a transcription factor with a putative binding site in the promoter region of *ccdc175* since it has been previously characterized to present a prominent role in mediating neural differentiation in planarians [49]. Several data agree with the hypothesis that *lhx1/5-1* could transcriptionally activate *ccdc175*. First, planarians lose part of nerve cords upon *ccdc175* inhibition coupled with a loss of neural subpopulations and accumulation of neural stem cells, a phenotype similar to the observed after *lhx1/5-1* RNAi. Second, coexpression of both transcripts at the single-cell level across neural lineages is observed. Finally, the conservation in several species of the presence of the *lhx* domain and promoter activity in the region upstream of *ccdc175* indicates its functional relevance (Table S3).

In agreement with the former results, the loss of differentiated neural tissue including dopaminergic neurons and chemoreceptors in *ccdc175* RNAi planarians coupled with a significant increase of neural committed neoblast populations reinforce the role that *ccdc175* is having in the progression of neural tissue differentiation. However, *ccdc175* could have a role outside of the nervous tissue as well. This is especially relevant due to the increase of *smedwi-1+/agat+* cells observed in *ccdc175* RNAi animals in comparison to controls. The fact that an increased number of epidermal cells present both a marker of stem cell and of late epidermal progenitor indicates a loss of the hierarchical steps in the establishment of the epidermal fate. This also results in the accumulation of epidermal cells in the epidermal monolayer with loss of nuclear circularity. Both results coupled with the defects in cell cycle progression strongly indicate that defects in cellular differentiation are also occurring in the epidermis of *ccdc175* RNAi animals. So far there have been no effects described in the epidermis of *lhx1/5-1* RNAi animals beside a loss of coordination of the motile ventral cilia[49]. However it would be worth exploring if we see similar defects in differentiation as in *ccdc175* RNAi animals to assess the extent of the putative promoter role of *lhx1/5-1* over *ccdc175* beyond the nervous system.

All in all, we present an evolutionary conserved coiled-coil gene, which is planarians is involved in the maintenance of the neural cellular balance through the regulation of the cell cycle, apoptosis and senescence. We show that it regulates differentiation of the nerve cords. We propose a model in which in an early stage of *ccdc175* RNAi inhibition planarians accumulate senescence markers (accumulation of SA- β -gal, increase of *p53* and *pRb*,

decrease of *Imnb1* and shortening of the telomeres) coupled with a decrease of apoptotic activity. This accumulation of senescence cells later leads to the emergence of the overgrowths and unpigmented regions together with the impairment of a proper cell cycle progression. These result in cellular dedifferentiation in the neural system coupled with an increase of epidermal cell density. Moreover, the TF *lhx1/5-1*, involved in neural differentiation, could act as a putative transcriptional regulator of *ccdc175* (Figure 5).

METHODS

Planarian culture

The planarians used in this study are the asexual clonal strain of *S. mediterranea* BCN-10 biotype and were maintained as previously described[55] in planarian artificial medium (PAM) water [56]. Animals were fed twice per week with liver, and those used in starvation experiments were starved for 1 week.

Sequence and phylogenetic analyses

For generating the phylogenetic trees, we first obtained the CCDC175 and LHX protein sequences from several different sources. In some of the cases we were able to collect them from the public databases, like in the case of *Hsa* or *Xtr* (Table S2). For the rest of the organisms a manual annotation was required. If the only resource available was a transcriptome, like in the case of *Tsol*, we used Transdecoder (v5.5.0) to obtain the translated proteins.

For the LHX tree we used HMMER with default parameters and a cutoff e-value of 1e-4 (v3.1b2) and the Pfam [57]motives of the LIM domain ([PF00412](#)) and HOX (PF00046) domain, we extracted the LIM and LIM+HOX containing proteins.

For the CCDC175 tree the hmmsearch tool was used with the planarian protein profile. Representative species with annotated references to *ccdc175* without discrepant homologies across relevant taxa were selected.

For the CCDC175 tree the whole set of translated proteins was aligned again using MAFFT [58]with the L-INS-i strategy. For the LHX tree the aligning of the LIM domain was selected using the same parameters. These alignments were the input used for IQ-TREE [59]to generate the phylogenetic trees. The options used to run the webserver of IQ-TREE were the ones by default, including the automatic substitution model selector and the ultrafast bootstrap analysis, except for the number of bootstrap alignments (set at 2500), the single branch test number of replicates (set at 2000) and the approximate Bayes test option (selected). The trees were visualized using Dendroscope3 v3.6.3 [60] with the default parameters.

Transcriptomic analysis

Transcriptomic analysis were performed on the Raw sequencing data in FASTQ format as well as the transcript abundances found in the NCBI Gene Expression Omnibus (GEO) [61]accessible using GEO Series accession number GSE95130.

Sequence analyses

ccdc175, *hpo* and *lhx1/5* were identified from the genome [62] and RNAi fragments were amplified using specific primers:

ccdc175 RNAi

F: TGAAGAATGGCAAGCACAAG

R:TCAGACACTTTGGCTGCACT

hippo RNAi

F: GAATCAGCTGCTCAGAGCCC

R: CTCGGCTTGCAAGTCTGAATC

lhx1/5 RNAi

F: CAGATGAACGCCGATTTCCC

R: CATTCCGGAGATGAAGCTGC

Sequence identity comparison was carried out using the pairwise alignment tool in Jalview suite v2.11[63]. Sequence comparison against the GenBank database was performed using the NCBI BLAST network server (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). Potential orthologs were searched for using tBLASTx where possible to allow a certain level of tolerance in case of a high degree of divergence. Domain characterization of each transcript was carried out using Scan prosite[64].

Single-Cell Sequencing Data

ccdc175 and *lhx1/5* gene expression profiles (dd_Smed_v6_9798_0 and dd_Smed_v6_11521_0 respectively) were obtained from the planaria single-cell database hosted by the Rajewsky lab at the Berlin Institute for Medical Systems Biology of the Max Delbrück Center, Berlin [65]. Single-cell Colocalizations were obtained from <https://compngen.bio.ub.edu/PlanNET>.

RNA interference analysis dsRNA

dsRNA was synthesized by in vitro transcription (Roche) and microinjection performed, as previously described [66], following the standard protocol of 3 × 32 nl injection of dsRNA for 3 consecutive days. To achieve stronger inhibition, we performed the same protocol over 3 consecutive weeks in starved planarians. Control animals were injected with dsRNA for green fluorescent protein (GFP), a gene not present in planarians.

Immunohistochemistry

Whole-mount immunohistochemistry was performed as previously described [67]. Animals were killed with 2% HCl and fixed with 4% formaldehyde. The primary antibody used were: mouse anti-SYNAPSIN (3C11) (anti-SYNORF-1, 1:50; Hybridoma Bank); rabbit anti-phospho-histone H3 (Ser10) (D2C8) (PH3) (1:500; Cell Signaling Technology; 9701) rabbit anti-β-catenin-2 (anti-Bcat2) (1:2,000) [68]; anti-actin JLA20 (1:2; Developmental Studies Hybridoma Bank). The secondary antibodies used were Alexa 568-conjugated goat anti-rabbit (1:1000; Molecular Probes; A-11011) and Alexa 488-conjugated goat anti-mouse (1:400; Molecular Probes; A-11001). Nuclei were stained with DAPI (1:5000; Sigma-Aldrich).

Caspase 3 activity assay

At the end of the second week of RNAi inhibition in starved condition and after 1,2 and 3 weeks under feeding, protein extraction was performed. BioRad protein reagent was used to obtain protein concentration of the cell lysates. Fluorometric analysis of caspase-3 activity was performed as described previously [29]. Fluorescence was measured in a luminescence spectrophotometer (Perkin- Elmer LS-50) using Fluostar Optima microplate fluorescence reader (BMG Labtech), applying the following settings: excitation, 380 nm; emission, 440 nm.

20 mg of protein extract was used to determine enzyme activity, incubating for 2 h at 37 °C with 20 µM caspase-3 substrate Ac-DEVD-AMC or 2 ml from a stock of 1 mg/ml for a final volume of 150 µl. Three technical replicates were analyzed per condition. Caspase-3 inhibitor Z-DEVD-FMK was used as a control.

SA-β-galactosidase staining

The β-galactosidase activity was analyzed following the protocols described previously [69, 70]. Planarians were fixed with 2% formaldehyde + 0.2% glutaraldehyde for 5 min, washed with PBS and stained with the staining solution containing: 20 mg/ml of X-gal (Roche;10703729001) in dimethylformamide, 0.2 M citric acid/sodium phosphate buffer pH=6, 100 mM potassium ferrocyanide, 5 M sodium chloride and 1 M magnesium chloride. Planarians were incubated for 1h and 40 min at 37 °C.

Quantitative real-time PCR

Total RNA was extracted from a pool of five planarians per condition using TRIzol reagent (Invitrogen). cDNA was synthesized as previously described [29]. Expression levels were normalized to that of the housekeeping gene *ura4*. All experiments were performed using three biological and three technical replicates for each condition. The design of specific primers corresponding to *ccdc175*, *lmnb1*, and the use of previously published ones for *p53*, *pRb* [71] allowed to check their levels of expression after the RNAi experiments. All designed primers used in this study are the following:

ccdc175 qPCR

F: GCAGCCAAAGTGTCTGATGT
R: CGGAAACAGTAGCTTCGTGA

lmnb1 qPCR

F:GGGAAATACGGTCTCGTGTG
R: CAAATTTCCCAGAGCAGTCG

Telomere quantitative PCR (telomere qPCR)

Telomere quantitative PCR was performed as previously described [72] in Supplemental Experimental Procedures.

Whole-mount BrdU staining

Whole-mount BrdU staining was performed as previously described [73].

Whole-mount *in situ* hybridization

Probes were synthesized *in vitro* using SP6 or T7 polymerase and DIG- or FITC- modified (Roche) from previously cloned fragments using the following primers:

Probe *ccdc175*

F: GAATCGGATCAATCGGAAAA
R: TTTTGCTTCGTCTTGAATTTGA

Probe *lmnb1*

F: AAGGCTGAACAGAGGGAACA

R: GCAACGCTAATATTACACCGAGT

RNA probes were purified by ethanol precipitation and the addition of 7.5 M ammonium acetate. For colorimetric whole-mount *in situ* hybridization, animals were sacrificed using 5% N-acetyl-L-cysteine (NAC), fixed with 4% formaldehyde and permeabilized with Reduction Solution (50 mM DTT, 1% NP-40, 0.5% SDS in 1× PBS). For fluorescent whole-mount double *in situ* hybridization, animals were sacrificed using 5% N-acetyl-L-cysteine (NAC), fixed with 4% formaldehyde and permeabilized with Reduction Solution (50 mM DTT, 1% NP-40, 0.5% SDS in 1× PBS). The fixative and whole-mount *in situ* hybridization protocol used has been previously described [74]. or whole-mount fluorescent *in situ* hybridization, animals were sacrificed with 7.5% NAC and fixed with 4% formaldehyde. Fluorescent *in situ* hybridization was carried out as described previously [75]. For double fluorescent *in situ* hybridization (dFISH) an azide step (150 mM sodium azide for 45 min at room temperature) was added. Nuclei were stained with DAPI (1:5000; Sigma).

Promoters

The “TF tools” plugin for the PlanNET web service [76] was used as a search tool for finding the putative promoter of *ccdc175* according to the presence of *lhx1* transcription factor binding motifs. The website, the source code of the plugin, and downloads for all the annotations are available at <https://compugen.bio.ub.edu/PlanNET>.

ACR/Promoter/Lhx1 binding site motif research in different species

The presence/absence of accessible chromatin regions and promoter regions in different species was determined by visualizing the ATAC-seq and DNase-seq, and H3K27ac and H3K4me1/2/3 tracks, respectively. Genome Browsers used were listed in Table S3.

The presence of the *Lhx1* binding site motif was determined using the FIMO tool (<https://meme-suite.org/meme/tools/fimo>) [77] using the default parameters. FASTA files containing DNA sequences corresponding to the *ccdc175* promoter region (1kb upstream the TSS) from different species were obtained from different sources, mostly using UCSC browser (Table S3).

Imaging

In vivo images were taken with sCMEX-3 High End Digital Microscope Camera DC.3000s (Visual Inspection Technology) in a Zeiss Stemi SV 6 binocular loupe. Whole mount Brightfield colorimetric *in situ* hybridization and immunostaining samples were observed with a *Leica* stereoscope and images were taken with *Progress C3 Jenoptik* camera (Jena, TH, Germany). A Zeiss LSM 880 confocal microscope (Zeiss, Oberkochen, Germany) was used to obtain confocal images of whole-mount fluorescent *in situ* hybridization samples, BrdU assays and epidermal immunostainings. Z-stacks of immunohistochemistry on dissociated cells were acquired with a *Leica Thunder* microscope (Carl Zeiss, Jena).

Quantification of H3P+ cells were done using freely available ImageJ software [78] with the analyze particle function and normalized by animal area determined with the measurement tool. To build 3D reconstructions, confocal sections were deconvolved using Huygens Deconvolution Software (Scientific Volume Imaging) and processed using Imaris Software (Bitplane). Fiji/ImageJ was also used to show representative confocal stacks for each experimental condition.

Colocalization quantification was performed using the equivalent areas using the “ROI-manager” tool in Fiji. Nuclear area in BrdU experiments was measured using the “threshold” tool and mask conversion for all samples. Quantification of BrdU+ cells and single-cell populations were carried using the analyze particle function.

The circularity index and mean nuclear area of cells was measured using at least 146 nuclei per animal following the same process.

The nuclear density Nuclear-stained (DAPI) images were transformed into a mask using the “threshold” tool and converted to a mask. The mask was used to obtain the nuclear signal with the analyze particle function.

To determine mean distance between the apical pole and to the base of epidermal cells, the distance between the adherent junctions (bcat2) to the basal lamina of the cell was measured. Measurements were taken in three different regions of the same section and the mean value was calculated.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 6. Two-sided Student's *t*-tests ($\alpha=0.05$) were performed to compare the means of two populations. Comparisons of more than 2 populations were done with ANOVA test. Previous confirmation of normality and homogeneity of data was performed with Shapiro–Wilk test and Bartlett test respectively. To determine the p-value accumulated for each pairwise comparison it was used the Tukey HSD test.

REFERENCES

1. Pearson BJ, Alvarado AS (2010) A planarian p53 homolog regulates proliferation and self-renewal in adult stem cell lineages. *Development* 137:213–221. <https://doi.org/10.1242/dev.044297>
2. Almuedo-Castillo M, Crespo X, Seebeck F, et al (2014) JNK Controls the Onset of Mitosis in Planarian Stem Cells and Triggers Apoptotic Cell Death Required for Regeneration and Remodeling. *PLoS Genetics* 10:1–15. <https://doi.org/10.1371/journal.pgen.1004400>
3. de Sousa N, Rodríguez-Esteban G, Rojo-Laguna JI, et al (2018) Hippo signaling controls cell cycle and restricts cell plasticity in planarians. *PLoS Biology* 16:1–28. <https://doi.org/10.1371/journal.pbio.2002399>
4. Lin AYT, Pearson BJ (2014) Planarian yorkie/YAP functions to integrate adult stem cell proliferation, organ homeostasis and maintenance of axial patterning. *Development (Cambridge)* 141:1197–1208. <https://doi.org/10.1242/dev.101915>
5. Oviedo NJ, Pearson BJ, Levin M, Alvarado AS (2008) Planarian PTEN homologs regulate stem cells and regeneration through TOR signaling. *DMM Disease Models and Mechanisms* 1:131–143. <https://doi.org/10.1242/dmm.000117>
6. González-Estévez C, Felix DA, Rodríguez-Esteban G, Aziz Aboobaker A (2012) Decreased neoblast progeny and increased cell death during starvation-induced planarian degrowth. *International Journal of Developmental Biology* 56:83–91. <https://doi.org/10.1387/ijdb.113452cg>
7. González-Estévez C, Felix DA, Smith MD, et al (2012) SMG-1 and mTORC1 Act

- Antagonistically to Regulate Response to Injury and Growth in Planarians. *PLoS Genetics* 8:e1002619. <https://doi.org/10.1371/journal.pgen.1002619>
8. Roten A Van, Barakat AZ, Wouters A, et al (2018) A carcinogenic trigger to study the function of tumor suppressor genes in *Schmidtea mediterranea*. *DMM Disease Models and Mechanisms* 11:. <https://doi.org/10.1242/dmm.032573>
 9. Yun MH (2018) Cellular senescence in tissue repair: Every cloud has a silver lining. *International Journal of Developmental Biology* 62:591–604. <https://doi.org/10.1387/ijdb.180081my>
 10. Antelo-Iglesias L, Piccallos-Rabina P, Estévez-Souto V, et al (2021) The role of cellular senescence in tissue repair and regeneration. *Mechanisms of Ageing and Development* 198:111528. <https://doi.org/10.1016/j.mad.2021.111528>
 11. McHugh D, Gil J (2018) Senescence and aging: Causes, consequences, and therapeutic avenues. *Journal of Cell Biology* 217:65–77. <https://doi.org/10.1083/jcb.201708092>
 12. Di Micco R, Krizhanovsky V, Baker D, d'Adda di Fagagna F (2021) Cellular senescence in ageing: from mechanisms to therapeutic opportunities. *Nature Reviews Molecular Cell Biology* 22:75–95. <https://doi.org/10.1038/s41580-020-00314-w>
 13. Yu Q, Walters HE, Pasquini G, et al (2023) Cellular senescence promotes progenitor cell expansion during axolotl limb regeneration. *Developmental Cell* 58:2416–2427.e7. <https://doi.org/10.1016/j.devcel.2023.09.009>
 14. Salinas-Saavedra M, Febrimarsa, Krasovec G, et al (2023) Senescence-induced cellular reprogramming drives cnidarian whole-body regeneration. *Cell Reports* 42:112687. <https://doi.org/10.1016/j.celrep.2023.112687>
 15. Campisi J, D'Adda Di Fagagna F (2007) Cellular senescence: When bad things happen to good cells. *Nature Reviews Molecular Cell Biology* 8:729–740. <https://doi.org/10.1038/nrm2233>
 16. Campisi J (2013) Aging, cellular senescence, and cancer. *Annual Review of Physiology* 75:685–705. <https://doi.org/10.1146/annurev-physiol-030212-183653>
 17. Da Silva-álvarez S, Collado M (2016) Cellular Senescence. *Encyclopedia of Cell Biology* 3:511–517. <https://doi.org/10.1016/B978-0-12-394447-4.30066-9>
 18. Rodriguez-Brenes IA, Wodarz D, Komarova NL (2015) Quantifying replicative senescence as a tumor suppressor pathway and a target for cancer therapy. *Scientific Reports* 5:1–13. <https://doi.org/10.1038/srep17660>
 19. Kumari R, Jat P (2021) Mechanisms of Cellular Senescence: Cell Cycle Arrest and Senescence Associated Secretory Phenotype. *Frontiers in Cell and Developmental Biology* 9:1–24. <https://doi.org/10.3389/fcell.2021.645593>
 20. Debacq-chainiaux F, Ameer R Ben, Bauwens E, et al (2016) Cellular Ageing and Replicative Senescence. Springer International Publishing, Cham
 21. Yousefzadeh M, Henpita C, Vyas R, et al (2021) DNA damage—how and why we age?

eLife 10:1–17. <https://doi.org/10.7554/eLife.62852>

22. Olan I, Narita M (2022) Senescence: An Identity Crisis Originating from Deep Within the Nucleus. *Annual Review of Cell and Developmental Biology* 38:1–21. <https://doi.org/10.1146/annurev-cellbio-120420-013537>
23. Rapp YG, Ransbotyn V, Grafi G (2015) Senescence meets dedifferentiation. *Plants* 4:356–368. <https://doi.org/10.3390/plants4030356>
24. Fraguas S, Barberán S, Ibarra B, et al (2012) Regeneration of neuronal cell types in *Schmidtea mediterranea*: An immunohistochemical and expression study. *International Journal of Developmental Biology* 56:143–153. <https://doi.org/10.1387/ijdb.113428sf>
25. Sarnat HB, Netsky MG (1985) The Brain of the Planarian as the Ancestor of the Human Brain. *Canadian Journal of Neurological Sciences / Journal Canadien des Sciences Neurologiques* 12:296–302. <https://doi.org/10.1017/S031716710003537X>
26. Huang Z, Whelan C V., Dechmann D, Teeling EC (2020) Genetic variation between long-lived versus short-lived bats illuminates the molecular signatures of longevity. *Aging* 12:15962–15977. <https://doi.org/10.18632/aging.103725>
27. Liu J, Guan D, Dong M, et al (2020) UFMylation maintains tumour suppressor p53 stability by antagonizing its ubiquitination. *Nature Cell Biology* 22:1056–1063. <https://doi.org/10.1038/s41556-020-0559-z>
28. Hendzel MJ, Wei Y, Mancini MA, et al (1997) Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. *Chromosoma* 106:348–360. <https://doi.org/10.1007/s004120050256>
29. González-Estévez C, Felix DA, Aboobaker AA, Saló E (2007) Gtdap-1 promotes autophagy and is required for planarian remodeling during regeneration and starvation. *Proceedings of the National Academy of Sciences of the United States of America* 104:13373–13378. <https://doi.org/10.1073/pnas.0703588104>
30. Freund A, Laberge RM, Demaria M, Campisi J (2012) Lamin B1 loss is a senescence-associated biomarker. *Molecular Biology of the Cell* 23:2066–2075. <https://doi.org/10.1091/mbc.E11-10-0884>
31. Shimi T, Butin-Israeli V, Adam SA, et al (2011) The role of nuclear lamin B1 in cell proliferation and senescence. *Genes and Development* 25:2579–2593. <https://doi.org/10.1101/gad.179515.111>
32. Chandra T, Ewels PA, Schoenfelder S, et al (2015) Global reorganization of the nuclear landscape in senescent cells. *Cell Reports* 10:471–483. <https://doi.org/10.1016/j.celrep.2014.12.055>
33. Rodier F, Campisi J (2011) Four faces of cellular senescence. *Journal of Cell Biology* 192:547–556. <https://doi.org/10.1083/jcb.201009094>
34. Sharpless NE, Sherr CJ (2015) Forging a signature of in vivo senescence. *Nature Reviews Cancer* 15:397–408. <https://doi.org/10.1038/nrc3960>

35. Beauséjour CM, Krtolica A, Galimi F, et al (2003) Reversal of human cellular senescence: Roles of the p53 and p16 pathways. *EMBO Journal* 22:4212–4222. <https://doi.org/10.1093/emboj/cdg417>
36. Perrigue PM, Najbauer J, Jozwiak AA, et al (2015) Planarians as a model of aging to study the interaction between stem cells and senescent cells in vivo. *Pathobiology of Aging & Age-related Diseases* 5:30052. <https://doi.org/10.3402/pba.v5.30052>
37. Tan TCJ, Rahman R, Jaber-Hijazi F, et al (2012) Telomere maintenance and telomerase activity are differentially regulated in asexual and sexual worms. *Proceedings of the National Academy of Sciences of the United States of America* 109:4209–4214. <https://doi.org/10.1073/pnas.1118885109>
38. Marión RM, López de Silanes I, Mosteiro L, et al (2017) Common Telomere Changes during In Vivo Reprogramming and Early Stages of Tumorigenesis. *Stem Cell Reports* 8:460–475. <https://doi.org/10.1016/j.stemcr.2017.01.001>
39. Puri D, Wagner W (2023) Epigenetic rejuvenation by partial reprogramming. *BioEssays* 45:1–8. <https://doi.org/10.1002/bies.202200208>
40. Eisenhoffer GT, Kang H, Alvarado AS (2008) Molecular Analysis of Stem Cells and Their Descendants during Cell Turnover and Regeneration in the Planarian *Schmidtea mediterranea*. *Cell Stem Cell* 3:327–339. <https://doi.org/10.1016/j.stem.2008.07.002>
41. Reddien PW, Oviedo NJ, Jennings JR, et al (2005) Developmental biology: SMEDWI-2 is a PIWI-like protein that regulates planarian stem cells. *Science* 310:1327–1330. <https://doi.org/10.1126/science.1116110>
42. Martin-Belmonte F, Perez-Moreno M (2012) Epithelial cell polarity, stem cells and cancer. *Nature Reviews Cancer* 12:23–38. <https://doi.org/10.1038/nrc3169>
43. Nishimura K, Kitamura Y, Agata K (2008) [Molecular mechanism of brain regeneration and reconstruction of dopaminergic neural network in planarians]. *Brain and nerve = Shinkei kenkyu no shinpo* 60:307–17
44. Oviedo NJ, Newmark PA, Sánchez Alvarado A (2003) Allometric scaling and proportion regulation in the freshwater planarian *Schmidtea mediterranea*. *Developmental Dynamics* 226:326–333. <https://doi.org/10.1002/dvdy.10228>
45. Cote LE, Simental E, Reddien PW (2019) Muscle functions as a connective tissue and source of extracellular matrix in planarians. *Nature Communications* 10:1592. <https://doi.org/10.1038/s41467-019-09539-6>
46. Currie KW, Molinaro AM, Pearson BJ (2016) Neuronal sources of hedgehog modulate neurogenesis in the adult planarian brain. *eLife* 5:1–18. <https://doi.org/10.7554/eLife.19735>
47. Yasuoka Y, Taira M (2021) LIM homeodomain proteins and associated partners: Then and now. *Current Topics in Developmental Biology* 145:113–166. <https://doi.org/10.1016/BS.CTDB.2021.04.003>
48. Singh N, Singh D, Modi D (2022) LIM Homeodomain (LIM-HD) Genes and Their Co-Regulators in Developing Reproductive System and Disorders of Sex Development.

49. Currie KW, Pearson BJ (2013) Transcription factors *lhx1/5-1* and *pitx* are required for the maintenance and regeneration of serotonergic neurons in planarians. *Development* (Cambridge, England) 140:3577–88. <https://doi.org/10.1242/dev.098590>
50. Donato L, Alibrandi S, Scimone C, et al (2022) The impact of modifier genes on cone-rod dystrophy heterogeneity: An explorative familial pilot study and a hypothesis on neurotransmission impairment. *PLoS ONE* 17:1–26. <https://doi.org/10.1371/journal.pone.0278857>
51. Ou HL, Hoffmann R, González-López C, et al (2021) Cellular senescence in cancer: from mechanisms to detection. *Molecular Oncology* 15:2634–2671. <https://doi.org/10.1002/1878-0261.12807>
52. Wei W, Ji S (2018) Cellular senescence: Molecular mechanisms and pathogenicity. *Journal of Cellular Physiology* 233:9121–9135. <https://doi.org/10.1002/jcp.26956>
53. Ito T, Igaki T (2016) Dissecting cellular senescence and SASP in *Drosophila*. *Inflammation and Regeneration* 36:1–8. <https://doi.org/10.1186/s41232-016-0031-4>
54. Schmitt CA, Wang B, Demaria M (2022) Senescence and cancer — role and therapeutic opportunities. *Nature Reviews Clinical Oncology* 19:619–636. <https://doi.org/10.1038/s41571-022-00668-4>
55. Fernández-Taboada E, Moritz S, Zeuschner D, et al (2010) Smed-SmB, a member of the LSm protein superfamily, is essential for chromatoid body organization and planarian stem cell proliferation. *Development* 137:1583–1583. <https://doi.org/10.1242/dev.051847>
56. Cebria F, Cebrià F, Newmark PA (2005) Planarian homologs of netrin and netrin receptor are required for proper regeneration of the central nervous system and the maintenance of nervous system architecture. *Development* 132:3691–3703. <https://doi.org/10.1242/dev.01941>
57. El-Gebali S, Mistry J, Bateman A, et al (2019) The Pfam protein families database in 2019. *Nucleic Acids Research* 47:D427–D432. <https://doi.org/10.1093/nar/gky995>
58. Katoh K, Rozewicki J, Yamada KD (2018) MAFFT online service: Multiple sequence alignment, interactive sequence choice and visualization. *Briefings in Bioinformatics* 20:1160–1166. <https://doi.org/10.1093/bib/bbx108>
59. Trifinopoulos J, Nguyen LT, von Haeseler A, Minh BQ (2016) W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis. *Nucleic Acids Research* 44:W232–W235. <https://doi.org/10.1093/NAR/GKW256>
60. Huson DH, Scornavacca C (2012) Dendroscope 3: An interactive tool for rooted phylogenetic trees and networks. *Systematic Biology* 61:1061–1067. <https://doi.org/10.1093/sysbio/sys062>
61. Barrett T, Wilhite SE, Ledoux P, et al (2012) NCBI GEO: archive for functional genomics data sets—update. *Nucleic Acids Research* 41:D991–D995. <https://doi.org/10.1093/nar/gks1193>

62. Grohme MA, Schloissnig S, Rozanski A, et al (2018) The genome of *Schmidtea mediterranea* and the evolution of core cellular mechanisms. *Nature* 554:56–61. <https://doi.org/10.1038/nature25473>
63. Waterhouse AM, Procter JB, Martin DMA, et al (2009) Jalview Version 2-A multiple sequence alignment editor and analysis workbench. *Bioinformatics* 25:1189–1191. <https://doi.org/10.1093/bioinformatics/btp033>
64. de Castro E, Sigrist CJA, Gattiker A, et al (2006) ScanProsite: Detection of PROSITE signature matches and ProRule-associated functional and structural residues in proteins. *Nucleic Acids Research* 34:362–365. <https://doi.org/10.1093/nar/gkl124>
65. Plass M, Solana J, Wolf FA, et al (2018) Cell type atlas and lineage tree of a whole complex animal by single-cell transcriptomics. *Science* 360:1–17. <https://doi.org/10.1126/science.aaq1723>
66. Sánchez Alvarado A, Newmark PA (1999) Double-stranded RNA specifically disrupts gene expression during planarian regeneration. *Proceedings of the National Academy of Sciences of the United States of America* 96:5049–54. <https://doi.org/10.1073/PNAS.96.9.5049>
67. Ross KG, Omuro KC, Taylor MR, et al (2015) Novel monoclonal antibodies to study tissue regeneration in planarians. *BMC Developmental Biology* 15:2. <https://doi.org/10.1186/s12861-014-0050-9>
68. Chai G, Ma C, Bao K, et al (2010) Complete functional segregation of planarian β -catenin-1 and -2 in mediating Wnt signaling and cell adhesion. *Journal of Biological Chemistry* 285:24120–24130. <https://doi.org/10.1074/jbc.M110.113662>
69. Dimri GP, Lee X, Basile G, et al (1995) A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proceedings of the National Academy of Sciences of the United States of America* 92:9363–9367. <https://doi.org/10.1073/pnas.92.20.9363>
70. Debacq-Chainiaux F, Erusalimsky JD, Campisi J, Toussaint O (2009) Protocols to detect senescence-associated beta-galactosidase (SA- β gal) activity, a biomarker of senescent cells in culture and in vivo. *Nature Protocols* 4:1798–1806. <https://doi.org/10.1038/nprot.2009.191>
71. Peiris TH, Ramirez D, Barghouth PG, et al (2016) Regional signals in the planarian body guide stem cell fate in the presence of genomic instability. *Development (Cambridge)* 143:1697–1709. <https://doi.org/10.1242/dev.131318>
72. Iglesias M, Felix DA, Gutiérrez-Gutiérrez Ó, et al (2019) Downregulation of mTOR Signaling Increases Stem Cell Population Telomere Length during Starvation of Immortal Planarians. *Stem Cell Reports* 13:405–418. <https://doi.org/10.1016/j.stemcr.2019.06.005>
73. Lin AYT, Pearson BJ (2017) Yorkie is required to restrict the injury responses in planarians. *PLoS Genetics* 13:1–30. <https://doi.org/10.1371/journal.pgen.1006874>
74. Currie KW, Brown DDDR, Zhu S, et al (2016) HOX gene complement and expression in the planarian *Schmidtea mediterranea*. *EvoDevo* 7:2663–2670.

<https://doi.org/10.1186/s13227-016-0044-8>

75. King RS, Newmark PA (2013) In situ hybridization protocol for enhanced detection of gene expression in the planarian *Schmidtea mediterranea*. *BMC developmental biology* 13:8. <https://doi.org/10.1186/1471-213X-13-8>
76. Castillo-Lara S, Abril JF (2018) PlanNET: Homology-based predicted interactome for multiple planarian transcriptomes. *Bioinformatics* 34:1016–1023. <https://doi.org/10.1093/bioinformatics/btx738>
77. Grant CE, Bailey TL, Noble WS (2011) FIMO: Scanning for occurrences of a given motif. *Bioinformatics* 27:1017–1018. <https://doi.org/10.1093/bioinformatics/btr064>
78. Schindelin J, Arganda-Carreras I, Frise E, et al (2012) Fiji: An open-source platform for biological-image analysis. *Nature Methods* 9:676–682. <https://doi.org/10.1038/nmeth.2019>

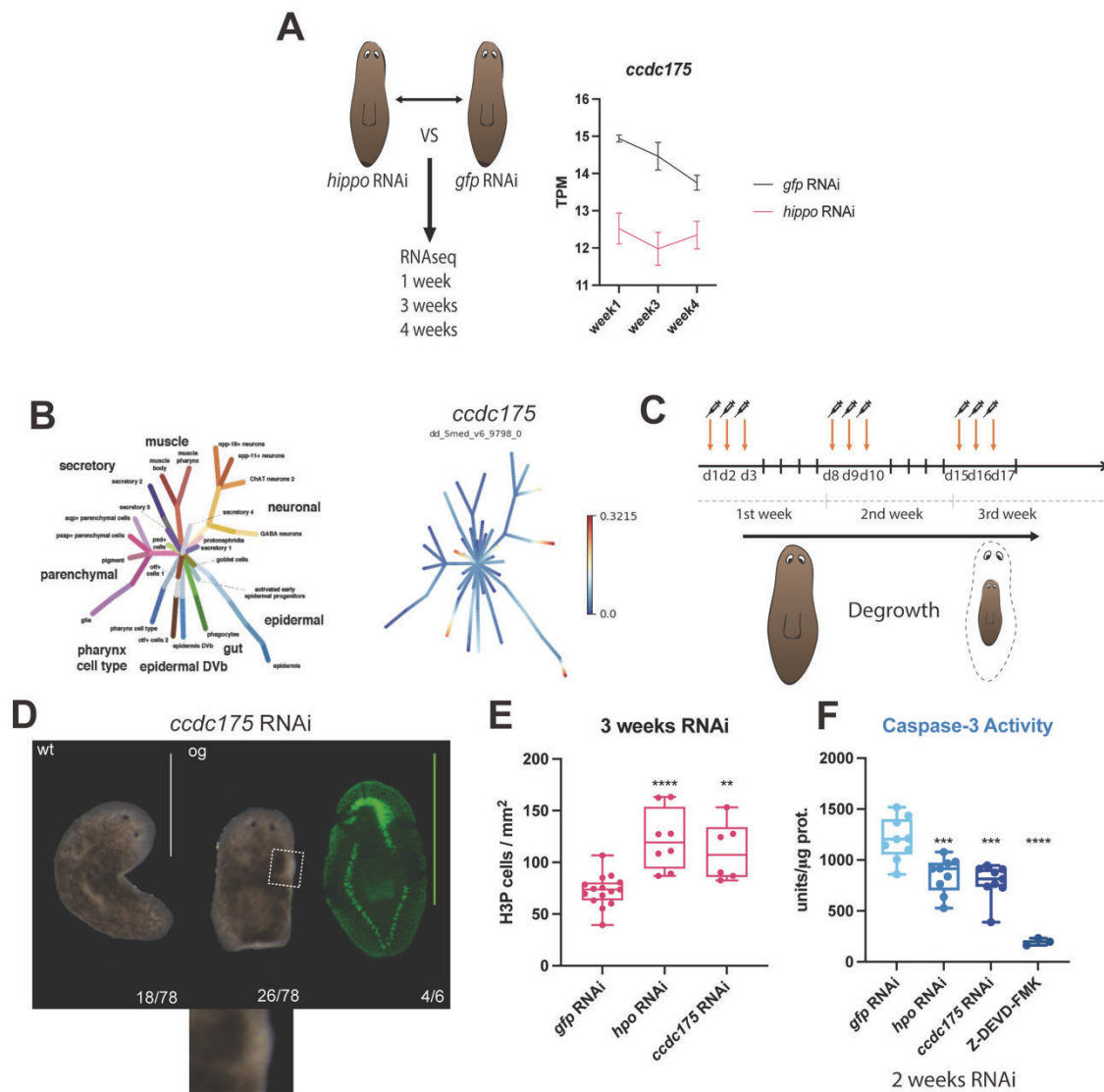


Figure 1: *ccdc175* is required for the progression of the cell cycle and apoptosis. (A) Cartoon depicting the experimental design of the *hippo* RNAi RNA-seq [3] and graph showing the transcripts per kilobase million (TPM) of *ccdc175* in the RNA-seq of *hippo* (RNAi) animals (pink) and controls (*gfp* RNAi, black). (B) Schematic representation of single-cell transcriptomic expression of *ccdc175* (according to [65]). (C) Schematic illustrating the RNAi experimental procedure of *ccdc175* inhibition. (D) Stereomicroscopic image of live *ccdc175* RNAi planarians depicting overgrowths (og) and wildtype (wt) phenotype (n=78) together with Anti-SYNAPSIN (3C11) immunostaining of *ccdc175* (RNAi) animals (n=6). The white discontinuous box and magnification highlight the overgrowth. (E) Quantification of mitotic cells (H3P+) normalized by area after rescue experiments of *gfp* (control) *hpo* and *ccdc175* RNAi animals. The experiments were performed in planarians subjected to 3 weeks of injections (n≥6). Data is represented as Box & whiskers min to max. Showing all points. **p < 0.01; ****p < 0.0001 and was analyzed with ANOVA test. (F) Quantification of caspase-3 enzymatic activity in *gfp*, *hippo* and *ccdc175* RNAi animals after 2 weeks of injections. Z-DEVD-FMK corresponds to the negative control. Data is represented as Box & whiskers min to max. Showing all points. ***p < 0.001; ****p < 0.0001 and was analyzed with ANOVA test.

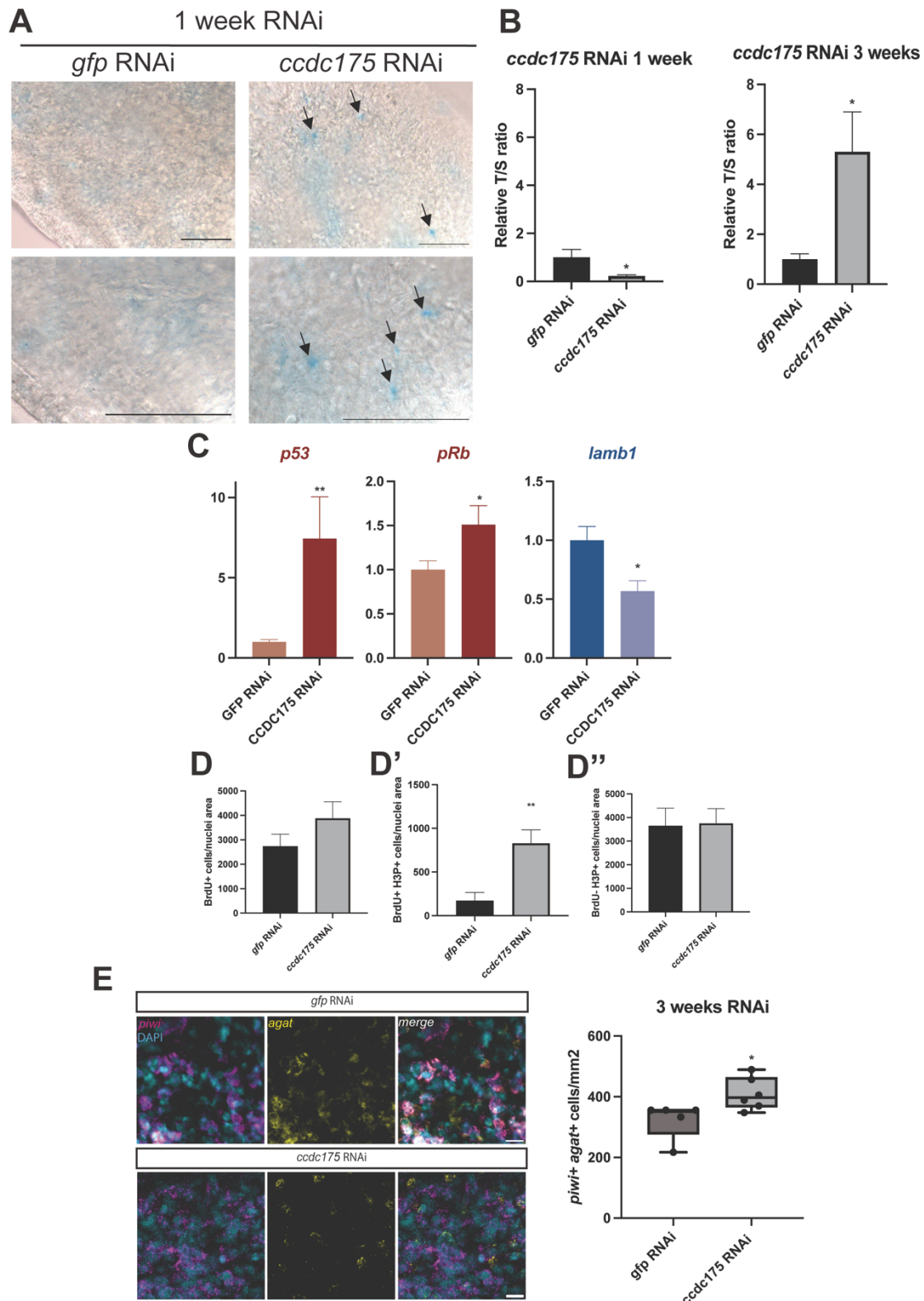


Figure 2: *ccdc175* links senescence and a switch in telomeric dynamics with impairment of the cell cycle and cellular dedifferentiation. (A) SA- β -gal activity staining in *gfp*, and *ccdc175* RNAi animals. Scale bar 50 μ m. All experiments were performed in planarians subjected to 1 week of RNAi. (B) Telomere quantitative PCR (qPCR) comparing control and *ccdc175* RNAi planarians respectively at 1 and 3 weeks of RNAi. qPCR was performed on genomic DNA for 3 biological replicates (5 planarians per replicate) per

condition. The qPCR determines the ratio of telomere (T) repeat copy number to a single-copy (S) gene (genomic DNA from dd_Smed_v6_2426_0_1) copy number (T/S ratio) compared with a reference DNA sample (*gfp* RNAi) (two-tailed Student's t-test, * $p < 0.05$). (C) qRT-PCR quantification of *Imnb1*, *p53* and *pRb* expression after 1 week of *ccdc175* RNAi compared with controls. In all the panels, relative expression is plotted as $2^{-\Delta\Delta CT}$ values. Data are were analyzed by Student t test and represented as mean $\pm$ s.e.m * $P < 0.05$; ** $P < 0.01$. (D) Quantification of BrdU+ cells in *ccdc175* (RNAi) animals and corresponding controls. (D') Quantification of double-positive BrdU+/H3P+ cells in *ccdc175* (RNAi) animals and corresponding controls. (D'') Quantification of BrdU-/H3P+ cells in *ccdc175* (RNAi) animals and corresponding controls. Experiments were performed after a 16-h BrdU pulse. All graphs correspond to animals subjected to RNAi for 3 weeks ($n \geq 9$). Error bars represent standard error. Data were analyzed by Student t test. ** $p < 0.05$. (E) Double FISH with the neoblast marker *smedwi-1* and late epidermal cell progenitors *agat* and corresponding quantification. Data is represented as mean $\pm$ s.e.m ** $p < 0.01$. Data was analyzed with Student t test ($n \geq 5$). Scale bars: 10 μ m.

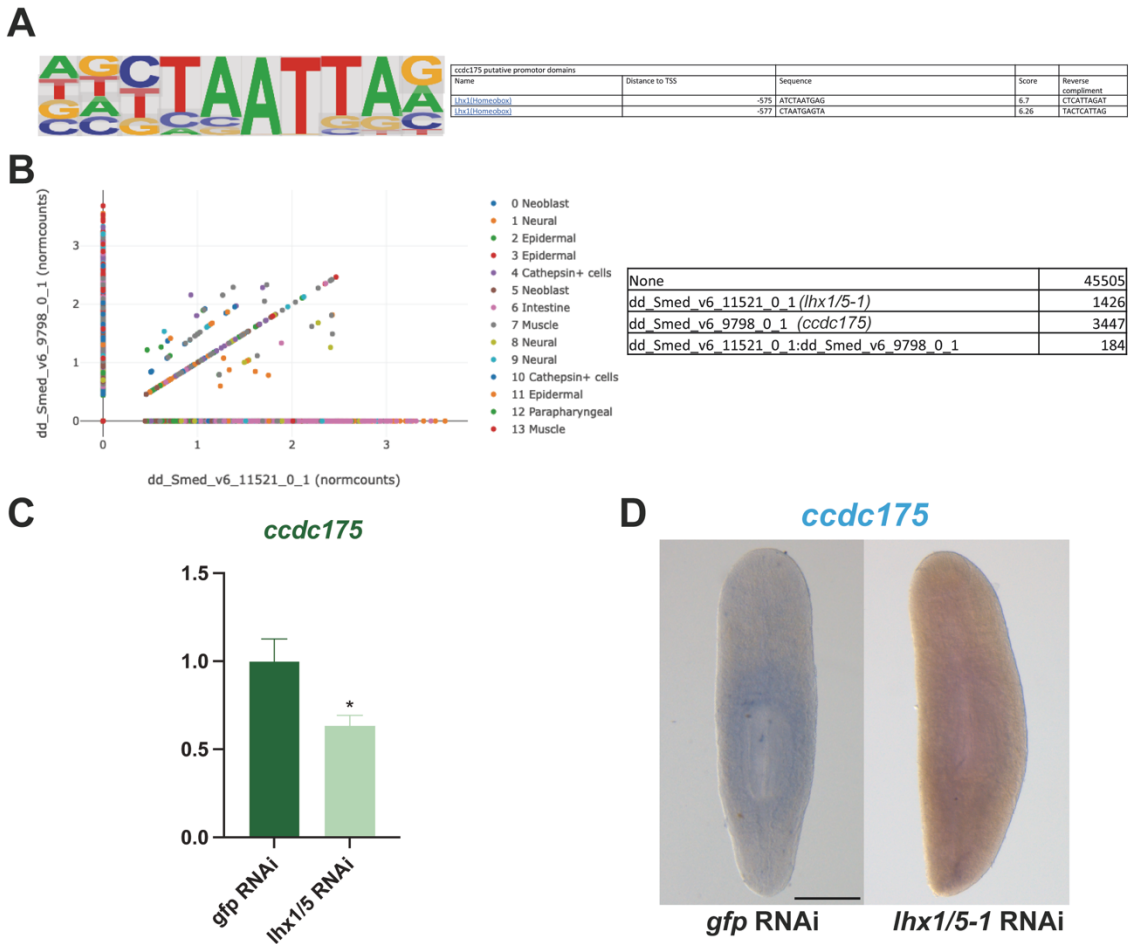


Figure 3: *lhx1/5-1* could bind to a cis-regulatory element found in *ccdc175* promoter region in planarians and across metazoans. (A) HOMER putative TF binding motifs of *lhx1* found in the promoter region of *ccdc175*. (B) Single cell coexpression representation and quantifications of *ccdc175* and *lhx1/5-1* across tissues in homeostatic conditions (according to [65]). (C) qRT-PCR quantification of *ccdc175* expression after 1 week of *lhx1/5-1* RNAi compared with controls. In all the panels, relative expression is plotted as $2^{-\Delta\Delta CT}$ values. Data were analyzed by Student t test and represented as mean $\pm$ s.e.m * $P < 0.05$. (D) Whole-mount *in situ* hybridization analysis of the expression of *ccdc175* at after 1 week of *lhx1/5-1* RNAi. Scale bar: 200 μ m.

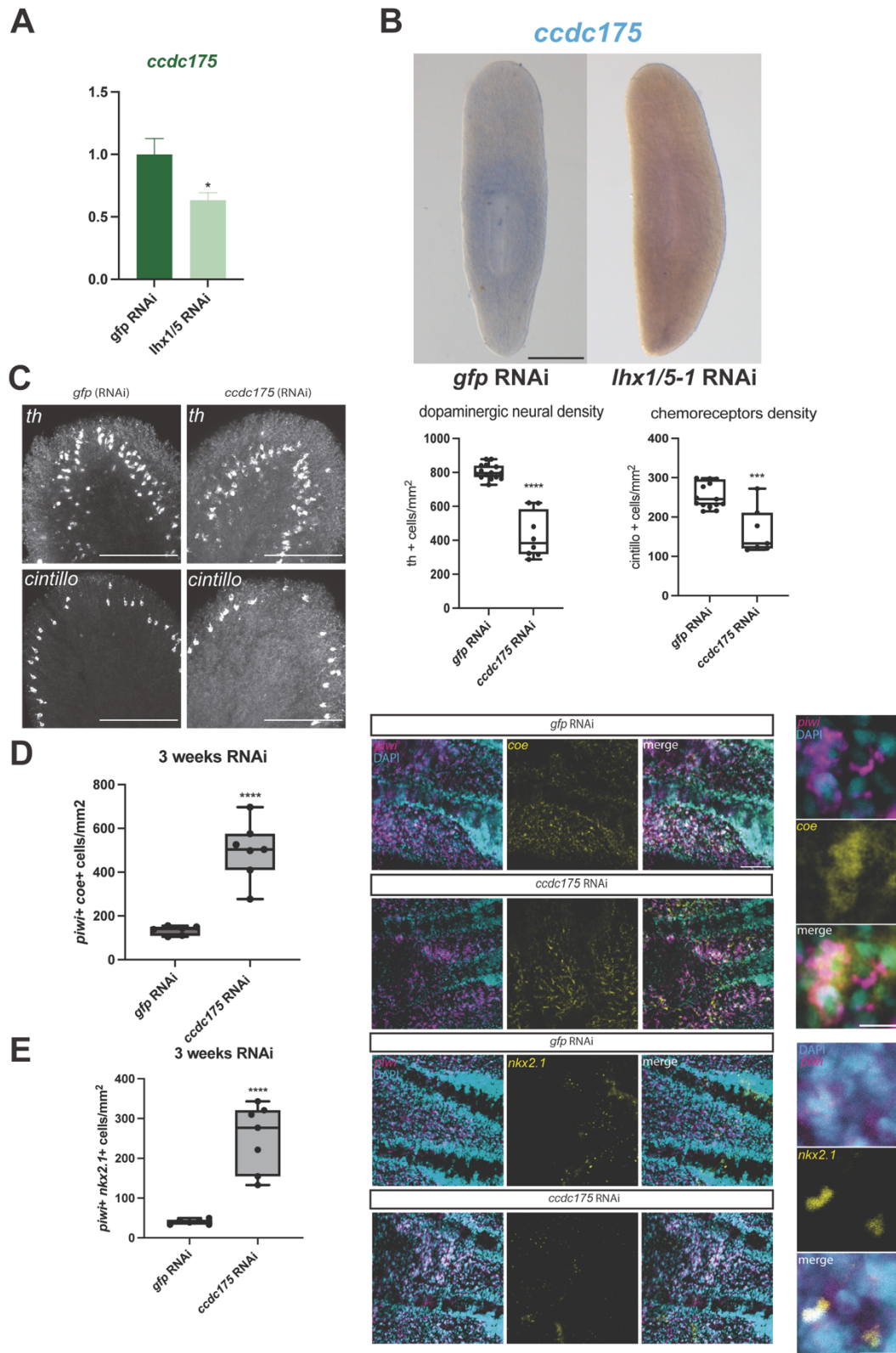


Figure 4: *ccdc175* plays a role in neural differentiation. (A) FISH for *th*⁺ and *cintillo*⁺ in control and *ccdc175* (RNAi) animals injected for 3 weeks. The corresponding quantification is shown (n≥7). The total number of cells was normalized with respect to the head area (from the anterior tip to the pharynx). Data were analyzed with Student t test (n≥5). ***p < 0.001; ****p < 0.0001. Scale bars: 150 μm. (B and C) Double FISH with markers of different neural cell progenitors: *smadwi-1/coe*, *smadwi-1/nkx2.1* and corresponding quantification. Scale bars:

100 and 10 μ m. Data were analyzed with Student t test ($n \geq 6$). $p < 0.0001$. Representative magnifications depicting both markers double colocalizations are depicted as well.

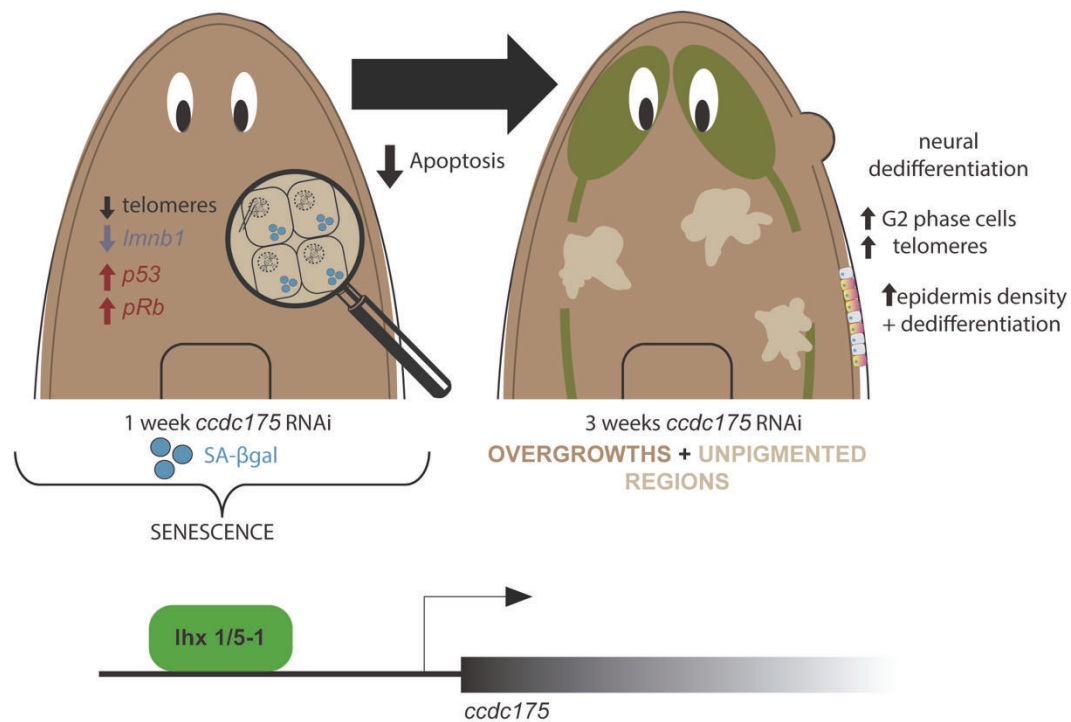


Figure 5: Model representing the role of *ccdc175* in planarians. In an early stage of *ccdc175* RNAi inhibition planarians accumulate a myriad of senescence markers such as an increase of the transcripts *p53* and *pRb* and a decrease of *Imnb1* and shortening of the telomeres coupled with a decrease of apoptotic activity and an accumulation of SA-βgal. This accumulation of senescence cells later leads to the emergence of the overgrowths and unpigmented regions together with impairment of a proper cell cycle progression after 3 weeks of *hippo* RNAi inhibition. Moreover the TF *lhx1/5-1* involved in neural differentiation could act as a putative promotor modulating the expression of *ccdc175*.

SUPPORTING INFORMATION

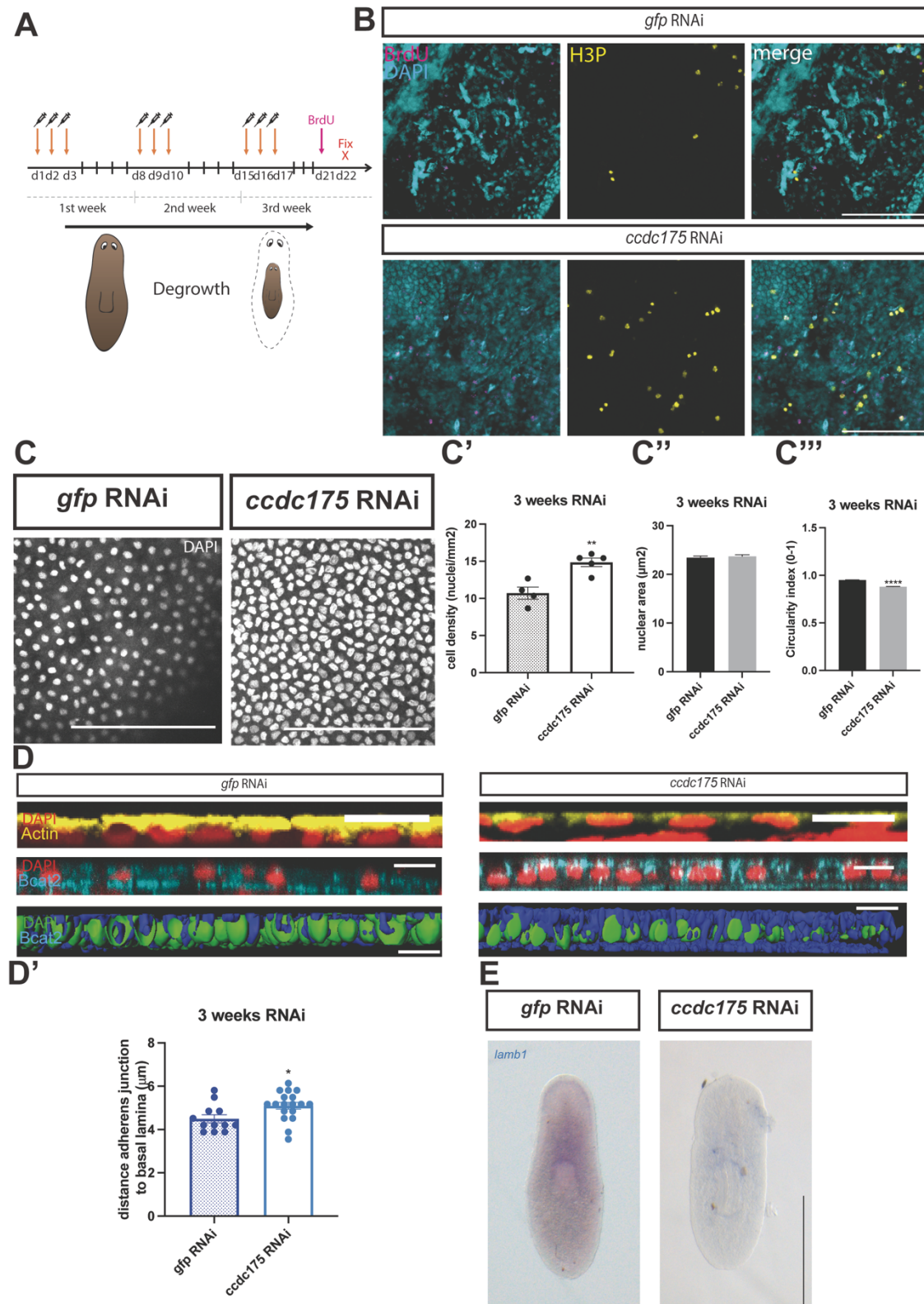


Figure S1: *ccdc175* is essential for G2-M, the maintenance of the epidermal cellular density and regulating senescence. (A) Cartoon illustrating the BrdU pulse procedure. Animals were starved for 1 week, injected with *hippo* dsRNA for 3 weeks, and then injected with BrdU and fixed 16 h later. (B) BrdU labeling combined with immunostaining with anti-H3P antibody in the pre-pharynx region in controls and in planarians subjected to *ccdc175* RNAi

for 3 weeks. Scale bar: 100 μ m. (C) DAPI staining of epithelial cells of the prepharyngeal region. Scale bar: 20 μ m (C') Quantification of the mean epidermal cell density. $n \geq 4$ (C'') Quantification of the mean nuclear cell area ($n \geq 6$) (C''') Quantification of the mean circularity index of the nuclei ($n \geq 6$). Data are represented as mean $\pm$ s.e.m (D) anti-Bcat2 and anti-Actin immunostaining and DAPI of *gfp*, and *ccdc175* RNAi planarians. The x-z confocal projections are shown together with the IMARIS reconstructions of the anti-Bcat2 and DAPI immunostainings depicting the position of the adherens junctions in the epidermis. Scale bars: 10 μ m. (D') Quantification of distance between the adherens junctions and the basal lamina ($n \geq 12$). (E) Whole-mount *in situ* hybridization analysis of the expression of *lmnb1* in *gfp* and *ccdc175* RNAi animals after 1 week of injections. Scale bars: 1mm.

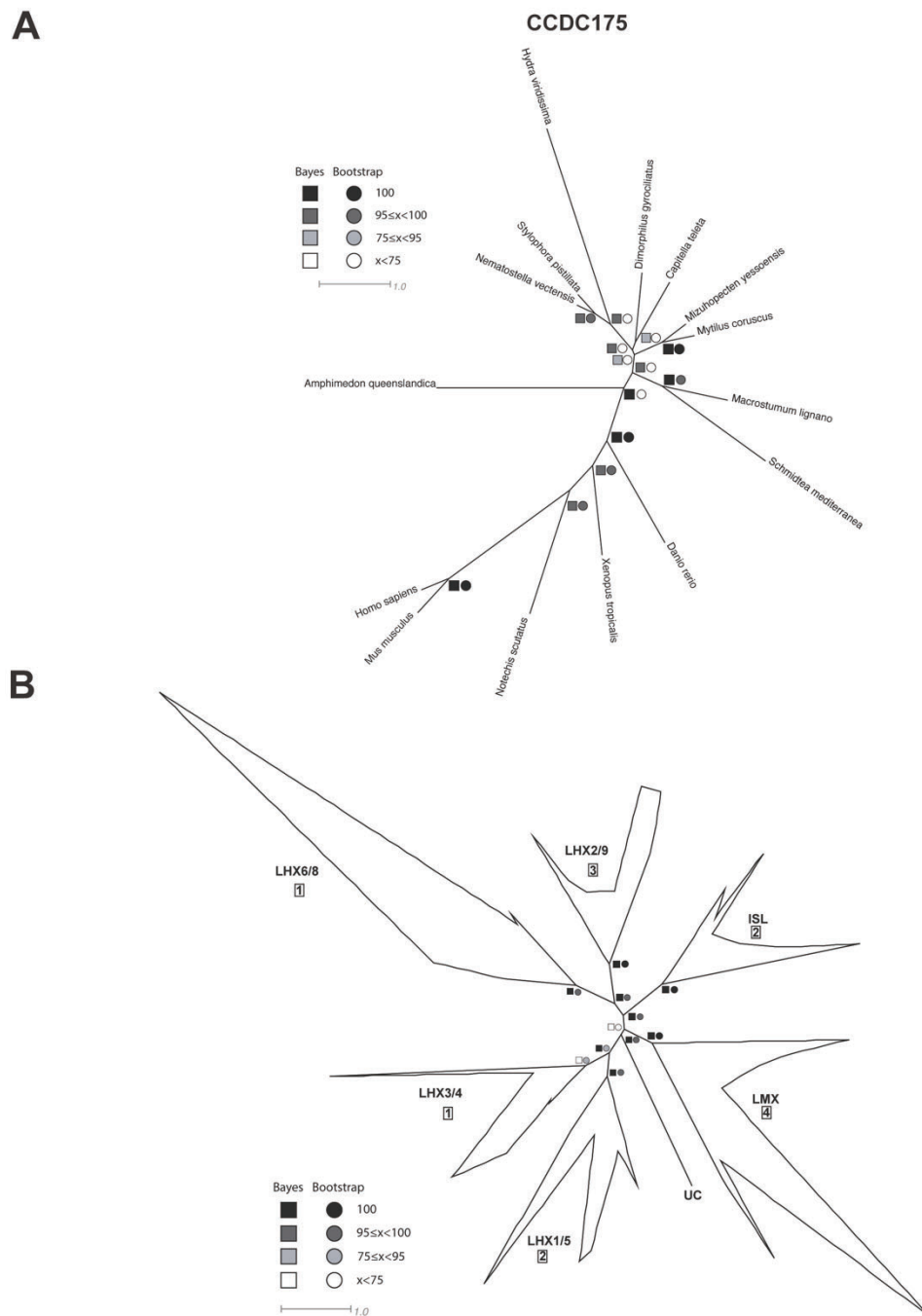


Figure S2: Phylogenetic trees of *ccdc175* and *lhx* family across metazoans. (A) Phylogenetic analysis of *ccdc175* using protein sequences. At nodes, values for the approximate Bayes (square) and Likelihood (circle) ratio test are shown. Scale indicates

[illegible]

Table 25. List of publications of 2022			
Publ. number	Publ. title	Publ. year	Publ. type
2022-001		2022	...
2022-002		2022	...
2022-003		2022	...
2022-004		2022	...
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2022-085		2022	...
2022-086		2022	...
2022-087		2022	...
2022-088		2022	...
2022-089		2022	...

Promoter region ccd175 gene							
	ACRs	Promoter activity	ACR and Promoter activity Sources	Presence	Ihx/Ixtd motifs n° Ihx1 (Hsap) n° Ihx1 (Mmus) Ihx3 (Hs)		
<i>Homo sapiens</i>	E	H3K27ac / H3K4me1 / H3K4me3	UCSC Genome Browser on Human Feb. 2009 (GRCh37/hg19) Assembly	N			
<i>Mus musculus</i>	E	H3K27ac / H3K4me1 / H3K4me1	UCSC Genome Browser on Mouse Dec. 2011 (GRChm38/mm10) Assembly	Y			
<i>Danio rerio</i>	E	H3K4me3 / H3K4me1	UCSC Genome Browser on Zebrafish Sep. 2014 (GRCz10/danRer10) Assembly/UCSC Genome Browser on Zebrafish Jul. 2010 (Zv9/danRefR7) Assembly	Y		1	1
<i>Xenopus tropicalis</i>	-	H3K4me3 / H3K4me1	UCSC Genome Browser on Xenbase - <i>Xenopus tropicalis</i> 8.0 Assembly (xtr8_0) / UCSC Genome Browser on Xenbase - <i>Xenopus tropicalis</i> 8.0 Assembly (xtr8_0)	Y			
<i>Schmidola mediterranea</i>	E	H3K27ac	Plannet	Y	1		
<i>Hydra vulgaris</i>	E	H3K27ac / H3K4me2 / H3K4me3	https://research.rhgh.nl/hydra/browse/browse.cgi	Y			2
<i>Acanthamoeba castellanii</i>	-	H3K4me3 / H3K4me1	http://www.h3k4me3.org/	Y			

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MFN2 IS NECESSARY FOR STEM CELL PROLIFERATION DURING GROWTH BUT DISPENSABLE DURING STARVATION

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Mfn2 is a gene required for mitochondrial fusion. Its role has been linked to the cellular energetic state, the progression of the cell cycle and also with cellular differentiation. Moreover, it has recently been suggested that *mfn2* could be operating upstream of the mTOR pathway. Here we use planarians as model system to investigate the role of *mfn2* in stem cell function. Planarians can regenerate any body part and are constantly resizing their bodies according to food availability. This active cellular turnover relies in a population of somatic stem cells capable of producing each of the cellular lineages of the animal. Recently we have showed that stem cell cycle progression has specific signaling requirements when the planarians are in starvation.

We have studied the function of *mfn2* in planarians at different metabolic contexts: feeding and starvation. In the feeding context, our results indicate that *mfn2* is necessary for the stem cell proliferation peak linked to growth after feeding through adjusting the ATP levels, since its RNAi inhibition causes planarian degrowth and a decrease in ATP. In the starvation context, *mfn2* is dispensable to maintain the basal proliferation rate of somatic stem cells but remains necessary to maintain cell identities. Both contexts alter the progression of the cell cycle but each does so by affecting different phases. Taken together, it suggests that the proliferation of somatic stem cells could have different energy requirements in different metabolic situations. We think that the study of *mfn2* function in planarian stem cells could provide new insights into the metabolic regulation of stem cells.

INTRODUCTION

Mitochondria, often described as the powerhouse of the cell, are placed at the center of cellular metabolism. Their symbiotic origin [1] coupled with their plasticity and network capabilities are key for the production of ATP (the main cellular energy token) [2]. They also play a major role in regulating cell death through modulating the extrinsic and intrinsic apoptotic pathways either through an amplification loop or through the liberation of cytochrome c respectively [3].

Changes in mitochondria morphology through fusion and fission mechanisms are key to modulate such endeavors. These processes are carried by dynamin GTPases highly conserved across species [4]. The fusion process is carried out first by the union of the outer mitochondrial membrane by Mitofusin 1 and 2 in humans (MFN1 and MFN2) [5], or the ortholog Fuzzy Onions in yeast (*fzo1p*) [6] and fruit flies (*fzo*) [7], followed by the formation of a complex with OPA1 which is responsible of the inner membrane fusion [8, 9].

This mitochondrial fusion dynamic has been linked with the progression of the cell cycle. Particularly, in the G1/S transition mitochondria enhance their fusion process in order to form hyperpolarized tubes prior to mitosis. This is due to reach the energy requirements and other ATP intermediates required for the DNA replication [10].

Mitochondrial dynamics and the cellular metabolic state have been associated with cellular differentiation. For instance, cells exiting quiescence and starting to proliferate appear to have less mitochondria fusion and a more glycolytic dependent metabolism. The same change occurs in iPSCs during the reprogramming [11,12]. Moreover, in pathological contexts such as cancer the phenomenon known as the Warburg effect leads to a similar outcome with trade off from OXPHOS metabolism to glycolysis [13].

Finally, a molecular pathway regulation downstream of *mfn2* has been recently suggested [14]. Such link allows to undergo one step further in the description of the mechanisms responsible to coordinate cell cycle, cellular differentiation and mitochondrial dynamics. Such pathway is the Akt/mTOR signaling network, a key pathway responsible for the regulation of the body size upon nutritional intake in response to amino acid levels.

Planarians are constantly readjusting their body size according to nutrient availability. This makes them an ideal model organism to assess the function of mitofusins in vivo. Planarian plasticity is accomplished by a population of stem cells (neoblasts) capable of producing each of the constitutive cellular lineages of the animal. The balance of cell proliferation and apoptosis is tightly regulated by robust signaling mechanisms (15-18). Also, it has been proven that planarians follow the Kleiber's law of metabolic rate scaling that states that the metabolic rate is proportional by a factor of $\frac{3}{4}$ to the body mass [15, 16]. Therefore, a disruption in size could be caused by an imbalance on the energy levels in these animals. Moreover, in regards to planarian growth and size regulation, the insulin/mTOR pathway has emerged as the predominant signaling modulator. Inhibition of insulin-like peptides or TOR diminishes cell proliferation, prevents planarian growth after feeding and accelerates shrinking during starvation [17, 18]. On the other hand, overactivation of mTOR using *pten* or *smg-1* RNA interference (RNAi) promotes an increase of proliferation and overgrowths formation [19–21].

Here we study the function of *smed-mfn2* (*mfn2* from now on) in planarians at different metabolic contexts: feeding and starvation. In the feeding context, our results indicate that *mfn2* is necessary for the stem cell proliferation peak linked to growth through adjusting the ATP levels since its RNAi inhibition causes planarian degrowth and a decrease in ATP. In the starvation context, we see that *mfn2* is dispensable for the maintenance of somatic stem cells basal proliferation rate but remains necessary for keeping cell identities. Taken together, these results suggest that *mfn2* could be a modulator of cell cycle progression affecting the growth of planarians and that the proliferation of somatic stem cells could have different energy requirements in different metabolic contexts.

RESULTS

***mfn2* RNAi leads to accelerated degrowth under starving conditions**

Mitofusin2 (*Smed-mfn2*; from now on *mfn2*) presents a ubiquitous pattern of expression across the whole body of the planarian (dd_Smed_v6_2168_0_1, Figure S1B). This pattern moderately decreases in intensity post-irradiation, a treatment that induces stem cell apoptosis [22]. This experiment indicates the presence of the transcript in the neoblasts (Figure S1A). Data from single cell RNA-seq experiments revealed that *mfn2* presents high levels of expression in goblet cells and mild expression in glia, some secretory and epidermis lineages and neoblasts (Figure S1B). To study the role of *mfn2* in planarians, we injected 1 week starved planarians with *mfn2* dsRNA for 3 consecutive days for a period of one or 2 weeks in order to see an associated phenotype. A presence of unpigmented regions was observed in some animals after three weeks of inhibition at 500 ng/ul of concentration. These are areas of the planarian epidermis that present a loss of pigmentation and have been related with processes of cellular dedifferentiation [23]. The same phenotype can be replicated with more penetrance after silencing *mfn2* injecting 1000 ng/ul of dsRNA for 2 weeks (Figure 1B). Moreover, downregulation of *mfn2* also results in an accelerated degrowth under starving conditions, which appears to be aggravated at higher doses of RNAi (1000 ng/ul) in comparison to lower ones (500 ng/ul) and to controls (*gfp* RNAi) (Figure 1E). The dose-dependent inhibition of *mfn2* with respect to the concentration of dsRNA injected was tested by qPCR (Figure S1C).

To investigate the number of mitoses following *mfn2* dsRNA injections, we used an antibody that recognizes Histone H3 phosphorylated at serine 10 (anti-H3P). This mark is present from the onset of mitosis to telophase [24]. We quantified the number of mitotic cells 3 days post injection after the second week of inhibition for the 500 ng/ul condition and 3 days after the third week for the 1000 ng/ul condition. We then compared the number of H3P+ cells with the respective *gfp* RNAi controls and found no increase in the mitotic rate after in either condition (Figure 1C).

Although we did not see differences in the mitotic figures, we decided to test the implication of *mfn2* in the regulation of the cell cycle, as this has been already described in in Hela cells [14]. In order to see possible differences across the progression of the cell cycle in *mfn2* RNAi conditions compared to *gfp* RNAi controls we injected planarians with 5-bromoethynyl-20-deoxyuridine (BrdU) and analyzed them by immunofluorescence with anti-H3P, 16 h after injection. The staining revealed an accumulation of H3P+BrdU+ and no increase of H3P+BrdU- cells in the *mfn2* RNAi animals compared to controls (Figure 1D and S2D). This result indicates cells cannot enter M phase from G2, however the ones in M phase before the BrdU pulse can progress through the different phases of mitosis.

All in all, we show that *mfn2* RNAi inhibition in starved planarians leads to the formation of unpigmented areas. This is coupled with an accelerated degrowth. Although we do not observe changes in the number of proliferating cells we still demonstrate defects in the progression of the cell cycle specifically associated with the progression from G2 to M phase.

***mfn2* is important for cellular differentiation in planarians under starving conditions**

In order to further characterize the potential defects in cellular differentiation associated with the unpigmented phenotype we decided to check by in situ hybridization the coexpression of a late progeny marker from an epidermal lineage (*smed-agat*; *agat* from now on) [25]) together with a stem cell one (*smedwi-1*; *piwi* from now on) [26]. More specifically, *agat* is expressed in differentiating adult stem cell descendants and is necessary for tissue maintenance and formation of new tissue during regeneration and plays a role in the interconversion of ATP and ADP to maintain adequate energy levels [27]. We found an aberrant increase of *agat*+/*piwi*+ cells upon 2 weeks of *mfn2* inhibition at 1000 ng/ul. The colocalizations quantified were

localized in the anterior portion of the animal, between the pharynx and the eyes (Figure 2A). These results indicate defects in cell differentiation in these animals.

Moreover, these results also correlate with a significant increase of necrotic rates upon 2 weeks of *mfn2* downregulation (Figure S2A). Necrosis was labelled by injecting a mixture of PI and Hoechst 33342 in the planarian in vivo. In this context PI is only able to label the nuclei with pores, corresponding to necrotic cells. This result juxtaposes a decrease in apoptotic rates in the same period (Figure S2B). Apoptosis activity is measured through a Fluorometric analysis of caspase-3 activity of planarian cell lysates by a luminescence spectrophotometer. These phenomena occur while the ATP levels in whole planarians display no significant changes compared to controls indicating no changes in catabolic activity (Figure S2C). ATP levels were measured by using an ATP Bioluminescence Assay kit on planarian cell lysates measuring the levels of Luciferase activity [28].

To sum up we describe that *mfn2* RNAi in starving conditions leads to defects in the epidermal differentiation of these animals. These results are coupled with an increase of necrotic rates and a decrease of apoptotic activity in a context in which ATP levels are low but present no significant changes compared with *gfp* RNAi control animals. The low ATP background could set the stage for the increase of necrosis and lack of apoptosis that contribute to the defects of epidermal differentiation. However more information of the metabolic status of these cells should be recollected to reinforce this connection.

Silencing of *mfn2* in fed planarians inhibits their growth, associated to a decrease in energy levels.

To analyze the role of *mfn2* in planarians during feeding periods, when there are growing, we injected dsRNA in planarians during 3 non-consecutive days, alternated with 2 days of feeding, for a period of 3 weeks. Our data shows that *mfn2* silencing results in planarian growth impairment under feeding conditions in comparison to the exponential growth visualized in control animals (Figure 3A). We checked the levels of proliferation by measuring the number of H3P+ cells at 1, 2 and 3 weeks of *mfn2* RNAi inhibition compared to the *gfp* RNAi controls in feeding conditions. The results indicated that the lack of growth was coupled with a decrease in the number of H3P+ cells under RNAi feeding conditions in comparison to the *gfp* RNAi fed controls (Figure 3C).

In order to assess the energy balance in these animals we determined general ATP levels in whole planarians upon 2 and 3 weeks of *mfn2* inhibition under feeding conditions (Figure 3D). The results showed decreased levels of ATP compared to controls at 3 weeks, indicating that *mfn2* is required for the maintenance of ATP levels associated with proliferation in fed planarians.

These results kept pointing towards a role of mTOR in regulating planarians growth. Therefore, we checked whether injections of Rapamycin, an inhibitor of mTOR, in planarians could affect the levels of proliferation. The results showed a decrease in proliferation, as had been previously reported [21] mimicking the previous results of *mfn2* under feeding conditions (Figure 3E). These results reinforced the idea that the lack of proliferation seen in *mfn2* RNAi fed planarians resembled the lack of proliferation associated with an inhibition of the mTOR pathway caused by Rapamycin.

To test possible differences across the progression of the cell cycle in *mfn2* RNAi conditions compared to *gfp* RNAi controls we injected planarians with 5-bromoethynyl- 20-deoxyuridine (BrdU) and performed an immunostaining for anti-H3P16 h post-injection. We detected a decrease of BrdU+ cells in *mfn2* RNAi conditions compared to controls indicating that a smaller number of cells are entering the cell cycle at the moment of the BrdU administration in *mfn2* RNAi animals. The double staining with H3P revealed no changes in H3P+BrdU+ cells. However it indicated a decrease of H3P+BrdU- cells in the *mfn2* RNAi animals compared to

controls (Figure 3F and S3D). This suggests that although stem cells can progress from G2 to M, they have trouble going through the different phases of mitosis.

Furthermore, *mfn2* fed RNAi animals do not show the 3h post-feeding mitotic peak previously described as a mitotic response to feeding [29] (Figure S3A) despite showing an increase in ATP levels (Figure S3B). Moreover, when we check the apoptotic levels by Caspase-3 enzymatic assay we see a persistent decrease of apoptosis in *mfn2* RNAi fed planarian in contrast to the peak at 2 weeks that we observe in the *gfp* RNAi fed animals (Figure S3C).

Together, our results suggest that the decrease in ATP production in fed planarians is translated to a lack of stem cell progression through the M phase of the cell cycle. This decrease in energy levels is also reflected in different apoptotic dynamics between the *mfn2* RNAi fed animals and the *gfp* RNAi fed controls.

***mfn2* RNAi downregulates the transcript levels of components of the mTOR signaling pathway and enriches the population of stem cells with long telomeres**

In order to see if the alterations in proliferation and the defects of the cell cycle were caused by alterations of the mTOR pathway we decided to check by qPCR significant differences in the transcriptomic levels of several components of the pathway. The idea stem from a previous work in planarians in which a novel negative regulator of the mTOR pathway was characterized by the same approach [30]. qPCR analysis revealed that *mfn2* downregulation in feeding conditions significantly downregulates *mtor* and induces overexpression of the negatively regulated targets *4ebp* and *Rad51*. We also check the transcriptomic levels of *akt* an upstream regulator of mTOR to assess if the transcriptomic downregulation of the pathway was also occurring more upstream. *akt* levels remain unchanged between control and *mfn2* RNAi feeding conditions (Figure 4A). This suggest that the transcriptional alterations derived from *mfn2* RNAi inhibition are occurring at the *mtor* and downstream levels of the pathway independently of *akt* transcriptional regulation.

In planarians it has been described that telomere elongation occurs in the stem cell populations under starving conditions. Moreover it is proved that such enrichment of stem cells with longer telomeres is dependent on the mTOR pathway [31]. To check whether telomere elongation due to deregulation of the mTOR pathway, characteristic of an starved context, could be detected in our *mfn2* fed RNAi animals, we checked the telomere length of *mfn2* RNAi fed animals by telomeric qPCR (TelqPCR). Our results indicate an elongation of the telomeres in 3 weeks *mfn2* RNAi fed planarians compared to *gfp* RNAi fed animals (Figure 4B).

Overall, these results revealed that under feeding conditions *mfn2* may be downregulating the Akt/mTOR pathway at the level of mTOR, affecting also its effectors. Moreover, this downregulation of the mTOR pathway leads to an elongation of the telomeres, mimicking a starving situation despite the animals being fed.

DISCUSSION

One of the main points in this project is that despite the fact that cells respond differently according to the nutritional conditions of the animals our results indicate that *mfn2* modulate the progression of the cell cycle in both feeding and starving contexts. This is transduced in a decrease in proliferation upon *mfn2* RNAi under feeding conditions. Moreover, *mfn2* inhibition during starvation also results in a significant decrease of apoptosis without changes in mitosis. In contrast, in feeding animals both apoptosis and proliferation decrease resulting in a growth impairment in contrast to controls, that grow exponentially. Also, *mfn2* RNAi under feeding conditions lowers ATP levels which overall suggest that these animals do not generate sufficient energy despite the input of nutrients. This lack of energy could explain the difficulties in the progression of the cell cycle these animals are experimenting. In yeast for example, the lack of nutrition leads to a cell cycle arrest at G1 until conditions improve and the cell-cycle is postponed [32]. This effect has been connected with the energy requirements and other ATP

intermediates necessary for DNA duplication. Moreover it has also been reported that in pea root tip meristems, cells in stationary phase after a period of carbohydrate starvation show a dependence in aerobic metabolism to properly progress towards the G2/M transition [33]. Nonetheless, at 3 hours post-feeding a peak of ATP is still seen under *mfn2* RNAi feeding conditions suggesting that lack of ATP hydrolysis could be occurring in the immediate hours after the food intake.

Both evidences are linked to the lack of proliferation in these conditions. A situation that has previously been observed in another context in planarians in which starving conditions CCT chaperonins regulate the ATP levels necessary for regeneration through the unfolded protein response of the endoplasmic reticulum [28]. Mitotic activity therefore is linked with ATP levels because mitosis is seen as a highly energy-demanding process [34]. Moreover, the disruption between energy levels and growth further emphasizes the physiological basis of Kleiber's law in planarians that state that specific increase in the mass/cell is due to size-dependent energy storage under feeding conditions. Also, the lack of ATP changes that we detect in starving conditions further correlates with the size-independence of the metabolic rate with size during starvation in planarians [16].

The differences in phenotypes in both contexts despite downregulating the same gene could be dependent on the different metabolism status the cells are experimenting. What we mean by that is that since the ATP levels are drastically different between both context their metabolic source might be as well and lead to further ramifications affecting differently the apoptosis activation as well as the proliferation levels.

Closer analysis of the cell cycle reinforce these evidences. Our BrdU labelling co-stained with H3P revealed that in starving conditions *mfn2* RNAi post 16h incubation presented a significant increase of H3P+BrdU+ cells without a significant increase of H3P+BrdU- cells nor in the total BrdU+ cells compared to controls indicating defects in the progression of the G2 phase. Striking, in feeding conditions *mfn2* RNAi animals presented less BrdU+ cells compared to controls coupled with a decrease of H3P+BrdU- cells and no difference in H3P+BrdU+ cells indicating an accumulation of cells in M phase.

The lack of progression in the cell cycle in spite of the feeding conditions was also coupled with a significant elongation of the telomeres such as the one happening upon caloric restriction conditions [31, 35]. This is due to the fact that in planarians it has been described that telomere elongation occurs in the stem cell populations under starving conditions. Moreover it is proved that such enrichment of stem cells with longer telomeres is dependent on the mTOR pathway. [31] This was further reinforced by the qPCR evidences showcasing that *mfn2* may be downregulating the Akt/mTOR pathway at the level of *mTOR* and further deregulating its effectors. At the end, this downregulation of the pathway coupled with an elongation of the telomeres in the same context indicates that the animals are mimicking a starving situation despite being fed. This agrees with the fact that mTOR has been regarded as a universal mechanism that is extracellularly activated by nutrient intake and activates the signals required for growth[36]. In planarians TORC-1 is downregulated under starvation. Furthermore, RNAi-mediated silencing of TOR has been described to decrease proliferation and increases cell death, impairing growth [37, 38]. On the other hand, *mTOR* is upregulated in response to feeding conditions and promotes an increase of proliferation [20, 21, 39]. In our context, these evidences reinforce the hypothesis of metabolic alterations being linked with the lack of proliferation observed upon *mfn2* RNAi. This is because in the context of feeding mTOR pathway should be activated triggering proliferation but in the context of *mfn2* RNAi inhibition we see a global downregulation of the pathway at the transcriptomic level transduced in a lack of proliferation and elongation of the telomeres. This context therefore resembles more a starving situation in which growth is not possible in spite of the nutritional intake.

It is plausible to indicate that the lack of proliferation after *mfn2* silencing in starved conditions may be due to an impaired entry into G2 phase due to energy restrictions as it is known that

mitochondrial dynamics regulated by several metabolic pathways coordinate cell division [40, 41]. Furthermore, mTOR has been stated to control cell cycle [42] and in our conditions Rapamycin injections (inhibitor of mTOR) are able to reproduce the proliferation impairment found in *mfn2* fed RNAi conditions similarly to previous accounts [21].

Another readout that could be associated with the fluctuations in metabolism is the suggested necrosis increase after *mfn2* RNAi under starving conditions that occur without changes on ATP levels. Previous data from mice revealed that *mfn2* knockdown caused alterations in mitochondrial metabolism characterized by reduced mitochondrial membrane potential and cellular oxygen consumption as well as depressed substrate oxidation. Its loss-of-function reduced the activity of the Krebs cycle and the electron transport chain which appeared compensated by a higher rate of glucose uptake and aerobic glycolysis and a lower rate of glycogen synthesis in order to compensate the ATP levels [43]. Therefore, we suggest that these restrictions in the production of ATP could be held responsible for the increase of necrotic cells in the starvation context of *mfn2* RNAi downregulation.

Interestingly, we have evidences of a similar context in planarians in which another family of genes modulate size regulation through regulation of the mTOR pathway. This was a *de novo* gene family of genes taxonomically restricted to the planarian clade named *blitzschnell* (*bls*). These genes were modulated according to food intake and were acting as inhibitors upstream of the mTOR pathway. As a consequence they were modulating the growth response upon feeding conditions in an opposite manner to *mfn2* thus leading to more proliferation and growth upon inhibition under feeding conditions. Intriguingly one of the defined aspects that changed between feeding and starving conditions observed was the size of the cells. This is because in starving conditions animals were shrinking due to a decrease in the cell volume despite presenting more accumulation of cells.[30] Therefore it might be worth exploring this variable in the *mfn2* RNAi context to see if similar decrease of cell size occur in our starving context.

Overall, the role of *mfn2* in this energy regulation leads to the hypothesis of the lack of ATP modulation as the factor for the planarian shrinkage and change in cell death dynamics. Following this train of thought, we found that the appearance of unpigmented regions in starved *mfn2* RNAi animals is derived from a loss of proper differentiated cellular status. This matches with the energetic restrictions detected in starvation coupled with a decrease of apoptotic activity. We think that this could be linked to a process of loss of cellular differentiation due to the fact that the conjunction of a decrease of apoptosis coupled with low levels of ATP is similar to the restrictions found imposed by the Warburg effect in cancer [13]. Another suggestion worth mentioning is that that these cells that are having troubles progressing the cell cycle could not die by apoptosis and enter cellular senescence.

Further experiments focused in the behavior of the mitochondrial chain under the previous conditions would reinforce the hypothesis of the metabolic implications of *mfn2* RNAi in the observed phenotypes [44]. Optimization on planarians of high-resolution imaging techniques such as electric microscopy to analyze mitochondria networks would also reinforce the hypothesis in their role on mitochondria dynamics.

Knowing that growth control in animals such as planarians that constantly undergo body size regulation is given according to nutrient availability, we present *mfn2* as an example of mitochondrial dynamics modulator that fulfil the requirement for such continuous regulation. This is mainly due to the fact that RNAi silencing of *mfn2* in feeding conditions revealed alterations in the planarian growth coupled with a decrease in ATP production. Furthermore, we were able to prove that *mfn2* transcriptomically regulates *mTOR* and its effectors, which in turn regulate size in response to food intake in planarians and in vertebrates. We also suggest that such metabolic changes associated with growth control also have consequences in the maintenance of cellular differentiation as these elements also appear deregulated in most cancers (Figure 5). Further unveiling such mechanism therefore would bring further insights in the design of novel cancer therapeutic strategies.

METHODS

Planarian culture

The planarians used in this study are the asexual clonal strain of *S. mediterranea* BCN-10 biotype and were maintained as previously described[45] in planarian artificial medium (PAM) water[46]. Animals were fed twice per week with liver, and those used in starvation experiments were starved for 1 week.

Sequence analyses

mfn2 was identified from the genome [47] and RNAi fragments were amplified using specific following primers:

mfn2 RNAi

F: **GAAGCAGCTGGGCAATAATC**

R: **ATTTTCTCGCCGTTTCTTT**

Sequence identity comparison was carried out using the pairwise alignment tool in Jalview suite v2.11 [48]. Sequence comparison against the GenBank database was performed using the NCBI BLAST network server (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). Potential orthologs were searched for using tBLASTx where possible to allow a certain level of tolerance in case of a high degree of divergence. Domain characterization of each transcript was carried out using Scan prosite[49].

RNA interference analysis dsRNA in starvation

dsRNA was synthesized by in vitro transcription (Roche) and microinjection performed, as previously described [50], following the standard protocol of 3 × 32 nl injection of dsRNA for 3 consecutive days. To achieve stronger inhibition, we performed the same protocol over 2 consecutive weeks in starved planarians. Control animals were injected with dsRNA for green fluorescent protein (GFP), a gene not present in planarians.

RNA interference analysis dsRNA in feeding conditions

Planarians were injected with dsRNA during 3 nonconsecutive days alternated with 2 days of feeding for a period of 3 weeks. Planarians were injected 3 times with 32,2 nl with a dsRNA in a concentration of 800 ng/μl during the first week and 1000 ng/ μl the following ones. Controls were injected with green fluorescent protein (*gfp*) at the same concentration of the experimental RNAi. Pictures to measure the size of the animals were taken the third day of injection every week. They were taken using grid millimeter paper for scale purposes.

Measurement of ATP levels

ATP levels were measured by using an ATP Bioluminescence Assay kit CLS II (Roche) according to the manufacturer's instructions. Planarians were incubated in cell lysis reagent and boiled for 15 min at 100°C. Then, samples were sonicated for 4 cycles of 3000. Luciferase reagent was added to the samples, and luminescence was measured by the Perkin-Elmer LS-50 spectrophotometer using the injection function. Three biological replicates consisting of five animals were used per condition, and each sample was replicated three times in each ATP experiment. Protein content was measured by the Bradford method (Micro BCA Protein Assay kit, Thermo Scientific).

In vivo PI incorporation analysis

Animals were injected with *gfp* or *mfn2* over 2 consecutive weeks. Following incorporation of PI and Hoechst in vivo was performed as previously described [23].

Caspase 3 activity assay

At the end of the second week of RNAi inhibition in starved condition and after 1,2 and 3 weeks under feeding, protein extraction was performed. BioRad protein reagent was used to obtain protein concentration of the cell lysates. Fluorometric analysis of caspase-3 activity was performed as described previously [51]. Fluorescence was measured in a luminescence spectrophotometer (Perkin- Elmer LS-50) using Fluostar Optima microplate fluorescence reader (BMG Labtech), applying the following settings: excitation, 380 nm; emission, 440 nm. 20 mg of protein extract was used to determine enzyme activity, incubating for 2 h at 37 °C with 20 µM caspase-3 substrate Ac-DEVD-AMC or 2 ml from a stock of 1 mg/ml for a final volume of 150 µl. Three technical replicates were analyzed per condition. Caspase-3 inhibitor Z-DEVD-FMK was used as a control.

Quantitative real-time PCR

Total RNA was extracted from a pool of five planarians per condition using TRIzol reagent (Invitrogen). cDNA was synthesized as previously described [51]. Expression levels were normalized to that of the housekeeping gene *ura4*. All experiments were performed using three biological and three technical replicates for each condition. The design of specific primers corresponding to *mfn2*, and the use of previously published ones for *mtor*, *akt* [30] and also *4ebp* and *Rad51* allowed to check their levels of expression after the RNAi experiments. All primers used in this study are the following:

mfn2 qPCR:

F: GCAGTTTGAGGAGTCTCGATG

R: ATATCTTCCAGGCGCTTGAC

rad51 qPCR:

F: ATGGCAGCAAAACAAATGGT

R: TGTGCACTTATACCAGCTCCT

4ebp qPCR:

F: AAACACCACCAAAATGTTTGAA

R: TGAAATTGGCTTTCATCATCA

Telomere quantitative PCR (telomere qPCR)

Animals were injected with *gfp* or *mfn2* over 3 consecutive weeks in feeding conditions. Following telomere quantification was done as described [31].

Whole-mount *in situ* hybridization

Probes were synthesized *in vitro* using SP6 or T7 polymerase and DIG- or FITC- modified (Roche) from previously cloned fragments. RNA probes were purified by ethanol precipitation and the addition of 7.5 M ammonium acetate. For colorimetric whole-mount *in situ* hybridization, animals were sacrificed using 5% N-acetyl-L-cysteine (NAC), fixed with 4% formaldehyde and permeabilized with Reduction Solution (50 mM DTT, 1% NP-40, 0.5% SDS in 1× PBS). The fixative and whole-mount *in situ* hybridization protocol used has been previously described [52].

Immunohistochemistry

Whole-mount immunohistochemistry was performed as previously described [53]. Animals were killed with 2% HCl and fixed with 4% formaldehyde. The primary antibody used was rabbit anti-phospho-histone H3 (Ser10) (D2C8) (PH3) (1:500; Cell Signaling Technology; 9701). The secondary antibody used was Alexa 568-conjugated goat anti-rabbit (1:1000; Molecular Probes; A-11011).

Whole-mount BrdU staining

Whole-mount BrdU staining was performed as previously described [54].

Irradiation

To deplete the neoblast population, planarians were irradiated with X rays in PAM water during 1h at 4,5 mA, 200 kV and 93'6 Gy. Afterwards they were fixated for in situ hybridization at 1 and 3 days after the irradiation.

Single-Cell Sequencing Data

mfn2 gene expression profile (dd_Smed_v6_2168_0_1) was obtained from the planaria single-cell database hosted by the [Rajewsky lab](#) at the [Berlin Institute for Medical Systems Biology](#) of the [Max Delbrück Center](#), Berlin [55].

Imaging

In vivo images were taken with sCMEX-3 High End Digital Microscope Camera DC.3000s (Visual Inspection Technology). Whole mount *in situ* hybridization and immunostaining samples were observed with a *Leica* stereoscope and images were taken with *Progress C3 Jenoptik* camera. A Zeiss LSM 880 confocal microscope (Zeiss, Oberkochen, Germany) was used to obtain confocal images of whole-mount fluorescent in situ hybridization sample. Quantification of H3P+ cells were done using freely available ImageJ software [56] with the analyze particle function and normalized by animal area determined with the measurement tool. Colocalization quantification was performed using the equivalent areas using the "ROI-manager" tool in Fiji.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 6. Two-sided Student's *t*-tests ($\alpha=0.05$) were performed to compare the means of two populations. Comparisons of more than 2 populations were done with ANOVA test. Previous confirmation of normality and homogeneity of data was performed with Shapiro–Wilk test and Bartlett test respectively. To determine the p-value accumulated for each pairwise comparison it was used the Tukey HSD test.

REFERENCES

1. Margulis L (1967) On the origin of mitosing cells. *Journal of theoretical biology* 14:255–74
2. Schmidt O, Pfanner N, Meisinger C (2010) Mitochondrial protein import: From proteomics to functional mechanisms. *Nature Reviews Molecular Cell Biology* 11:655–667. <https://doi.org/10.1038/nrm2959>
3. Osellame LD, Blacker TS, Duchon MR (2012) Cellular and molecular mechanisms of mitochondrial function. *Best Practice and Research: Clinical Endocrinology and Metabolism* 26:711–723. <https://doi.org/10.1016/j.beem.2012.05.003>

4. Hoppins S, Lackner L, Nunnari J (2007) The machines that divide and fuse mitochondria. *Annual Review of Biochemistry* 76:751–780. <https://doi.org/10.1146/annurev.biochem.76.071905.090048>
5. Eura Y, Ishihara N, Yokota S, Mihara K (2003) Two Mitofusin Proteins, Mammalian Homologues of FZO, with Distinct Functions Are Both Required for Mitochondrial Fusion. *Journal of Biochemistry* 134:333–344. <https://doi.org/10.1093/jb/mvg150>
6. Rapaport D, Brunner M, Neupert W, Westermann B (1998) Fzo1p is a mitochondrial outer membrane protein essential for the biogenesis of functional mitochondria in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* 273:20150–20155. <https://doi.org/10.1074/jbc.273.32.20150>
7. Hales KG, Fuller MT (1997) Developmentally regulated mitochondrial fusion mediated by a conserved, novel, predicted GTPase. *Cell* 90:121–129. [https://doi.org/10.1016/S0092-8674\(00\)80319-0](https://doi.org/10.1016/S0092-8674(00)80319-0)
8. Pelloquin L, Belenguer P, Menon Y, Ducommun B (1998) Identification of a fission yeast dynamin-related protein involved in mitochondrial DNA maintenance. *Biochemical and Biophysical Research Communications* 251:720–726. <https://doi.org/10.1006/bbrc.1998.9539>
9. Alexander C, Votruba M, Pesch UEA, et al (2000) OPA1, encoding a dynamin-related GTPase, is mutated in autosomal dominant optic atrophy linked to chromosome 3q28. *Nature Genetics* 26:211–215. <https://doi.org/10.1038/79944>
10. Mitra K, Wunder C, Roysam B, et al (2009) A hyperfused mitochondrial state achieved at G1-S regulates cyclin E buildup and entry into S phase. *Proceedings of the National Academy of Sciences of the United States of America* 106:11960–11965. <https://doi.org/10.1073/pnas.0904875106>
11. Bhattacharya D, Scimè A (2020) Mitochondrial Function in Muscle Stem Cell Fates. *Frontiers in Cell and Developmental Biology* 8:1–15. <https://doi.org/10.3389/fcell.2020.00480>
12. Cha Y, Kim T, Jeon J, et al (2021) SIRT2 regulates mitochondrial dynamics and reprogramming via MEK1-ERK-DRP1 and AKT1-DRP1 axes. *Cell Reports* 37:110155. <https://doi.org/10.1016/j.celrep.2021.110155>
13. Warburg O (1956) On the origin of cancer cells. *Science* 123:309–314. <https://doi.org/10.1126/science.123.3191.309>
14. Liu X, Sun J, Yuan P, et al (2019) Mfn2 inhibits proliferation and cell-cycle in Hela cells via Ras-NF- κ B signal pathway. *Cancer Cell International* 19:1–10. <https://doi.org/10.1186/s12935-019-0916-9>
15. Kleiber M (1932) California Agricultural Experiment Station Body Size and Metabolism. *Hilgardia Journal of Agricultural Science* 6:315–353
16. Thommen A, Werner S, Frank O, et al (2019) Body size-dependent energy storage causes Kleiber's law scaling of the metabolic rate in planarians. *eLife* 8:1–29. <https://doi.org/10.7554/eLife.38187>
17. Miller CM, Newmark PA (2012) An insulin-like peptide regulates size and adult stem cells in planarians. *International Journal of Developmental Biology* 56:75–82. <https://doi.org/10.1387/ijdb.113443cm>
18. Tu KC, Pearson BJ, Sánchez Alvarado A (2012) TORC1 is required to balance cell proliferation and cell death in planarians. *Developmental Biology* 365:458–469. <https://doi.org/10.1016/j.ydbio.2012.03.010>

19. Oviedo NJ, Pearson BJ, Levin M, Alvarado AS (2008) Planarian PTEN homologs regulate stem cells and regeneration through TOR signaling. *DMM Disease Models and Mechanisms* 1:131–143. <https://doi.org/10.1242/dmm.000117>
20. González-Estévez C, Felix DA, Rodríguez-Esteban G, Aziz Aboobaker A (2012) Decreased neoblast progeny and increased cell death during starvation-induced planarian degrowth. *International Journal of Developmental Biology* 56:83–91. <https://doi.org/10.1387/ijdb.113452cg>
21. González-Estévez C, Felix DA, Smith MD, et al (2012) SMG-1 and mTORC1 Act Antagonistically to Regulate Response to Injury and Growth in Planarians. *PLoS Genetics* 8:e1002619. <https://doi.org/10.1371/journal.pgen.1002619>
22. Shiroor DA, Bohr TE, Adler CE (2020) Injury Delays Stem Cell Apoptosis after Radiation in Planarians. *Current Biology* 30:2166–2174.e3. <https://doi.org/10.1016/j.cub.2020.03.054>
23. de Sousa N, Rodríguez-Esteban G, Rojo-Laguna JI, et al (2018) Hippo signaling controls cell cycle and restricts cell plasticity in planarians. *PLoS Biology* 16:1–28. <https://doi.org/10.1371/journal.pbio.2002399>
24. Hendzel MJ, Wei Y, Mancini MA, et al (1997) Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. *Chromosoma* 106:348–360. <https://doi.org/10.1007/s004120050256>
25. Eisenhoffer GT, Kang H, Alvarado AS (2008) Molecular Analysis of Stem Cells and Their Descendants during Cell Turnover and Regeneration in the Planarian *Schmidtea mediterranea*. *Cell Stem Cell* 3:327–339. <https://doi.org/10.1016/j.stem.2008.07.002>
26. Reddien PW, Oviedo NJ, Jennings JR, et al (2005) Developmental biology: SMEDWI-2 is a PIWI-like protein that regulates planarian stem cells. *Science* 310:1327–1330. <https://doi.org/10.1126/science.1116110>
27. Lee H, Kim JH, Chae YJ, et al (1998) Creatine synthesis and transport systems in the male rat reproductive tract. *Biology of Reproduction* 58:1437–1444. <https://doi.org/10.1095/biolreprod58.6.1437>
28. Gutiérrez-Gutiérrez Ó, Felix DA, Salvetti A, et al (2021) Regeneration in starved planarians depends on TRiC/CCT subunits modulating the unfolded protein response. *EMBO reports* 22:e52905. <https://doi.org/10.15252/embr.202152905>
29. Baguñà J (1974) Dramatic mitotic response in planarians after feeding, and a hypothesis for the control mechanism. *The Journal of experimental zoology* 190:117–22. <https://doi.org/10.1002/jez.1401900111>
30. Pascual-Carreras E, Marin-Barba M, Herrera-Úbeda C, et al (2020) Planarian cell number depends on Blitzschnell , a novel gene family that balances cell proliferation and cell death. *Development* 147:. <https://doi.org/10.1242/dev.184044>
31. Iglesias M, Felix DA, Gutiérrez-Gutiérrez Ó, et al (2019) Downregulation of mTOR Signaling Increases Stem Cell Population Telomere Length during Starvation of Immortal Planarians. *Stem Cell Reports* 13:405–418. <https://doi.org/10.1016/j.stemcr.2019.06.005>
32. Smets B, Ghillebert R, De Sniijder P, et al (2010) Life in the midst of scarcity: Adaptations to nutrient availability in *Saccharomyces cerevisiae*
33. Webster PL, Hof J V (1969) Dependence of energy and aerobic metabolism of initiation of DNA synthesis and mitosis by G1 and G2 cells. *Experimental cell research* 55:88–

94. [https://doi.org/10.1016/0014-4827\(69\)90461-3](https://doi.org/10.1016/0014-4827(69)90461-3)
34. Kalucka J, Missiaen R, Georgiadou M, et al (2015) Metabolic control of the cell cycle. *Cell Cycle* 14:3379–3388. <https://doi.org/10.1080/15384101.2015.1090068>
35. Vera E, Bernardes de Jesus B, Foronda M, et al (2013) Telomerase Reverse Transcriptase Synergizes with Calorie Restriction to Increase Health Span and Extend Mouse Longevity. *PLoS ONE* 8:. <https://doi.org/10.1371/journal.pone.0053760>
36. Laplante M, Sabatini DM (2012) mTOR Signaling in Growth Control and Disease. *Cell* 149:274–293. <https://doi.org/10.1016/j.cell.2012.03.017>
37. Peiris TH, Weckerle F, Ozamoto E, et al (2012) TOR signaling regulates planarian stem cells and controls localized and organismal growth. *Journal of Cell Science* 125:1657–1665. <https://doi.org/10.1242/jcs.104711>
38. Tu KC, Pearson BJ, Sánchez Alvarado A (2012) TORC1 is required to balance cell proliferation and cell death in planarians. *Developmental Biology* 365:458–469. <https://doi.org/10.1016/j.ydbio.2012.03.010>
39. Oviedo NJ, Newmark PA, Sánchez Alvarado A (2003) Allometric scaling and proportion regulation in the freshwater planarian *Schmidtea mediterranea*. *Developmental Dynamics* 226:326–333. <https://doi.org/10.1002/dvdy.10228>
40. Salazar-Roa M, Malumbres M (2017) Fueling the Cell Division Cycle. *Trends in Cell Biology* 27:69–81. <https://doi.org/10.1016/j.tcb.2016.08.009>
41. Molinaro AM, Lindsay-mosher N, Pearson BJ (2021) Identification of TOR-responsive slow-cycling neoblasts in planarians. 1–14. <https://doi.org/10.15252/embr.202050292>
42. Fingar DC, Salama S, Tsou C, et al (2002) Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4EBP1/eIF4E. *Genes and Development* 16:1472–1487. <https://doi.org/10.1101/gad.995802>
43. Zorzano A, Liesa M, Sebastián D, et al (2010) Mitochondrial fusion proteins: Dual regulators of morphology and metabolism. *Seminars in Cell and Developmental Biology* 21:566–574. <https://doi.org/10.1016/j.semcdb.2010.01.002>
44. Brand MD, Nicholls DG (2011) Assessing mitochondrial dysfunction in cells. *Biochemical Journal* 435:297–312. <https://doi.org/10.1042/BJ20110162>
45. Fernández-Taboada E, Moritz S, Zeuschner D, et al (2010) Smed-SmB, a member of the LSm protein superfamily, is essential for chromatoid body organization and planarian stem cell proliferation. *Development* 137:1583–1583. <https://doi.org/10.1242/dev.051847>
46. Cebria F, Cebrià F, Newmark PA (2005) Planarian homologs of netrin and netrin receptor are required for proper regeneration of the central nervous system and the maintenance of nervous system architecture. *Development* 132:3691–3703. <https://doi.org/10.1242/dev.01941>
47. Grohme MA, Schloissnig S, Rozanski A, et al (2018) The genome of *Schmidtea mediterranea* and the evolution of core cellular mechanisms. *Nature* 554:56–61. <https://doi.org/10.1038/nature25473>
48. Waterhouse AM, Procter JB, Martin DMA, et al (2009) Jalview Version 2-A multiple sequence alignment editor and analysis workbench. *Bioinformatics* 25:1189–1191. <https://doi.org/10.1093/bioinformatics/btp033>
49. de Castro E, Sigrist CJA, Gattiker A, et al (2006) ScanProsite: Detection of PROSITE signature matches and ProRule-associated functional and structural residues in

- proteins. *Nucleic Acids Research* 34:362–365. <https://doi.org/10.1093/nar/gkl124>
50. Sánchez Alvarado A, Newmark PA (1999) Double-stranded RNA specifically disrupts gene expression during planarian regeneration. *Proceedings of the National Academy of Sciences of the United States of America* 96:5049–54. <https://doi.org/10.1073/PNAS.96.9.5049>
 51. González-Estévez C, Felix DA, Aboobaker AA, Saló E (2007) Gtdap-1 promotes autophagy and is required for planarian remodeling during regeneration and starvation. *Proceedings of the National Academy of Sciences of the United States of America* 104:13373–13378. <https://doi.org/10.1073/pnas.0703588104>
 52. Font-Martín D, Pascual-Carreras E, Saló E (2023) Combining Fluorescent In Situ Hybridization with Immunofluorescence and Lectin Staining in Planarians. In: *Methods in Molecular Biology*. pp 67–79
 53. Ross KG, Omuro KC, Taylor MR, et al (2015) Novel monoclonal antibodies to study tissue regeneration in planarians. *BMC Developmental Biology* 15:2. <https://doi.org/10.1186/s12861-014-0050-9>
 54. Lin AYT, Pearson BJ (2017) Yorkie is required to restrict the injury responses in planarians. *PLoS Genetics* 13:1–30. <https://doi.org/10.1371/journal.pgen.1006874>
 55. Plass M, Solana J, Wolf FA, et al (2018) Cell type atlas and lineage tree of a whole complex animal by single-cell transcriptomics. *Science* 360:1–17. <https://doi.org/10.1126/science.aag1723>
 56. Schindelin J, Arganda-Carreras I, Frise E, et al (2012) Fiji: An open-source platform for biological-image analysis. *Nature Methods* 9:676–682. <https://doi.org/10.1038/nmeth.2019>

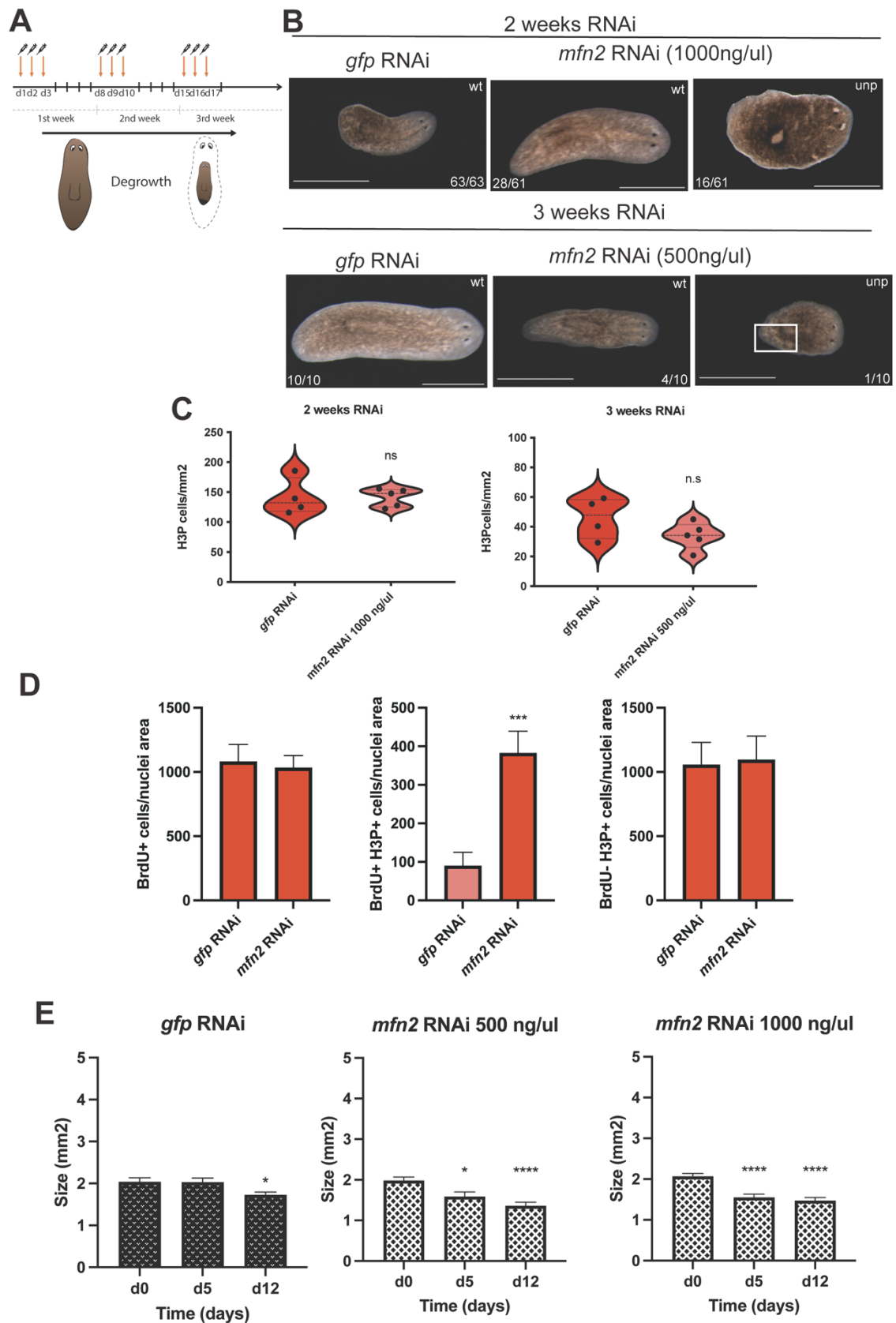


Figure 1: *mfn2* RNAi in starvation leads to the formation of unpigmented regions , accelerated degrowth and problems progressing in cell cycle from G2 to M. (A) Schematic illustrating the RNAi experimental procedure in starving conditions. **(B)** Stereomicroscopic image of live *gfp* and *mfn2* RNAi planarians at 1000 ng/ul for 2 weeks and

500 ng/ul for 3 weeks respectively depicting unpigmented regions ($n \geq 61$ and $n=10$ respectively). The white box mark an unpigmented region. (C) Quantification of mitotic cells (H3P+) normalized by area after 2 weeks of *mfn2* RNAi at 1000 ng/ul and 3 weeks of *mfn2* RNAi at 500 ng/ul and the respective *gfp* RNAi (control) animals ($n \geq 4$). (D) Quantification of BrdU+ cells, double-positive BrdU+/H3P+ cells and BrdU-/H3P+ cells in *gfp* and *mfn2* (RNAi) animals. Experiments were performed after a 16-h BrdU pulse post 2 weeks of RNAi ($n=7$). Error bars represent standard error. Data were analyzed by Student *t* test. *** $p < 0.001$. (E) Size quantification of the *mfn2* RNAi animals at 1000 ng/ul and at 500 ng/ul compared to the *gfp* RNAi controls after 0, 5 and 12 days of starvation. Data is represented as mean $\pm$ s.e.m * $p < 0.05$; **** $p < 0.0001$ ($n \geq 16$). Scale bars: 1mm. RNAi, RNA interference; *gfp*, green fluorescent protein; *mfn2*, mitofusin-2; unp, unpigmented regions; wt, wild type.

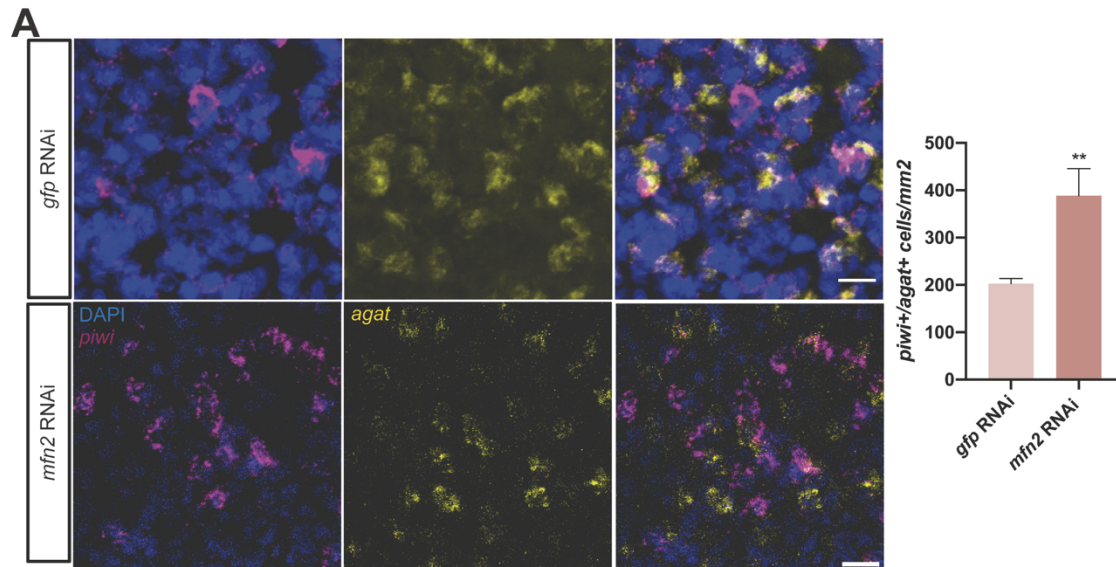


Figure 2: *mfn2* RNAi in starvation cause defects in epidermal differentiation. (A) Double FISH with the neoblast marker *smcdwi-1* (*piwi*) and late epidermal cell progenitors *agat* and corresponding quantification. Data is represented as mean $\pm$ s.e.m ** $p < 0.01$. ($n \geq 6$). Scale bars: 10 μ m. FISH, fluorescent in situ hybridization; RNAi, RNA interference; *gfp*, green fluorescent protein; *mfn2*, mitofusin-2.

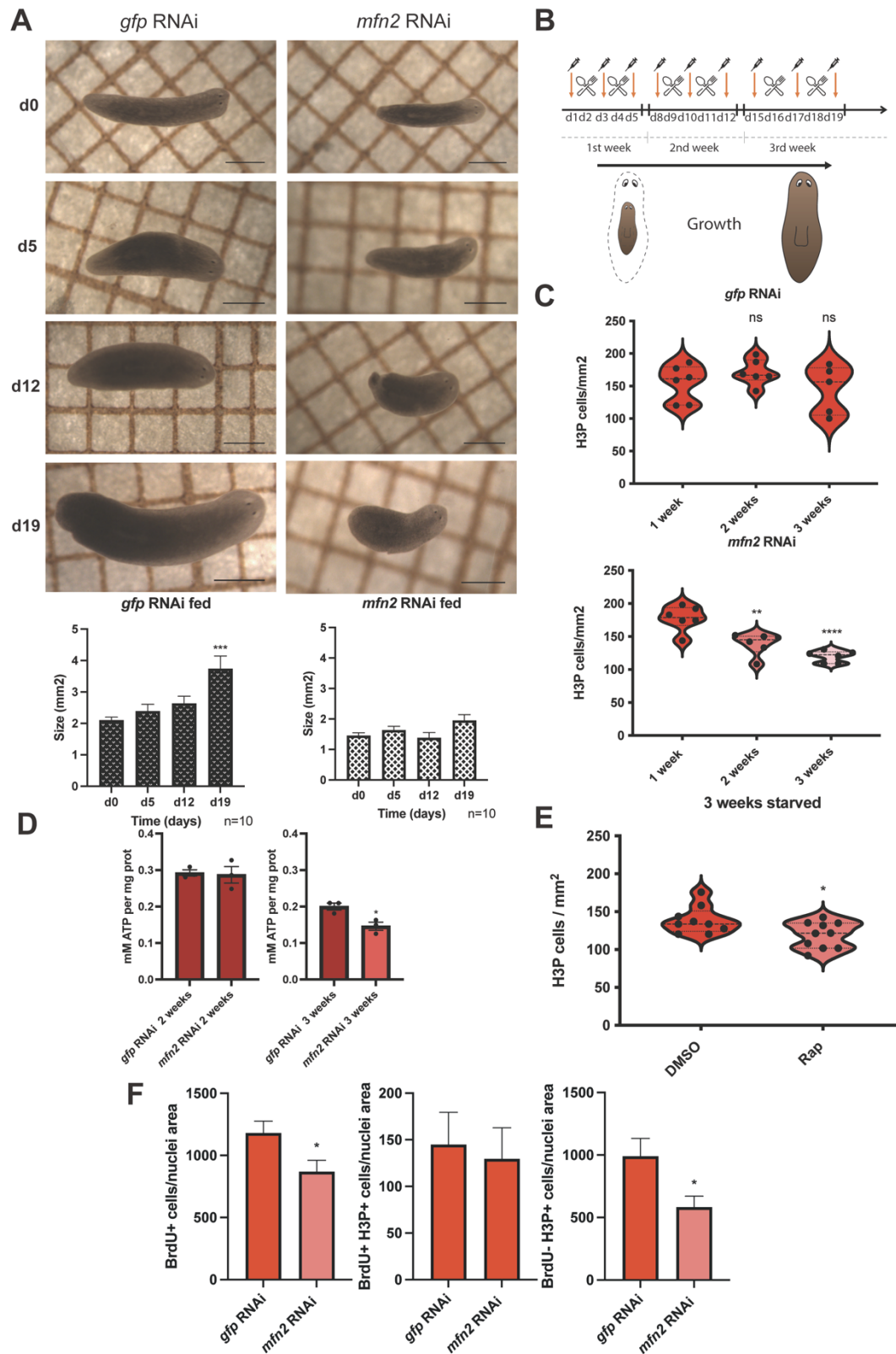


Figure 3: *mfn2* RNAi in feeding conditions impairs growth and proliferation, mimicking *mtor* inhibition, decreases the number of stem cells that are able to enter S phase and progress through the different M phases (A) Size quantification of the *mfn2* RNAi animals compared to the *gfp* RNAi controls after 0 5 12 and 19 days under feeding conditions. Representative images of the animal sizes after each week of RNAi silencing are shown. Data is represented as mean $\pm$ s.e.m * $p < 0.05$; **** $p < 0.0001$, ($n=10$). Scale bars: 1mm. (B)

Schematic illustrating the RNAi experimental procedure in feeding conditions. (C) Quantification of mitotic cells (H3P+) normalized by area after 1, 2 and 3 weeks of *mfn2* RNAi and *gfp* RNAi (control) animals (n≥5). (D) ATP measurement on whole planarian extracts after *mfn2* or *gfp* RNAi under feeding conditions after 2 or 3 weeks respectively. Data are mean±s.e.m. *P<0.05 (E) Quantification of mitotic cells (H3P+) normalized by area 3 weeks of Rapamycin treatment and DMSO as drug control in starvation (n≥9). (F) Quantification of BrdU+ cells, double-positive BrdU+/H3P+ cells and BrdU-/H3P+ cells in *gfp* and *mfn2* (RNAi) animals. Experiments were performed after a 16-h BrdU pulse (n=7) post 3 weeks of RNAi. Error bars represent standard error. Data were analyzed by Student *t* test. *p < 0.05.

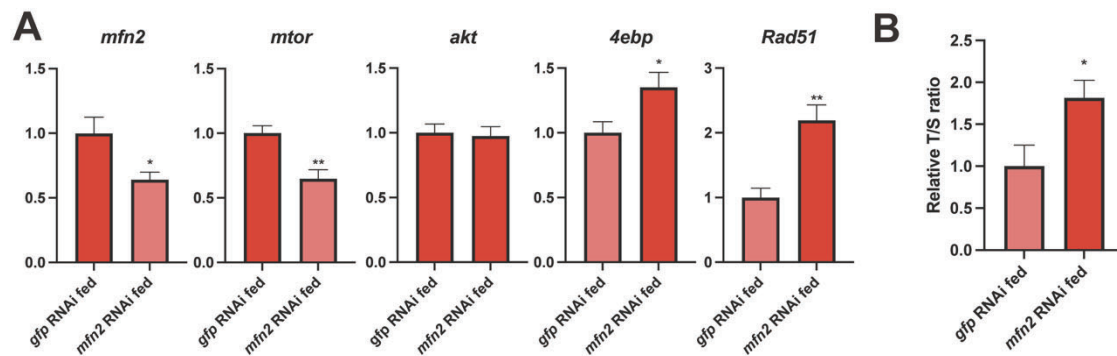
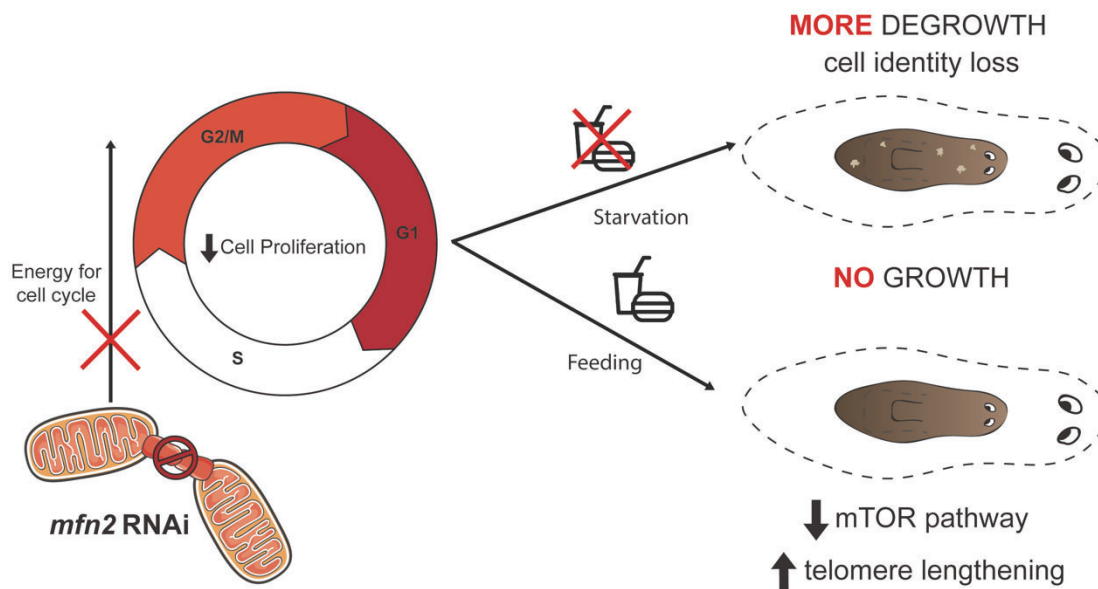


Figure 4: *mfn2* RNAi regulates *mtor*, its effectors and telomere length in feeding conditions. (A) Bar charts represents qRT-PCR quantifications of *mfn2*, *mtor*, *akt*, *4ebp*, *rad51* after 3 weeks of fed *GFP* and *mfn2* RNAi. In all the panels, relative expression is plotted as $2^{-\Delta\Delta CT}$ values. Data is represented as mean±s.d. *P<0.05; **P<0.01. (B) Telomere quantitative PCR (qPCR) comparing *GFP* RNAi and *mfn2* RNAi planarians. Data is represented as mean±s.d. *P<0.05;



SUPPORTING INFORMATION

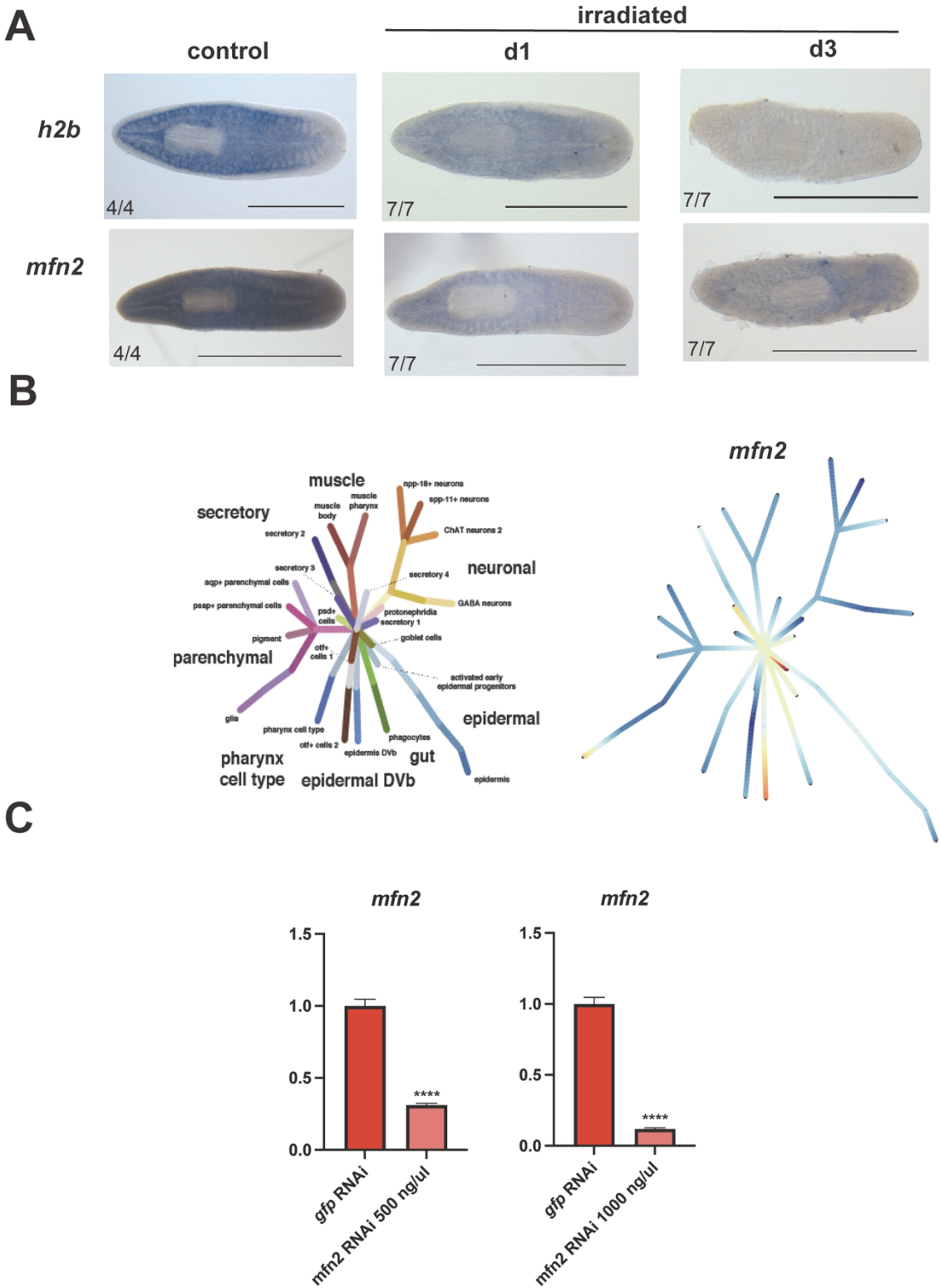


Figure S1: *mfn2* pattern of expression and downregulated levels. (A) Whole-mount *in situ* hybridization analysis of the expression of *h2b* and *mfn2* in wildtype animals in control conditions and after 1 and 3 days of lethal irradiation. (B) Schematic representation of single-cell transcriptomic expression of *mfn2* genes (according to [55]).(C) Bar charts represents qRT-PCR quantifications of *mfn2* transcript levels after 2 weeks of starved *gfp* and *mfn2* RNAi

at 500 ng/ul and 1000 ng/ul. In all the panels, relative expression is plotted as $2^{-\Delta\Delta CT}$ values. Data are mean $\pm$ s.d. ****P<0.0001.

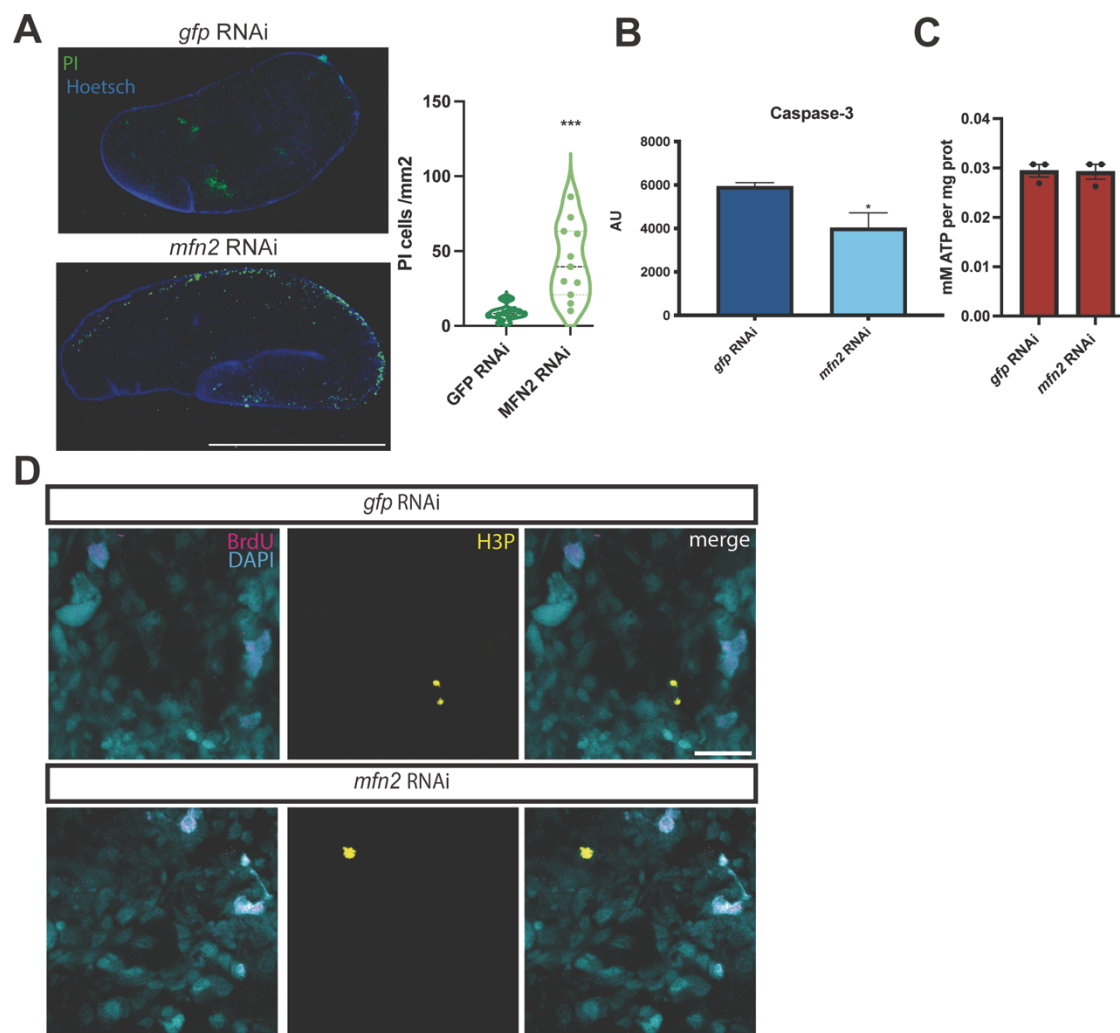


Figure S2: *mfn2* RNAi in starvation cause an increase of necrosis and decrease of apoptosis. (A) Staining of dead cells using PI in live control and *mfn2* (RNAi) animals. Nuclei are stained with Hoechst. Quantification of the PI+ cells per area is shown. Data were analyzed by Student *t* test ($n \geq 11$). *** $p < 0.001$. Scale bar 1mm. PI, propidium iodide; RNAi, RNA interference. (B) Quantification of caspase-3 enzymatic activity in *gfp* and *mfn2* RNAi animals after 2 weeks of injections. Data is represented as mean $\pm$ s.e.m * $p < 0.05$. (C) ATP measurement on whole planarian extracts of *mfn2* RNAi versus controls under starved conditions after 2 weeks. (D) BrdU labeling combined with immunostaining with anti-H3P antibody in the pre-pharynx region in controls and in planarians subjected to *mfn2* and *gfp* RNAi for 2 weeks. Scale bars: 25 μ m.

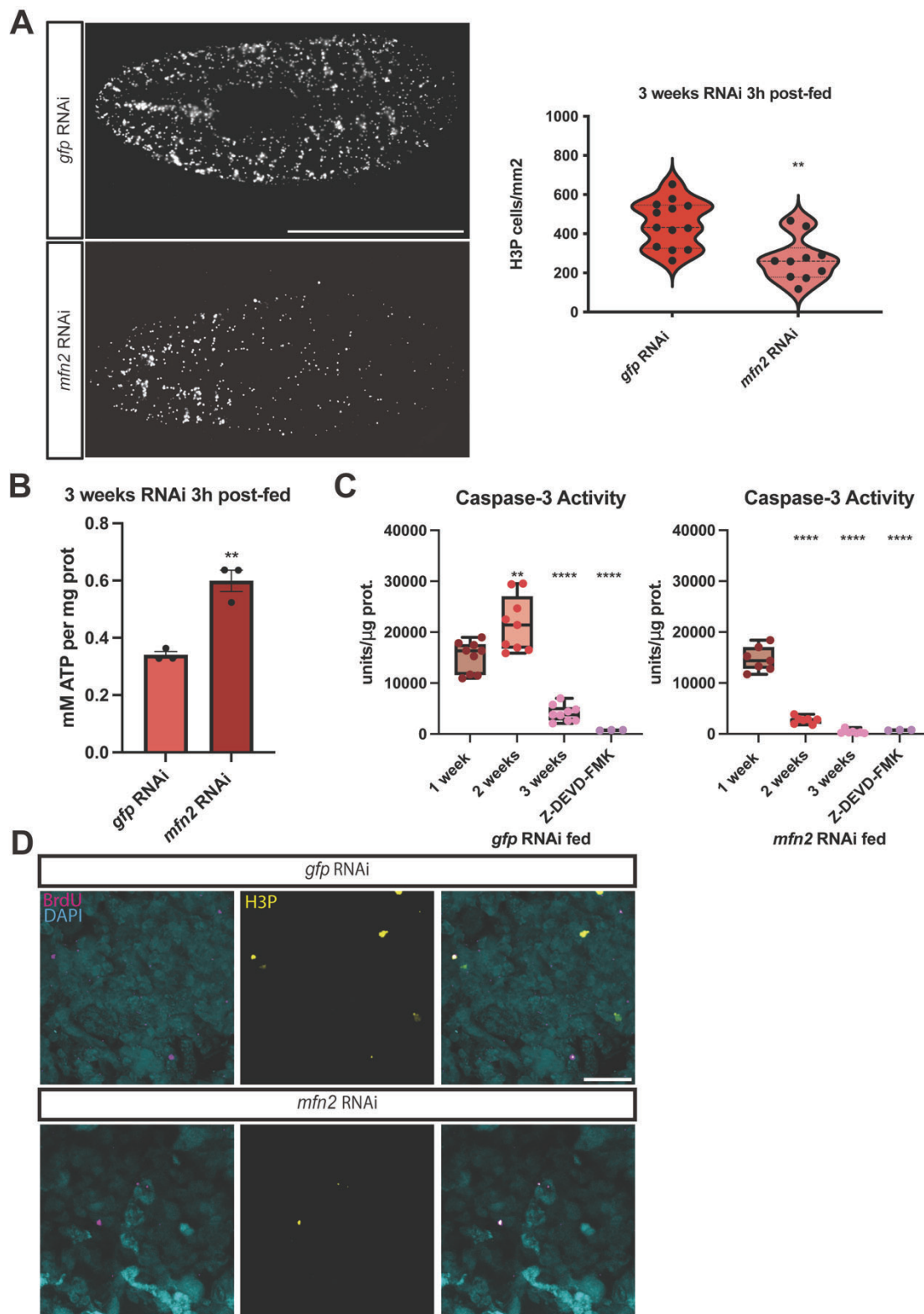


Figure S3: *mfn2* RNAi in feeding conditions avoids the 3h post-fed mitotic peak and decreases apoptosis. (A) Staining of cycling cells using H3P in control and *mfn2* (RNAi) animals 3h post-fed after 4 weeks in feeding conditions. Quantification of mitotic cells (H3P+) normalized by area are shown ($n \geq 10$). (B) ATP measurement on whole planarian extracts of *mfn2* RNAi versus controls under fed conditions after 3 weeks 3 hours post fed. Data is represented as mean $\pm$ s.e.m. $^{**}p < 0.001$. (C) Quantification of caspase-3 enzymatic activity in *mfn2* and *gfp* RNAi animals after 1, 2 and 3 weeks of injections in feeding conditions. Z-DEVD-

FMK corresponds to the negative control. Data is represented as mean $\pm$ s.e.m **p < 0.001; ****p < 0.0001. (D) BrdU labeling combined with immunostaining with anti-H3P antibody in the pre-pharynx region in controls and in planarians subjected to *mfn2* and *gfp* RNAi for 3 weeks. Scale bars: 25 μ m.

GENERAL DISCUSSION:

The integration of cell proliferation and apoptosis is essential for proper cell fate specification during animal development and homeostasis. In this Thesis, different aspects of these processes have been approached through the study of the animal model *Schmidtea mediterranea*, which is continuously renewing their tissues in a stem-cell based manner. In a previous study, *hippo* inhibition by RNAi was characterized in planarians [82]. Those results indicated cellular dedifferentiation and the formation of overgrowths which correlated with a decrease in cell death, an arrest in mitosis and the increase in cell plasticity. With this model in mind, we studied several putative transcription factors found downregulated after inhibiting *hippo*, a pathway essential to maintain the differentiated fate in planarians, with the idea that each of them reproduces some aspect of the *hippo* function.

Cellular senescence could mediate the formation of overgrowths after *hippo* silencing in planarians

We have shown that the overgrowths and cellular dedifferentiation that occur in planarians after *hippo* RNAi happen after an accumulation of senescent signals. We think that these signals could trigger the phenotype since it has been described that although it is traditionally viewed as a tumor suppressor mechanism to prevent cell cycle progression, it has also been described to present a paracrine tumor inducing effect aiding in the proliferation, metastasis and migration of tumorigenic cells [123]. This effect is mainly driven by the senescence associated secretory phenotype (SASP) that triggers the activation of pro-tumorigenic factors. Specifically, the SASP has been described to be composed of a mixture of pro-inflammatory cytokines, growth-factor molecules and matrix remodeling enzymes (including metalloproteases) [2, 123]. It has been reported that changes in the extracellular matrix composition often impair proper mechanotransduction. This context leads to the disruption of integrin-mediated signaling processes that end up affecting the cytoskeletal and nuclear composition of the surrounding cells. As a consequence the epigenetic landscape and chromatin organization of these cells change and in turn modify gene expression patterns and ultimately cellular identity [165].

We think that in the planarian context senescence plays an active role in triggering cellular dedifferentiation. This is reinforced by the fact that the inhibition of *syne1* and *trrap*, two genes described to present an active role in cellular senescence [166, 167], phenocopy *hippo* RNAi. Their inhibition also cause an increase at the transcriptomic level of some bona fide senescent markers (*p53*, *pRb* and *Imnb1*) Nonetheless, it is important to emphasize that the proteomic levels of expression of these markers and phosphorylation activation of p53 and pRb could provide more direct evidence of cellular senescence than the transcript levels [168, 169]. In line with these evidences, the accumulation of senescence aggregates in all three cases (*hippo*, *syne1* and *med17* RNAi conditions) was also detected by performing SA- β -gal staining. Still, we require a method to empirically quantify such accumulations of SA- β -gal positive cells and elucidate the type of cell populations positive for the staining. To that avail we are currently optimizing the use of SPiDER- β Gal dye combined with FACS in order to quantify the number of senescent cells between neoblasts (X1), progenitors (X2) and differentiated cells (Xins). Setting up all these preliminary evidences opens the door to expand the field of senescence in planarians in other contexts and encourage us to adapt more accurate techniques to the model.

Moreover, the treatment with senolytic and senomorphic drugs we performed with Rapamycin and Navitoclax after *hippo* RNAi prevented the rise of the overgrowth phenotype. This gave us some evidences in favor of the hypothesis that the accumulation of senescent cells is

required in order to trigger cellular dedifferentiation in this *hippo* RNAi context. However, other experiments are required to support such evidences. One example would be to check if the SA- β -gal staining in *hippo* RNAi animals is prevented by co-injection assays with Rapamycin or Navitoclax combined with *hippo* RNAi. Moreover, the transcript levels of the senescent markers *p53*, *pRb* and *Imnb1* should also be assessed in both drug rescues by qPCR.

Overall, the results in this first chapter suggests that cellular senescence triggered after *hippo* RNAi plays an important role in planarian cellular dedifferentiation.

The Hippo pathway controls tissue renewal through cytoskeleton maintenance and transcriptional regulation in planarians

We also studied specifically the formation of the overgrowths and the cell dedifferentiation process after *hippo* RNAi. By analyzing the transcriptomic footprint of *hippo* RNAi in planarians and its different expression dynamics we detected and validated genes associated with microtubule maintenance and transcriptomic regulators as important players in the emergence of the phenotype. The *hippo* RNAi-like phenotype was defined by the formation of overgrowths and unpigmented regions and the accumulation of H3P positive cells. We characterized in more detail *syne1* due to its role in the maintenance of the cellular cytoarchitecture and *med17* which is a subunit of the mediator complex involved in the maintenance of the epigenetic landscape for a proper transcription. We found in both cases that their RNAi mimic the *hippo* phenotype. Specifically, the impairment in the progression of the cell cycle at different phases upon *syne1*, *med17* and *hippo* RNAi. In *syne1* RNAi animals, cells are having trouble completing the M phase as previously described in *hippo* RNAi animals [82]. On the other hand, in *med17* RNAi animals cells are having trouble surpassing the G2 phase. This could stem from the fact that *med17* is required for the active transcription of the elements that compose the cell cycle impairing cells to surpass the G2 phase where transcription is most active [170]. On the other hand, *syne1* being a connector of the cytoskeleton and the nuclei plays a more prominent role in the assemble of the mitotic spindle during the mitosis.[171] We also found evidences supporting a role of these genes in cellular dedifferentiation mechanisms. These were the elongation of the telomeres found in all three RNAi conditions. Telomere elongation has been seen in other models as an indicator of cellular dedifferentiation both in vivo and in vitro [132, 133]. Further evidences linking *syne1* and *med17* with *hippo* and in support of the dedifferentiation role were also collected. In the case of the role of the pathway in maintenance of the cytoskeletal architecture, we found evidences of loss of epidermal polarity as a sign of a loss of the cellular differentiated state in planarians inhibited with *syne1* and *hippo*. These results mimicked the effects of *Igf2* RNAi, a known upstream regulator of the Hippo pathway required for epidermal polarity [12,13]. For more evidences of loss of differentiation due to alterations in transcription regulation we also used a synthetic Cdk8/19 inhibitor which acts as an activator of the mediator complex. The results of this inhibition ended up phenocopying the overgrowths and cellular dedifferentiation found in *hippo* and *med17* RNAi animals further supporting that modulating the epigenetic landscape is essential for proper cellular differentiation [174, 175]. Overall, we highlighted that Hippo maintains the cellular differentiated state through two distinctive mechanisms: maintenance of the stability of the cytoskeleton and regulation of transcriptional programs.

However, it is important to remark that despite all the results pointing towards cellular dedifferentiation as a cause of the appearance of dedifferentiated cells and overgrowths in Hippo, *syne1* and *med17* RNAi animals, we still cannot rule out the possibility that cellular differentiation might be defective and triggering the incorrect expression of lineage markers. Some experiments would help to contrast both hypotheses. One of them would imply to quantify the total number of cells of *syne1* and *med17* RNAi animals in comparison to controls. If there are no changes detected this would reinforce the idea of differentiated cells reentering cell cycle in opposition to an accumulation of proliferating progenitors. Another interesting

approach would be the depletion of neoblasts by sublethal irradiation. This would allow us to work in a background with almost no proliferating cells. RNAi of *syne1* and *med17* in irradiated animals could promote the expression of neoblasts markers such as *smedwi-1*, demonstrating a process of dedifferentiation. Both of these experiments were already done in *hippo* RNAi planarians where the dedifferentiation model was first proposed [82]. Still, recent publications have defined the planarian neoblasts as a highly heterogeneous cellular population from the molecular and functional perspective. This cell type has been described to be composed of different subsets of specialized neoblasts that retain less multipotent capacity and present transcription factors associated to multiple specific fates for the formation of each differentiated cell type [176, 177]. Therefore, finding contexts in planarians in which dedifferentiation might occur could be quite remarkable and go in line with the recently described flexibility in the path of neoblast commitment towards differentiation.

We would like also to emphasize that the regulation that we perceive in planarians between *hippo* and the putative effectors we describe is of an indirect nature. At the end of the day the changes we see in the candidate effectors we study originate from transcript downregulations found downstream of *hippo* RNAi inhibition. We therefore acknowledge that the molecular pathway connecting them with *hippo* remains unknown and require of further studies. We also want to point out that the direct inhibition of *syne1* and *med17* by RNAi could affect further cell types beyond the ones affected by *hippo* RNAi. On the other hand since these effectors act downstream of *hippo*, we expect that some of the repercussions attributed to the *hippo* RNAi phenotype are triggered by other effectors beside the ones we characterized. Therefore, we take into consideration that the phenocopying of the *hippo* RNAi model can be partial.

In order to pursue further experiments in regards to the changes in cellular identity we should expand to other experimental models. A very interesting approach would be attempting to perform in vitro reprogramming of differentiated cells by transient down-regulation of *syne1* and *med17* by siRNA in several types of differentiated cultured cells or progenitors and check the expression of markers that indicted a partial dedifferentiation towards less mature cell types. Previous work has shown that transient expression of YAP is able to in vitro reprogram several lines of differentiated cells [49]. [178]. This approach would indicate that these genes are necessary to maintain the differentiated state and would further consolidate the cellular dedifferentiation hypothesis found in planarians. Furthermore it would suggest an evolutionary function conservation of these genes in planarians and mammals. We don't expect to achieve the level of reprogramming mentioned by overexpressing YAP but minor shifts in cellular identity closely resembling the ones presented in planarians.

To further elucidate the role of *med17* in global transcription activity and the changes it may cause in global enhancers conformations and cellular identity it could be very interesting to include the Yeast (*Saccharomyces cerevisiae*) as an experimental model. This is due to the abundance of literature in their field related to transcription regulation. It has already been described that the Mediator complex presents a global role as a cofactor activating RNA Pol II [179]. Moreover, ChEC-seq profiles mapping protein-DNA interactions revealed specific binding landscapes of Mediator-RNA Pol II linked to specific transcriptomic profiles of some proximal genes [180]. Therefore, it would be interesting to perform the same ChEC-seq technique in a context where Med17 (SRB4 in yeasts) is depleted and check if an increase in the Mediator-RNA Pol II-DNA global interactions is observed as well as in a specific binding profile. This information could be further enriched with a Hi-C approach that could reveal hotspots of structural genomic reconfiguration. Afterwards, it would be very interesting to see if these hotspots correlate with enhancer regions connected to genes that appear in the previous specific Mediator-RNA Pol II binding profile.

This data would be of high value since it could be cross-referenced with already published Chip-seq data of RNA Pol II binding sites in an in vitro reprogramming context overexpressing

Mediator activity. Published work has shown that CDK8/19 inhibition increased Mediator-driven recruitment of RNA Pol II to enhancers triggering the transcription of naïve enhancer-derived RNAs (eRNAs) like *Pramel6* (*PRAME like 6*), *Tbx3* (*T-Box Transcription Factor 3*) and *Gjb3* (*Gap Junction Protein Beta 3*) and recruitment of RNA Pol II to promoter regions of genes such as *Nanog*, *Rex1/Zfp42* (*Zinc Finger Protein 42*), *Klf4* (*Kruppel-Like Factor 4*), *Tbx3* and *Tfcp2l1* (*Transcription Factor CP2 Like 1*) efficiently stabilizing the naive transcriptional program[181]. The end goal of this cross-referencing would be to find hotspots of evolutionary conserved genetic programs implicated in cellular differentiation. This could culminate in a list of putative candidate genes that would be later screened by RNAi and linked with the phenotypes we observe upon *med17* RNAi in planarians and potentially dedifferentiation.

***ccdc175* regulates the tissue renewal in planarians through cellular senescence and telomere dynamics.**

Furthermore, we characterized the role of *ccdc175* in planarian tissue renewal. This is an evolutionary conserved rather unstudied coiled-coil protein downregulated after *hippo* inhibition. At early stages of *ccdc175* RNAi inhibition, prior to the emergence of the phenotype, we detected a peak of senescent signals coupled with a decrease of apoptosis and a shortening of the telomeres as we described in *hippo* RNAi animals. This was immediately followed by the rise of the overgrowths, signs of cellular dedifferentiation and telomere elongation as described in *hippo* downregulation. Telomere dynamics has been a hot research topic in planarians since its characterization in the context of regeneration. This is due to the fact that asexual planarians maintain telomere length during reproduction by fission or after regenerating. This telomere maintenance is attributed to infinite telomerase activity of proliferating adult stem cells during regeneration capable of providing planarians its alleged immortality. However, the sexual strain does not maintain telomere length during regeneration. This is because sexual planarians present an alternate splicing of the protein subunit of the telomerase enzyme [14] Interestingly, the catalytic subunit of telomerase known as telomerase reverse transcriptase (TERT) appear highly conserved within phyla across metazoans. However some motif loses and alternative splicing events occur among specific lineages beyond Tricladida planarians. [14] These alternative splicing variants could be associated to important non-canonical functions related with high regenerative capabilities and potential immortal lifespans such as in the case of planarians. However there is still a lack of experimental models of TERT dynamic activity in these organisms associated to a specific biological role to support this hypothesis [182]. A similar alternative splicing could be occurring between close species in the case of *ccdc175*. Therefore it could be interesting to assess the evolutionary conservation of the *ccdc175* sequence across metazoans focusing on differential splicing events and sequence changes between close species to see if the pattern is similar to the one described in TERT. Moreover, *ccdc175* is one of the few fast-evolving genes found to exhibit significant positive selection in one species of long-lived bats (*Myotis myotis*) (37.1 years of maximum lifespan) compared to a short-lived species (*M. Molossus*) (5.6 years of maximum lifespan) indicating that they present an enrichment of allelic variant of *ccdc175* correlated with longevity [183]. Therefore, it would be interesting to see if a correlation between species with long life spans and non-canonical forms of *ccdc175* exist. It would also be of special interest to see if the length of the telomeres in these long-lived species appears elongated in comparison to their shorter-lived counterpart cousins.

An interesting approach with *ccdc175* would be a to attempt to test by shRNA if its downregulation *in vitro* on mature human neural cell cultures induces cellular dedifferentiation.[184] The experimental design would involve checking the expression of markers that indicate a partial dedifferentiation towards less mature cell types as we see in planarians. Any results indicating defects in the neuron conformation or identity would be of high value due to the lack of experimental evidences reported for this gene function.

In summary, we discovered a role of *ccdc175* in maintaining the cellular balance through the regulation of the cell cycle and cellular senescence with an emphasis on telomere modulation.

***mfn2* is necessary for stem cell proliferation during growth but dispensable during starvation**

mfn2 is a transcript found downregulated at later stages of *hippo* RNAi (4 weeks). We found interesting to study the function of *mfn2* in planarians due to potential connections with mTOR pathway and the consequences this could present to the constant growth and degrowth in planarian size dynamics. In this chapter we established that the inhibition of *mfn2*, a key protein involved in mitochondrial fusion, is able to impair proliferation in both feeding and starving conditions leading to a lack of growth upon feeding. This is coupled with a decrease in ATP production in feeding conditions and with cellular dedifferentiation in starving conditions similar to what happens in cancer progression [185]. The fact that we see different phenotypes depending on the nutritional status of the animal further reinforces the idea that the effects are dependent of the ATP levels the cells have at their disposal to proliferate. Therefore, it is highly probable that changes in metabolism between feeding and starvation impose a background energy threshold to these animals which is further affected upon *mfn2* RNAi inhibition and later cause the difference in the phenotypes between feeding and starvation.

However, further experiments focused in the behavior of the mitochondrial respiratory chain under the previous conditions would reinforce the hypothesis of the metabolic implications of *mfn2* RNAi in the observed phenotypes [186]. We think that the optimization of high-resolution imaging techniques such as transmission electron microscopy (TEM) to analyze mitochondria networks would bring more information about their fusion and fission state in planarians cells at specific timepoints and indicate if it mimics the reported results from rat GTPase-domain mutant of Mfn2 (Mfn2K109T) in HeLa cells displaying mitochondrial fragmentation [187]. Moreover, we could focus on quantifying the number stem cells and their fused/fissioned mitochondria ratios per cell. More specific biochemical assays would also be necessary to implement though in order to check the status of the mitochondrial respiratory chain in these conditions. One idea would be to perform Seahorse Real-Time Cell Metabolic Analysis after FACS sorting of live cells to check the oxygen consumption rate of the planarians *mfn2* RNAi in the context of feeding or starvation [188]. Electron microscopy images would indicate if the mitochondria are more fused or fissioned and the Seahorse Real-Time Cell Metabolic Analysis would provide an indication of the ATP production levels associated with each situation as long as we are able to detect differences by Seahorse after the planarian cell sorting by FACS. Thus, they would also provide relevant data that would aid in explaining the defects we observe in the cell cycle progression with the different ATP levels we see between the feeding and starved situations. This is because we would have a direct cause between the molecular function of *mfn2* to induce mitochondrial fusion and the ability of the cells to produce more ATP.

Moreover, we also demonstrate that *mfn2* inhibition downregulates *mTOR* transcript levels and deregulates its effectors *4ebp* (*Eukaryotic Translation Initiation Factor 4E Binding Protein 1*) and *Rad51* (*RAD51 Recombinase*) uncoupling the aforementioned pathway from the nutrient input provided with the feeding conditions. This causes an elongation of the telomeres typically associated with caloric restriction [189]. The lack of growth in feeding conditions is tied to defects in the progression of the cell cycle. It has been reported that changes in mitochondrial dynamics modulate the cell cycle progression. During the G1/S transition in normal rat kidney (NRK) cells an hyper fusion event occurs in order to increase the ATP production capabilities of the mitochondria network required for the DNA replication [142]. This conformation is later reverted by fission at the entry of the M phase in order to ensure a

distribution of mitochondria among the daughter cells post mitosis [190]. It would be interesting to assess the mitochondrial conformations by transmission electronic microscopy (TEM) in both feeding and starved *mfn2* RNAi conditions since we reported accumulations of cells at different points of the cycle (M phase and G2 phase). This is even more interesting due to the fact that in feeding conditions mitochondria tend to fission in many tissues (except the nervous system and the musculature) since are less dependent on OXPHOS metabolism whereas the opposite occur in starvation leading to more fusion [191]. Therefore, these differences in fusion and fission according to nutritional availability could set an already different mitochondrial network background in fed and starved planarians on top of the *mfn2* RNAi.

Still, the hypothesis exposed in regards to growth and the modulation of mTOR presents some parallelisms with previous work from the lab in which a similar phenotype was described involving the regulation of the mTOR pathway. In that project we characterized a *de novo* gene family of genes taxonomically restricted to the planarian clade named *blitzschnell* (*bls*). In this context, we indicated that this family of genes, which expression is modulated according to food intake, were acting as inhibitors upstream of the mTOR pathway. As a consequence, they were modulating the growth response upon feeding conditions in an opposite manner to *mfn2* thus leading to more proliferation and growth upon inhibition under feeding conditions.

To end up this thesis, we would like to emphasize the following idea. By down-playing single genes from different functional niches we are able to trigger cellular dedifferentiation through interrelated but yet different cellular processes. It is of special importance that this occurs in the context of an *in vivo* organism such as planarians since their cell turnover is based in their huge pool of stem cells, where a process of dedifferentiation has not been described to play an active role [192]. It is important to emphasize that just with the overactivation of YAP (the target of Hippo) *in vitro* cells have been able to recapitulate several pluripotent stem cell populations [49]. Nonetheless no approximation *in vivo* has yielded such effects yet. This is in spite of its role as an oncogene being described to increase genomic instability, trigger EMT together with an increase in cell migration and metastasis. Such evidence indicates that regulation of elements of the Hippo pathway together with the maintenance of proper transcriptomic programs and cytoskeleton configuration are required to make a cell retain its identity. Nonetheless other elements that exist in a tissue such as cell-cell interactions and a complex extracellular matrix also play a role in shaping such identities in many cases through interactions with the Hippo pathway itself. Therefore, these cell-cell interactions together with the scaffold provided by the extracellular matrix could add an extra layer of regulation *in vivo* that modulates the extent of the described dedifferentiation effects associated with the Hippo pathway *in vitro*. Still, we believe that our context is encased in a pathological system phenocopying *hippo* RNAi and resemble events of diseases that present tissue dysplasia's or when pushing it to an extreme, cancer [193]. We therefore think that in order to continue this work is worth exploring how these events differ from physiological dedifferentiation processes that have been observed in other organisms such as in the life cycle of some cnidarians [194] and some forms of tissue regeneration [195, 196] as they provide a great frame of reference to filter the distinctive footprints that make pathological cellular dedifferentiation unique. In a similar way Hippo also has a physiological role regulating the growth of organs during development and regeneration [52] in contrast to its pathological role associated with cancer [50]. Therefore, based in our results, it would be worth exploring more in depth how the telomeric machinery is linked to the dedifferentiation process, specifically in regards to the progression and speed of the cell cycle upon reactivation as many of its components remain poorly studied to this day [197]. Our approach also highlights the importance of focusing on poorly studied evolutionary conserved genes in planarians as a tool that allows us to help bring forward novel elements from a non-canonical organism perspective to uncover

processes that we were unaware before. In the long run, we expect this line of research to provide us with a deeper and better understanding of the establishment of cellular identity across different organisms and how its failure can lead to pathologies such as cancer.

CONCLUSIONS:

After re-analyzing a previously published RNA-seq that compared *hippo* RNAi with controls, we found *syne1* and *trrap* as DEGs that have been described to have a role in cellular senescence in other model systems. We also found *syne1* and *med17* as representative of the two most prevalent GO Ontology Terms in the RNA-seq: cytoskeleton regulation and transcriptional regulation, respectively. Additionally, we have identified *ccdc175* and *mfn2* as genes required for cell renewal and for growth regulation, respectively.

1. CELLULAR SENESCENCE MEDIATES THE FORMATION OF OVERGROWTHS AFTER HIPPO SILENCING IN PLANARIANS

1.1. We established several markers for the detection of senescence in planarians: accumulation of SA- β -Gal, a decrease of *Imnb1* expression levels and increase of *p53* and *pRb*.

1.1. The downregulation of *hippo*, *syne1* or *trrap* trigger an increase in senescence markers.

1.2. The senomorphic drug Rapamycin and the senolytic drug Navitoclax rescue the *hippo* RNAi phenotype by preventing the formation of overgrowths.

2. THE HIPPO PATHWAY CONTROLS TISSUE RENEWAL THROUGH CYTOSKELETON MAINTENANCE AND TRANSCRIPTIONAL REGULATION IN PLANARIANS

2.1. RNAi of *syne1* and *med17* phenocopy the *hippo* RNAi phenotype, with the formation of overgrowths, appearance of unpigmented regions and disruptions in the nervous system.

2.2. *syne1* and *med17* are required for the maintenance of the neural and epidermal differentiated state.

2.3. *syne1* and *med17* regulate cell cycle progression in a different manner: *syne1* regulates the transition through the different phases of M while *med17* regulates the entrance from G2 to M.

2.4. *syne1*, representing the function of cytoskeleton regulation is required for the maintenance of the epidermal cellular polarity, supporting that cytoskeleton stability is a function of the Hippo pathway to maintain tissue homeostasis.

2.5. RNAi of *med17* representing the function of transcriptional regulation combined with the enhancement of the mediator complex activity by treatment with specific drugs aggravates *hippo* RNAi phenotype, indicating that transcription regulation is an important effector of the Hippo pathway to maintain tissue homeostasis.

3. CCDC175 REGULATES THE RENEWAL OF THE NEURAL TISSUE IN PLANARIANS.

3.1. *ccdc175*, a gene with no known function in any model system, was found as a downregulated gene in *hippo* RNAi planarians compared to controls

3.2. RNAi of *ccdc175* phenocopy the *hippo* RNAi phenotype, with the formation of overgrowths, appearance of unpigmented regions and disruptions in the nervous system.

3.3. *ccdc175* regulates the renewal of the neural tissue in planarians.

3.4. *ccdc175* is necessary for G2-M transition and its inhibition increases senescence and reduces apoptosis.

4. MFN2 IS NECESSARY FOR STEM CELL PROLIFERATION DURING GROWTH BUT DISPENSABLE DURING STARVATION

4.1. *mfn2* downregulation in starved planarians promotes the appearance of unpigmented regions and produces an accelerated degrowth.

4.2. *mfn2* in starved planarians is necessary for the G2 to M transition in the cell cycle.

4.3. *mfn2* downregulation under feeding conditions reveals a lack of growth of planarians, a decrease in energy levels and a decrease in the transcriptomic levels of mTOR pathway components.

4.4. *mfn2* in fed planarians is necessary for the transition through the different phases of mitosis.

BIBLIOGRAPHY

1. Penzo-me AI, Stanger BZ (2015) Organ-Size Regulation in Mammals. Cold Spring Harbor Perspectives in Biology 1–12
2. Nardella C, Clohessy JG, Alimonti A, Pandolfi PP (2011) Pro-senescence therapy for cancer treatment. Nature Reviews Cancer 11:503–511. <https://doi.org/10.1038/nrc3057>
3. Baguñà J, Romero R (1981) Quantitative analysis of cell types during growth, degrowth and regeneration in the planarians *Dugesia mediterranea* and *Dugesia tigrina*. Hydrobiologia 84:181–194. <https://doi.org/10.1007/BF00026179>
4. Baguñà J, Riutort M (2004) Molecular phylogeny of the Platyhelminthes. Canadian Journal of Zoology 82:168–193. <https://doi.org/10.1139/z03-214>
5. Vila-Farré M, Rink JC (2018) The ecology of freshwater planarians
6. Lázaro EM, Harrath A, Stocchino GA, et al (2011) *Schmidtea mediterranea* phylogeography: An old species surviving on a few Mediterranean islands? BMC Evolutionary Biology 11:1–15. <https://doi.org/10.1186/1471-2148-11-274>
7. Saló E (2006) The power of regeneration and the stem-cell kingdom: Freshwater planarians (Platyhelminthes). BioEssays 28:546–559. <https://doi.org/10.1002/bies.20416>
8. Morgan TH (1898) Experimental studies of the regeneration of *Planaria maculata*. Archiv für Entwicklungsmechanik der Organismen 7:364–397. <https://doi.org/10.1007/BF02161491>
9. Child CM (1911) Studies on the dynamics of morphogenesis and inheritance in experimental reproduction. III. The formation of new zooids in *Planaria* and other forms. Journal of Experimental Zoology 11:221–280. <https://doi.org/10.1002/jez.1400110303>
10. Reddien PW, Alvarado AS (2004) Fundamentals of Planarian Regeneration. Annual Review of Cell and Developmental Biology 20:725–757. <https://doi.org/10.1146/annurev.cellbio.20.010403.095114>
11. Reddien PW, Oviedo NJ, Jennings JR, et al (2005) Developmental biology: SMEDWI-2 is a PIWI-like protein that regulates planarian stem cells. Science 310:1327–1330. <https://doi.org/10.1126/science.1116110>
12. Solana J, Kao D, Mihaylova Y, et al (2012) Defining the molecular profile of planarian pluripotent stem cells using a combinatorial RNAseq, RNA interference and irradiation approach. Genome biology 13:R19. <https://doi.org/10.1186/gb-2012-13-3-r19>
13. Molinaro AM, Pearson BJ (2016) In silico lineage tracing through single cell transcriptomics identifies a neural stem cell population in planarians. Genome Biology 17:1–17. <https://doi.org/10.1186/s13059-016-0937-9>
14. Tan TCJ, Rahman R, Jaber-Hijazi F, et al (2012) Telomere maintenance and telomerase activity are differentially regulated in asexual and sexual worms. Proceedings of the National Academy of Sciences of the United States of America 109:4209–4214. <https://doi.org/10.1073/pnas.1118885109>
15. Plass M, Solana J, Wolf FA, et al (2018) Cell type atlas and lineage tree of a whole complex animal by single-cell transcriptomics. Science 360:1–17. <https://doi.org/10.1126/science.aaq1723>

16. Van Wolfswinkel JC, Wagner DE, Reddien PW (2014) Single-cell analysis reveals functionally distinct classes within the planarian stem cell compartment. *Cell Stem Cell* 15:326–339. <https://doi.org/10.1016/j.stem.2014.06.007>
17. Wenemoser D, Reddien PW (2010) Planarian regeneration involves distinct stem cell responses to wounds and tissue absence. *Developmental Biology* 344:979–991. <https://doi.org/10.1016/j.ydbio.2010.06.017>
18. Newmark PA, Sánchez Alvarado A (2000) Bromodeoxyuridine specifically labels the regenerative stem cells of planarians. *Developmental Biology* 220:142–153. <https://doi.org/10.1006/dbio.2000.9645>
19. Baguñà J (2012) The planarian neoblast: The rambling history of its origin and some current black boxes. *International Journal of Developmental Biology* 56:19–37. <https://doi.org/10.1387/ijdb.113463jb>
20. Almuedo-Castillo M, Crespo X, Seebeck F, et al (2014) JNK Controls the Onset of Mitosis in Planarian Stem Cells and Triggers Apoptotic Cell Death Required for Regeneration and Remodeling. *PLoS Genetics* 10:1–15. <https://doi.org/10.1371/journal.pgen.1004400>
21. Pellettieri J, Fitzgerald P, Watanabe S, et al (2010) Cell death and tissue remodeling in planarian regeneration. *Dev Biol* 338:76–85. <https://doi.org/10.1016/j.ydbio.2009.09.015>
22. Molina MD, Saló E, Cebrià F (2007) The BMP pathway is essential for re-specification and maintenance of the dorsoventral axis in regenerating and intact planarians. *Developmental Biology* 311:79–94. <https://doi.org/10.1016/j.ydbio.2007.08.019>
23. Clark EG, Petersen CP (2023) BMP suppresses WNT to integrate patterning of orthogonal body axes in adult planarians. *PLoS Genetics* 19:1–25. <https://doi.org/10.1371/journal.pgen.1010608>
24. Iglesias M, Gomez-Skarmeta JL, Saló E, Adell T (2008) Silencing of *Smed-βcatenin1* generates radial-like hypercephalized planarians. *Development* 135:1215–1221. <https://doi.org/10.1242/dev.020289>
25. Yamamoto H, Agata K (2011) Optic chiasm formation in planarian I: Cooperative netrin- and robo-mediated signals are required for the early stage of optic chiasm formation. *Development Growth and Differentiation* 53:300–311. <https://doi.org/10.1111/j.1440-169X.2010.01234.x>
26. Fraguas S, Barberán S, Ibarra B, et al (2012) Regeneration of neuronal cell types in *Schmidtea mediterranea*: An immunohistochemical and expression study. *International Journal of Developmental Biology* 56:143–153. <https://doi.org/10.1387/ijdb.113428sf>
27. Cote LE, Simental E, Reddien PW (2019) Muscle functions as a connective tissue and source of extracellular matrix in planarians. *Nature Communications* 10:1592. <https://doi.org/10.1038/s41467-019-09539-6>
28. Nishimura K, Kitamura Y, Inoue T, et al (2007) Reconstruction of Dopaminergic Neural Network and Locomotion Function in Planarian Regenerates. *Developmental Neurobiology* 67:1059–1078. <https://doi.org/10.1002/dneu>
29. Cebrià F, Kudome T, Nakazawa M, et al (2002) The expression of neural-specific genes reveals the structural and molecular complexity of the planarian central nervous system. *Mechanisms of Development* 116:199–204. [https://doi.org/10.1016/S0925-4773\(02\)00134-X](https://doi.org/10.1016/S0925-4773(02)00134-X)
30. Nishimura K, Kitamura Y, Inoue T, et al (2008) Characterization of tyramine β-

- hydroxylase in planarian *Dugesia japonica*: Cloning and expression. *Neurochemistry International* 53:184–192. <https://doi.org/10.1016/j.neuint.2008.09.006>
31. Nishimura K, Kitamura Y, Umesono Y, et al (2008) Identification of glutamic acid decarboxylase gene and distribution of GABAergic nervous system in the planarian *Dugesia japonica*. *Neuroscience* 153:1103–1114. <https://doi.org/10.1016/j.neuroscience.2008.03.026>
 32. Nishimura K, Kitamura Y, Taniguchi T, Agata K (2010) Analysis of motor function modulated by cholinergic neurons in planarian *Dugesia japonica*. *Neuroscience* 168:18–30. <https://doi.org/10.1016/j.neuroscience.2010.03.038>
 33. Sarnat HB, Netsky MG (1985) The Brain of the Planarian as the Ancestor of the Human Brain. *Canadian Journal of Neurological Sciences / Journal Canadien des Sciences Neurologiques* 12:296–302. <https://doi.org/10.1017/S031716710003537X>
 34. Nishimura K, Kitamura Y, Agata K (2008) [Molecular mechanism of brain regeneration and reconstruction of dopaminergic neural network in planarians]. *Brain and nerve = Shinkei kenkyu no shinpo* 60:307–17
 35. Oviedo NJ, Newmark PA, Sánchez Alvarado A (2003) Allometric scaling and proportion regulation in the freshwater planarian *Schmidtea mediterranea*. *Developmental Dynamics* 226:326–333. <https://doi.org/10.1002/dvdy.10228>
 36. Currie KW, Molinaro AM, Pearson BJ (2016) Neuronal sources of hedgehog modulate neurogenesis in the adult planarian brain. *eLife* 5:1–18. <https://doi.org/10.7554/eLife.19735>
 37. Currie KW, Pearson BJ (2013) Transcription factors *lhx1/5-1* and *pitx* are required for the maintenance and regeneration of serotonergic neurons in planarians. *Development (Cambridge, England)* 140:3577–88. <https://doi.org/10.1242/dev.098590>
 38. Molina MD, Abduljabbar D, Guixeras A, et al (2023) LIM-HD transcription factors control axial patterning and specify distinct neuronal and intestinal cell identities in planarians. *Open Biology* 13:. <https://doi.org/10.1098/rsob.230327>
 39. Forsthoefel DJ, Park AE, Newmark PA (2011) Stem cell-based growth, regeneration, and remodeling of the planarian intestine. *Developmental Biology* 356:445–459. <https://doi.org/10.1016/j.ydbio.2011.05.669>
 40. Rink JC, Vu HTK, Alvarado AS (2011) The maintenance and regeneration of the planarian excretory system are regulated by EGFR signaling. *Development* 138:3769–3780. <https://doi.org/10.1242/dev.066852>
 41. Scimone ML, Wurtzel O, Malecek K, et al (2018) *foxF-1* Controls Specification of Non-body Wall Muscle and Phagocytic Cells in Planarians. *Current Biology* 28:3787–3801.e6. <https://doi.org/10.1016/j.cub.2018.10.030>
 42. Rieger RM, Tyler S, Smith JPI, Rieger GE (1991) *Platyhelminthes: Turbellaria. A Microscopic Anatomy of Invertebrates, Vol. 3: Platyhelminthes and Nemertinea*. Wiley-Liss, New York
 43. SKAER RJ (1965) the Origin and Continuous Replacement of Epidermal Cells in the. *Journal of embryology and experimental morphology* 13:129–139
 44. Tu KC, Cheng LC, Vu HTK, et al (2015) *Egr-5* is a post-mitotic regulator of planarian epidermal differentiation. *eLife* 4:1–27. <https://doi.org/10.7554/eLife.10501>
 45. Eisenhoffer GT, Kang H, Alvarado AS (2008) Molecular Analysis of Stem Cells and Their Descendants during Cell Turnover and Regeneration in the Planarian *Schmidtea*

- mediterranea. *Cell Stem Cell* 3:327–339. <https://doi.org/10.1016/j.stem.2008.07.002>
46. Xu T, Wang W, Zhang S, et al (1995) Identifying tumor suppressors in genetic mosaics: the *Drosophila* *lats* gene encodes a putative protein kinase. *Development* 121:1053–63. <https://doi.org/10.1101/gad.3.9.1273>
 47. Lu L, Li Y, Kim SM, et al (2010) Hippo signaling is a potent in vivo growth and tumor suppressor pathway in the mammalian liver. *Proceedings of the National Academy of Sciences* 107:1437–1442. <https://doi.org/10.1073/pnas.0911427107>
 48. Sarikaya DP, Extavour CG (2015) The Hippo Pathway Regulates Homeostatic Growth of Stem Cell Niche Precursors in the *Drosophila* Ovary. *PLoS Genetics* 11:1–28. <https://doi.org/10.1371/journal.pgen.1004962>
 49. Panciera T, Azzolin L, Fujimura A, et al (2016) Induction of Expandable Tissue-Specific Stem/Progenitor Cells through Transient Expression of YAP/TAZ. *Cell Stem Cell* 19:725–737. <https://doi.org/10.1016/j.stem.2016.08.009>
 50. Yin MX, Zhang L (2014) Hippo signaling in epithelial stem cells. *Acta Biochimica et Biophysica Sinica* 47:39–45. <https://doi.org/10.1093/abbs/gmu111>
 51. Edgar BA (2006) From cell structure to transcription: Hippo Forges a new path. *Cell* 124:267–273. <https://doi.org/10.1016/j.cell.2006.01.005>
 52. Zhao B, Tumaneng K, Guan K-L (2011) The Hippo pathway in organ size control, tissue regeneration and stem cell self-renewal. *Nature Cell Biology* 13:877–883. <https://doi.org/10.1038/ncb2303>
 53. Dupont S (2016) Role of YAP/TAZ in cell-matrix adhesion-mediated signalling and mechanotransduction. *Experimental Cell Research* 343:42–53. <https://doi.org/10.1016/j.yexcr.2015.10.034>
 54. Dupont S, Morsut L, Aragona M, et al (2011) Role of YAP/TAZ in mechanotransduction. *Nature* 474:179–184. <https://doi.org/10.1038/nature10137>
 55. Currie KW, Brown DDDR, Zhu S, et al (2016) HOX gene complement and expression in the planarian *Schmidtea mediterranea*. *EvoDevo* 7:2663–2670. <https://doi.org/10.1186/s13227-016-0044-8>
 56. Silva E, Tsatskis Y, Gardano L, et al (2006) The Tumor-Suppressor Gene *fat* Controls Tissue Growth Upstream of *Expanded* in the Hippo Signaling Pathway. *Current Biology* 16:2081–2089. <https://doi.org/10.1016/j.cub.2006.09.004>
 57. Peng HW, Slattery M, Mann RS (2009) Transcription factor choice in the Hippo signaling pathway: Homothorax and yorkie regulation of the microRNA *bantam* in the progenitor domain of the *Drosophila* eye imaginal disc. *Genes and Development* 23:2307–2319. <https://doi.org/10.1101/gad.1820009>
 58. Goulev Y, Fauny JD, Gonzalez-Marti B, et al (2008) SCALLOPED Interacts with YORKIE, the Nuclear Effector of the Hippo Tumor-Suppressor Pathway in *Drosophila*. *Current Biology* 18:435–441. <https://doi.org/10.1016/j.cub.2008.02.034>
 59. Chen Y, Han H, Seo G, et al (2020) Systematic analysis of the Hippo pathway organization and oncogenic alteration in evolution. *Scientific reports* 10:3173. <https://doi.org/10.1038/s41598-020-60120-4>
 60. Sebe-Pedrs A, Zheng Y, Ruiz-Trillo I, Pan D (2012) Premetazoan Origin of the Hippo Signaling Pathway. *Cell Reports* 1:13–20. <https://doi.org/10.1016/j.celrep.2011.11.004>
 61. Martin-Belmonte F, Perez-Moreno M (2012) Epithelial cell polarity, stem cells and cancer. *Nature Reviews Cancer* 12:23–38. <https://doi.org/10.1038/nrc3169>

62. Grzeschik NA, Parsons LM, Allott ML, et al (2010) Lgl, aPKC, and Crumbs Regulate the Salvador/Warts/Hippo Pathway through Two Distinct Mechanisms. *Current Biology* 20:573–581. <https://doi.org/10.1016/j.cub.2010.01.055>
63. Lehman K, Rossi G, Adamo JE, Brennwald P (1999) Yeast Homologues of Tomosyn and lethal giant larvae Function in Exocytosis and Are Associated with the Plasma Membrane SNARE , Sec9. *Journal of Cell Biology* 146:125–140
64. Zhang X, Wang P, Gangar A, et al (2005) Lethal giant larvae proteins interact with the exocyst complex and are involved in polarized exocytosis. *Journal of Cell Biology* 170:273–283. <https://doi.org/10.1083/jcb.200502055>
65. Barros CS, Phelps CB, Brand AH (2003) Drosophila nonmuscle myosin II promotes the asymmetric segregation of cell fate determinants by cortical exclusion rather than active transport. *Developmental Cell* 5:829–840. [https://doi.org/10.1016/S1534-5807\(03\)00359-9](https://doi.org/10.1016/S1534-5807(03)00359-9)
66. Kagami M, Toh-e A, Matsui Y (1998) Protein , Is Related to Myosins in Function
67. Vasioukhin V (2006) Lethal Giant Puzzle of Lgl. *Developmental Neuroscience* 28:13–24. <https://doi.org/10.1159/000090749>
68. Baggiolini A, Varum S, Mateos JM, et al (2015) Premigratory and migratory neural crest cells are multipotent in vivo. *Cell Stem Cell* 16:314–322. <https://doi.org/10.1016/j.stem.2015.02.017>
69. Buitrago-Delgado E, Nordin K, Rao A, et al (2015) Shared regulatory programs suggest retention of blastula-stage potential in neural crest cells. *Science* 348:1332–1335. <https://doi.org/10.1126/science.aaa3655>
70. Nieto MA, Huang RYYJ, Jackson RAA, Thiery JPP (2016) Emt: 2016. *Cell* 166:21–45. <https://doi.org/10.1016/j.cell.2016.06.028>
71. Nieto MA, Sargent MG, Wilkinson DG, Cooke J (1994) Control of cell behavior during vertebrate development by Slug, a zinc finger gene. *Science* 264:835–839. <https://doi.org/10.1126/science.7513443>
72. Zhao B, Lei QY, Guan KL (2008) The Hippo-YAP pathway: new connections between regulation of organ size and cancer. *Current Opinion in Cell Biology* 20:638–646. <https://doi.org/10.1016/j.ceb.2008.10.001>
73. Moroishi T, Hansen CG, Guan KL (2015) The emerging roles of YAP and TAZ in cancer. *Nature Reviews Cancer* 15:73–79. <https://doi.org/10.1038/nrc3876>
74. O'Hayre M, Vázquez-Prado J, Kufareva I, et al (2013) The emerging mutational landscape of G proteins and G-protein-coupled receptors in cancer. *Nature Reviews Cancer* 13:412–424. <https://doi.org/10.1038/nrc3521>
75. Kandoth C, McLellan MD, Vandin F, et al (2013) Mutational landscape and significance across 12 major cancer types. *Nature* 502:333–339. <https://doi.org/10.1038/nature12634>
76. Barry ER, Morikawa T, Butler BL, et al (2013) Restriction of intestinal stem cell expansion and the regenerative response by YAP. *Nature* 493:106–110. <https://doi.org/10.1038/nature11693>
77. Janse van Rensburg HJ, Yang X (2016) The roles of the Hippo pathway in cancer metastasis. *Cellular Signalling* 28:1761–1772. <https://doi.org/10.1016/j.cellsig.2016.08.004>
78. Johnson R, Halder G (2014) The two faces of Hippo: Targeting the Hippo pathway for

- regenerative medicine and cancer treatment. *Nature Reviews Drug Discovery* 13:63–79. <https://doi.org/10.1038/nrd4161>
79. Zheng Y, Pan D (2019) The Hippo Signaling Pathway in Development and Disease. *Developmental Cell* 50:264–282. <https://doi.org/10.1016/j.devcel.2019.06.003>
 80. Demircan T, Berezikov E (2013) The Hippo pathway regulates stem cells during homeostasis and regeneration of the flatworm *Macrostomum lignano*. *Stem cells and development* 22:2174–85. <https://doi.org/10.1089/scd.2013.0006>
 81. Lin AYT, Pearson BJ (2014) Planarian yorkie/YAP functions to integrate adult stem cell proliferation, organ homeostasis and maintenance of axial patterning. *Development* 141:1197–1208. <https://doi.org/10.1242/dev.101915>
 82. de Sousa N, Rodríguez-Esteban G, Rojo-Laguna JI, et al (2018) Hippo signaling controls cell cycle and restricts cell plasticity in planarians. *PLoS biology* 16:e2002399. <https://doi.org/10.1371/journal.pbio.2002399>
 83. Gorgoulis V, Adams PD, Alimonti A, et al (2019) Cellular Senescence: Defining a Path Forward. *Cell* 179:813–827. <https://doi.org/10.1016/j.cell.2019.10.005>
 84. Campisi J, D'Adda Di Fagagna F (2007) Cellular senescence: When bad things happen to good cells. *Nature Reviews Molecular Cell Biology* 8:729–740. <https://doi.org/10.1038/nrm2233>
 85. Campisi J (2013) Aging, cellular senescence, and cancer. *Annual Review of Physiology* 75:685–705. <https://doi.org/10.1146/annurev-physiol-030212-183653>
 86. Da Silva-álvarez S, Collado M (2016) Cellular Senescence. *Encyclopedia of Cell Biology* 3:511–517. <https://doi.org/10.1016/B978-0-12-394447-4.30066-9>
 87. Rodriguez-Brenes IA, Wodarz D, Komarova NL (2015) Quantifying replicative senescence as a tumor suppressor pathway and a target for cancer therapy. *Scientific Reports* 5:1–13. <https://doi.org/10.1038/srep17660>
 88. Kumari R, Jat P (2021) Mechanisms of Cellular Senescence: Cell Cycle Arrest and Senescence Associated Secretory Phenotype. *Frontiers in Cell and Developmental Biology* 9:1–24. <https://doi.org/10.3389/fcell.2021.645593>
 89. Debacq-chainiaux F, Ameer R Ben, Bauwens E, et al (2016) Cellular Ageing and Replicative Senescence. Springer International Publishing, Cham
 90. Yousefzadeh M, Henpita C, Vyas R, et al (2021) DNA damage—how and why we age? *eLife* 10:1–17. <https://doi.org/10.7554/eLife.62852>
 91. Olan I, Narita M (2022) Senescence: An Identity Crisis Originating from Deep Within the Nucleus. *Annual Review of Cell and Developmental Biology* 38:1–21. <https://doi.org/10.1146/annurev-cellbio-120420-013537>
 92. Rapp YG, Ransbotyn V, Grafi G (2015) Senescence meets dedifferentiation. *Plants* 4:356–368. <https://doi.org/10.3390/plants4030356>
 93. Milanovic M, Fan DNY, Belenki D, et al (2018) Senescence-associated reprogramming promotes cancer stemness. *Nature* 553:96–100. <https://doi.org/10.1038/nature25167>
 94. He S, Sharpless NE (2017) Senescence in Health and Disease. *Cell* 169:1000–1011. <https://doi.org/10.1016/j.cell.2017.05.015>
 95. Rodier F, Campisi J (2011) Four faces of cellular senescence. *Journal of Cell Biology* 192:547–556. <https://doi.org/10.1083/jcb.201009094>

96. Sharpless NE, Sherr CJ (2015) Forging a signature of in vivo senescence. *Nature Reviews Cancer* 15:397–408. <https://doi.org/10.1038/nrc3960>
97. Beauséjour CM, Krtolica A, Galimi F, et al (2003) Reversal of human cellular senescence: Roles of the p53 and p16 pathways. *EMBO Journal* 22:4212–4222. <https://doi.org/10.1093/emboj/cdg417>
98. Kuilman T, Peeper DS (2009) Senescence-messaging secretome: SMS-ing cellular stress. *Nature Reviews Cancer* 9:81–94. <https://doi.org/10.1038/nrc2560>
99. Acosta JC, Banito A, Wuestefeld T, et al (2013) A complex secretory program orchestrated by the inflammasome controls paracrine senescence. *Nature Cell Biology* 15:978–990. <https://doi.org/10.1038/ncb2784>
100. Wiley CD, Flynn JM, Morrissey C, et al (2017) Analysis of individual cells identifies cell-to-cell variability following induction of cellular senescence. *Aging Cell* 16:1043–1050. <https://doi.org/10.1111/ace.12632>
101. Shay JW, Wright WE (2019) Telomeres and telomerase: three decades of progress. *Nature Reviews Genetics* 20:299–309. <https://doi.org/10.1038/s41576-019-0099-1>
102. De Lange T (2018) Shelterin-mediated telomere protection. *Annual Review of Genetics* 52:223–247. <https://doi.org/10.1146/annurev-genet-032918-021921>
103. D'Adda Di Fagagna F (2008) Living on a break: Cellular senescence as a DNA-damage response. *Nature Reviews Cancer* 8:512–522. <https://doi.org/10.1038/nrc2440>
104. Robbins E, Levine EM, Eagle H (1970) Morphologic changes accompanying senescence of cultured human diploid cells. 148–162
105. Hernandez-Segura A, Nehme J, Demaria M (2018) Hallmarks of Cellular Senescence. *Trends in Cell Biology* 28:436–453. <https://doi.org/10.1016/j.tcb.2018.02.001>
106. McHugh D, Gil J (2018) Senescence and aging: Causes, consequences, and therapeutic avenues. *Journal of Cell Biology* 217:65–77. <https://doi.org/10.1083/jcb.201708092>
107. Freund A, Laberge RM, Demaria M, Campisi J (2012) Lamin B1 loss is a senescence-associated biomarker. *Molecular Biology of the Cell* 23:2066–2075. <https://doi.org/10.1091/mbc.E11-10-0884>
108. Shimi T, Butin-Israeli V, Adam SA, et al (2011) The role of nuclear lamin B1 in cell proliferation and senescence. *Genes and Development* 25:2579–2593. <https://doi.org/10.1101/gad.179515.111>
109. Salama R, Sadaie M, Hoare M, Narita M (2014) Cellular senescence and its effector programs. *Genes and Development* 28:99–114. <https://doi.org/10.1101/gad.235184.113>
110. Chandra T, Ewels PA, Schoenfelder S, et al (2015) Global reorganization of the nuclear landscape in senescent cells. *Cell Reports* 10:471–483. <https://doi.org/10.1016/j.celrep.2014.12.055>
111. Criscione SW, Teo YV, Neretti N (2016) The Chromatin Landscape of Cellular Senescence. *Trends in Genetics* 32:751–761. <https://doi.org/10.1016/j.tig.2016.09.005>
112. Adams PD, Ivanov A, Pawlikowski J, et al (2013) Lysosome-mediated processing of chromatin in senescence. *Journal of Cell Biology* 202:129–143. <https://doi.org/10.1083/jcb.201212110>
113. González-Gualda E, Baker AG, Fruk L, Muñoz-Espín D (2021) A guide to assessing

- cellular senescence in vitro and in vivo. *The FEBS Journal* 288:56–80. <https://doi.org/10.1111/febs.15570>
114. Da Silva-Álvarez S, Picallos-Rabina P, Antelo-Iglesias L, et al (2019) The development of cell senescence. *Experimental Gerontology* 128:110742. <https://doi.org/10.1016/j.exger.2019.110742>
 115. Muñoz-Espín D, Cañamero M, Maraver A, et al (2013) Programmed cell senescence during mammalian embryonic development. *Cell* 155:1104. <https://doi.org/10.1016/j.cell.2013.10.019>
 116. Storer M, Mas A, Robert-Moreno A, et al (2013) Senescence is a developmental mechanism that contributes to embryonic growth and patterning. *Cell* 155:1119. <https://doi.org/10.1016/j.cell.2013.10.041>
 117. Da Silva-Álvarez S, Guerra-Varela J, Sobrido-Cameán D, et al (2020) Cell senescence contributes to tissue regeneration in zebrafish. *Aging Cell* 19:1–5. <https://doi.org/10.1111/ace.13052>
 118. Yun MH, Davaapil H, Brookes JP (2015) Recurrent turnover of senescent cells during regeneration of a complex structure. *eLife* 4:1–16. <https://doi.org/10.7554/eLife.05505>
 119. Azevedo AS, Grotek B, Jacinto A, et al (2011) The regenerative capacity of the zebrafish caudal fin is not affected by repeated amputations. *PLoS ONE* 6:1–8. <https://doi.org/10.1371/journal.pone.0022820>
 120. Antelo-Iglesias L, Picallos-Rabina P, Estévez-Souto V, et al (2021) The role of cellular senescence in tissue repair and regeneration. *Mechanisms of Ageing and Development* 198:. <https://doi.org/10.1016/j.mad.2021.111528>
 121. Ritschka B, Knauer-Meyer T, Gonçalves DS, et al (2020) The senotherapeutic drug ABT-737 disrupts aberrant p21 expression to restore liver regeneration in adult mice. *Genes and Development* 34:489–494. <https://doi.org/10.1101/gad.332643.119>
 122. Mylonas KJ, O'Sullivan ED, Humphries D, et al (2021) Cellular senescence inhibits renal regeneration after injury in mice, with senolytic treatment promoting repair. *Science Translational Medicine* 13:1–13. <https://doi.org/10.1126/scitranslmed.abb0203>
 123. Da Silva-Álvarez S, Collado M (2021) The Jekyll and Hyde of Senescence in Cancer: TIMP1 Controls the Switch from Tumor-Controlling to Tumor-Promoting Senescence. *Cancer Cell* 39:13–15. <https://doi.org/10.1016/j.ccell.2020.12.013>
 124. Chen B, Zhu R, Hu H, et al (2022) Elimination of Senescent Cells by Senolytics Facilitates Bony Endplate Microvessel Formation and Mitigates Disc Degeneration in Aged Mice. *Frontiers in Cell and Developmental Biology* 10:1–13. <https://doi.org/10.3389/fcell.2022.853688>
 125. Chen J, Jin S, Abraham V, et al (2011) The Bcl-2/Bcl-X L/Bcl-w inhibitor, navitoclax, enhances the activity of chemotherapeutic agents in vitro and in vivo. *Molecular Cancer Therapeutics* 10:2340–2349. <https://doi.org/10.1158/1535-7163.MCT-11-0415>
 126. Wang R, Yu Z, Sunchu B, et al (2017) Rapamycin inhibits the secretory phenotype of senescent cells by a Nrf2-independent mechanism. *Aging Cell* 16:564–574. <https://doi.org/10.1111/ace.12587>
 127. Perrigue PM, Najbauer J, Jozwiak AA, et al (2015) Planarians as a model of aging to study the interaction between stem cells and senescent cells in vivo. *Pathobiology of Aging & Age-related Diseases* 5:30052. <https://doi.org/10.3402/pba.v5.30052>
 128. Shiroor DA, Wang K, Sanketi BD, et al (2023) Inhibition of ATM kinase rescues

- planarian regeneration after lethal radiation . *EMBO reports* 24:1–15.
<https://doi.org/10.15252/embr.202256112>
129. Yang Y, Akinci E, Dutton JR, et al (2013) Stage specific reprogramming of mouse embryo liver cells to a beta cell-like phenotype. *Mechanisms of Development* 130:602–612. <https://doi.org/10.1016/j.mod.2013.08.002>
 130. Roostaei A, Benoit YD, Boudjadi S, Beaulieu JF (2016) Epigenetics in Intestinal Epithelial Cell Renewal. *Journal of Cellular Physiology* 231:2361–2367. <https://doi.org/10.1002/jcp.25401>
 131. Raff M (2003) Adult Stem Cell Plasticity: Fact or Artifact? *Annual Review of Cell and Developmental Biology* 19:1–22. <https://doi.org/10.1146/annurev.cellbio.19.111301.143037>
 132. Marión RM, López de Silanes I, Mosteiro L, et al (2017) Common Telomere Changes during In Vivo Reprogramming and Early Stages of Tumorigenesis. *Stem Cell Reports* 8:460–475. <https://doi.org/10.1016/j.stemcr.2017.01.001>
 133. Puri D, Wagner W (2023) Epigenetic rejuvenation by partial reprogramming. *BioEssays* 45:1–8. <https://doi.org/10.1002/bies.202200208>
 134. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126:663–76. <https://doi.org/10.1016/j.cell.2006.07.024>
 135. Patel SH, Camargo FD, Yimlamai D (2017) Hippo Signaling in the Liver Regulates Organ Size, Cell Fate, and Carcinogenesis. *Gastroenterology* 152:533–545. <https://doi.org/10.1053/j.gastro.2016.10.047>
 136. Jopling C, Boue S, Belmonte JCI (2011) Dedifferentiation, transdifferentiation and reprogramming: Three routes to regeneration. *Nature Reviews Molecular Cell Biology* 12:79–89. <https://doi.org/10.1038/nrm3043>
 137. Waddington CH (2014) *The Strategy of the Genes*, 1st Editio. Routledge
 138. Rinkevich B, Ballarin L, Martinez P, et al (2022) A pan-metazoan concept for adult stem cells: the wobbling Penrose landscape. *Biological Reviews* 97:299–325. <https://doi.org/10.1111/brv.12801>
 139. Alberts, B., Johnson A., Lewis J., Raff, M., Roberts, K., and Walter P (2002) *Molecular Biology of the Cell*, 4th Editio. Garland Science, New York
 140. Cooper GM (2000) *The Cell: A Molecular Approach*, 2nd Editio. Sinauer Associates, Sunderland (MA)
 141. Smets B, Ghillebert R, De Snijder P, et al (2010) Life in the midst of scarcity: Adaptations to nutrient availability in *Saccharomyces cerevisiae*
 142. Mitra K, Wunder C, Roysam B, et al (2009) A hyperfused mitochondrial state achieved at G1-S regulates cyclin E buildup and entry into S phase. *Proceedings of the National Academy of Sciences of the United States of America* 106:11960–11965. <https://doi.org/10.1073/pnas.0904875106>
 143. Schafer KA (1998) The cell cycle: A Review.pdf. *Vet Pathol* 35:461–478. <https://doi.org/10.1177/030098589803500601>
 144. Grohme MA, Schloissnig S, Rozanski A, et al (2018) The genome of *Schmidtea mediterranea* and the evolution of core cellular mechanisms. *Nature* 554:56–61. <https://doi.org/10.1038/nature25473>

145. Zhu SJ, Pearson BJ (2013) The Retinoblastoma pathway regulates stem cell proliferation in freshwater planarians. *Developmental Biology* 373:442–452. <https://doi.org/10.1016/j.ydbio.2012.10.025>
146. Pearson BJ, Alvarado AS (2010) A planarian p53 homolog regulates proliferation and self-renewal in adult stem cell lineages. *Development* 137:213–221. <https://doi.org/10.1242/dev.044297>
147. Drummond-barbosa D, Spradling AC (2001) Stem Cells and Their Progeny Respond to Nutritional Changes during *Drosophila* Oogenesis. *Developmental Biology* 278:265–278. <https://doi.org/10.1006/dbio.2000.0135>
148. Narbonne P, Roy R (2006) Regulation of germline stem cell proliferation downstream of nutrient sensing. *Cell Division* 1:1–9. <https://doi.org/10.1186/1747-1028-1-29>
149. Wullschlegel S, Loewith R, Hall MN (2006) TOR Signaling in Growth and Metabolism. *Cell* 124:471–484. <https://doi.org/10.1016/j.cell.2006.01.016>
150. Fingar DC, Blenis J (2004) Target of rapamycin (TOR): an integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression. *Oncogene* 3151–3171. <https://doi.org/10.1038/sj.onc.1207542>
151. Jorgensen P, Rupeš I, Sharom JR, et al (2004) A dynamic transcriptional network communicates growth potential to ribosome synthesis and critical cell size. *Genes & Development* 2491–2505. <https://doi.org/10.1101/gad.1228804>.)
152. Laplante M, Sabatini DM (2012) mTOR Signaling in Growth Control and Disease. *Cell* 149:274–293. <https://doi.org/10.1016/j.cell.2012.03.017>
153. Saxton RA, Sabatini DM (2017) mTOR Signaling in Growth, Metabolism, and Disease. *Cell* 168:960–976. <https://doi.org/10.1016/j.cell.2017.03.035>
154. Song MS, Salmena L, Pandolfi PP (2012) The functions and regulation of the PTEN tumour suppressor. *Nature Reviews Molecular Cell Biology* 13:283–296. <https://doi.org/10.1038/nrm3330>
155. Chen H, Ma Z, Vanderwaal RP, et al (2011) The mTOR Inhibitor Rapamycin Suppresses DNA Double-Strand Break Repair. 224:214–224. <https://doi.org/10.1667/RR2323.1>
156. Plo I, Laulier C, Gauthier L, et al (2008) AKT1 Inhibits Homologous Recombination by Inducing Cytoplasmic Retention of BRCA1 and RAD51. 5:9404–9412. <https://doi.org/10.1158/0008-5472.CAN-08-0861>
157. Baguñà J (1976) Mitosis in the Intact and Regenerating Planarian *Dugesia mediterranea* n . sp . *Journal of Experimental Zoology* 195:53–64
158. González-Estévez C, Felix DA, Rodríguez-Esteban G, Aziz Aboobaker A (2012) Decreased neoblast progeny and increased cell death during starvation-induced planarian degrowth. *International Journal of Developmental Biology* 56:83–91. <https://doi.org/10.1387/ijdb.113452cg>
159. Pellettieri J, Fitzgerald P, Watanabe S, et al (2010) Cell death and tissue remodeling in planarian regeneration. *Developmental Biology* 338:76–85. <https://doi.org/10.1016/j.ydbio.2009.09.015>
160. Miller CM, Newmark PA (2012) An insulin-like peptide regulates size and adult stem cells in planarians. *International Journal of Developmental Biology* 56:75–82. <https://doi.org/10.1387/ijdb.113443cm>
161. González-Estévez C, Felix DA, Smith MD, et al (2012) SMG-1 and mTORC1 Act

- Antagonistically to Regulate Response to Injury and Growth in Planarians. *PLoS Genetics* 8:e1002619. <https://doi.org/10.1371/journal.pgen.1002619>
162. Peiris TH, Weckerle F, Ozamoto E, et al (2012) TOR signaling regulates planarian stem cells and controls localized and organismal growth. *Journal of Cell Science* 125:1657–1665. <https://doi.org/10.1242/jcs.104711>
 163. Oviedo NJ, Pearson BJ, Levin M, Alvarado AS (2008) Planarian PTEN homologs regulate stem cells and regeneration through TOR signaling. *DMM Disease Models and Mechanisms* 1:131–143. <https://doi.org/10.1242/dmm.000117>
 164. Felix DA, Gutiérrez-gutiérrez Ó, Espada L, et al (2019) Seminars in Cell & Developmental Biology It is not all about regeneration: Planarians striking power to stand starvation. *Seminars in Cell and Developmental Biology* 87:169–181. <https://doi.org/10.1016/j.semcd.2018.04.010>
 165. Ferrai C, Schulte C (2024) Mechanotransduction in stem cells. *European Journal of Cell Biology* 103:151417. <https://doi.org/10.1016/j.ejcb.2024.151417>
 166. Sur-Erdem I, Hussain MS, Asif M, et al (2020) Nesprin-1 impact on tumorigenic cell phenotypes. *Molecular Biology Reports* 47:921–934. <https://doi.org/10.1007/s11033-019-05184-w>
 167. Kwan S, Sheel A, Song C, et al (2020) Depletion of TRRAP Induces p53-Independent Senescence in Liver Cancer by Down-Regulating Mitotic Genes. *Hepatology* 71:275–290. <https://doi.org/10.1002/hep.30807>
 168. Rodier F, Muñoz DP, Teachenor R, et al (2011) DNA-SCARS: Distinct nuclear structures that sustain damage-induced senescence growth arrest and inflammatory cytokine secretion. *Journal of Cell Science* 124:68–81. <https://doi.org/10.1242/jcs.071340>
 169. Ben-Porath I, Weinberg RA (2005) The signals and pathways activating cellular senescence. *International Journal of Biochemistry and Cell Biology* 37:961–976. <https://doi.org/10.1016/j.biocel.2004.10.013>
 170. Agrawal R, Singh A, Giri J, et al (2023) MEDIATOR SUBUNIT17 is required for transcriptional optimization of root system architecture in Arabidopsis. *Plant physiology* 192:1548–1568. <https://doi.org/10.1093/plphys/kiad129>
 171. Fan J, Beck KA (2004) A role for the spectrin superfamily member syne-1 and kinesin II in cytokinesis. *Journal of Cell Science* 117:619–629. <https://doi.org/10.1242/jcs.00892>
 172. Parsons L, Grzeschik N, Richardson H (2014) Igl Regulates the Hippo Pathway Independently of Fat/Dachs, Kibra/Expanded/Merlin and dRASSF/dSTRIPAK. *Cancers* 6:879–896. <https://doi.org/10.3390/cancers6020879>
 173. Cao F, Miao Y, Xu K, Liu P (2015) Lethal (2) giant larvae: An indispensable regulator of cell polarity and cancer development. *International Journal of Biological Sciences* 11:380–389. <https://doi.org/10.7150/ijbs.11243>
 174. Whyte WA, Orlando DA, Hnisz D, et al (2013) Master transcription factors and mediator establish super-enhancers at key cell identity genes. *Cell* 153:307–319. <https://doi.org/10.1016/j.cell.2013.03.035>
 175. Thandapani P (2019) Super-enhancers in cancer. *Pharmacology and Therapeutics* 199:129–138. <https://doi.org/10.1016/j.pharmthera.2019.02.014>
 176. King HO, Owusu-Boaitey KE, Fincher CT, Reddien PW (2024) A transcription factor

- atlas of stem cell fate in planarians. *Cell Reports* 43:113843. <https://doi.org/10.1016/j.celrep.2024.113843>
177. Park C, Owusu-Boaitey KE, Valdes GM, Reddien PW (2023) Fate specification is spatially intermingled across planarian stem cells. *Nature Communications* 14:7422. <https://doi.org/10.1038/s41467-023-43267-2>
 178. Kato Y, Takatori S, Akahori A, et al (2024) Protocol for gene knockdown using siRNA in primary cultured neonatal murine microglia. *STAR Protocols* 5:102867. <https://doi.org/10.1016/j.xpro.2024.102867>
 179. Fischer V, Schumacher K, Tora L, Devys D (2019) Global role for coactivator complexes in RNA polymerase II transcription. *Transcription* 10:29–36. <https://doi.org/10.1080/21541264.2018.1521214>
 180. Plaschka C, Larivière L, Wenzek L, et al (2015) Architecture of the RNA polymerase II-Mediator core initiation complex. *Nature* 518:376–380. <https://doi.org/10.1038/nature14229>
 181. Lynch CJ, Bernad R, Calvo I, Serrano M (2020) Manipulating the Mediator complex to induce naïve pluripotency. *Experimental Cell Research* 395:.. <https://doi.org/10.1016/j.yexcr.2020.112215>
 182. Lai AG, Pouchkina-Stantcheva N, Di Donfrancesco A, et al (2017) The protein subunit of telomerase displays patterns of dynamic evolution and conservation across different metazoan taxa. *BMC Evolutionary Biology* 17:1–21. <https://doi.org/10.1186/s12862-017-0949-4>
 183. Huang Z, Whelan C V., Dechmann D, Teeling EC (2020) Genetic variation between long-lived versus short-lived bats illuminates the molecular signatures of longevity. *Aging* 12:15962–15977. <https://doi.org/10.18632/aging.103725>
 184. Ritter B, Ferguson SM, De Camilli P, McPherson PS (2017) A lentiviral system for efficient knockdown of proteins in neuronal cultures. *MNI Open Research* 1:2. <https://doi.org/10.12688/mniopenres.12766.1>
 185. Warburg O (1956) On the origin of cancer cells. *Science* 123:309–314. <https://doi.org/10.1126/science.123.3191.309>
 186. Brand MD, Nicholls DG (2011) Assessing mitochondrial dysfunction in cells. *Biochemical Journal* 435:297–312. <https://doi.org/10.1042/BJ20110162>
 187. Eura Y, Ishihara N, Yokota S, Mihara K (2003) Two Mitofusin Proteins, Mammalian Homologues of FZO, with Distinct Functions Are Both Required for Mitochondrial Fusion. *Journal of Biochemistry* 134:333–344. <https://doi.org/10.1093/jb/mvg150>
 188. Osuma EA, Riggs DW, Gibb AA, Hill BG (2018) High throughput measurement of metabolism in planarians reveals activation of glycolysis during regeneration. *Regeneration* 5:78–86. <https://doi.org/10.1002/reg2.95>
 189. Vera E, Bernardes de Jesus B, Foronda M, et al (2013) Telomerase Reverse Transcriptase Synergizes with Calorie Restriction to Increase Health Span and Extend Mouse Longevity. *PLoS ONE* 8:.. <https://doi.org/10.1371/journal.pone.0053760>
 190. Lee S, Park YY, Kim SH, et al (2014) Human mitochondrial Fis1 links to cell cycle regulators at G2/M transition. *Cellular and Molecular Life Sciences* 71:711–725. <https://doi.org/10.1007/s00018-013-1428-8>
 191. Zorzano A, Liesa M, Sebastián D, et al (2010) Mitochondrial fusion proteins: Dual regulators of morphology and metabolism. *Seminars in Cell and Developmental Biology*

21:566–574. <https://doi.org/10.1016/j.semcd.2010.01.002>

192. Ge XY, Han X, Zhao YL, et al (2022) An insight into planarian regeneration. *Cell Proliferation* 55:1–14. <https://doi.org/10.1111/cpr.13276>
193. Senga SS, Grose RP (2021) Hallmarks of cancer—the new testament. *Open Biology* 11:0–2. <https://doi.org/10.1098/rsob.200358>
194. Pascual-Torner M, Carrero D, Perez-Silva JG, et al (2022) Comparative genomics of mortal and immortal cnidarians unveils novel keys behind rejuvenation. *Proceedings of the National Academy of Sciences of the United States of America* 119:1–8. <https://doi.org/10.1073/pnas.2118763119>
195. Lin TY, Gerber T, Taniguchi-Sugiura Y, et al (2021) Fibroblast dedifferentiation as a determinant of successful regeneration. *Developmental Cell* 56:1541–1551.e6. <https://doi.org/10.1016/j.devcel.2021.04.016>
196. Wang H, Simon A (2016) Skeletal muscle dedifferentiation during salamander limb regeneration. *Current Opinion in Genetics and Development* 40:108–112. <https://doi.org/10.1016/j.gde.2016.06.013>
197. Mason-Osann E, Gali H, Flynn RL (2019) Resolving roadblocks to telomere replication. *Methods in Molecular Biology* 1999:31–57. https://doi.org/10.1007/978-1-4939-9500-4_2

ANNEX: PLANARIAN CELL NUMBER DEPENDS ON *blitzschnell*, A NOVEL GENE FAMILY THAT BALANCES CELL PROLIFERATION AND CELL DEATH

STEM CELLS AND REGENERATION

RESEARCH ARTICLE

Planarian cell number depends on *blitzschnell*, a novel gene family that balances cell proliferation and cell death

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ABSTRACT

Control of cell number is crucial to define body size during animal development and to restrict tumoral transformation. The cell number is determined by the balance between cell proliferation and cell death. Although many genes are known to regulate those processes, the molecular mechanisms underlying the relationship between cell number and body size remain poorly understood. This relationship can be better understood by studying planarians, flatworms that continuously change their body size according to nutrient availability. We identified a novel gene family, *blitzschnell* (*bls*), that consists of *de novo* and taxonomically restricted genes that control cell proliferation: cell death ratio. Their silencing promotes faster regeneration and increases cell number during homeostasis. Importantly, this increase in cell number leads to an increase in body size only in a nutrient-rich environment; in starved planarians, silencing results in a decrease in cell size and cell accumulation that ultimately produces overgrowths. *bls* expression is downregulated after feeding and is related to activity of the insulin/Akt/mTOR network, suggesting that the *bls* family evolved in planarians as an additional mechanism for restricting cell number in nutrient-fluctuating environments.

KEY WORDS: Body size, Cell number, Growth, Regeneration, Overgrowth, mTOR

INTRODUCTION

During embryonic development, all species undergo dramatic increases in size until reaching a definitive body size, which is strikingly similar across species. The definitive body size of an organism is reached by increasing either cell number or cell size. Changes in cell size have been described in specific organs, e.g. imaginal discs in *Drosophila* (Miyaoaka et al., 2012; Hariharan, 2015). However, regulation of cell number, achieved by modulating the balance between cell death and cell proliferation, is the main mechanism by which animals reach their definitive body size (Guertin and Sabatini, 2006). Although conceptually simple, the developmental mechanisms that control cell number constitute one of the more intriguing issues in biology. In general, the main signaling pathways thought to control growth regulate cell proliferation and cell death in response to the nutritional

environment. Studies in multiple species have identified the same key signaling pathways that appear to regulate body size: the JNK pathway, the Hippo pathway and the insulin/Akt/mTOR signaling network. The JNK signaling pathway controls cell death and proliferation, mainly in response to cellular stress (Dhanasekaran and Reddy, 2017). The Hippo signaling pathway regulates proliferation, apoptosis and cell differentiation in response to mechanical stimuli (Udan et al., 2003; Zeng and Hong, 2008). Genetic perturbation of JNK or Hippo signaling leads to overgrowths or organ size changes, but does not affect overall body size (Tumaneng et al., 2012; Willsey et al., 2016). In contrast, activation of the insulin/Akt/mTOR signaling network leads to increases in body size in animals as distant as *Drosophila* and mice (Saxton and Sabatini, 2017). Insulin/Akt/mTOR signaling is the most conserved molecular mechanism that relates nutrient intake to cell proliferation (Gokhale and Shingleton, 2015), and it is the main regulator of cell size in response to cellular amino acid levels (González and Hall, 2017; Wolfson and Sabatini, 2017).

The study of planarians, flatworms that display extraordinary cell plasticity, can help further our understanding of the molecular mechanisms that control body size during development. In most animals, adult body size is determined by growth during embryonic and juvenile stages, while the adult stage consists of tissue renewal. However, long-living species, such as planarians, change their body size according to nutrient availability during their entire lives. These alterations in planarian body size are mediated by changes in cell number (Baguña and Romero, 1981; Thommen et al., 2019) resulting from modulation of the balance between cell proliferation and apoptosis. Thus, the ratio of proliferation to apoptosis decreases in starvation conditions and increases in times of nutritional abundance (Baguña, 1976; Pellettieri et al., 2010). Planarian plasticity is sustained by a population of pluripotent adult stem cells (neoblasts) that can differentiate into any planarian cell type. Furthermore, the balance between the stem cell population and all types of differentiated cells relies on robust signaling mechanisms that allow continuous adjustment of cell proliferation, cell death and cell differentiation. The signaling pathways that determine cell fate and tissue patterning in planarians, including the Wnt and BMP pathways, have been extensively studied (Molina et al., 2011; Sureda-Gómez et al., 2016). However, the mechanisms that control planarian body size and growth remain to be fully elucidated. The insulin/mTOR pathway is the only pathway demonstrated to control planarian body size. Inhibition of insulin-like peptides or TOR attenuates cell proliferation, prevents planarian growth after feeding and accelerates shrinking during starvation (Miller and Newmark, 2012; Tu et al., 2012). Hyper-activation of mTOR using *PTEN* or *smg-1* RNA interference (RNAi) does not give rise to larger organisms but does promote overproliferation and outgrowth formation (Oviedo et al., 2008; González-Estévez et al., 2012a,b). In planarians, *JNK* is required for organ remodeling through the

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induction of apoptotic cell death (Almuedo-Castillo et al., 2014). Moreover, *hippo* inhibition increases mitosis, inhibits apoptosis and promotes dedifferentiation, leading to the formation of overgrowths but not to changes in body size or cell number (de Sousa et al., 2018). Planarian plasticity not only facilitates adaptation to the environment but also enables reproduction: most species reproduce asexually through fissioning the tail, producing two segments from which two complete organisms regenerate. Amputation in planarians triggers tightly controlled apoptotic and mitotic responses (Baguña and Salo, 1984; Pellettieri et al., 2010; Wenemoser and Reddien, 2010). Silencing of several signaling pathways, such as the JNK or TOR pathways, results in the formation of smaller blastemas in which cell proliferation, apoptosis and/or differentiation is impaired (Peiris et al., 2012; Tu et al., 2012; Almuedo-Castillo et al., 2014). Interestingly, TOR hyper-activation gives rise to larger blastemas, although they remain undifferentiated (González-Estévez et al., 2012a,b), and Hippo hyper-activation enhances the wound response, promoting the expansion of cell populations (Lin and Pearson, 2017).

Here, we describe the identification of a novel gene family that we have named *blitzschnell* (*bls*), which controls cell proliferation and cell death in intact and regenerating planarians. The *bls* family consists of 11 genes and four pseudogenes that can be classified into five subfamilies (*bls1*–*bls5*). The family is composed of *de novo* genes, which appear to be taxonomically restricted to the order Tricladida (commonly known as planarians). Silencing of *bls2*/*bls3*/*bls5* promotes faster regeneration and increases cell number during homeostasis. However, this increase in cell number leads to an increase in body size only in nutrient-rich environments: during starvation cells are unable to maintain their normal size and become smaller than those of control animals. Importantly, expression of *bls2*, *bls3* and *bls5* is downregulated after nutrient intake, and it was found to be related to the insulin/Akt/mTOR network, suggesting that in planarians the *bls* family may have evolved as an additional mechanism to restrict cell number in nutrient-fluctuating environments.

RESULTS

blitzschnell is a new gene family organized in two clusters of tandem repeats in *Schmidtea mediterranea*

We performed an RNAi screen to find candidate genes involved in planarian eye regeneration, and identified an unknown gene whose inhibition resulted in faster regeneration of the eyes after head amputation. We named this gene *blitzschnell* (*bls*), which means ‘quick as a flash’ in German. Surprisingly, upon attempting to identify the genomic locus of this gene in *Schmidtea mediterranea*, we found that *bls* belongs to a gene family composed of 15 members distributed on two distinct genomic scaffolds (Fig. 1A, Table S1).

Although all *bls* sequences shared more than 70% identity (Table S2), a phylogenetic analysis using the nucleotide sequence allowed us to classify *bls* genes into five subfamilies (Fig. S1A). Four of these subfamilies (*bls1*, *bls2*, *bls4* and *bls5*) contained two putative genes each (named a and b), apparently derived from duplications. Subfamily *bls3* contained seven *bls* sequences (named *bls3a*–*bls3g*). These were also apparently derived from recent successive tandem duplications, as suggested by their genomic organization (Fig. 1A) and near identical DNA sequences (Table S1; Table S2). One band of the expected size was successfully amplified using primers spanning the junction between *bls3a* and *bls3b*, confirming the existence of at least two repeats (Fig. 1A, Fig. S1B, Table S3). Interestingly, in the repeated block harboring *bls3* members and in the vicinity of other *bls* genes, we identified complete or fragmentary transposon-related genes, including the genes encoding the reverse transcriptase RNase H and integrase (Fig. 1A).

By mapping reads from the transcriptome of intact planarians (de Sousa et al., 2018) against the genome of *S. mediterranea* (Grohme et al., 2018), we detected transcripts for subfamilies *bls2*, *bls3* and *bls5*, but not for subfamilies *bls1* or *bls4* (Fig. S1C). Furthermore, the predicted open reading frame (ORF) for *bls2*, *bls3* and *bls5* encoded peptides containing a N-terminal signal peptide (SP), suggesting that they could be secreted, and a highly conserved C-terminal coiled-coil (CC) domain (Fig. 1B, Table S4). Non-detectable transcription,

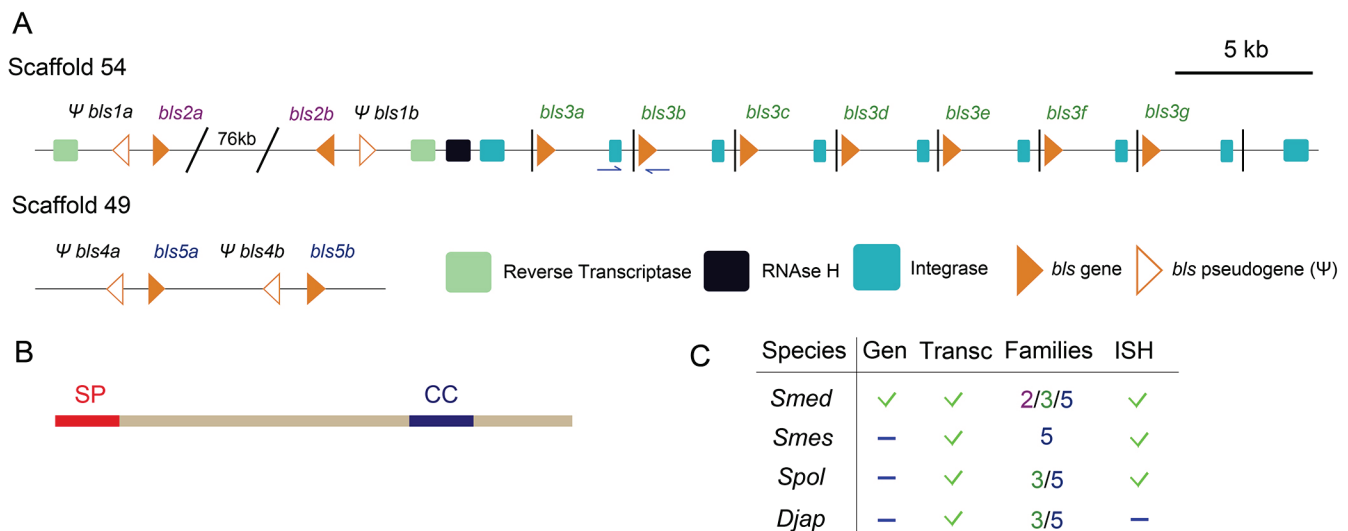


Fig. 1. The *bls* family is composed of 11 genes and four pseudogenes. (A) Cartoon illustrating the genomic organization of *Bls* family members. Subfamilies *bls1*, *bls2* and *bls3* are found in scaffold 54, and subfamilies *bls4* and *bls5* in scaffold 49. Primers used to amplify the junction of the first *bls3* repeats are indicated in blue. *bls* genes are represented by orange triangles. *bls* pseudogenes (Ψ) are represented by white and orange triangles. Transposon elements are indicated with squares. Scale bar indicates base pairs. (B) *Bls* protein domains: red, signal peptide (SP); blue, coiled coil (CC). (C) *bls* homologs found in the available genomic (Gen) and transcriptomic (Transc) datasets for planarian species. Expression as detected by *in situ* hybridization is indicated. Green tick indicates presence of *bls* homolog; blue line indicates no available data. ISH, *in situ* hybridization; *Smed*, *Schmidtea mediterranea* (asexual strain); *Smes*, *Schmidtea mediterranea* (sexual strain); *Spol*, *Schmidtea polychroa*; *Djap*, *Dugesia japonica*.

together with a much shorter ORF, strongly suggests that subfamilies *bls1* and *bls4* are made up of pseudogenes (Ψ).

Taken together, these data demonstrate that *bls* is a new gene family consisting of 11 genes and four pseudogenes. The genes encode very similar peptides that may be released into the extracellular space, as suggested by the presence of a signal peptide.

The *bls* family is taxonomically restricted to Tricladida

A BLAST search using *S. mediterranea* *bls* sequences against non-redundant transcriptomic and proteomic databases of all species (NCBI) produced no significant results. More-specific BLAST searches against genomic and transcriptomic datasets for Platyhelminth species (Egger et al., 2015) (NCBI and Planmine) indicated that homologs of the *bls* family are found only in species of the order Tricladida (planarians) (Fig. S1D; Table S5): *Schmidtea polychroa* (*Spol*), *Dugesia japonica* (*Djap*) and the sexual *S. mediterranea* strain (*Smes*). Although genomic databases are available for only a few Lophotrochozoa species, this result suggests that the *bls* family is taxonomically restricted to order Tricladida. Interestingly, a BLAST search of the available transcriptomic databases for Tricladida species returned more than one hit for those species (Fig. 1C), with a high degree of similarity at the nucleotide level (Table S6). Phylogenetic analysis performed with amino acid sequences (Table S7) revealed that the *bls5* subfamily was present in all Tricladida species studied; the *bls3* subfamily was present in *Smed*, *Spol*, and *Djap*; and the *bls2* subfamily was present only in *Smed* (Fig. 1C, Fig. S1E). However, it should be borne in mind that transcriptomic databases for Tricladida species other than *Smed* are incomplete. These findings suggest that the *bls* family is taxonomically restricted to Tricladida.

Subfamilies *bls2*, *bls3* and *bls5* are expressed in secretory cells

Although the three transcribed *bls* subfamilies (*bls2*, *bls3* and *bls5*) shared a high percentage of sequence identity at nucleotide level (Table S2), we designed riboprobes spanning different gene regions (Table S3, Table S8) to specifically detect genes from each subfamily. Whole-mount *in situ* hybridization with each riboprobe revealed the same pattern of expression and labelled specific dorsal-prepharyngeal cells (Fig. 2A, Fig. S2A). Double fluorescence *in situ* hybridization (FISH) revealed co-expression of genes from the three families in the same cells, although not presenting identical subcellular localization (Fig. S2B), confirming riboprobe specificity.

The *bls3* riboprobe revealed that *bls3*⁺ cells were located dorsally and in the marginal cells throughout the body (Fig. 2A). These *bls3*⁺ cells were differentiated, as they were insensitive to irradiation (Fig. S2C), and corresponded to secretory cells, as they co-expressed *dd4277*, a secretory and parenchymal cell marker (Fincher et al., 2018; Plass et al., 2018) (Fig. 2B). *bls3* was not expressed in blastemas during regeneration, but re-established its expression pattern according to the remodeling of the fragment in question (Fig. S2D). Interestingly, whole-mount *in situ* hybridization in sexual *S. mediterranea* (*Smes*) and *S. polychroa* (*Spol*) revealed the same expression pattern as observed for *Smed* (Fig. S2E), supporting a conserved function among Tricladida species (Fig. 1C). Taken together, our data indicate that genes from subfamilies *bls2*, *bls3* and *bls5* are expressed in a specific subpopulation of secretory prepharyngeal cells in planarians.

Inhibition of *bls* genes promotes faster regeneration

Because *bls2*, *bls3* and *bls5* genes share a high percentage of identity (Table S2), specific inhibition of any of these genes using

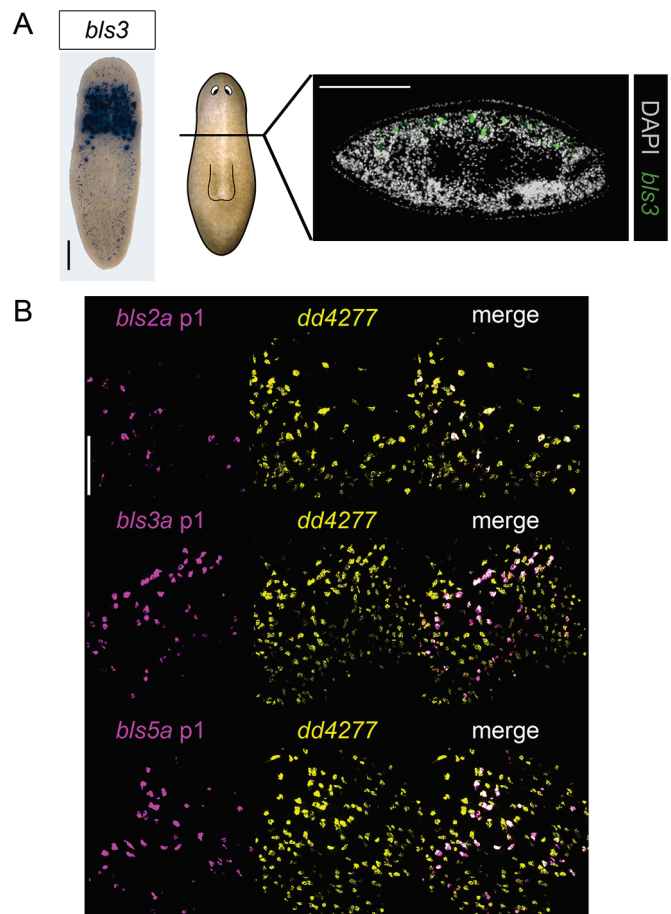


Fig. 2. *bls* expression pattern. (A) *bls3* expression in a transverse section, as detected by whole-mount *in situ* hybridization (blue) and fluorescent *in situ* hybridization (green). Nuclei are stained with DAPI. (B) *bls2*, *bls3* and *bls5* co-expression with *dd4277*. Scale bars: 200 μ m.

RNAi was technically impossible. Furthermore, the high level of shared identity and cellular colocalization (Fig. S2B) suggested that at least some paralogs may perform similar functions. For this reason we designed double-stranded RNAs (dsRNAs) corresponding to a highly conserved region in order to inhibit genes of each of the three subfamilies (Fig. S3B, Table S8). qPCR analysis using primers specific to each subfamily (Fig. S3B) showed that expression levels of each of the three subfamilies were downregulated after RNAi (Fig. S3A,C). Sequencing of the fragments amplified by each qPCR corroborated inhibition of the genes of each of the three subfamilies (see Materials and Methods; Fig. S3C). These animals are referred to henceforth as *bls2/3/5*(RNAi) animals.

RNAi of *bls2*, *bls3* and *bls5* confirmed our initial observation of faster regeneration after head amputation in planarians. We observed earlier differentiation of the eye spots (Fig. S3D), and earlier differentiation of photoreceptor cells (identified by anti-arrestin immunostaining): after 3 days of regeneration (3dR, the optic chiasm was visible in most *bls2/3/5*(RNAi) animals but not in control animals (Fig. 3). In addition to the visual system, other anterior structures, such as the brain branches and chemoreceptors, regenerated faster than controls (Fig. 3), as evidenced by quantification of *gpas*⁺ (Cebrià et al., 2002) and *cinillo*⁺ (Oviedo et al., 2003) cells, respectively. Quantification of *pitx*⁺ cells (Currie and Pearson, 2013; März et al., 2013) revealed an increase the

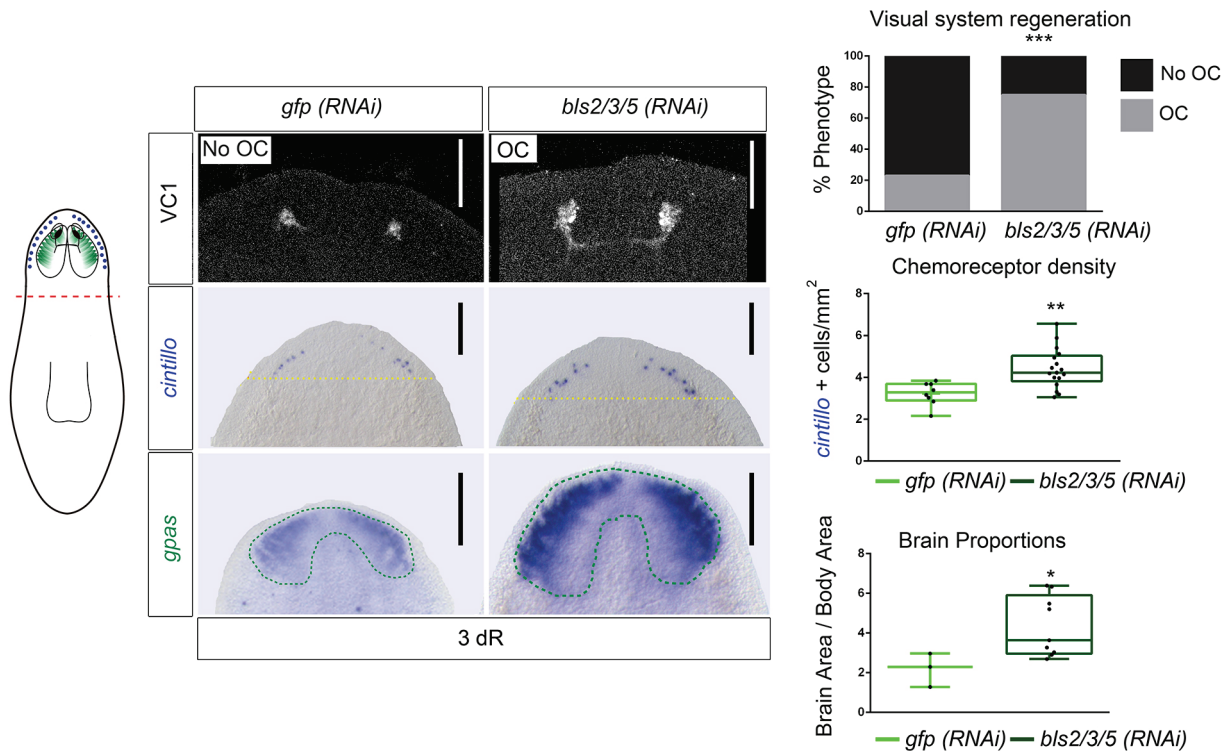


Fig. 3. Regeneration is accelerated in *bls2/3/5* RNAi planarians. Immunohistochemistry with anti-arrestin antibody (VC1), labelling the visual system; whole-mount *in situ* hybridization showing *cintillo* (chemoreceptors) and *gpas* (brain branches) expression. Illustration indicates areas of *gpas* and *cintillo* expression and positive arrestin (VC1) staining; dashed red line represents the level at which amputation was performed. Graphs show quantification of the appearance of the optic chiasm (controls, $n=23$; RNAi, $n=23$; *** $P<0.001$), *cintillo*⁺ cells/mm² (the area quantified is indicated with a yellow dashed line) (controls, $n=8$; RNAi, $n=17$, ** $P<0.01$) and *gpas*⁺ area/body area (*gpas*⁺ area is indicated with a green dashed line) (controls, $n=3$; RNAi, $n=9$; * $P<0.05$) in *gfp*(RNAi) and *bls2/3/5*(RNAi) animals at 3 days of regeneration (3 dR). Scale bars: 100 μ m.

number of differentiated neural cells in the blastema of *bls2/3/5*(RNAi) planarians as early as 18 h of regeneration (hR) (Fig. S3E). These results demonstrate that inhibition of *Smed-bls2/3/5* promotes faster regeneration.

***bls* genes attenuate cell proliferation and promotes cell death after injury**

To understand the mechanism by which *Smed-bls2/3/5*(RNAi) promotes faster regeneration, we analyzed the proliferative and apoptotic responses triggered by amputation. In planarians, amputation triggers a general proliferative response, which peaks at 6 hR, and a local response that peaks at 48 hR. Quantification of mitotic cells using an anti-phospho-histone 3 (PH3) antibody (Wenemoser and Reddien, 2010) revealed an increase in the mitotic response at both 6 hR and 48 hR in *bls2/3/5*(RNAi) versus control animals (Fig. 4A). The apoptotic response after amputation consists of two apoptotic peaks: one at 4 hR, which occurs close to the wound, and a second at 3 days of regeneration (dR), which is generalized (Pellettieri et al., 2010). Using a TUNEL assay (Fig. 4B) and by quantifying caspase 3 enzymatic activity (Fig. 4C), we demonstrated lower rates of apoptosis in *bls2/3/5*(RNAi) versus control planarians at both time points.

Distinct molecular and cellular responses are induced during healing of amputated tissue, notches (which imply tissue loss) and incisions (in which no tissue is removed). While control of cell proliferation and cell death is required in all scenarios, incision gives rise to just the first proliferative and apoptotic peaks. To examine how general the role of *bls2/3/5* is in attenuating cell proliferation and promoting cell death, we analyzed the response to notching and

incision in *bls2/3/5*(RNAi) animals. In both situations, when compared with control RNAi, animals showed an increase in the number of mitotic cells (Fig. S4A,C) and a decrease in apoptosis (Fig. S4B,D), indicating that *bls2/3/5* attenuates proliferation and promotes cell death. These findings indicate that *Smed-bls2/3/5* attenuates cell proliferation and promotes cell death after any injury type, regardless of whether tissue is removed.

Cells are more numerous but smaller in starved *bls*(RNAi) planarians, resulting in no overall change in body size

The pattern of *bls2*, *bls3* and *bls5* expression in secretory cells in the prepharyngeal region and along the planarian margin suggests that these peptides may play a role in controlling cell proliferation and cell death, not only after injury but also during homeostasis, as planarians undergo continuous growth and degrowth according to nutrient availability. These changes in size are thought to be primarily due to modulation of cell number (Baguña and Romero, 1981; Thommen et al., 2019) through regulation of the balance between proliferation and apoptosis (Pellettieri et al., 2010; González-Estévez et al., 2012a,b). In nutrient-poor environments planarians shrink by decreasing mitosis and increasing cell death. To determine whether *bls2/3/5* participates in regulating the proliferation/apoptosis equilibrium and body size during degrowth, we injected starved animals with *bls2/3/5* dsRNA for 3 weeks (Fig. 5A, Fig. S5A). The number of PH3⁺ nuclei found in starved *bls2/3/5*RNAi animals versus control was only increased after the third week (Fig. 5B, Fig. S5C). Importantly, starved *bls2/3/5*(RNAi) planarians showed decreased apoptosis as the first week of inhibition compared with controls (Fig. 5C, Fig. S5D).

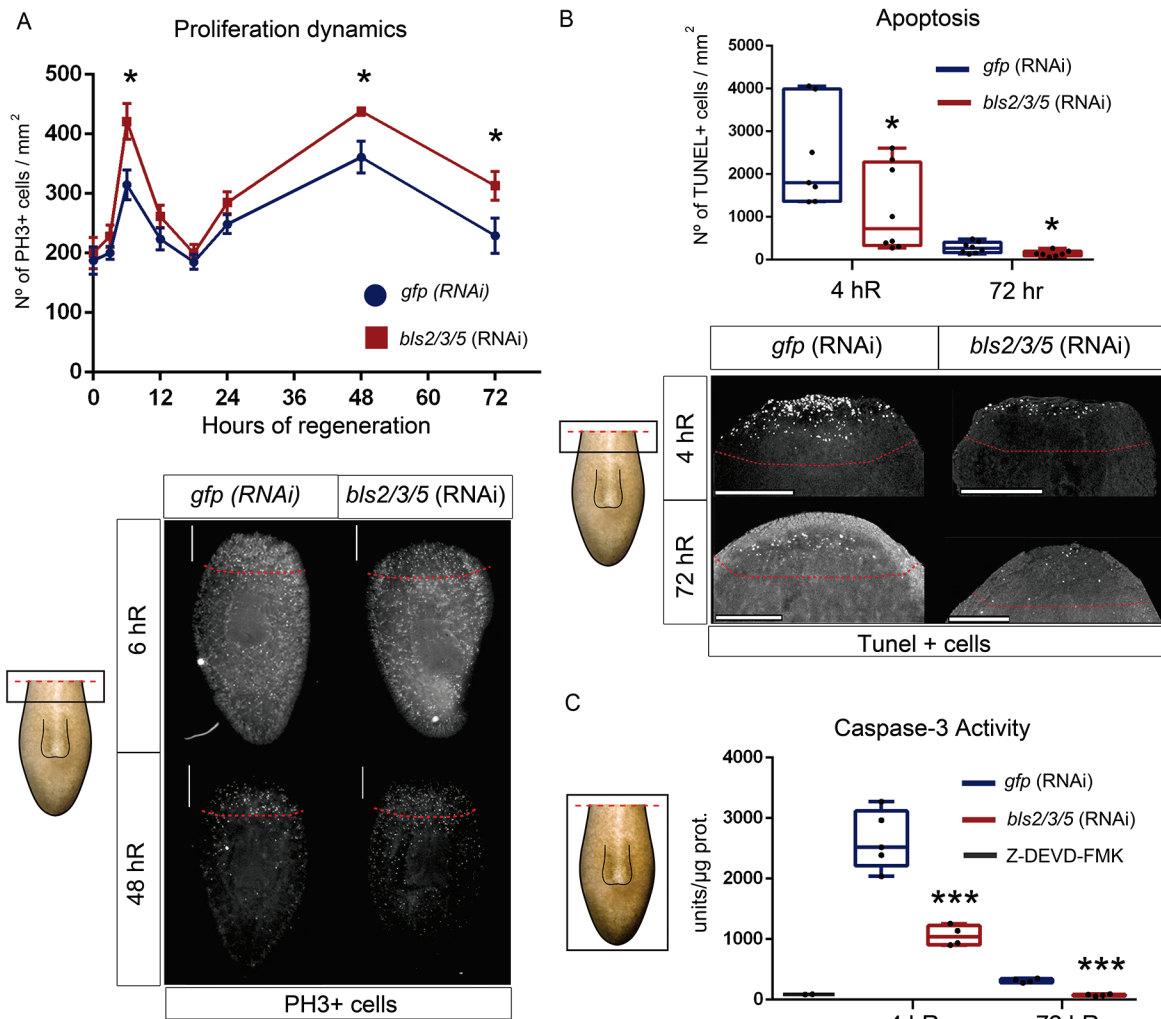


Fig. 4. After *bls2/3/5* RNAi, planarians exhibit increased proliferation and decreased apoptosis during anterior regeneration. (A) Quantification of PH3⁺ cells at different stages of regeneration (controls, $n > 5$; RNAi, $n > 5$; * $P < 0.05$). Lower panel shows anti-PH3 immunostaining of *gfp*(RNAi) and *bls2/3/5*(RNAi) animals. (B) Quantification of TUNEL⁺ cells in *bls2/3/5*(RNAi) and control animals (controls, $n > 7$; RNAi, $n > 7$; * $P < 0.05$). Lower panel shows TUNEL images. Images correspond to z projections. (C) Quantification of caspase 3 activity in *bls2/3/5*(RNAi) animals and controls (controls, $n = 4$; RNAi, $n = 4$; *** $P < 0.001$). In the schematic drawings, the red dashed line represents the amputation plane and the square indicates the region analyzed. In the images, the red dashed line outlines the area quantified. Scale bars: 500 μ m in A; 200 μ m in B.

Although this alteration in the proliferation/apoptosis equilibrium did not give rise to larger animals (Fig. 5D), total cell number was higher in RNAi-injected animals versus controls (Fig. 5E). The fact that total cell number but not body size was increased in *bls2/3/5*(RNAi) animals implies a decrease in cell size. To examine changes in cell size, we focused our analysis on the epidermis, as epidermal cells form a monolayer that can be easily imaged in three dimensions. Nuclear staining revealed a higher density of epithelial cells in *bls2/3/5*(RNAi) animals (Fig. 5F, Fig. S5E), and quantification of mean epidermal cell area confirmed that this parameter was reduced in *bls2/3/5*(RNAi) animals when compared with controls (Fig. 5F'). A decrease in mean epidermal cell area could be due not to a reduction in the total cell volume but to narrowing of the cells in *bls2/3/5*(RNAi) animals. To quantify epidermal cell volume, we measured epidermal cell height (i.e. the mean distance from the apical to the basal margin of the cell) in animals immunostained with 6G10 antibody. We observed no differences in apical-basal distance in *bls2/3/5*(RNAi) animals with respect to controls (Fig. 5G,G'). Multiplication of mean cell area by

mean cell height confirmed a decrease in epidermal cell volume in *bls2/3/5*(RNAi) animals versus controls (Fig. 5H,I). Changes in specific neural populations were evaluated by confocal imaging (Fig. 5J, Fig. S5F) and qPCR (Fig. S5G). The density of serotonergic (*pitx*⁺) (Currie and Pearson, 2013; März et al., 2013), octapaminergic (*tbh*⁺) (Eisenhoffer et al., 2008) and dopaminergic (*th*⁺) (Fraguas et al., 2011) neurons, and of chemoreceptors (*cintillo*⁺) (Oviedo et al., 2003) was increased in *bls2/3/5*(RNAi) animals. Given the increase in cell density found in *bls2/3/5*(RNAi) animals after 3 weeks of inhibition, it could be that the increase in PH3⁺ cells found at this time point results from the cell accumulation rather than from an effect of *bls2/3/5*(RNAi) on mitotic activity. This hypothesis is supported by the finding that the number of PH3⁺ cells/area normalized by epidermal cell density of *bls2/3/5*(RNAi) is not significantly different from controls after 2 and 3 weeks of inhibition (Fig. S5H).

Importantly, continuous inhibition of *bls2/3/5*(RNAi) for 4 weeks resulted in the formation of overgrowths that were all located in the posterior part of the animal, mainly in the dorso-

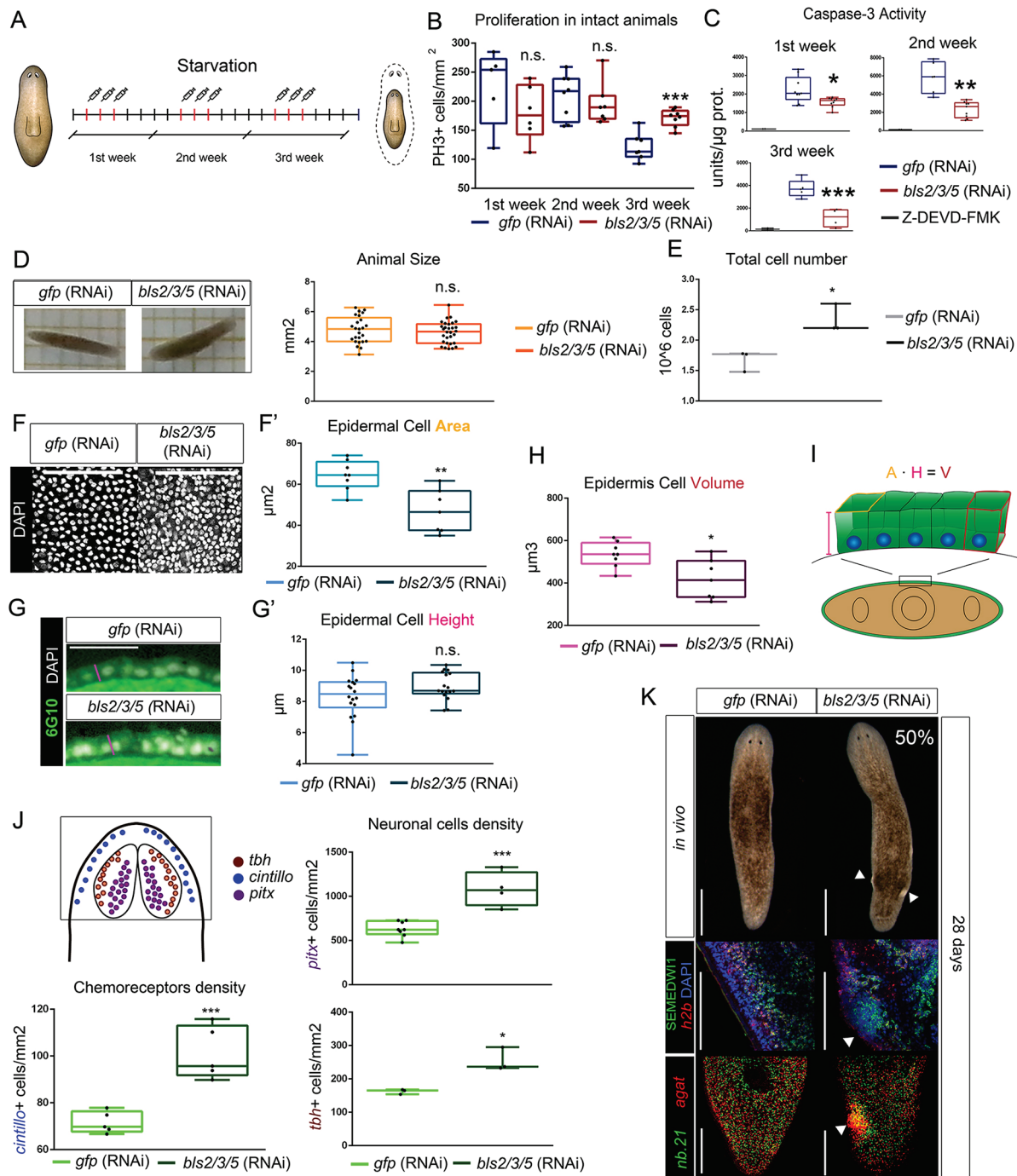


Fig. 5. In starving conditions, *bls2/3/5*(RNAi) animals show decreased cell death and cell accumulation but not an increase in body size. (A) Schematic depicting RNAi procedure. (B) Quantification of PH3+ cells after 1 week of RNAi treatment (controls, n=5; RNAi, n=6; n.s.); 2 weeks of RNAi treatment (controls, n=7; RNAi, n=6; n.s.); and 3 weeks of RNAi treatment (controls, n=7; RNAi, n=9; ***P<0.001). Quantification is based in the number of PH3+ cells per body area. (C) Quantification of caspase 3 after 1 week of RNAi treatment (controls, n=9; RNAi, n=9; *P<0.05); 2 weeks of treatment (controls, n=6; RNAi, n=9; **P<0.01); and 3 weeks of treatment (controls, n=10; RNAi, n=8; ***P<0.001). (D) Quantification of body area *in vivo* animals (controls, n=25; RNAi, n=30; n.s.). (E) Quantification of cell number (controls, n=3; RNAi, n=3; *P<0.05). Each biological replicate represents five animals. (F) DAPI staining of epithelial cells of the prepharyngeal region. (F') Quantification of the mean epidermal cell area (A) (controls, n=8; RNAi, n=7; **P<0.01). (G) Transverse sections of planarian epidermis immunostained using anti-6G10. The distance from the basal to the apical part of the cells (epidermal cell height, H) is indicated with a pink line. (G') Height quantification (controls, n=18; RNAi, n=17; n.s.). (H) Quantification of epidermal cell volume (V) (controls, n=8; RNAi, n=7; *P<0.05). (I) Schematic illustration of the measurements performed to quantify V. (J) Illustration depicts the expression of *cintillo*, *pitx* and *tbh*. The square indicates the area quantified. Quantification of *cintillo*+ cells/head area (controls, n=5; RNAi, n=5; ***P<0.001), *pitx*+ cells/head area (controls, n=8; RNAi, n=4; ***P<0.001), *tbh*+ cells/head area (controls, n=3; RNAi, n=3; *P<0.05). (K) Half of the animals developed overgrowths after 4 weeks of *bls2/3/5* inhibition in starved conditions. Fluorescence *in situ* hybridization of *h2b* riboprobe combined with anti-SMEDWI-1 immunostaining, and fluorescence *in situ* hybridization with *agal*+ and *nb.21* riboprobes (controls, n=60; RNAi, n=58). White arrowheads indicate overgrowths. Images from F and K correspond to z projections. Scale bars: 1 mm squares in D; 20 μm in F,G; 500 μm in K.

ventral margin (Fig. 5K). The molecular analysis of those overgrowths demonstrates the accumulation of postmitotic cells ($PIWI^+$ and $h2b^-$) (Solana et al., 2012), which express epidermal progenitor cell markers ($nb21^+$ and $agat1^+$) (Eisenhoffer et al., 2008) (Fig. 5K, Fig. S5I).

These data indicate that *bls2/3/5* promotes cell death during periods of shrinkage. *bls2/3/5* inhibition in starved planarians prevents the necessary reduction in cell number. Because cell size is reduced in *bls2/3/5*(RNAi) versus control animals, the increase in cell number observed in the former does not translate to larger body size. However, the accumulation of cells following long term inhibition does lead to overgrowths.

***bls* RNAi in fed planarians results in increases in cell number and body size**

Planarians grow in size in nutrient-rich environments. This growth is due to an increase in cell number resulting from an increase in the mitosis:apoptosis ratio (Pellettieri et al., 2010; González-Estévez et al., 2012a,b). Our previous findings suggest that *bls2/3/5* inhibition in continuously fed planarians may lead to an increase in cell number and possibly also in body size. To test this hypothesis, planarians fed twice per week were injected with *bls2/3/5* dsRNA for 3 weeks (Fig. 6A, Fig. S6A,B). Compared with controls, these animals showed an increase in the rate of mitosis from the first week of inhibition (Fig. S6B,C), together with a decrease in the rate of apoptosis (Fig. 6C, Fig. S6E). Furthermore, during this 3-week period RNAi animals grew faster and reached a larger size (Fig. 6D) than controls (Fig. 6E). Quantification of dissociated cells revealed an increase in total cell number in *bls2/3/5*(RNAi) animals after 3 weeks of RNAi (Fig. 6F). In contrast to the results obtained for starved planarians, no differences in epidermal cell area or volume were observed in fed animals with respect to controls (Fig. 6G–J, Fig. S6F). Furthermore, quantification of neural and chemoreceptor cells revealed no differences in cell density between RNAi and control planarians (Fig. 6K, Fig. S6G).

These data indicate that *Smed-bl2/3/5* also promotes cell death and attenuates the rate of mitosis during growth periods, resulting in an increase in cell number. Remarkably, in fed animals this increase in cell number translates to an increase in body size, as cell size is maintained in this nutrient-rich context.

***bls* transcription depends on nutrient intake and mTOR signaling**

Our results demonstrate that *bls2/3/5* subfamilies control the balance of cell proliferation and cell death in planarians not only after injury but also during normal homeostasis. We hypothesize that *bls2/3/5*-mediated signaling may constitute a general mechanism required to balance cell proliferation:cell death ratio in response to nutrient availability in planarians. According to our hypothesis, *bls2/3/5* activity would be required in nutrient-poor environments but not when food is readily available. As previously mentioned, planarian growth is sustained by increasing mitosis and decreasing cell death. After feeding, apoptosis remains very low and changes little (Fig. S7A), but proliferation increases and mitosis peaks at 3 h post-feeding (hpf) (Baguña, 1974; Newmark and Sánchez Alvarado, 2000) (Fig. S7B). Thus, according to our hypothesis, *bls* expression should be actively downregulated a few hours after food ingestion to enable subsequent growth. Quantification of mRNA levels of *bls2*, *bls3* and *bls5* by qPCR at 3 hpf and 24 hpf revealed downregulation of all three *bls* mRNAs (Fig. 7A). This downregulation was also confirmed by

FISH expression analysis: after feeding (24 hpf) expression of all three genes had decreased and/or the expression pattern had expanded and delocalized with respect to starved conditions (Fig. 7B, Fig. S7C,D).

To further understand the mechanism by which *bls2/3/5* balances cell proliferation:cell death ratio in response to nutrient intake, we analyzed its possible functional interaction with the mTOR pathway, a central regulator of cell metabolism (Saxton and Sabatini, 2017). In planarians, mTOR is upregulated in response to food intake, and its inhibition decreases proliferation and increases cell death, impeding growth (Peiris et al., 2012; Tu et al., 2012). Quantification of *bls2*, *bls3* and *bls5* expression levels in growing mTOR (RNAi) planarians shows a significant increase of *bls2*, *bls3* and *bls5* mRNA expression levels (Fig. 7C). Furthermore, quantification of mTOR expression levels in growing *bls2/3/5* (RNAi) animals shows upregulation of mTOR mRNA levels (Fig. 7D). Akt is a serine threonine protein kinase downstream of insulin and upstream of mTOR pathway (Yoon, 2017), inhibition of which in planarians increases cell death, decreases proliferation and impedes planarian growth (Peiris et al., 2016). qPCR quantification of *Akt* levels also demonstrates its upregulation in *bls2/3/5* RNAi planarians (Fig. 7D).

Overall, these suggest that expression of *bls2/3/5* subfamilies are constantly regulated in planarians to balance the cell proliferation: cell death ratio, being downregulated by nutrient intake to allow planarian growth. *bls2/3/5* is a novel gene that regulates cell number in response to nutrient intake, but it interacts with the evolutionary conserved insulin/Akt/mTOR metabolic network.

DISCUSSION

***bls* is a *de novo* gene family taxonomically restricted to the order Tricladida (planarians)**

In this study, we have identified a new gene family, *blitzschnell* (*bls*), which appears to be an evolutionary novelty of Tricladida (planarians), and is essential for the control of cell number in response to nutrient intake. In *S. mediterranea*, the *bls* family is composed of 15 members, grouped in five subfamilies (*bls1-bl5*). Members of *bls1* and *bls4* subfamilies are pseudogenes, while members of *bls2*, *bls3* and *bls5* subfamilies encode short peptides that contain a signal peptide (SP) and a coiled coil domain (CC). Fluorescent *in situ* hybridization analysis with specific riboprobes, demonstrates that *bls2*, *bls3* and *bls5* are all expressed in a subset of secretory cells, seeming tissue specific. Furthermore, we have only been able to find homologs of *bls* in species of the Tricladida order. Although the genomic databases of Platyhelminthes are incomplete, the *bls* family appears to be taxonomically restricted (Wilson et al., 2005). All described features: gene duplication and presence of pseudogenes (Tautz and Domazet-Lošo, 2011), short open reading frame with a signal peptide and a ISD (Neme and Tautz, 2013; Palmieri et al., 2014; Wilson et al., 2017; Werner et al., 2018), being expressed in specific cell types (Toll-Riera et al., 2009; Carvunis et al., 2012; Zhao et al., 2014), and being taxonomically restricted (Van Oss and Carvunis, 2019), are shared by genes that originated *de novo* during evolution. *De novo* genes, previously known as orphan genes (Schlötterer, 2015), could originate from an existing gene in the genome (Long et al., 2003), from non coding genomic regions (Schlötterer, 2015) or from transposon domestication (McLysaght and Hurst, 2016). Although further phylogenetic studies are required to understand the origin of the *bls* family, our data favor the last two possibilities, as we could not find any homolog in species outside Tricladida, and we found transposable elements in the same genomic region where the *bls* family is found.

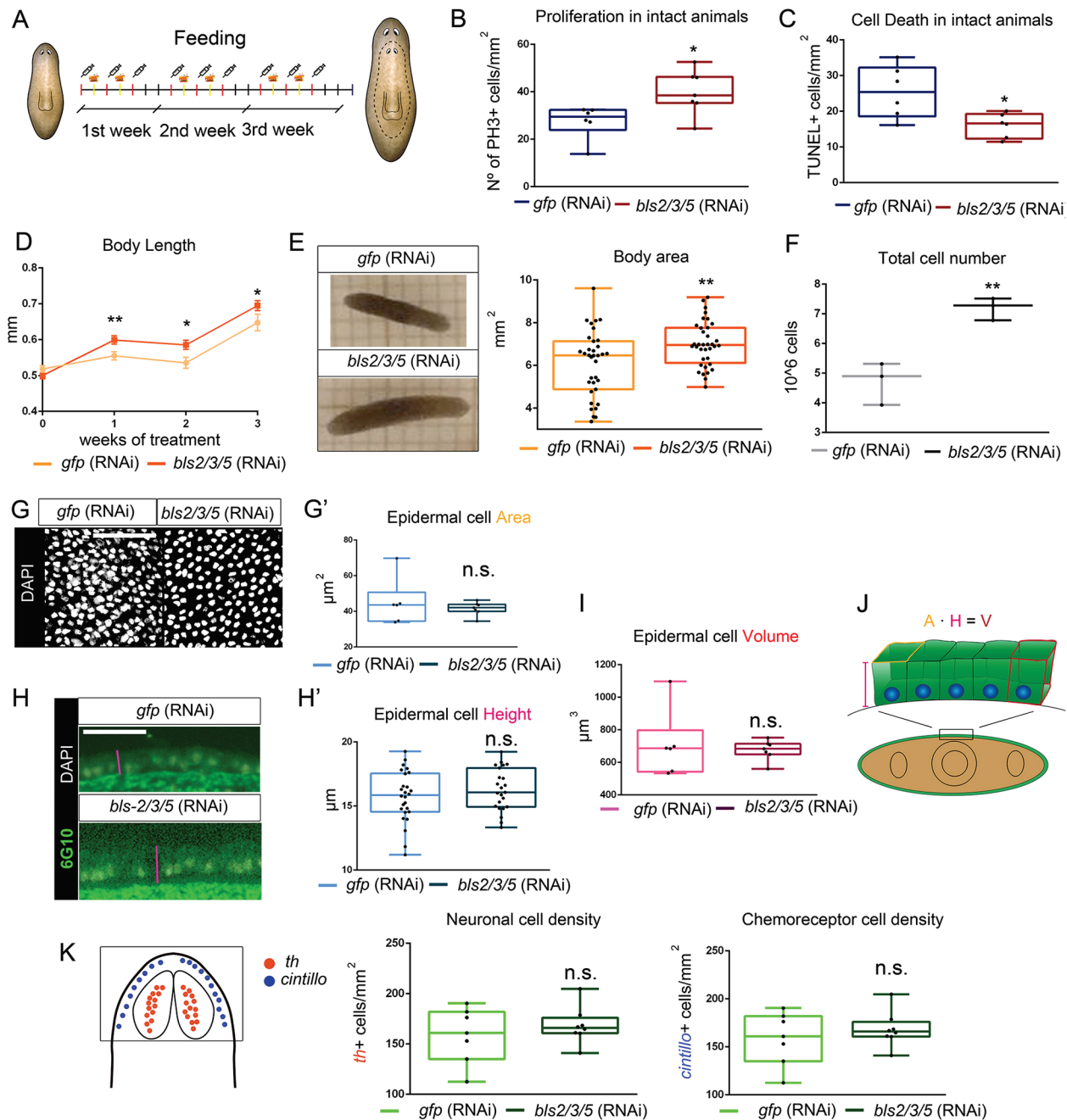


Fig. 6. In fed conditions, *bls2/3/5*(RNAi) animals show an increase in proliferation and a decrease in cell death, which results in larger animals. (A) Schematic depicting the RNAi procedure. (B) Quantification of PH3⁺ cells after 3 weeks of RNAi treatment (controls, *n*=6; RNAi, *n*=7; **P*<0.05). Quantification is based in the number of PH3⁺ cells per body area. (C) Quantification of TUNEL⁺ cells after 3 weeks of RNAi treatment (controls, *n*=6; RNAi, *n*=6; **P*<0.05) (see Materials and Methods and Fig. S6E for details of quantification). (D) Length of control and RNAi animals (controls, *n*>35; RNAi, *n*>35; **P*<0.05, ***P*<0.01). (E) Quantification of body area in live animals (controls, *n*=35; RNAi, *n*=36; ***P*<0.01). (F) Quantification of cell number (controls, *n*=3; RNAi, *n*=3; ***P*<0.01). Each biological replicate represents five animals. (G) DAPI staining of epithelial cells of the prepharyngeal region. (G') Quantification of mean epidermal cell area (A) (controls, *n*=6; RNAi, *n*=7; n.s.). (H) Transverse sections of planarian epidermis immunostained using anti-6G10. The distance from the basal to the apical part of the cell (epidermal cell height, H) is indicated with a pink line. (H') Quantification of H (controls, *n*=26; RNAi, *n*=23; n.s.). (I) Quantification of epidermal cell volume (V) (controls, *n*=6; RNAi, *n*=7; n.s.). (J) Illustration showing measurements used to quantify V. (K) Quantification of *th*⁺ cells/head area (controls, *n*=9; RNAi, *n*=6; n.s.) and *cintillo*⁺ cells/head area (controls, *n*=7; RNAi, *n*=8; n.s.). Illustration depicts the expression of *th* and *cintillo*, and the square indicates the area quantified. Images from G' correspond to z projections. Scale bars: 1 mm squares in D'; 20 µm in G,H.

The appearance of *bls* in Tricladida may be linked to the requirement for continuous and rapid modulation of cell number in response to nutrient availability

Our results demonstrate that *bls2/3/5* regulates the mitosis:apoptosis ratio in all scenarios analyzed. In homeostatic animals, the

imbalance in this ratio led to an increase in cell number. Strikingly, this increase in cell number resulted in normal body size but smaller cell size in starved planarians, and in larger body size and normal cell size in fed animals (Fig. 8), suggesting an energy-dependent role for this gene. In other organisms, *de novo*

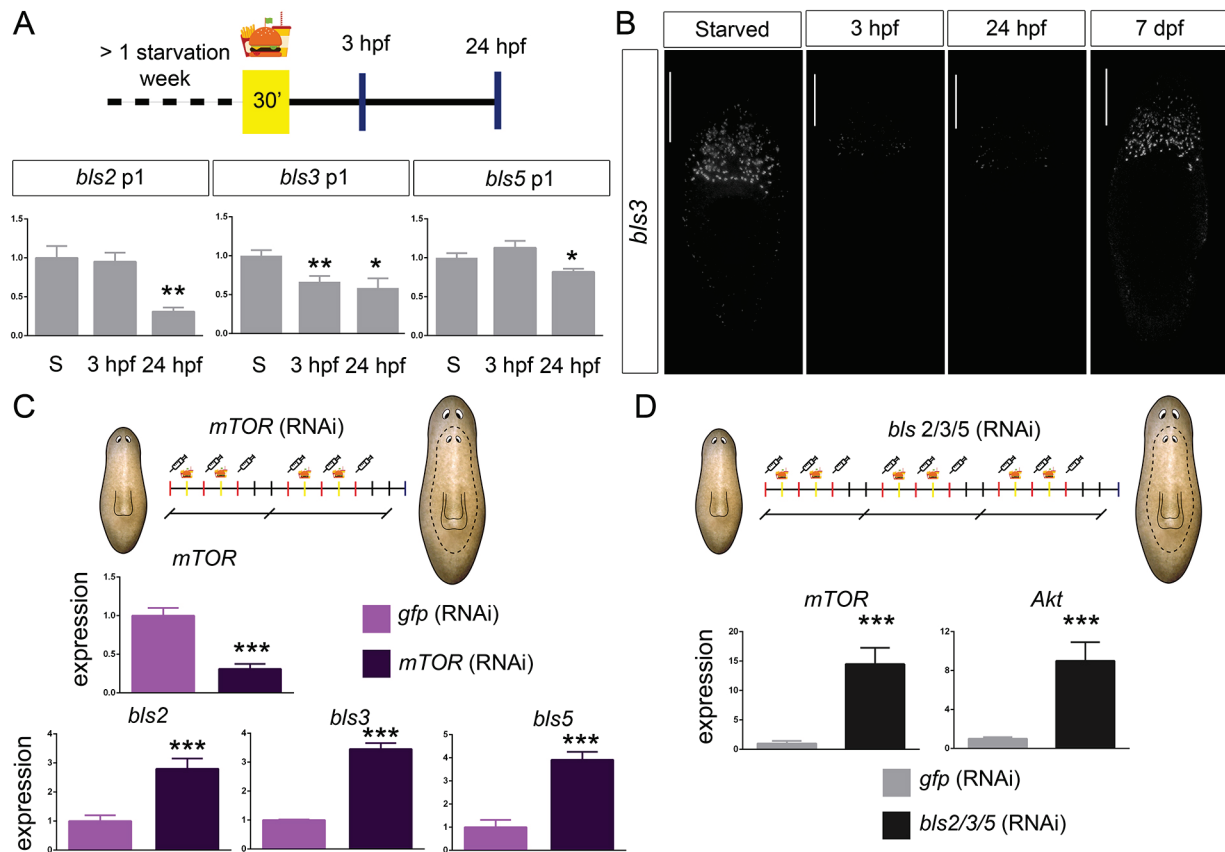


Fig. 7. *bls2*, *bls3* and *bls5* levels are regulated by food ingestion and by mTOR signaling. (A) Schematic depicting the experimental procedure. Animals were starved for more than 1 week and then fed for 30 min and fixed at different time points thereafter. Bar charts represent qRT-PCR quantification of *bls2*, *bls3* and *bls5* expression in starved animals (S) and in fed animals 3 and 24 h post-feeding (hpf). (B) Representative fluorescence *in situ* hybridization images of *bls3* before feeding and at different time points after feeding, demonstrating its reduced expression at 3 hpf and 24 hpf, and its recovery at 7 dpf. (C) qRT-PCR quantification of *bls2*, *bls3* and *bls5* expression in fed *mTOR* (RNAi) animals. Scheme depicts the RNAi procedure. (D) qRT-PCR quantification of *mTOR* and *Akt* expression in fed *bls2/3/5*(RNAi) animals. Scheme depicts the RNAi procedure. In all the panels, relative expression is plotted as $2^{-\Delta\Delta CT}$ values. Data are mean $\pm$ s.d. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

genes have been shown to play an important role in the response to biotic and/or abiotic stresses (Colbourne et al., 2011; Donoghue et al., 2011; Zhao et al., 2014). The observation that *bls2*, *bls3* and *bls5* are downregulated a few hours after food ingestion suggests that those genes can function as sensors of cell energy status. According to this hypothesis, *bls* expression is required to restrict cell number (and maintain cell size) in starvation conditions, but is downregulated after nutrient intake to allow for increases in cell number and body size (Fig. 8). The appearance of *bls* in Tricladida during evolution may be linked to the requirement for continuous modulation of cell number in response to nutrient availability in these organisms. The increase in the number of copies of *bls* family members and their tandem disposition suggest that they may be regulated by the same promoter, facilitating rapid regulation of their protein levels according to cell energy status.

Because *bls* genes share 70–100% of identity at the nucleotide level, we were unable to inhibit specific copies using RNAi, and were therefore unable to determine which gene copies perform the described function. For this reason, in this study we have ascribed this function to '*bls2/3/5*'. However, because all *bls* genes appear to follow the same expression dynamics and share almost identical amino acid sequences, we hypothesize that the gene copies encoding the SP and CC domains may perform the same function. This is in agreement with the aforementioned hypothesis of simultaneous regulation enabling rapid changes in expression.

Nonetheless, we cannot rule out the possibility that copies that do not encode the CC domain may act as inhibitors.

***bls* genes could control the mitosis:apoptosis ratio and the cell size through interacting with the insulin/Akt/mTOR network**

It has been described that *de novo* and TRG lack catalytic domains and normally interact with proteins in conserved networks (Arendsee et al., 2014). The presence of a SP suggests that *bls2*, *bls3* and *bls5* may be secreted and interact with components of those conserved pathways. Our results suggest that *bls2/3/5* may interact with members of the insulin/Akt/mTOR pathways, a universal mechanism that is activated by the extracellular nutrients and activates the signals required for growth. In planarians TORC-1 is downregulated during starvation and its inhibition decreases proliferation without affecting cell death. mTOR is upregulated in response to food intake in planarians, and its inhibition decreases proliferation and increases cell death, impeding growth (Peiris et al., 2012; Tu et al., 2012). mTOR hyper-activation, through PTEN or *smg-1* RNAi, promotes overproliferation and outgrowths (Oviedo et al., 2003; González-Estévez et al., 2012a,b). As *bls2/3/5* is expressed in nutrient-deprived conditions, it could be acting as an inhibitor of the insulin/Akt/mTOR network, which is in fact what our results suggest; mTOR and Akt are upregulated when *bls2/3/5* is inhibited, and vice versa.

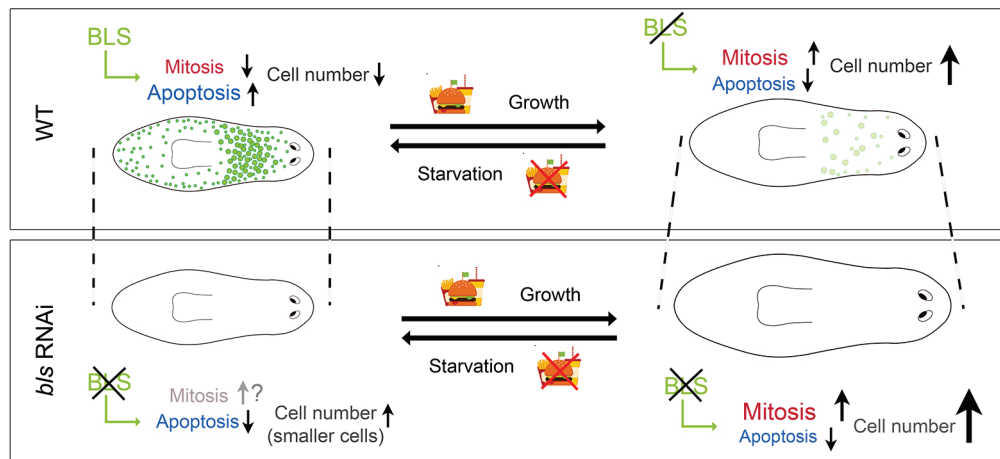


Fig. 8. Model representing *bls*-mediated control of cell number. In starving conditions *bls* is expressed and limits cell number and body size by promoting apoptosis without significantly affecting mitotic index. After feeding *bls* is downregulated, allowing an increase in mitotic cells and a decrease in cell death, which results in an increase in cell number. In starved *bls* RNAi animals, the mitosis:apoptosis ratio increases, as does total cell number. However, cells cannot maintain their size and body size does not increase. In fed *bls* RNAi animals, the mitosis:apoptosis ratio is even higher than in controls and cell number increases. This is accompanied by an increase in body size, as cells maintain their normal size.

An important result in this study is the finding that cellular responses are different according to the energetic status of the animals. *bls2/3/5* inhibition during starvation results in an early decrease in apoptosis and, with our methods, we could not detect an increase in mitotic index. In contrast, in growing animals both parameters seem to be affected. This could be also true when modulating the mTOR pathway, and the reason why different studies show opposite results regarding the mitotic rates mTOR silencing during homeostasis (Peiris et al., 2012; Tu et al., 2012). Cell death and proliferation analysis from specific nutritional context are required to understand the metabolic role of those signals.

Similarly, only fed animal cells are able to maintain the size after *bls2/3/5* inhibition. One possible explanation for the inability of starved animals to maintain cell size is that *bls2/3/5* silencing in these conditions may promote entry into M phase before cells reach their proper size. It is possible that, in wild-type planarians, cell cycle length varies according to nutritional status. Recent data suggest the existence of crosstalk between cell division and mitochondrial dynamics and metabolic pathways (Salazar-Roa and Malumbres, 2017). For example, yeast grown in nutrient-poor conditions adjust their cell-cycle duration to accommodate slower growth, so that the size at which cells divide is similar to that observed in nutrient-rich environments (Lloyd, 2013). It is possible that the duration of the cell cycle is longer in starved than fed animals, thereby ensuring that daughter cells reach the appropriate size. Promoting entry into M phase after *bls2/3/5* silencing could give rise to smaller cells in starved but not in fed animals. Because a mechanism through which mTOR signals regulate cell size is by controlling cell cycle (Fingar et al., 2002), and, as described above, planarian mTOR activity is regulated by food intake, interaction of *bls2/3/5* with this pathway could account for the smaller size of cells in starved animals. Analysis of the cell cycle in each of these different conditions will be required to test this hypothesis.

***bls* genes are tumor suppressors, inhibition of which favors regeneration**

The appearance of overgrowths in starved *bls2/3/5* RNAi animals could be a consequence of the increase in cell density promoted after

sustained inhibition cell death, as observed in tumoral processes (Lowe and Lin, 2000). *bls* genes as tumor suppressors during planarian degrowth. This observation presents us with a paradox: although caloric restriction extends lifespan (Pifferi and Aujard, 2019), in *bls2/3/5*(RNAi) planarians, food deprivation promotes hyperplasia and the formation of overgrowths, while fed *bls2/3/5*(RNAi) animals only increase body size with no apparent changes in patterning. A second key observation is that while *bls2/3/5* inhibition in starved animals promotes overgrowths, it favors regeneration after any kind of injury. This is consistent with the view that tumor suppressors evolved not to suppress tumor growth but to control cellular processes such as proliferation, cell death and cell differentiation, which are essential during embryogenesis and are activated during regeneration of complex tissues (Nacu and Tanaka, 2011). Perturbation of tumor suppressor function can enhance the regeneration of somatic stem cells in the hematopoietic system or endocrine cells (Pomerantz and Blau, 2013). Furthermore, inhibition of the Hippo pathway and consequent YAP/TAZ activation results in increases in organ size and promotes tumor formation in adult mice, but also promotes regeneration of the liver, gut, muscle and heart in mouse models (Moya and Halder, 2019).

Silencing of several known vertebrate tumor suppressors, including mTOR, p53 and Hippo, also induces the formation of overgrowths in planarians, but despite increasing proliferation does not promote proper regeneration. While TOR hyper-activation results in larger blastemas, these remain undifferentiated (González-Estévez et al., 2012a,b). Hippo hyper-activation also enhances the wound response and promotes expansion of the epidermal and muscle cell populations, and regeneration of larger structures such as the eyes (Lin and Pearson, 2017). However, this new tissue is not properly patterned (de Sousa et al., 2018). *bls* is the first family of genes described whose inhibition promotes faster but apparently normal regeneration. One possible explanation is that *bls* genes specifically control cell number (through regulation of the cell proliferation:cell death ratio) but not cell differentiation, as described for other signaling pathways such as Hippo (de Sousa et al., 2018). In this scenario, an increase in the number of cells during early stages of regeneration could accelerate the expression of wound-induced genes (Wenemoser et al., 2012), as we observed for *pitx*, and thereby promote more rapid appearance of regenerated structures.

Conclusions

In most animal species, the adult stage is distinguished from the embryonic stage by the maintenance of body size, cell number and proportions. However, long-lived animals, such as planarians, continuously regulate body size in adulthood by controlling cell number according to nutrient availability. Thus, the mechanisms described for other organisms, such as *Drosophila*, in which tissues 'know' their final size, may not apply to planarians (Nowak et al., 2013). Given that nutrient availability always fluctuates in nature, the *bls* family may represent an example of *de novo* genes that evolved in planarians to fulfil the requirement for continuous regulation of cell number according to nutrient availability. Other *de novo* genes have been implicated in increasing the fitness of the organism (Reinhardt et al., 2013; Schlötterer, 2015). Examples are described in cnidarians, in which *Hym301* regulates tentacle number (Khalturin et al., 2008), and in mollusks, in which each species expresses a unique set of secreted proteins that drives shell diversity (Aguilera et al., 2017). *De novo* genes are usually integrated into existing pathways, adding additional levels of regulation. *bls* genes may interact with members of the insulin/Akt/mTOR signaling pathways, which regulate growth in response to nutrient intake in planarians and in vertebrates. RNAi of components of these pathways does not fully phenocopy *bls2/3/5* RNAi. However, this signaling pathway should be thought of as a network in which each of these signals functions in a complex and dynamic manner, as opposed to a linear pathway. Future studies will need to determine whether the primary function of *bls* is to control proliferation or apoptosis, or both, and to elucidate the molecular integration of *bls* genes within the insulin/Akt/mTOR network. Given that the molecular signals controlling body and organ growth are also key players in most human cancers, understanding the mechanism by which *bls* genes act as tumor suppressors would help identify novel targets for the design of therapeutic strategies to modulate tissue growth.

MATERIALS AND METHODS

Planarian culture

The planarians used in this study are the asexual clonal strain of *S. mediterranea* BCN-10 biotype and were maintained as previously described (Fernández-Taboada et al., 2010) in planarian artificial medium (PAM) water (Cebrià and Newmark, 2005). Animals were fed twice per week with liver, and those used in starvation experiments were starved for 1 week.

RNAi screening

To identify genes involved in eye regeneration we performed a high density DNA microarray. We ran a gene expression profile of planarian tissues with control animals (control RNAi) and planarians without eyes (*Smed-sine oculis* RNAi) during early stages of head regeneration using the 'array star' software from Nymbelgene. Sixty-one candidate genes differentially expressed in those conditions were analyzed by RNAi (Eckelt, 2011). Among them we isolated *Smed-bl3*, which inhibition produced a faster eye regeneration.

Sequence and phylogenetic analyses

A fragment of *Smed-bl3* was identified from Eckelt (2011). Other members of the families were identified from the genome (Grohme et al., 2018) and amplified using specific primers (Table S3). The signal peptide was identified with SigalP v5.0 (Almagro Armenteros et al., 2019) and the coiled-coil domain was characterized using the tool available online from PRABI [Pole Rhone-Alpes de Bioinformatique; https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_lupas.html (Lupas et al., 1991)]. Sequence identity comparison was carried out using the pairwise alignment tool in Jalview suite v2.11 (Waterhouse et al., 2009).

To determine which members of each family were expressed, we mapped the RNAseq paired reads from adult wild-type animals (de Sousa et al., 2018) against assembly 2 of the *S. mediterranea* genome (Grohme et al., 2018) using Bowtie2 (Langmead et al., 2009) v2.3.4, selecting the *-end-to-*

end option. After alignment, we extracted the reads mapping the scaffolds of interest using *samtools view* (Li et al., 2009) v1.9. The final assessment was performed manually using the Integrative Genomics Viewer (Robinson et al., 2011) (IGV v2.4.4) to verify the families with mapped reads.

Sequence comparison against the GenBank database was performed using the NCBI BLAST network server (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). Potential orthologs were searched for using tBLASTx where possible to allow a certain level of tolerance in case of a high degree of divergence. The search for orthologs was performed against transcriptomes and genomes from several Platyhelminthes species (Table S4) (Egger et al., 2015).

The IQ-tree web server (Trifinopoulos et al., 2016) was used to reconstruct the phylogenetic relationships between *Smed-bl3* families. The nucleotide or protein sequences were first aligned using the alignment servers in Jalview suite (MUSCLE for nucleotides and MAFFT for amino acids). Substitution model selection was performed automatically by the software, the number of bootstrap iterations was set to 1500 and default options were selected for the remaining parameters. The trees were visualized using FigTree v1.4.4 (<http://tree.bio.ed.ac.uk/software/figtree/>) with the default parameters.

Whole-mount *in situ* hybridization

Probes were synthesized *in vitro* using SP6 or T7 polymerase and DIG- or FITC- modified (Roche). RNA probes were purified by ethanol precipitation and the addition of 7.5 M ammonium acetate. For colorimetric whole-mount *in situ* hybridization, animals were sacrificed using 5% N-acetyl-L-cysteine (NAC), fixed with 4% formaldehyde and permeabilized with Reduction Solution (50 mM DTT, 1% NP-40, 0.5% SDS in 1× PBS). The fixative and whole-mount *in situ* hybridization protocol used has been previously described (Currie et al., 2016). For whole-mount fluorescent *in situ* hybridization, animals were sacrificed with 7.5% NAC and fixed with 4% formaldehyde. Fluorescent *in situ* hybridization was carried out as described previously (King and Newmark, 2013). For double fluorescent *in situ* hybridization (dFISH) an azide step (150 mM sodium azide for 45 min at room temperature) was added. Nuclei were stained with DAPI (1:5000; Sigma). For fluorescent *in situ* hybridization of paraffin sections, animals were sacrificed with 2% HCl and fixed with 4% PFA. Paraffin embedding and sectioning were carried out as previously described (Cardona et al., 2005), slides were de-waxed and re-hydrated, and antigen retrieval step was performed as previously described (Sureda-Gómez et al., 2016). Sections were hybridized with the corresponding probes for 16 h and incubated with antibody diluted 1% BSA for 16 h. Both steps were carried out in a humidified chamber (Cardona et al., 2005).

Immunohistochemistry

Whole-mount immunohistochemistry was performed as previously described (Ross et al., 2015). Animals were killed with 2% HCl and fixed with 4% formaldehyde. The following antibodies used in these experiments: mouse anti-synapsin (anti-SYNORF1, 1:50; Developmental Studies Hybridoma Bank), mouse anti-VC1 (anti-arrestin, 1:15,000, kindly provided by Professor Hidefumi Orii, University of Hyogo, Kobe, Japan), rabbit anti-phosphohistone H3 (Ser10) (D2C8) (PH3) (1:500; Cell Signaling Technology; 9701) and anti-SMEDWI-1 antibody (1:1000, kindly provided by Professor Kerstin Bartscherer, Hubrecht Institute, Utrecht, The Netherlands). The secondary antibodies used were Alexa 488-conjugated goat anti-mouse (1:400; Molecular Probes; A28175) and Alexa 568-conjugated goat anti-rabbit (1:1000; Molecular Probes; A-11011). Nuclei were stained with DAPI (1:5000). For immunohistochemistry of paraffin sections, animals were killed and treated as described above. Sections were blocked in 1% bovine serum albumin (BSA) in 1×PBS for 1 h at room temperature and then incubated with primary antibodies diluted in blocking solution (mouse anti-muscle fiber antibody, 6G10, 1:400; Developmental Studies Hybridoma Bank) for 16 h at 4°C in a humidified chamber. Subsequently, sections were washed in 1×PBS and incubated with secondary antibodies (anti-mouse Alexa 488-conjugated antibody, 1:400; Molecular Probes; A28175) in blocking solution for 3 h at room temperature in a humidified chamber. Nuclei were stained with DAPI (1:5000; Sigma-Aldrich).

TUNEL assay

For the whole-mount TUNEL assay, animals were sacrificed with 10% NAC, fixed with 4% FA and permeabilized with 1% sodium dodecyl sulfate (SDS) solution. A TUNEL assay was carried out as described previously (Pellettieri et al., 2010) using the ApopTag Red *In situ* Apoptosis Detection Kit (CHEMICON, S7165). Nuclei were stained with DAPI (1:5000; Sigma-Aldrich). For TUNEL assay on paraffin sections, animals were killed and treated as described above. Sections were treated as described previously (Pellettieri et al., 2010) and after the dewaxing step a proteinase K step was added for permeabilization. Next, we used the ApopTag Red *In situ* Apoptosis Detection Kit (CHEMICON, S7165). Positive cells were counted in at least 9 representative sagittal sections per animal, and the overall mean value was determined. Six animals were analyzed per condition.

RNA interference analysis

Double-strand RNA (dsRNA) was synthesized by *in vitro* transcription (Roche) as previously described (Sanchez Alvarado and Newmark, 1999). dsRNA (3×32.2 nl) was injected into the digestive system of each animal on three consecutive days (1 round). The experiments in which regeneration was studied consisted of two consecutive rounds of injections and an amputation at the end of each round. In experiments in which planarians were starved, animals underwent three or four consecutive rounds of injection, without amputation. In experiments involving fed animals, planarians received dsRNA injections on three non-consecutive days per week and were fed on the two intervening days. This process was repeated for 3 weeks in total. All control animals were injected with dsRNA of green fluorescent protein (GFP). RNAi of subfamilies *bls2*, *bls3* and *bls5* was carried out using two different RNA sequences, both of which produced the same phenotype when injected in regenerating planarians (Table S3, Table S8). The sequences corresponded to the full *bls3* sequence and to a smaller region showing the greatest similarity between the *bls2*, *bls3* and *bls5* subfamilies. In the case of the second RNA sequence, inhibition of all members of the transcribed families was demonstrated by qPCR analysis (Figs S3B,C, S5B, S6B).

Feeding experiments

In long-term growth experiments involving RNAi, animals were fed twice per week: food was provided in the morning and removed at the end of the day (Fig. 5A, Fig. S5A). PAM water was replenished three times per week. To study gene expression after feeding, we analyzed planarians that had been starved for 1 week and then fed for 30 min. Next, we removed the food and replenished the PAM water (Fig. 7A). Hours post feeding (hpf) were counted from the moment of removal of the last piece of food.

Quantitative real-time PCR

Total RNA was extracted from a pool of five planarians per condition using TRIzol reagent (Invitrogen). cDNA was synthesized as previously described (Almuedo-Castillo et al., 2014). Expression levels were normalized to that of the housekeeping gene *ura4*. All experiments were performed using three biological and three technical replicates for each condition. The design of specific primers corresponding to the 5' region for subfamilies *bls2*, *bls3* and *bls5* allowed verification of the inhibition of the three gene families after RNAi. All primers used in this study are shown in Table S3.

Caspase 3 activity assay

For each condition, protein extraction was performed in five planarians. The protein concentration of the cell lysates was measured using Bio-Rad protein reagent. Fluorometric analysis of caspase 3 activity was performed as described previously (González-Estévez et al., 2007) using 20 mg of protein extract, which was incubated for 2 h at 37°C with 20 µM caspase 3 substrate Ac-DEVD-AMC or 2 ml from a stock of 1 mg/ml for a final volume of 150 µl. Using a Fluostar Optima microplate fluorescence reader (BMG Labtech), fluorescence was measured in a luminescence spectrophotometer (Perkin-Elmer LS-50), applying the following settings: excitation, 380 nm; emission, 440 nm. Three technical replicates were analyzed per condition.

Cell number and cell volume analyses

To quantify total cell number, planarian cells were dissociated with trypsin and the nuclei stained with DAPI (Moritz et al., 2012). The cell suspension

was transferred to a Neubauer chamber, cells were manually counted on three occasions, and the mean value calculated. Five planarians were analyzed per biological replicate, and three replicates were analyzed per condition. Mean cell volume (V) was calculated by multiplying mean epidermal cell area (A) by epidermal cell height (H). To quantify the mean epidermal cell area, the prepharyngeal epidermal area was imaged and the number of nuclei per area was quantified. To determine mean epidermal cell height, the distance between the apical to the basal part of the cell was measured. Measurements were taken in three different regions of the same section and the mean value obtained.

Imaging and quantification

Whole-mount and fluorescent *in situ* hybridization, and immunohistochemistry images were captured with a ProgRes C3 camera from Jenoptik (Jena). A Leica MZ16F microscope (Leica Microsystems) was used to observe the samples and obtain fluorescent *in situ* hybridization, immunostaining and TUNEL images. A Leica TCS SPE confocal microscope (Leica Microsystems) was used to obtain confocal images of whole-mount fluorescent *in situ* hybridization, immunostaining and TUNEL assays. Representative confocal stacks for each experimental condition are shown. Cell counting of PH3⁺, TUNEL⁺ and specific cell types was carried out by eye quantification in a previous defined area of each animal. Areas are schematically indicated in each figure. The total number of positive cells was divided by these areas. Images were blind analyzed and later grouped according to each genotype. To calculate the ratio of PH3⁺ cells:epidermal cell density, the number of PH3⁺ cells/mm² counted per animal was divided by the epidermal cell density quantified in a predetermined prepharyngeal region.

Statistical analyses

Statistical analyses were performed using GraphPad Prism 6. Two-sided Student's *t*-tests ($\alpha=0.05$) were performed to compare the means of two populations. Two-sided Fisher's exact tests were used to compare two phenotypic variants between two populations. Fisher.test from the R package was used to compare more than two phenotypic variants between two populations.

Statistical data presentation

Results were plotted using GraphPad Prism 6. To compare two populations, we used box plots depicting the median, the 25th and 75th percentiles (box), and all included data points (black dots). Whiskers extend to the largest data point within the 1.5 interquartile range of the upper quartile and to the smallest data point within the 1.5 interquartile lower range of the quartile. To plot data points over time, we used *xy* plots, in which each dot represents the mean and bars represent the standard error. Each dot is connected with the next in an arbitrary manner. To visualize the percentage phenotype in each population we used the Stacked Bars plot in R. Each phenotype is assigned a distinct color.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: E.P.-C., E.S., T.A.; Methodology: E.P.-C., E.S., T.A.; Formal analysis: E.P.-C., T.A.; Investigation: E.P.-C., M.M.-B., C.H.-Ú, D.F.-M., K.E., N.d.S.; Data curation: E.P.-C.; Writing - original draft: E.P.-C., T.A.; Writing - review & editing: E.P.-C., T.A.; Supervision: E.S., T.A.; Project administration: J.G.-F., E.S., T.A.; Funding acquisition: J.G.-F., E.S., T.A.

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Data availability

Nucleotide sequence data reported are available in the Third Party Annotation Section of the DDBJ/ENA/GenBank databases under the accession numbers TPA: BK010973-BK010987. GenBank accession numbers of *bls* sequences are as follows: *bls1a* (BK010973), *bls1b* (BK010974), *bls2a* (BK010975), *bls2b* (BK010976), *bls3a* (BK010977), *bls3b* (BK010978), *bls3c* (BK010979), *bls3d* (BK010980), *bls3e* (BK010981), *bls3f* (BK010982), *bls3g* (BK010983), *bls4a* (BK010984), *bls4b* (BK010985), *bls5a* (BK010986) and *bls5b* (BK010987).

Supplementary information

Supplementary information available online at <http://dev.biologists.org/lookup/doi/10.1242/dev.184044.supplemental>

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References

- Aguilera, F., McDougall, C. and Degnan, B. M. (2017). Co-option and de novo gene evolution underlie molluscan shell diversity. *Mol. Biol. Evol.* **34**, 779–792. doi:10.1093/molbev/msw294
- Almagro Armenteros, J. J., Tsirigos, K. D., Sønderby, C. K., Petersen, T. N., Winther, O., Brunak, S., von Heijne, G. and Nielsen, H. (2019). SignalP 5.0 improves signal peptide predictions using deep neural networks. *Nat. Biotechnol.* **37**, 420–423. doi:10.1038/s41587-019-0036-z
- Almuedo-Castillo, M., Crespo, X., Seebeck, F., Bartscherer, K., Saló, E. and Adell, T. (2014). JNK controls the onset of mitosis in planarian stem cells and triggers apoptotic cell death required for regeneration and remodeling. *PLoS Genet.* **10**, e1004400. doi:10.1371/journal.pgen.1004400
- Arendsee, Z. W., Li, L. and Wurtele, E. S. (2014). Coming of age: orphan genes in plants. *Trends Plant Sci.* **19**, 698–708. doi:10.1016/j.tplants.2014.07.003
- Baguñá, J. (1974). Dramatic mitotic response in planarians after feeding, and a hypothesis for the control mechanism. *J. Exp. Zool.* **190**, 117–122. doi:10.1002/jez.1401900111
- Baguñá, J. (1976). Mitosis in the intact and regenerating planarian *Dugesia mediterranea* n.sp. I. Mitotic studies during growth, feeding and starvation. *J. Exp. Zool.* **195**, 53–64. doi:10.1002/jez.1401950106
- Baguñá, J. and Saló, E. (1984). Regeneration and pattern formation in planarians I. The pattern of mitosis in anterior and posterior regeneration in *Dugesia* (G) *tigrina*, and a new proposal for blastema formation. *Development* **83**, 63–80.
- Baguñá, J. and Romero, R. (1981). Quantitative analysis of cell types during growth, degrowth and regeneration in the planarians *Dugesia mediterranea* and *Dugesia tigrina*. *Hydrobiologia* **84**, 181–194. doi:10.1007/BF00026179
- Cardona, A., Fernández, J., Solana, J. and Romero, R. (2005). An in situ hybridization protocol for planarian embryos: monitoring myosin heavy chain gene expression. *Dev. Genes Evol.* **215**, 482–488. doi:10.1007/s00427-005-0003-1
- Carvunis, A.-R., Rolland, T., Wapinski, I., Calderwood, M. A., Yildirim, M. A., Simonis, N., Charlotiaux, B., Hidalgo, C. A., Barbette, J., Santhanam, B. et al. (2012). Proto-genes and de novo gene birth. *Nature* **487**, 370–374. doi:10.1038/nature11184
- Cebrià, F., Nakazawa, M., Mineta, K., Ikeo, K., Gojobori, T. and Agata, K. (2002). Dissecting planarian central nervous system regeneration by the expression of neural-specific genes. *Dev. Growth Differ.* **44**, 135–146. doi:10.1046/j.1440-169x.2002.00629.x
- Cebrià, F. and Newmark, P. A. (2005). Planarian homologs of netrin and netrin receptor are required for proper regeneration of the central nervous system and the maintenance of nervous system architecture. *Development* **132**, 3691–3703. doi:10.1242/dev.01941
- Colbourne, J. K., Pfrender, M. E., Gilbert, D., Thomas, W. K., Tucker, A., Oakley, T. H., Tokishita, S., Aerts, A., Arnold, G. J., Basu, M. K. et al. (2011). The ecoresponsive genome of *daphnia pulex*. *Science* **331**, 555–561. doi:10.1126/science.1197761
- Currie, K. W. and Pearson, B. J. (2013). Transcription factors *lhx1/5-1* and *pitx* are required for the maintenance and regeneration of serotonergic neurons in planarians. *Development* **140**, 3577–3588. doi:10.1242/dev.098590
- Currie, K. W., Brown, D. D. R., Zhu, S., Xu, C. J., Voisin, V., Bader, G. D. and Pearson, B. J. (2016). HOX gene complement and expression in the planarian *Schmidtea mediterranea*. *EvoDevo* **7**, 7. doi:10.1186/s13227-016-0044-8
- de Sousa, N., Rodríguez-Esteban, G., Rojo-Laguna, J. I., Saló, E. and Adell, T. (2018). Hippo signaling controls cell cycle and restricts cell plasticity in planarians. *PLoS Biol.* **16**, e2002399. doi:10.1371/journal.pbio.2002399
- Dhanasekaran, D. N. and Reddy, E. P. (2017). JNK-signaling: a multiplexing hub in programmed cell death. *Genes Cancer* **8**, 682–694. doi:10.18632/genesandcancer.155
- Donoghue, M. T., Keshavaiah, C., Swamidatta, S. H. and Spillane, C. (2011). Evolutionary origins of Brassicaceae specific genes in *Arabidopsis thaliana*. *BMC Evol. Biol.* **11**, 47. doi:10.1186/1471-2148-11-47
- Eckelt, K. (2011). *Multi-Approach Analysis for Identification and Functional Characterization of Eye Regeneration Related Genes of Schmidtea mediterranea*. Barcelona, Spain: University of Barcelona.
- Egger, B., Lapraz, F., Tomiczek, B., Müller, S., Dessimoz, C., Girstmair, J., Skunca, N., Rawlinson, K., Cameron, C., Beli, E. et al. (2015). A transcriptomic-phylogenomic analysis of the evolutionary relationships of flatworms. *Curr. Biol.* **25**, 1347–1353. doi:10.1016/j.cub.2015.03.034
- Eisenhoffer, G. T., Kang, H. and Alvarado, A. S. (2008). Molecular analysis of stem cells and their descendants during cell turnover and regeneration in the planarian *Schmidtea mediterranea*. *Cell Stem Cell* **3**, 327–339. doi:10.1016/j.stem.2008.07.002
- Fernández-Taboada, E., Moritz, S., Zeuschner, D., Stehling, M., Scholer, H. R., Saló, E. and Gentile, L. (2010). Smed-SmB, a member of the LSM protein superfamily, is essential for chromatoid body organization and planarian stem cell proliferation. *Development* **137**, 1055–1065. doi:10.1242/dev.042564
- Fincher, C. T., Wurtzel, O., de Hoog, T., Kravarik, K. M. and Reddien, P. W. (2018). Cell type transcriptome atlas for the planarian *Schmidtea mediterranea*. *Science* **360**, eaaq1736. doi:10.1126/science.aaq1736
- Fingar, D. C., Salama, S., Tsou, C., Harlow, E. and Blenis, J. (2002). Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4EBP1/eIF4E. *Genes Dev.* **16**, 1472–1487. doi:10.1101/gad.995802
- Fraguas, S., Barberán, S. and Cebrià, F. (2011). EGFR signaling regulates cell proliferation, differentiation and morphogenesis during planarian regeneration and homeostasis. *Dev. Biol.* **354**, 87–101. doi:10.1016/j.ydbio.2011.03.023
- Gokhale, R. H. and Shingleton, A. W. (2015). Size control: the developmental physiology of body and organ size regulation. *Wiley Interdiscip. Rev. Dev. Biol.* **4**, 335–356. doi:10.1002/wdev.181
- González, A. and Hall, M. N. (2017). Nutrient sensing and TOR signaling in yeast and mammals. *EMBO J.* **36**, 397–408. doi:10.15252/embj.201696010
- González-Estévez, C., Felix, D. A., Aboobaker, A. A. and Saló, E. (2007). Gtdap-1 promotes autophagy and is required for planarian remodeling during regeneration and starvation. *Proc. Natl. Acad. Sci. USA* **104**, 13373–13378. doi:10.1073/pnas.0703588104
- González-Estévez, C., Felix, D. A., Rodríguez-Esteban, G. and Aboobaker, A. A. (2012a). Decreased neoblast progeny and increased cell death during starvation-induced planarian degrowth. *Int. J. Dev. Biol.* **56**, 83–91. doi:10.1387/ijdb.113452cg
- González-Estévez, C., Felix, D. A., Smith, M. D., Paps, J., Morley, S. J., James, V., Sharp, T. V. and Aboobaker, A. A. (2012b). SMG-1 and mTORC1 act antagonistically to regulate response to injury and growth in planarians. *PLoS Genet.* **8**, e1002619. doi:10.1371/journal.pgen.1002619
- Grohme, M. A., Schloissnig, S., Rozanski, A., Pippel, M., Young, G. R., Winkler, S., Brandl, H., Henry, I., Dahl, A., Powell, S., et al. (2018). The genome of *Schmidtea mediterranea* highlights the plasticity of cellular core mechanisms. *Nature* **554**, 56–61. doi:10.1038/nature2547
- Guertin, D. A. and Sabatini, D. M. (2006). Cell size control. *eLS* **1–10**. doi:10.1038/npg.els.0003359
- Hariharan, I. K. (2015). Organ size control: lessons from *Drosophila*. *Dev. Cell* **34**, 255–265. doi:10.1016/j.devcel.2015.07.012
- Khalturin, K., Anton-Erxleben, F., Sassmann, S., Wittlieb, J., Hemmrich, G. and Bosch, T. C. G. (2008). A novel gene family controls species-specific morphological traits in *Hydra*. *PLoS Biol.* **6**, 2436–2449. doi:10.1371/journal.pbio.0060278
- King, R. S. and Newmark, P. A. (2013). In situ hybridization protocol for enhanced detection of gene expression in the planarian *Schmidtea mediterranea*. *BMC Dev. Biol.* **13**, 8. doi:10.1186/1471-213X-13-8
- Langmead, B., Trapnell, C., Pop, M. and Salzberg, S. L. (2009). Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol.* **10**, R25. doi:10.1186/gb-2009-10-3-r25
- Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G. and Durbin, R. (2009). The sequence alignment/map format and SAMtools. *Bioinformatics* **25**, 2078–2079. doi:10.1093/bioinformatics/btp352
- Lin, A. Y. T. and Pearson, B. J. (2017). Yorkie is required to restrict the injury responses in planarians. *PLoS Genet.* **13**, e1006874. doi:10.1371/journal.pgen.1006874
- Lloyd, A. C. (2013). The regulation of cell size. *Cell* **154**, 1194–1205. doi:10.1016/j.cell.2013.08.053
- Long, M., Betrán, E., Thornton, K. and Wang, W. (2003). The origin of new genes: glimpses from the young and old. *Nat. Rev. Genet.* **4**, 865–875. doi:10.1038/nrg1204
- Lowe, S. W. and Lin, A. W. (2000). Apoptosis in cancer. *Carcinogenesis* **21**, 485–495. doi:10.1093/carcin/21.3.485
- Lupas, A., Van Dyke, M. and Stock, J. (1991). Predicting coiled coils from protein sequences. *Science* **252**, 1162–1164. doi:10.1126/science.252.5009.1162

- März, M., Seebeck, F. and Bartscherer, K. (2013). A Pitx transcription factor controls the establishment and maintenance of the serotonergic lineage in planarians. *Development* **140**, 4499–4509. doi:10.1242/dev.100081
- McIsaght, A. and Hurst, L. D. (2016). Open questions in the study of de novo genes: what, how and why. *Nat. Rev. Genet.* **17**, 567–578. doi:10.1038/nrg.2016.78
- Miller, C. M. and Newmark, P. A. (2012). An insulin-like peptide regulates size and adult stem cells in planarians. *Int. J. Dev. Biol.* **56**, 75–82. doi:10.1387/ijdb.113443cm
- Miyaoka, Y., Ebato, K., Kato, H., Arakawa, S., Shimizu, S. and Miyajima, A. (2012). Hypertrophy and unconventional cell division of hepatocytes underlie liver regeneration. *Curr. Biol.* **22**, 1166–1175. doi:10.1016/j.cub.2012.05.016
- Molina, M. D., Neto, A., Maeso, I., Gómez-Skarmeta, J. L., Saló, E. and Cebrià, F. (2011). Noggin and noggin-like genes control dorsoventral axis regeneration in planarians. *Curr. Biol.* **21**, 300–305. doi:10.1016/j.cub.2011.01.016
- Moritz, S., Stöckle, F., Ortmeier, C., Schmitz, H., Rodríguez-Esteban, G., Key, G. and Gentile, L. (2012). Heterogeneity of planarian stem cells in the S/G2/M phase. *Int. J. Dev. Biol.* **56**, 117–125. doi:10.1387/ijdb.113440sm
- Moya, I. M. and Halder, G. (2019). Hippo–YAP/TAZ signalling in organ regeneration and regenerative medicine. *Nat. Rev. Mol. Cell Biol.* **20**, 211–226. doi:10.1038/s41580-018-0086-y
- Nacu, E. and Tanaka, E. M. (2011). Limb regeneration: a new development? *Annu. Rev. Cell Dev. Biol.* **27**, 409–440. doi:10.1146/annurev-cellbio-092910-154115
- Neme, R. and Tautz, D. (2013). Phylogenetic patterns of emergence of new genes support a model of frequent de novo evolution. *BMC Genomics* **14**, 117. doi:10.1186/1471-2164-14-117
- Newmark, P. A. and Sánchez Alvarado, A. (2000). Bromodeoxyuridine specifically labels the regenerative stem cells of planarians. *Dev. Biol.* **220**, 142–153. doi:10.1006/dbio.2000.9645
- Nowak, K., Seisenbacher, G., Hafen, E. and Stocker, H. (2013). Nutrient restriction enhances the proliferative potential of cells lacking the tumor suppressor PTEN in mitotic tissues. *eLife* **2**, 1–21. doi:10.7554/eLife.00380.044
- Oviedo, N. J., Newmark, P. A. and Sánchez Alvarado, A. (2003). Allometric scaling and proportion regulation in the freshwater planarian Schmidtea mediterranea. *Dev. Dyn.* **226**, 326–333. doi:10.1002/dvdy.10228
- Oviedo, N. J., Pearson, B. J., Levin, M. and Sanchez Alvarado, A. (2008). Planarian PTEN homologs regulate stem cells and regeneration through TOR signaling. *Dis. Model. Mech.* **1**, 131–143. doi:10.1242/dmm.000117
- Palmieri, N., Kosiol, C. and Schlötterer, C. (2014). The life cycle of Drosophila orphan genes. *eLife* **3**, e01311. doi:10.7554/eLife.01311
- Peiris, T. H., Weckerle, F., Ozamoto, E., Ramirez, D., Davidian, D., Garcia-Ojeda, M. E. and Oviedo, N. J. (2012). TOR signaling regulates planarian stem cells and controls localized and organismal growth. *J. Cell Sci.* **125**, 1657–1665. doi:10.1242/jcs.104711
- Peiris, T. H., Ramirez, D., Barghouth, P. G. and Oviedo, N. J. (2016). The Akt signaling pathway is required for tissue maintenance and regeneration in planarians. *BMC Dev. Biol.* **16**, 7. doi:10.1186/s12861-016-0107-z
- Pellettieri, J., Fitzgerald, P., Watanabe, S., Mancuso, J., Green, D. R. and Sánchez Alvarado, A. (2010). Cell death and tissue remodeling in planarian regeneration. *Dev. Biol.* **338**, 76–85. doi:10.1016/j.ydbio.2009.09.015
- Pifferi, F. and Aujard, F. (2019). Caloric restriction, longevity and aging: Recent contributions from human and non-human primate studies. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **95**, 109702. doi:10.1016/j.pnpbp.2019.109702
- Plass, M., Solana, J., Wolf, F. A., Ayoub, S., Misios, A., Glažar, P., Obermayer, B., Theis, F. J., Kocks, C. and Rajewsky, N. (2018). Cell type atlas and lineage tree of a whole complex animal by single-cell transcriptomics. *Science* **360**, eaag1723. doi:10.1126/science.aag1723
- Pomerantz, J. H. and Blau, H. M. (2013). Tumor suppressors: enhancers or suppressors of regeneration? *Development* **140**, 2502–2512. doi:10.1242/dev.084210
- Reinhardt, J. A., Wanjiru, B. M., Brant, A. T., Saelao, P., Begun, D. J. and Jones, C. D. (2013). De Novo ORFs in drosophila are important to organismal fitness and evolved rapidly from previously non-coding sequences. *PLoS Genet.* **9**, 1003860. doi:10.1371/journal.pgen.1003860
- Robinson, J. T., Thorvaldsdóttir, H., Winckler, W., Guttman, M., Lander, E. S., Getz, G. and Mesirov, J. P. (2011). Integrative genomics viewer. *Nat. Biotechnol.* **29**, 24–26. doi:10.1038/nbt.1754
- Ross, K. G., Omuro, K. C., Taylor, M. R., Munday, R. K., Hubert, A., King, R. S. and Zayas, R. M. (2015). Novel monoclonal antibodies to study tissue regeneration in planarians. *BMC Dev. Biol.* **15**, 2. doi:10.1186/s12861-014-0050-9
- Salazar-Roa, M. and Malumbres, M. (2017). Fueling the cell division cycle. *Trends Cell Biol.* **27**, 69–81. doi:10.1016/j.tcb.2016.08.009
- Sanchez Alvarado, A. and Newmark, P. A. (1999). Double-stranded RNA specifically disrupts gene expression during planarian regeneration. *Proc. Natl Acad. Sci. USA* **96**, 5049–5054. doi:10.1073/pnas.96.9.5049
- Saxton, R. A. and Sabatini, D. M. (2017). mTOR signaling in growth, metabolism, and disease. *Cell* **168**, 960–976. doi:10.1016/j.cell.2017.02.004
- Schlötterer, C. (2015). Genes from scratch - the evolutionary fate of de novo genes. *Trends Genet.* **31**, 215–219. doi:10.1016/j.tig.2015.02.007
- Solana, J., Kao, D., Mihaylova, Y., Jaber-Hijazi, F., Malla, S., Wilson, R. and Aboobaker, A. (2012). Defining the molecular profile of planarian pluripotent stem cells using a combinatorial RNAseq, RNA interference and irradiation approach. *Genome Biol.* **13**, R19. doi:10.1186/gb-2012-13-3-r19
- Sureda-Gómez, M., Martín-Durán, J. M. and Adell, T. (2016). Localization of planarian β -CATENIN-1 reveals multiple roles during anterior-posterior regeneration and organogenesis. *Development* **143**, 4149–4160. doi:10.1242/dev.135152
- Tautz, D. and Domazet-Lošo, T. (2011). The evolutionary origin of orphan genes. *Nat. Rev. Genet.* **12**, 692–702. doi:10.1038/nrg3053
- Thommen, A., Werner, S., Frank, O., Philipp, J., Knittelfelder, O., Quek, Y., Fahmy, K., Shevchenko, A., Friedrich, B. M., Jülicher, F. et al. (2019). Body size-dependent energy storage causes Kleiber's law scaling of the metabolic rate in planarians. *eLife* **8**, 1–29. doi:10.7554/eLife.38187
- Toll-Riera, M., Bosch, N., Bellora, N., Castelo, R., Armengol, L., Estivill, X. and Mar Alba, M. (2009). Origin of primate orphan genes: a comparative genomics approach. *Mol. Biol. Evol.* **26**, 603–612. doi:10.1093/molbev/msn281
- Trifunopoulos, J., Nguyen, L.-T., von Haeseler, A. and Minh, B. Q. (2016). W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis. *Nucleic Acids Res.* **44**, W232–W235. doi:10.1093/nar/gkw256
- Tu, K. C., Pearson, B. J. and Sánchez Alvarado, A. (2012). TORC1 is required to balance cell proliferation and cell death in planarians. *Dev. Biol.* **365**, 458–469. doi:10.1016/j.ydbio.2012.03.010
- Tumaneng, K., Schlegelmilch, K., Russell, R. C., Yimlamai, D., Basnet, H., Mahadevan, N., Fitamant, J., Bardeesy, N., Camargo, F. D. and Guan, K.-L. (2012). YAP mediates crosstalk between the Hippo and PI(3)K-TOR pathways by suppressing PTEN via miR-29. *Nat. Cell Biol.* **14**, 1322–1329. doi:10.1038/ncb2615
- Van Oss, S. B. and Carvunis, A. R. (2019). De novo gene birth. *PLoS Genet.* **15**, e1008160. doi:10.1371/journal.pgen.1008160
- Udan, R. S., Kango-Singh, M., Nolo, R., Tao, C. and Halder, G. (2003). Hippo promotes proliferation arrest and apoptosis in the Salvador/Warts pathway. *Nat. Cell Biol.* **5**, 914–920. doi:10.1038/ncb1050
- Waterhouse, A. M., Procter, J. B., Martin, D. M. A., Clamp, M. and Barton, G. J. (2009). Jalview Version 2—a multiple sequence alignment editor and analysis workbench. *Bioinformatics* **25**, 1189–1191. doi:10.1093/bioinformatics/btp033
- Wenemoser, D. and Reddien, P. W. (2010). Planarian regeneration involves distinct stem cell responses to wounds and tissue absence. *Dev. Biol.* **344**, 979–991. doi:10.1016/j.ydbio.2010.06.017
- Wenemoser, D., Lapan, S. W., Wilkinson, A. W., Bell, G. W. and Reddien, P. W. (2012). A molecular wound response program associated with regeneration initiation in planarians. *Genes Dev.* **26**, 988–1002. doi:10.1101/gad.187377.112
- Werner, M. S., Sieriebriennikov, B., Prabh, N., Loschko, T., Lanz, C. and Sommer, R. J. (2018). Young genes have distinct gene structure, epigenetic profiles, and transcriptional regulation. *Genome Res.* **28**, 1675–1687. doi:10.1101/gr.234872.118
- Willsey, H. R., Zheng, X., Carlos Pastor-Pareja, J., Willsey, A. J., Beachy, P. A. and Xu, T. (2016). Localized JNK signaling regulates organ size during development. *eLife* **5**, 1–18. doi:10.7554/eLife.11491
- Wilson, G. A., Bertrand, N., Patel, Y., Hughes, J. B., Feil, E. J. and Field, D. (2005). Orphans as taxonomically restricted and ecologically important genes. *Microbiology* **151**, 2499–2501. doi:10.1099/mic.0.28146-0
- Wilson, B. A., Foy, S. G., Neme, R. and Masel, J. (2017). Young genes are highly disordered as predicted by the preadaptation hypothesis of de novo gene birth. *Nat. Ecol. Evol.* **1**, 0146. doi:10.1038/s41559-017-0146
- Wolfson, R. L. and Sabatini, D. M. (2017). The dawn of the age of amino acid sensors for the mTORC1 Pathway. *Cell Metab.* **26**, 301–309. doi:10.1016/j.cmet.2017.07.001
- Yoon, M. S. (2017). The role of mammalian target of rapamycin (mTOR) in insulin signaling. *Nutrients* **9**, E1176. doi:10.3390/nu9111176
- Zeng, Q. and Hong, W. (2008). The emerging role of the hippo pathway in cell contact inhibition, organ size control, and cancer development in mammals. *Cancer Cell* **13**, 188–192. doi:10.1016/j.ccr.2008.02.011
- Zhao, L., Saelao, P., Jones, C. D. and Begun, D. J. (2014). Origin and spread of de novo genes in Drosophila melanogaster populations. *Science* **343**, 769–772. doi:10.1126/science.1248286

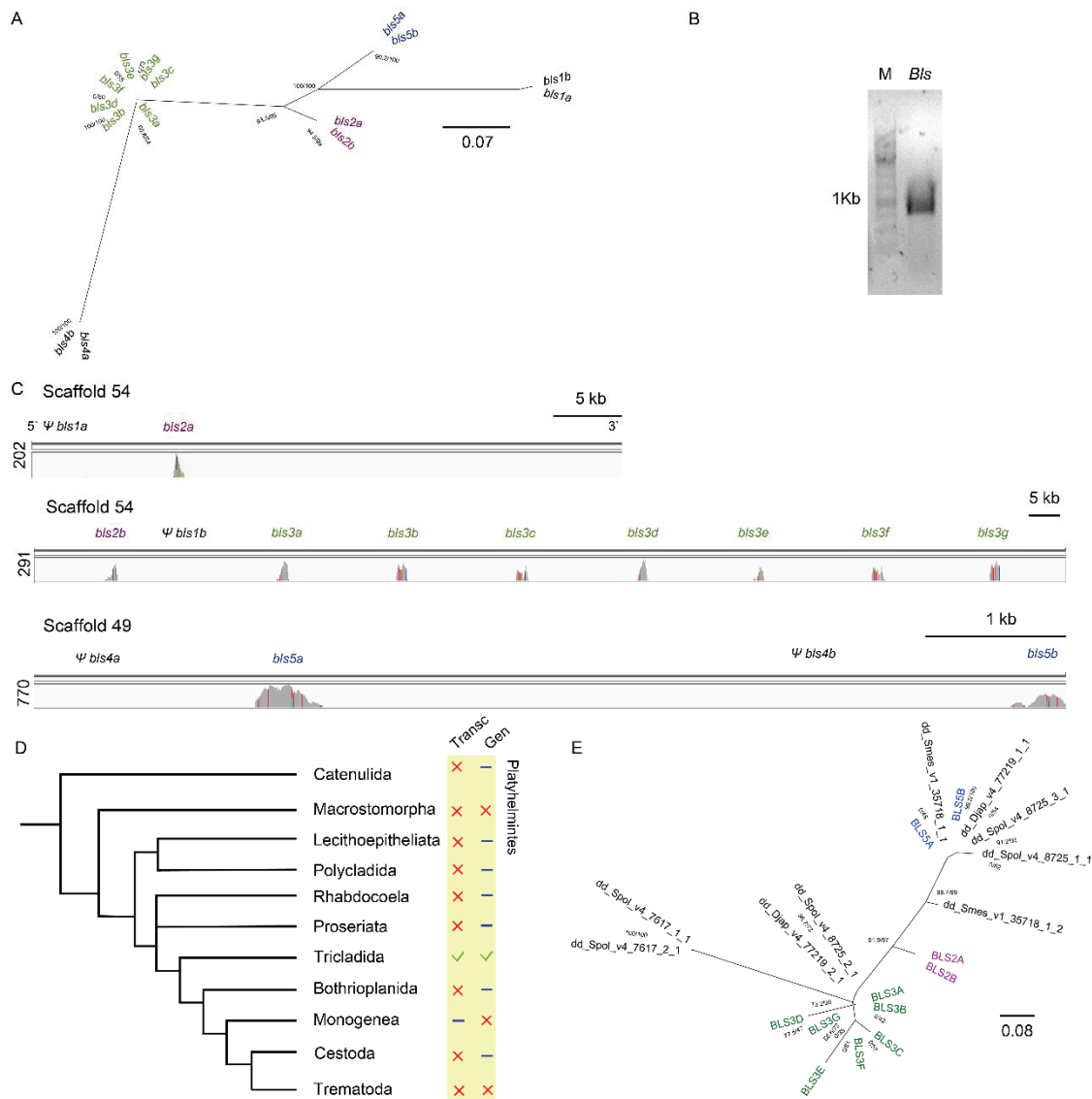


Figure S1. Genomic and evolutionary features of *bls* subfamilies. (A) Phylogenetic analysis of all members of *Smed bls* family using nucleotide sequences. They group into 5 subfamilies. Scale indicates expected nucleotide substitution per site. (B) PCR analysis using primers flanking the junction of the first *bls3* repeat (between *bls3a* and *b*) showing the expected 1 Kb band. (C) Transcriptomic reads mapping in the three non-consecutive parts of the two scaffolds where *bls* genes are located. Peaks represent reads accumulation. The absence of peaks is considered a lack of expression. Lateral number represents the higher summit in each track. (D) Presence of *bls* homologs in the transcriptomic (Transc) and genomic (Gen) available databases from different Platyhelminth species. Green check means that presence of some homolog, red cross indicates that no homologs have been identified and blue line indicates no available data. (E) Phylogenetic tree of the *bls* homologs in the Tricladida Order using amino acid sequences. *bls5* subfamily is present in all species, *bls3* was not found in *Smes*, and *bls2* was only found in *Smed*. Scale indicates expected amino acid substitution per site.

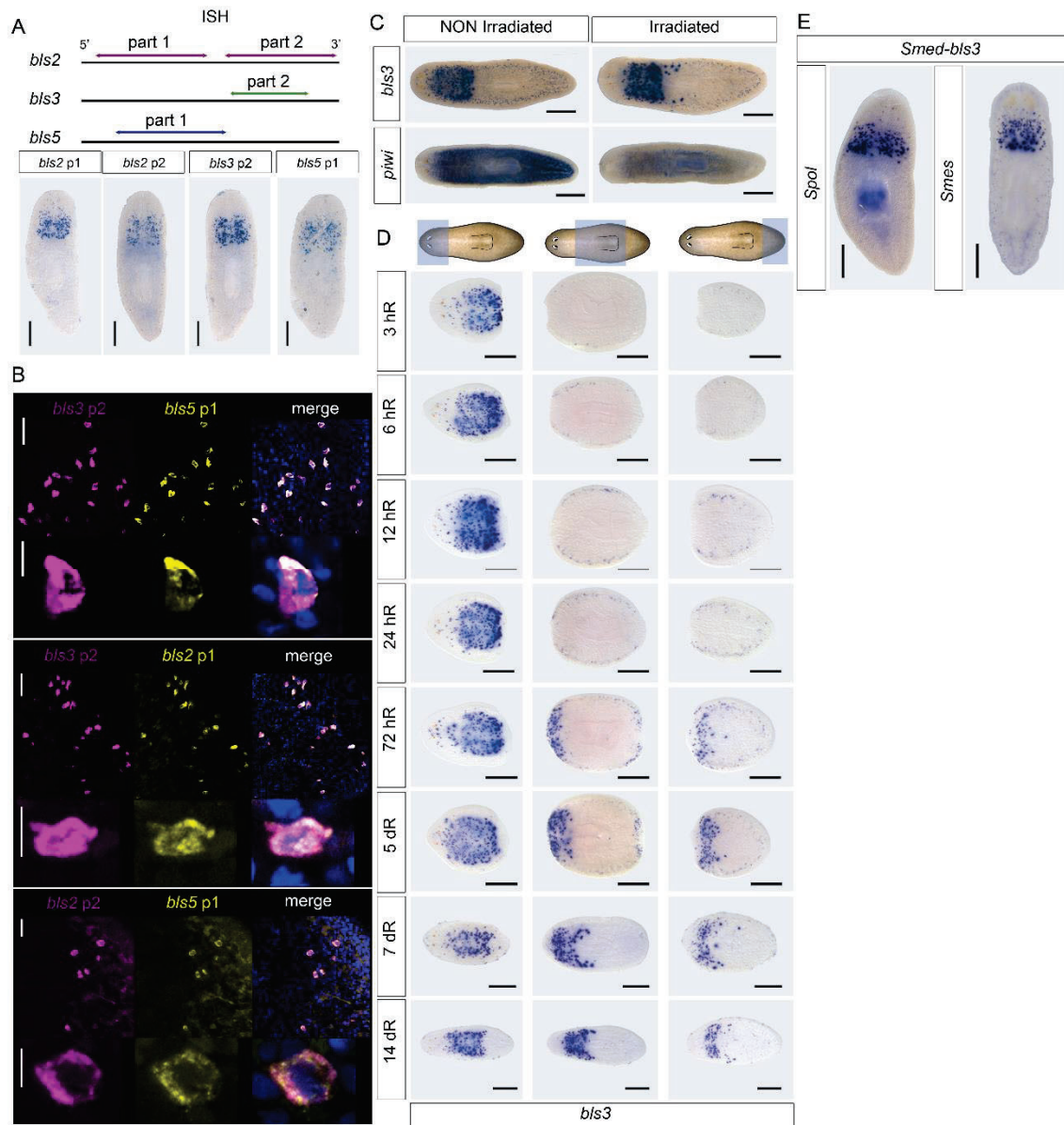


Figure S2. *bls2/3/5* expression in intact and regenerating animals. (A) Scheme indicating the riboprobes designed for each gene family. WISH with the different riboprobes in intact animals showing the similar expression pattern. (B) Double FISH combining all specific riboprobes. Each panel represents each gene combination. A magnification is also shown. All riboprobes colocalize in most of the cells, and magnifications demonstrate that riboprobes present a different cellular distribution. (C) WISH of *bls3* and *piwi* in non-irradiated and irradiated animals. After irradiation the neoblast marker (*piwi*) expression decreases but not *bls3*, corroborating that *bls* gene family are localized in differentiated cells. (D) WISH of *bls3* during regeneration at different time points. From 3hR to 24hR no *bls3* expression is observed in the blastemas. From 72hR to 14dR the new expression and redistribution of *bls3* is observed. (E) WISH of *bls3* in *Schmidtea polychroa* (Spol) and *Schmidtea mediterranea* sexual strain (Smes), showing the same expression pattern than in *Smed*. Scale bars: A, C, D and E are 500 μ m. In B are 50 μ m and 10 μ m in magnifications.

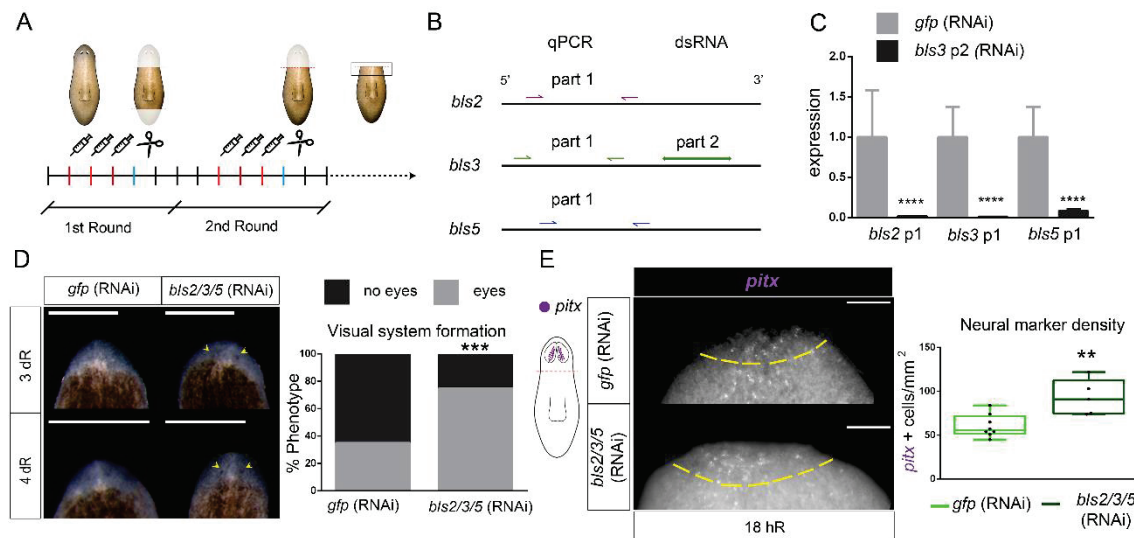


Figure S3. *bls2/3/5* inhibition after RNAi during regeneration, and faster regeneration of *bls2/3/5* (RNAi) animals. (A) Cartoon illustrating the protocol of RNAi inhibition during planarians regeneration. One week starved planarians were injected 3 consecutive days and amputated the following day. The following 3 days, planarians were let to regenerate. Planarians were amputated anterior and posteriorly, and a second week of inhibition was performed only with trunk fragments. The second week trunks were amputated just anteriorly. (B) Scheme indicating the fragments used for RNAi and qPCR analysis. (C) qRT-PCR analysis quantifying *bls2*, *bls3* and *bls5* expression after *bls3* inhibition at 3dR, demonstrating that all three subfamilies were down-regulated after injection of *bls3* dsRNA. Relative expression is plotted as $2^{-\Delta\Delta CT}$ values. Data are plotted as mean and error bars represent s. d. (**** $P < 0.0001$). (D) *in vivo* images of planarians showing that in *bls2/3/5* (RNAi) animals regenerating eyes are more evident (yellow arrows) than in controls at 3 and 4dR (n of controls=23, n of RNAi=23, *** $P < 0.001$). (E) Representation of *pitx* expression in *wt* animals. In the drawing, red dashed line represents amputation level. FISH of *pitx* shows an increase of *pitx*+ cells in the blastema at 18 hR. Quantification of *pitx*+ cells / mm^2 is showed (n of controls=8, n of RNAi=5, ** $P < 0.01$). Yellow dashed line in the images indicates the area quantified. Scale bars: 250 μm in (D), 100 μm in (E).

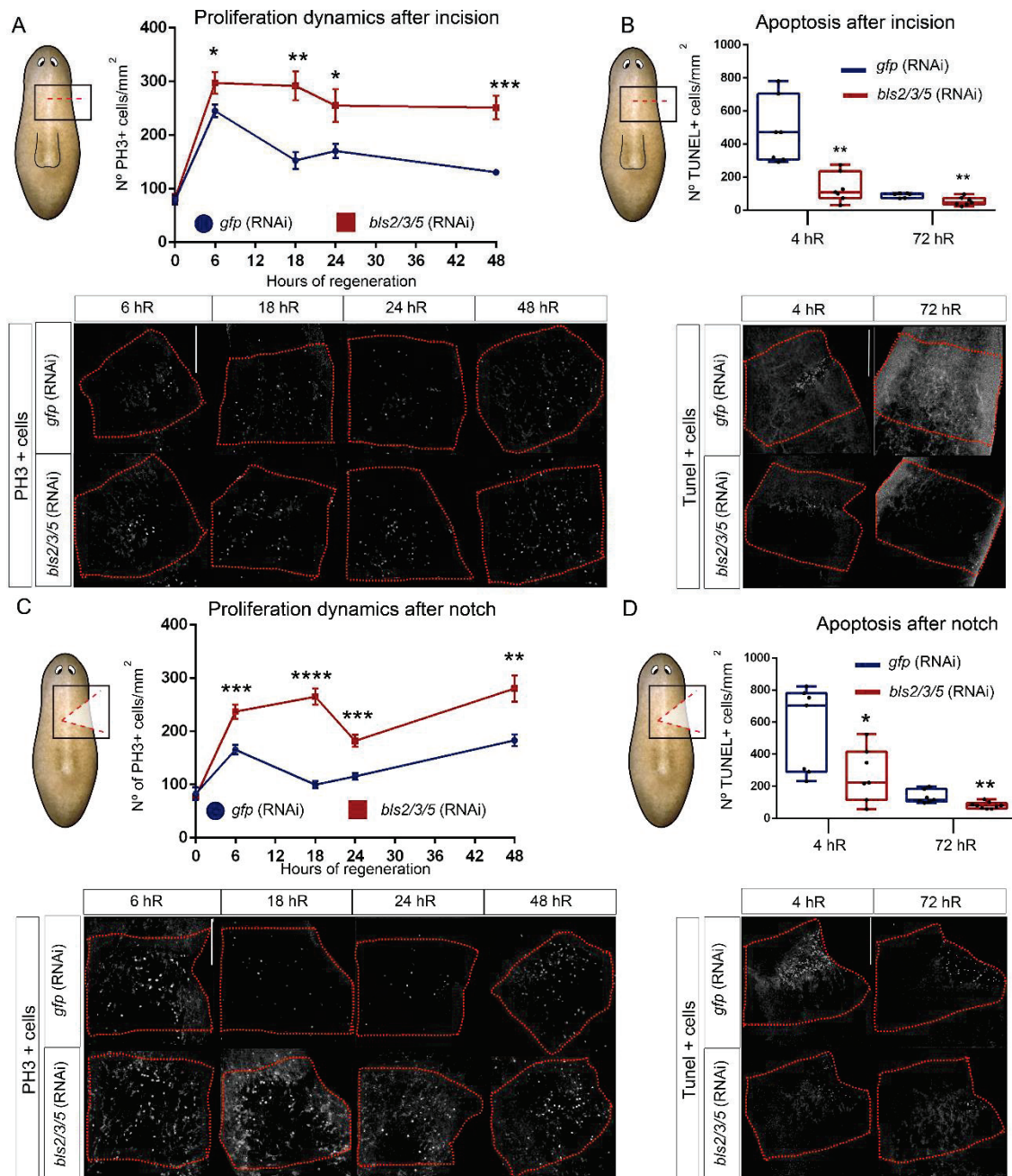


Figure S4. *bIs2/3/5* (RNAi) animals show an increase in proliferation and a decrease of apoptosis after any injury. (A) Quantification of PH3+ cells at different time points after incision shows an increment of mitotic cells/mm² in *bIs2/3/5* (RNAi) animals. Time points were 6 hR (n of controls=5, n of RNAi=7, **P*<0.05), 18 hR (n of controls=6, n of RNAi=7, ***P*<0.01), 24 hR (n of controls=6, n of RNAi=8, **P*<0.05) and 48 hR (n of controls=8, n of RNAi=8, ****P*<0.001). anti-PH3 immunostaining images are shown below. (B) Quantification of TUNEL+ cells show a decrease of apoptotic cells in *bIs2/3/5* (RNAi) animals after incision at 4 hR (n of controls=7, n of RNAi=7, ***P*<0.01) and 72 hR (n of controls=6, n of RNAi=7, ***P*<0.01). TUNEL images are shown below. (C) Quantification of PH3+ cells at different time points after notching shows an

increment of mitotic cells/mm² in *bls2/3/5* (RNAi) animals at 6 hR (n of controls=8, n of RNAi=9, *** $P<0.001$), 18 hR (n of controls=8, n of RNAi=8, **** $P<0.0001$), 24 hR (n of controls=8, n of RNAi=9, *** $P<0.001$) and 48 hR (n of controls=8, n of RNAi=8, ** $P<0.01$). anti-PH3 immunostaining images are shown below. D) Quantification of TUNEL+ cells show a decrease of apoptotic cells in *bls2/3/5* (RNAi) animals after notching at 4 hR (n of controls=7, n of RNAi=7, * $P<0.05$) and 72 hR (n of controls=7, n of RNAi=9, ** $P<0.01$). TUNEL images are shown below. All images correspond to Z projections. Illustrations show the amputation plane and the area analyzed (dashed red line and black square, respectively). Red dashed line in the images indicate the area analyzed. Scale bars: 200 μ m in all panels.

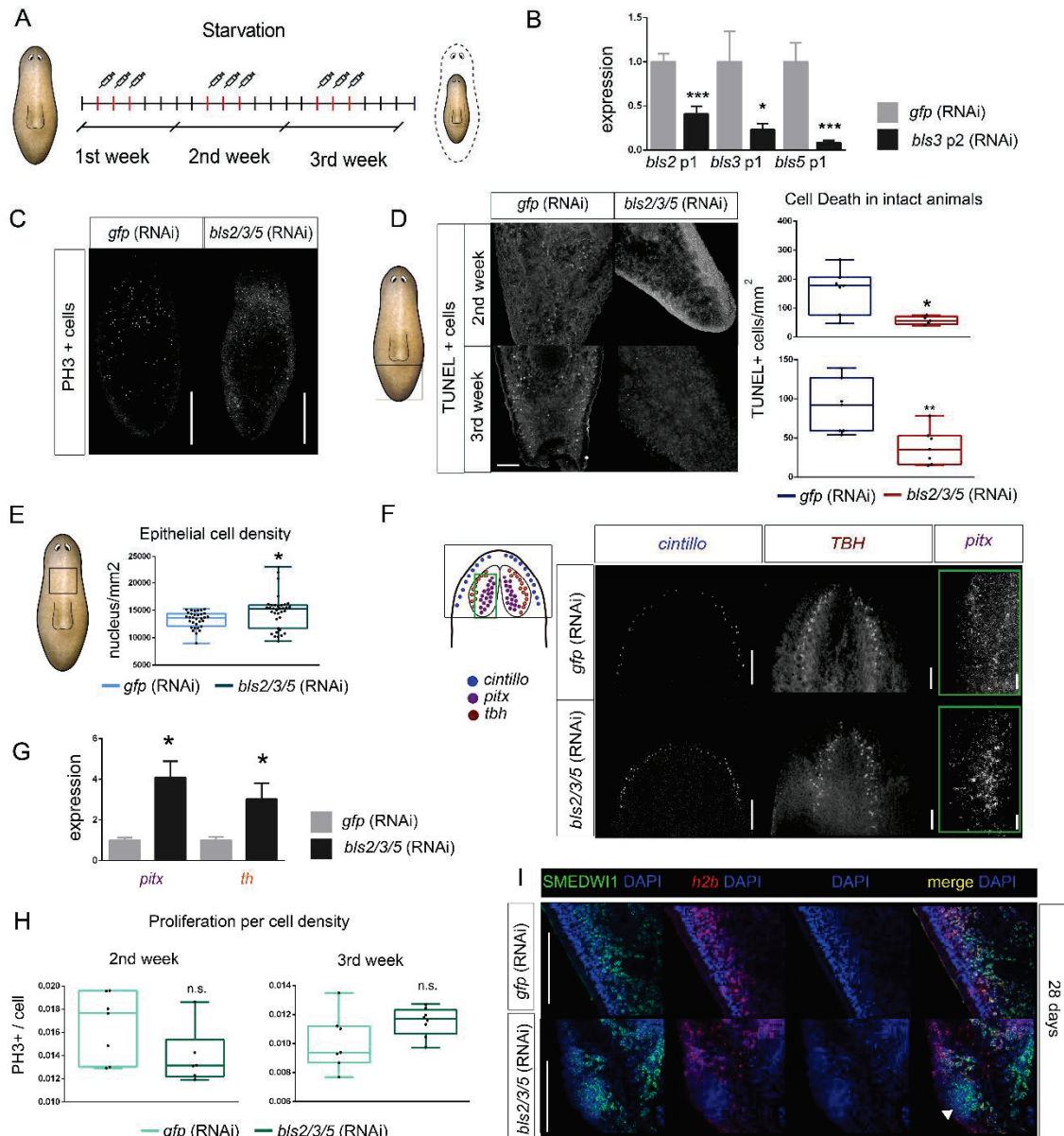


Figure S5. In starved conditions, *bIs2/3/5* (RNAi) animals show a decrease in cell death, which leads to cell accumulation and overgrowth formation. (A) Scheme of the RNAi procedure to inhibit *bIs2/3/5* in starved planarians. Each week, animals were injected 3 consecutive days. At the end of the third week, animals were fixed and analyzed. (B) qRT-PCR analysis measuring *bIs2*, *bIs3* and *bIs5* expression after 3 weeks of *bIs3* p2 inhibition demonstrated that all three genes are downregulated. Relative expression is plotted as $2^{-\Delta\Delta CT}$ values. Data is plotted as mean and error bars represent s.d. (* $P < 0.05$; *** $P < 0.001$). (C) anti-PH3 immunostaining images of *bIs2/3/5* (RNAi) animals and controls (D) TUNEL assay images of *bIs2/3/5* RNAi animals and controls, corresponding to the posterior region of the animals, which was the area quantified (indicated with a square in the schematic drawing). Images correspond to Z projections after the second and the third week of treatment. Quantification of TUNEL+

cells /area in *bls2/3/5*(RNAi) and control animals; after the second week of treatment (controls, n=7; RNAi, n=5, $*P<0.05$); and after the third week of treatment (controls, n=7; RNAi, n=7, $**P<0.01$). (E) Quantification of Nuclei of epidermal cells stained with DAPI per area (n of controls=15, n of RNAi=15, $*P<0.05$). The illustration indicates the area quantified with a black square. (F) Confocal images of the expression of neural (*tbh* and *pitx*) and chemoreceptor (*cintillo*) markers in *bls2/3/5* (RNAi) animals and controls. The illustration shows the expression of *tbh*, *pitx* and *cintillo*. *Cintillo* and *tbh*+ cells were quantified with respect to the head area (indicated with a square in the schematic drawing). *pitx*+ cells were quantified in a region of the head (green square in the images). (G) qRT-PCR quantification of the expression of neural markers (*pitx* and *th*). Relative expression is plotted as $2^{-\Delta\Delta CT}$ values. Data is plotted as mean and error bars represent s.d. ($*P<0.05$). (H) Normalization of PH3+cells/area by epidermal cell density (quantified as showed in E) in *bls2/3/5*(RNAi) and control animals. After 2 weeks of RNAi treatment (controls, n=7; RNAi, n=6; n.s.) and 3 weeks of RNAi treatment (controls, n=7; RNAi, n=8; n.s.). (I) Overgrowths of *bls2/3/5*(RNAi) stained with anti-SMEDWI and *h2b* riboprobe. Nuclei are in blue. White arrows indicate overgrowths. Images from D, G and I correspond to Z projections. Scale bars: 500 μ m in (C), 100 μ m in (D), 200 μ m in (G) and 500 μ m in (I).

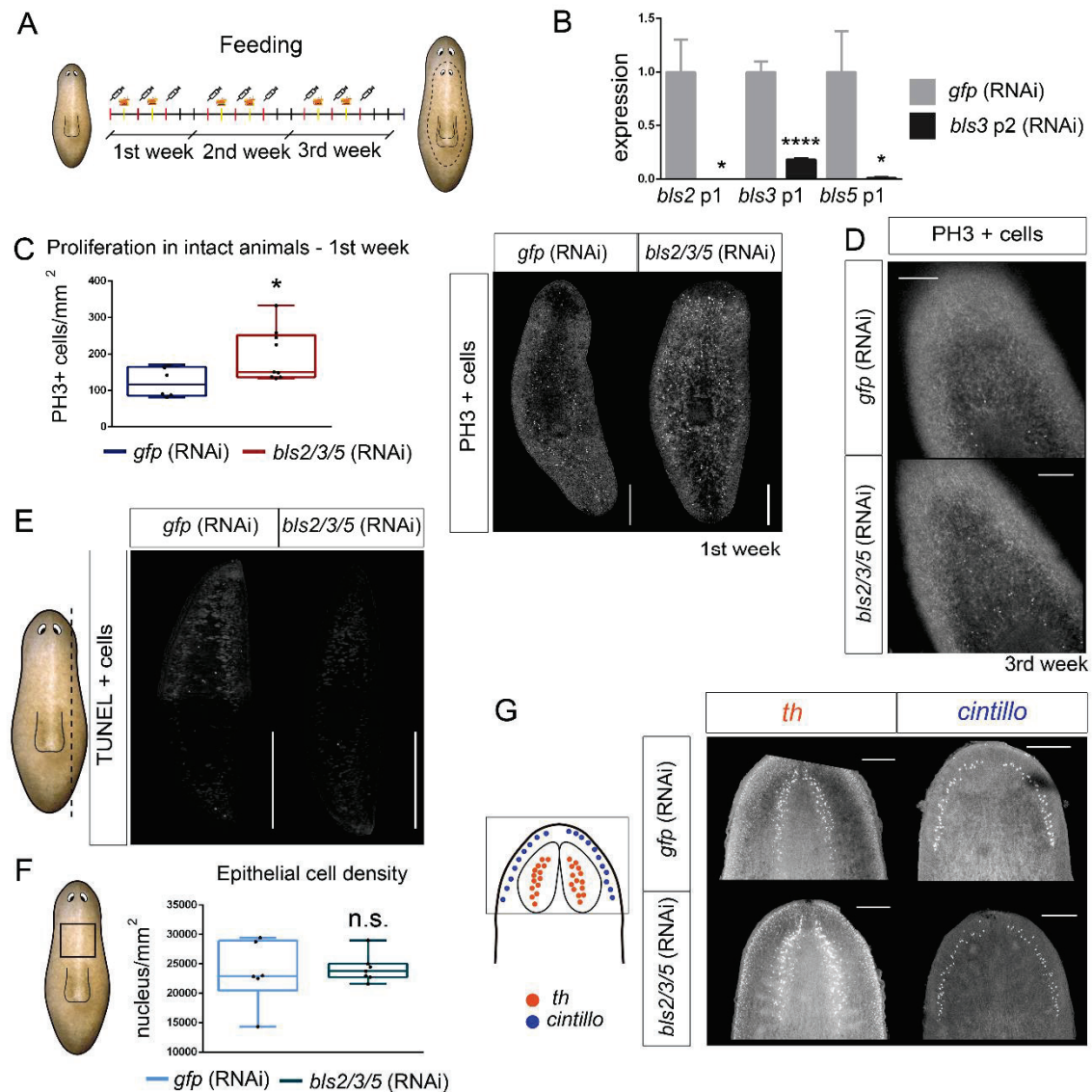


Figure S6. *B/s* inhibition during growth conditions decreases cell proliferation and increases cell death, but does not promote cell accumulation. (A) Scheme of the RNAi procedure to inhibit *bls2/3/5* in fed planarians. Each week, animals were injected 3 non consecutive days, being feed the other two days. At the end of the third week, animals were fixed (three days after the last injection) and analyzed. (B) qRT-PCR quantifying *bls2*, *bls3* and *bls5* expression after 3 rounds of *bls3* inhibition, demonstrating that all three genes are downregulated. Relative expression is plotted as $2^{-\Delta\Delta CT}$ values. Data is plotted as mean and error bars represent s.d. (* $P < 0.05$; *** $P < 0.001$). (C) Quantification of PH3+ cells/body area after 1 week of *bls2/3/5* RNAi treatment (n of controls=5, n of RNAi=9, * $P < 0.05$), and the corresponding anti-PH3 immunostaining images showing the increment of the mitotic cells. (D) Anti-PH3 immunostaining images of *bls2/3/5* RNAi animals and controls showing the increment of the mitotic cells after 3 weeks of the treatment. (E) TUNEL assay images of *bls2/3/5*

RNAi animals and controls, corresponding to transversal sections of the animals. TUNEL+ cells/section area was quantified. At least 9 sections per animal and at least 5 animals per condition were analyzed (F) Quantification of nuclei of epidermal cells stained with DAPI per area (n of controls=15, n of RNAi=15, *** $P<0.001$). The illustration indicates the area quantified with a black square. (G) Confocal images of the expression of neural (*th*) and chemoreceptor (*cintillo*) markers in *bls2/3/5* RNAi animals and controls. The illustration shows the expression of *th* and *cintillo*, and the square indicates the area analyzed. Images from C and F correspond to Z projections. Scale bars: 200 μm in the entire panel.

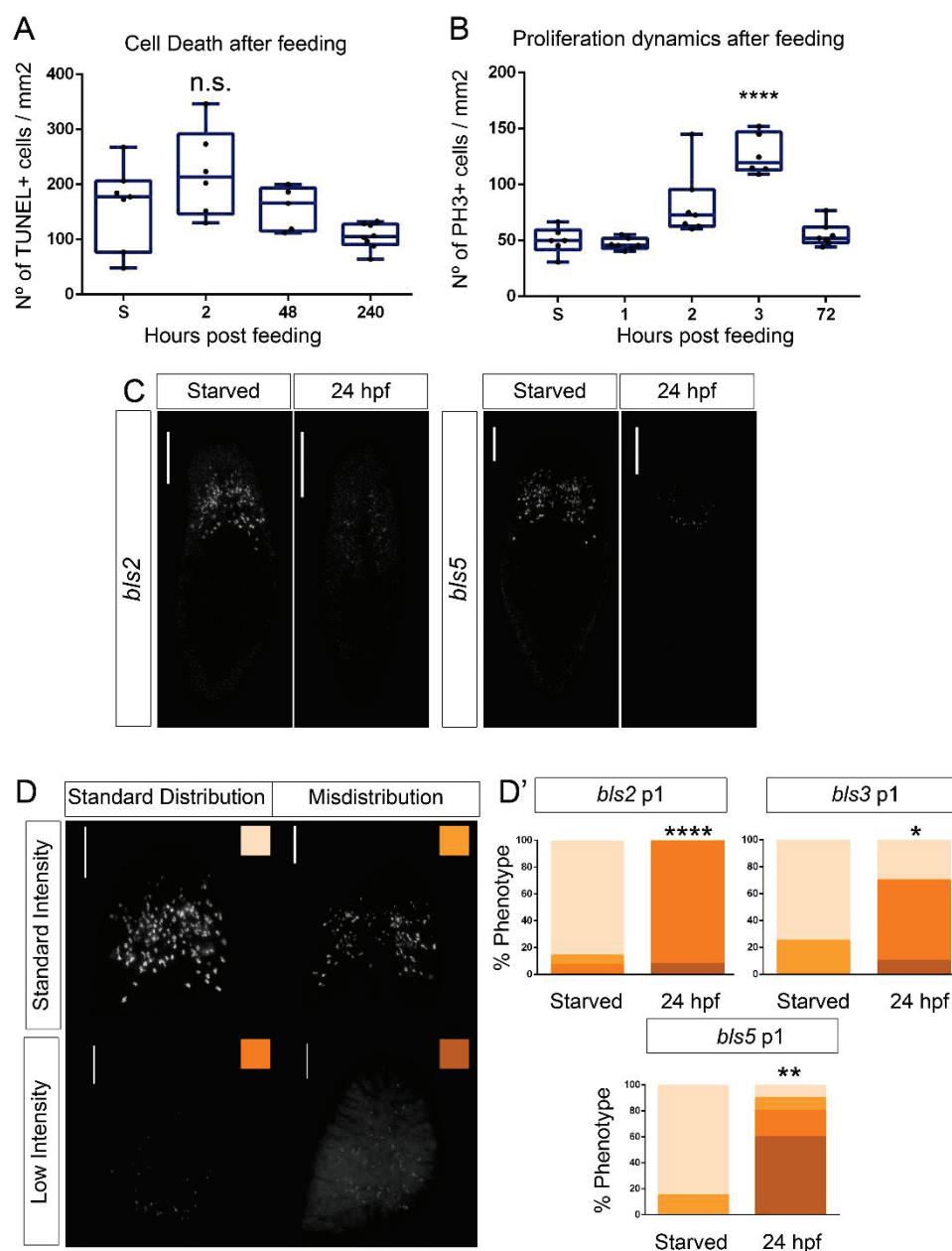


Figure S7. *bls2*, *bls3* and *bls5* are down-regulated by food ingestion. (A) TUNEL assay at different time points after feeding reveals no changes in wild type planarians (n of starved=7, n of 2hpf=6, n.s.). (B) PH3+ cells/body area quantification at different time points after feeding reveals a proliferative peak at 3hpf (n of starved=6, n of 3hpf=6, **** $P<0.0001$). (C) Representative FISH images of *bls2* and *bls5* before feeding and after feeding, demonstrating its reduced expression at 24 hpf. (D) Representative FISH images showing the downregulation and delocalization of *bls2*, *bls3*, and *bls5* expression observed after feeding. Four patterns of *bls2*, *bls3*, and *bls5* expression could be observed: high intensity and localized; high intensity and

delocalized; low intensity and localized; low intensity and delocalized. (The brightness and contrast of the images is not equivalent, since in the low density ones it had to be adjusted to show the expression). The percentages corresponding to each category are shown in D' (starvation conditions, $n > 7$; 24 hpf, $n > 10$; * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$).

Table S1. *Schmidtea mediterranea* *bls* nucleotide sequences.

[Click here to Download Table S1](#)

Table S2. Nucleotide subfamily identity.

[Click here to Download Table S2](#)

Table S3. Primers used to clone *bls* genes; and qPCR primers.

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Table S4. ORF of the *bls* genes in *Schmidtea mediterranea*.

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Table S5. Platyhelminthes species.

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Table S6. Nucleotide sequences of *bls* homologs in Tricladida species.

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Table S7. ORF of the *bls* homologs in Tricladida species.

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Table S8. Alignments of ISH and dsRNA sequences

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