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Short communication

Dissecting the function of Cullin-RING ubiquitin ligase complex genes in planarian regeneration

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ABSTRACT

The ubiquitin system plays a role in nearly every aspect of eukaryotic cell biology. The enzymes responsible for transferring ubiquitin onto specific substrates are the E3 ubiquitin ligases, a large and diverse family of proteins, for which biological roles and target substrates remain largely undefined. Studies using model organisms indicate that ubiquitin signaling mediates key steps in developmental processes and tissue regeneration. Here, we used the freshwater planarian, *Schmidtea mediterranea*, to investigate the role of Cullin-RING ubiquitin ligase (CRL) complexes in stem cell regulation during regeneration. We identified six *S. mediterranea cullin* genes, and used RNAi to uncover roles for homologs of Cullin-1, −3 and −4 in planarian regeneration. The *cullin-1* RNAi phenotype included defects in blastema formation, organ regeneration, lesions, and lysis. To further investigate the function of *cullin-1*-mediated cellular processes in planarians, we examined genes encoding the adaptor protein Skp1 and F-box substrate-recognition proteins that are predicted to partner with Cullin-1. RNAi against *skp1* resulted in phenotypes similar to *cullin-1* RNAi, and an RNAi screen of the F-box genes identified 19 genes that recapitulated aspects of *cullin-1* RNAi, including ones that in mammals are involved in stem cell regulation and cancer biology. Our data provides evidence that CRLs play discrete roles in regenerative processes and provide a platform to investigate how CRLs regulate stem cells *in vivo*.

1. Introduction

Planarians have emerged as an important model organism to examine gene function in stem cell-based tissue regeneration (Elliott and Sánchez Alvarado, 2012; Roberts-Galbraith and Newmark, 2015; Ross et al., 2017). These animals can restore lost or damaged tissues from a population of adult pluripotent stem cells, termed neoblasts (Baguñà, 2012; Rink, 2013; Wagner et al., 2011; Zhu and Pearson, 2016). Recent studies have provided insights into the molecular mechanisms that regulate regeneration in planarians at the genetic level (Elliott and Sánchez Alvarado, 2012; Roberts-Galbraith and Newmark, 2015; Wurtzel et al., 2015). However, the dynamic regulation of proteins during regeneration remains an open area of investigation. An essential cellular pathway in protein regulation is the ubiquitin system, in which cells utilize the highly conserved small ubiquitin polypeptide as a post-translational modification of other proteins, which can lead to degradation of target proteins (Ciechanover et al., 1984, 1980; Finley et al., 1984; Hershko et al., 1980). The ubiquitin-system plays a crucial role in diverse cellular processes, including DNA repair, transcription, synaptic plasticity, and regulation of the cell cycle, wherein ubiquitin-mediated proteolysis is a key regulatory step (Bennett and Harper, 2008; Dhananjayan et al., 2005; Finley et al., 2004; Glickman and Ciechanover, 2002; Hershko and Ciechanover, 1998; Hershko et al., 2000; Kawabe and Brose, 2011; Nakayama and Nakayama, 2005; Pickart, 2004; Varshavsky, 2005).

Ubiquitin is directed onto specific substrate proteins by E3 ubiquitin ligases (Ardley and Robinson, 2005; Dikic and Robertson, 2012; Glickman and Ciechanover, 2002; Hershko and Ciechanover, 1998), a large class of enzymes (over 600 predicted genes in humans; Li et al., 2008) for which many of the target specificity and function remain poorly understood. Identification of the biological roles of the E3s has been facilitated by siRNA screens using human cells *in vitro*, and by genetic screens in model organisms, such as *Drosophila* and *C. elegans* (Williamson et al., 2013). Specific roles for ubiquitin ligases have been demonstrated in embryonic stem cell fate determination (Werner et al., 2017; Xu et al., 2009), eye development (Ou et al., 2003), and neural development (Boix-Perales et al., 2007; Bury et al., 2008; J. Chen et al., 2012; D'Arca et al., 2010; Hoeck et al., 2010; Sobieszczuk et al., 2010;

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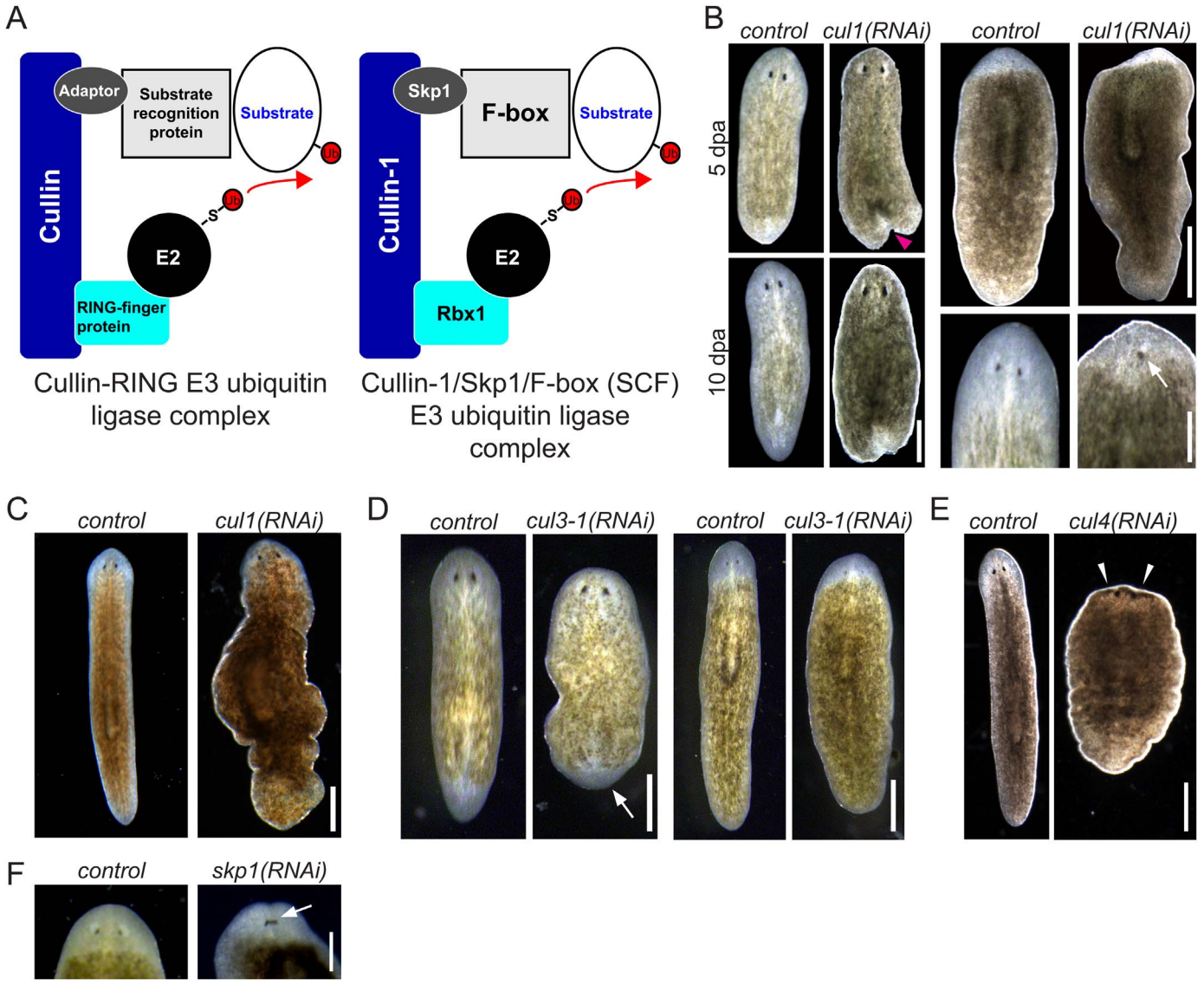

Fig. 1. Planarians possess multiple Cullin genes with distinct roles in regeneration and tissue homeostasis. A) Diagram summarizing the general organization of Cullin-RING E3 ubiquitin ligase complexes or the prototypical Skp1/Cullin-1/F-box (SCF) E3 ubiquitin ligase complex. E3 ubiquitin ligases transfer ubiquitin to the substrate by forming an isopeptide bond. B) Animals were fed dsRNA 6 times over 3 weeks against *gfp* (*controls*; n = 22) or *cul1* (n = 22), amputated pre-pharyngeally and allowed to regenerate for 10 days. Magenta arrowhead in head regenerate at 5 dpa indicates indented blastema in *cul1(RNAi)* planarian. White arrow marks abnormal regeneration in a single eye spot in the head of a *cul1(RNAi)* trunk regenerate. C) Animals were fed dsRNA over 6–8 weeks against *gfp* (*controls*; n = 38) or *cul1* (n =38). All *cul1(RNAi)* worms showed loss of mobility, formed lesions, and subsequently lysed. D) Animals were fed dsRNA 5 times over 3 weeks against *gfp* (*control*; n = 30) or *cul3-1* (n = 21), amputated pre-pharyngeally, and allowed to regenerate for 10 days. *cul3-1(RNAi)* planarians showed delayed regeneration and differentiation when compared to the controls. White arrow marks the small blastema observed in head regenerates. E) Animals were fed dsRNA 5 times over 3 weeks against *gfp* (*control*; n = 30) or *cul4* (n = 21), amputated pre-pharyngeally, and allowed to regenerate for 10 days. F) Animals were fed dsRNA 6 times over 3 weeks against *gfp* (*control*; n = 50) or *skp1* (n = 45), amputated pre-pharyngeally, and allowed to regenerate for 10 days. White arrow denotes abnormal eye regeneration. Scale bars = 500 μm.

Voigt and Papalopulu, 2006; Zhao et al., 2008; Zhu et al., 2005). Ubiquitin system components regulate regeneration in nematodes, flies, and mice, and are specifically upregulated during regeneration in sea cucumbers and axolotls (Hindi and Kumar, 2016; Pasten et al., 2012; Rao et al., 2009; Tian and Wu, 2013).

We are utilizing planarians as a model system to investigate the roles of E3 ubiquitin ligases in tissue regeneration. Previously, our lab demonstrated that members of the HECT E3 ligase gene family, which directly catalyze ubiquitin transfer onto a substrate via a ubiquitin-HECT complex intermediate (Metzger et al., 2012), are required for diverse aspects of regeneration in the planarian *Schmidtea mediterranea* (Henderson et al., 2015). In contrast to the HECT family, most E3 enzymes do not directly bind and transfer ubiquitin but rather coordinate the transfer of ubiquitin from an E2 onto a substrate, often through multimeric complexes, including the Cullin-RING ligase (CRL) family (Sarikas et al., 2011). Cullin proteins act as molecular scaffolds that organize the binding of other elements to form an E3 complex that requires a substrate recognition subunit (Fig. 1A). These recognition subunits confer target specificity for ubiquitylation and their differential utilization allows modularity within CRL classes, thereby enabling function in multiple aspects of cellular biology.

In this study, we analyzed CRL function in tissue regeneration by inhibiting genes encoding major components of these complexes in planarians. First, we identified and performed RNAi against *cullin* genes present in *S. mediterranea* and found that homologs of Cullin-1, -3 and -4 are involved in regulating tissue homeostasis and regeneration. Robust *cullin-1* RNAi phenotypes included lesions, lysis, and defects in blastema formation, organ regeneration, and homeostatic tissue maintenance. Cullin-1 is a core component of the canonical CRL, the SCF (Skp1/Cullin-1/F-box)-E3 ubiquitin ligase complex (Fig. 1A)

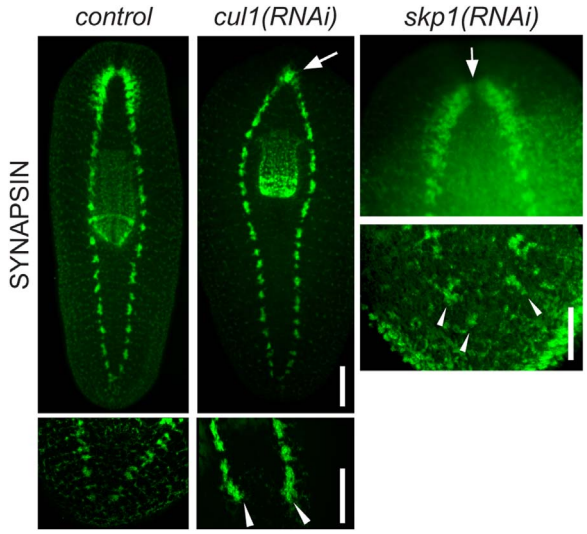

Fig. 2. *cullin-1* and *skp1* are required for normal nervous system regeneration. Animals were fed dsRNA 6 times over 3 weeks against *gfp*, *cul1* or *skp1*, amputated pre-pharyngeally, allowed to regenerate for 10 days, and stained with anti-SYNAPSIN to visualize nervous system regeneration. White arrow marks small mass of neuropil adjoining the ventral nerve cords or lack of anterior commissure formation in trunk regenerates and the arrowheads mark the ventral nerve cord stumps of head regenerates in *cul1(RNAi)* and *skp1(RNAi)* planarians. Scale bars: 250 μm for whole worm and 100 μm for tail inset for *cul1* RNAi; 250 μm for *skp1* RNAi.

(Sarikas et al., 2011). By combining *in situ* hybridizations and RNAi screens targeting SCF-complex member genes that encode homologs of Skp1 and F-box proteins, we gained insight into the function of *cullin-1*-mediated cellular processes. We found that *skp1* is ubiquitously expressed while *f-box* genes are expressed in diverse tissue-types. Knockdown of *skp1* recapitulated *cullin-1* RNAi phenotypes and knockdown of various *f-box* genes recapitulated aspects of the *cullin-1* RNAi phenotype. Our study provides evidence that perturbation of elements within multimeric E3 ubiquitin ligase complexes can identify discrete functions for E3 ubiquitin ligases in regenerative processes and demonstrates conserved biological roles for a subset of the *f-box* genes, which are involved in stem cell regulation and cancer biology in other organisms.

2. Results

2.1. Cullin homologs are broadly expressed in stem cells and differentiated tissues of S. mediterranea

To examine the role of CRLs in planarian regeneration, we first searched the *S. mediterranea* genome and transcriptomes using human Cullin protein sequences and found six genes predicted to encode homologs of Cullin-1 through −5: *Smed-cullin-1, Smed-cullin-2, Smed-cullin-3-1, Smed-cullin-3-2, Smed-cullin-4* and *Smed-cullin-5* (see Table S1; hereon the *S. mediterranea* homologs will be referred to as *cul1*, etc.). In addition, we performed phylogenetic analysis to establish the relationship of the *S. mediterranea cullin* genes to well-characterized homologs in other species (Fig. S1). These genes are predicted to have broad expression in multiple *S. mediterranea* cell types by single-cell RNA-seq data, including neoblasts and differentiated tissues (Fig. S2A) (Wurtzel et al., 2015). Consistent with the single-cell expression data, whole-mount *in situ* hybridization (WISH) showed that *cullin* genes are broadly expressed in planarians (Fig. S2A-B), suggesting roles for these genes in multiple tissues.

2.2. Cullin gene knockdown leads to pleiotropic defects in regeneration and tissue homeostasis

Amputation of planarian tissues provides a simple paradigm to assess the role of genes in stem cell-based tissue renewal. We examined the function of individual *cullin* genes using RNAi, amputating the worms pre-pharyngeally and allowing the animals to regenerate for up to 10 days (a time-point by which the worms have fully regenerated and patterned their tissues). *cul1(RNAi)* worms had defects in blastema formation, cell differentiation (e.g., delayed optic pigment cup regeneration), and possible disruption of midline patterning signaling (e.g., single observable eye spot) compared to control worms (Fig. 1B). Extended *cul1(RNAi)* treatment resulted in defects in uninjured worms, including slow inching-like locomotion, lesions, and eventual lysis (Fig. 1C). *cul3-1(RNAi)* worms exhibited defects in blastema formation and photoreceptor regeneration (Fig. 1D), and *cul4(RNAi)* worms displayed ventral curling, lesions, and subsequent lysis of the worms after amputation (Fig. 1E), as well as during homeostasis (n = 21; not shown). These data identify roles for multiple *cullin* genes in planarian regeneration, and indicate that *cul1* and *cul4* are essential for planarian survival.

Motivated by our interest in the robust *cul1* phenotypes, we sought to investigate further the potential association of this gene to the canonical SCF complex (Fig. 1A). Skp1 mediates the interaction between Cullin-1 and F-box proteins (Ardley and Robinson, 2005; Sarikas et al., 2011). We identified a planarian homolog of Skp1, *Smed-skp1* (*skp1*) (dd_Smed_v6_1337_0_1; *Rattus norvergicus*, Q6PEC4, BLASTX = $5.4e^{-91}$), and found that, similar to *cul1*, *skp1* has broad expression in the animal (Fig. S2A-B). Furthermore, we reasoned that *skp1* RNAi should phenocopy *cul1* inhibition. Indeed, *skp1(RNAi)* planarians showed a delay in blastema formation and photoreceptor regeneration by 10 days post amputation (dpa) (Fig. 1F). We further examined their function in tissue differentiation. *cul1* and *skp1* are expressed in neurons (Fig. S2A-B); thus, we hypothesized that these genes regulate neuronal regeneration. To visualize the central nervous system, we labeled *control(RNAi)*, *cul1(RNAi)*, and *skp1(RNAi)* worms with anti-SYNAPSIN (Fig. 2). During anterior regeneration, a drastic impairment in brain formation, with absent or narrowed cephalic ganglia, was observed in both *cul1(RNAi)* and *skp1(RNAi)* worms. Similarly, the ventral nerve cords did not regenerate in *cul1(RNAi)* and *skp1(RNAi)* head regenerates (Fig. 2). These data support the hypothesis that *cul1* and *skp1* function together in putative SCF complexes of *S. mediterranea*. Therefore, we sought to dissect the roles of specific SCF complexes by examining the function and expression of genes homologous to the F-box substrate recognition partners.

2.3. Analysis of F-box genes in S. mediterranea

To screen for specific roles of SCF complexes in planarians, we identified F-box-encoding genes in *S. mediterranea*. F-box proteins are defined by an F-box domain, which is necessary for SKP1-F-box protein binding (Bai et al., 1996). BLAST searches were performed using F-box domains from diverse species (see Materials and Methods) and identified 35 *S. mediterranea f-box* genes (Table S2), all of which contain an F-box domain and were classified by homology to one of three categories that are defined by the presence of other protein-protein interacting domains: F-box “only” (FBX), F-box leucine-rich repeat containing (FXL), or F-box WD40-repeat containing (FBW) (Table S2).

We hypothesized that F-box proteins, which confer SCF target specificity, mediate discrete roles for *cullin-1* in planarian tissues and that these functions can be genetically dissected using RNAi. Thus, we performed an RNAi screen against 30 *f-box* genes to determine if these genes can phenocopy aspects of the *cul1* and *skp1* phenotypes. Worms were treated with dsRNA and observed for defects in homeostasis and mobility before being amputated pre-pharyngeally and observed

Table 1
Functional analysis of F-box encoding genes in *Schmidtea mediterranea*.

***Smed* Gene ID**	**Phenotype**	**Nervous system patterning phenotype**
Smed-btrcp/fbw1a	Lesions/lysis (13/13)	Not analyzed
Smed-ect2-like	Delayed regeneration/patterning (14/27)	Not analyzed
Smed-fbw-3	Delayed regeneration/patterning (10/43), loss of mobility (10/43)	None observed
Smed-fbw7-like-1	Delayed regeneration/patterning (12/26)	Cephalic ganglia anterior commissure defect (7/14)
Smed-fbw7-like-2	Delayed regeneration/patterning (28/48)	Cephalic ganglia anterior commissure defect (7/14) and decreased neuropil density (7/14)
Smed-fbx-10	Delayed regeneration/patterning (25/31)	None observed
Smed-fbx-11	Delayed regeneration/patterning (27/42)	None observed
Smed-fbx-2	Delayed regeneration/patterning (15/15)	None observed
Smed-fbx-4	Delayed regeneration/patterning (13/30)	Cephalic ganglia anterior commissure defect (7/16)
Smed-fbx36	Delayed regeneration/patterning (8/27)	None observed
Smed-fbx38	Delayed regeneration/patterning (64/86)	Ventral nerve cord defect (4/5), reduced or absent cephalic ganglia (14/22)
Smed-fbx8	Delayed regeneration/patterning (12/43)	None observed
Smed-fxl-2	Delayed regeneration/patterning (10/29)	None observed
Smed-fxl-3	Delayed regeneration/patterning (8/22)	Cephalic ganglia anterior commissure defect (8/14) and decreased neuropil density (12/14)
Smed-fxl13	Delayed regeneration/patterning (10/32)	None observed
Smed-fxl16	Delayed regeneration/patterning (12/20)	None observed
Smed-fxl2-1	Delayed regeneration/patterning (24/47)	Reduced neuropil density of cephalic ganglia (7/26)
Smed-fxl20	Delayed regeneration/patterning (24/32)	Reduced neuropil density of cephalic ganglia (8/11)
Smed-morgue/ubc2	Delayed regeneration/patterning (27/36)	Cephalic ganglia anterior commissure defect (9/13), decreased neuropil density (4/13)

through regeneration for 10 days. We found that RNAi knockdown of 19 *f-box* genes led to defects in homeostasis or regeneration, like delayed regeneration (e.g., small blastemas), defects in blastema patterning (abnormal eye regeneration), mobility defects, lesions, and lysis (Table 1 and S2), which are similar to the phenotypes observed in *cul1* and *skp1* RNAi planarians. Worms fed RNAi against *btrcp* displayed body shape defects with tissue outgrowths, lesions, ventral curling, and lysis (Fig. 3A). These phenotypes were similar to those observed in *cul1(RNAi)* worms, suggesting that a planarian β-TrCP-containing SCF complex (i.e., $SCF^{\beta\text{-}TrCP}$) regulates cell signaling and survival. Additional RNAi phenotypes included loss of mobility (*fbw-3*) and blastema defects (*fbw7-like-1*, *fbw7-like-2*, *fbx38*, *fxl-3*, *fxl2-1*, and *fxl20*) (Table 1 and S2, Fig. 3B).

To further examine how *f-box* genes are involved in stem cell function or organ regeneration/patterning, we repeated RNAi experiments for 17 f-box genes and stained the worms with markers for the nervous system or mitosis. Anti-SYNAPSIN staining revealed defects in nervous system regeneration in eight of the RNAi treatment groups, such as a failure of the cephalic ganglia to connect at the anterior commissure, small or absent cephalic ganglia, and decreased neuropil density (Table 1, Fig. 3B). We also stained control and F-box RNAi planarians with anti-phospho-Histone H3 (PH3) to label mitotic cells. We found that nine genes displayed a decrease in the number of $PH3^+$ cells, while one gene, *fxl2-1*, showed an increase in $PH3^+$ cells at 10 dpa (Fig. 3C and S3A).

To determine if the expression of F-box encoding genes correlates with the cell- or tissue-specific phenotypes in planarians, we performed WISH against the 19 genes that showed phenotypes during screening. Six of the eight genes involved in nervous system regeneration had strong expression in the central nervous system (*fbw7l-1*, *fbw7l-2*, *fbx38*, *fxl2-1*, *fxl20*, and *morgue*) (Fig. 3D). We also observed tissue-specific gene expression patterns, such as *fbx-10* and *fxl13*, which displayed neural and pharyngeal expression patterns (Fig. 3D). Importantly, single-cell RNA-seq data support that *f-box* genes are expressed in diverse tissue types; furthermore, all 19 *f-box* genes producing RNAi phenotypes are predicted to have expression in *cul1*-expressing cells (Fig. S3B). Combined with the results from the RNAi screen, these data further indicate that *f-box* expression may be dictating tissue-specific roles of SCF complexes, including neoblast proliferation and differentiation, blastema formation, and organ patterning.

3. Discussion

In this study, we examined the function of Cullin-RING ligase (CRL) genes in planarian regeneration. Our work revealed definitive roles for three Cullin genes, *cul1*, *cul3-1* and *cul4,* in planarian regeneration and survival. Our results are consistent with extensive studies implicating these genes in stem cell biology and cell cycle regulation (Nakayama and Nakayama, 2005; Werner et al., 2017). We did not examine the function of the Anaphase Promoting Complex (APC) Cullin-domain subunit (Apc2) since previous studies indicate the APC has a conserved role in cell division (RNAi knockdown of a planarian CDC23 homolog, a component of the APC, blocks mitosis) (Azimzadeh et al., 2012; Reddien et al., 2005a). We had two major objectives in this work: to determine if multimeric E3 complexes could be studied using RNAi methodology in planarians, and further, to dissect the specific functions of E3 complex genes in stem cell regulation and tissue regeneration. Based on the results of screening the *cullin* genes, we focused on analyzing the highly conserved Skp1/Cullin-1/F-box (SCF) complex components. Knocking down *cul1* or *skp1* led to homeostasis and regeneration-specific phenotypes, like defects in blastema formation and nervous system regeneration. Due to the expected pleiotropic nature of the phenotypes observed in *cul1* and *skp1* RNAi worms, we subsequently analyzed F-box protein-encoding genes and found that knockdown of 19 genes, all of which are predicted to have expression in $cul1^+$ cells, phenocopied aspects of the *cul1*/*skp1* RNAi phenotypes. Moreover, our results implicate a subset of *f-box* genes in cell cycle regulation.

Because of their well-appreciated roles in stem cell biology and cancer (Maneix and Catic, 2016; Strikoudis et al., 2014; Werner et al., 2017), F-box proteins are important candidates for mechanistic evaluation and drug design. For example, $SCF^{\beta\text{-}TrCP}$ ubiquitylates substrates with key roles in signal transduction pathways that underlie many aspects cell division, development, and tumorigenesis (Fuchs et al., 2004; Willems et al., 2004). $SCF^{\beta\text{-}TrCP}$ is involved in regulating β-Catenin stability (Stamos and Weis, 2013) and it is intriguing that *btrcp* inhibition in *S. mediterranea* did not result in the obvious patterning defects characteristic of RNAi knockdown against canonical Wnt signaling components (Almuedo-Castillo et al., 2012). This could be due to the strength of the phenotype following *btrcp* RNAi, which included tissue outgrowths, ventral curling, and lysis. Although these phenotypes are consistent with a function of $SCF^{\beta\text{-}TrCP}$ in stem cell biology (Werner et al., 2017), it would be interesting to resolve a

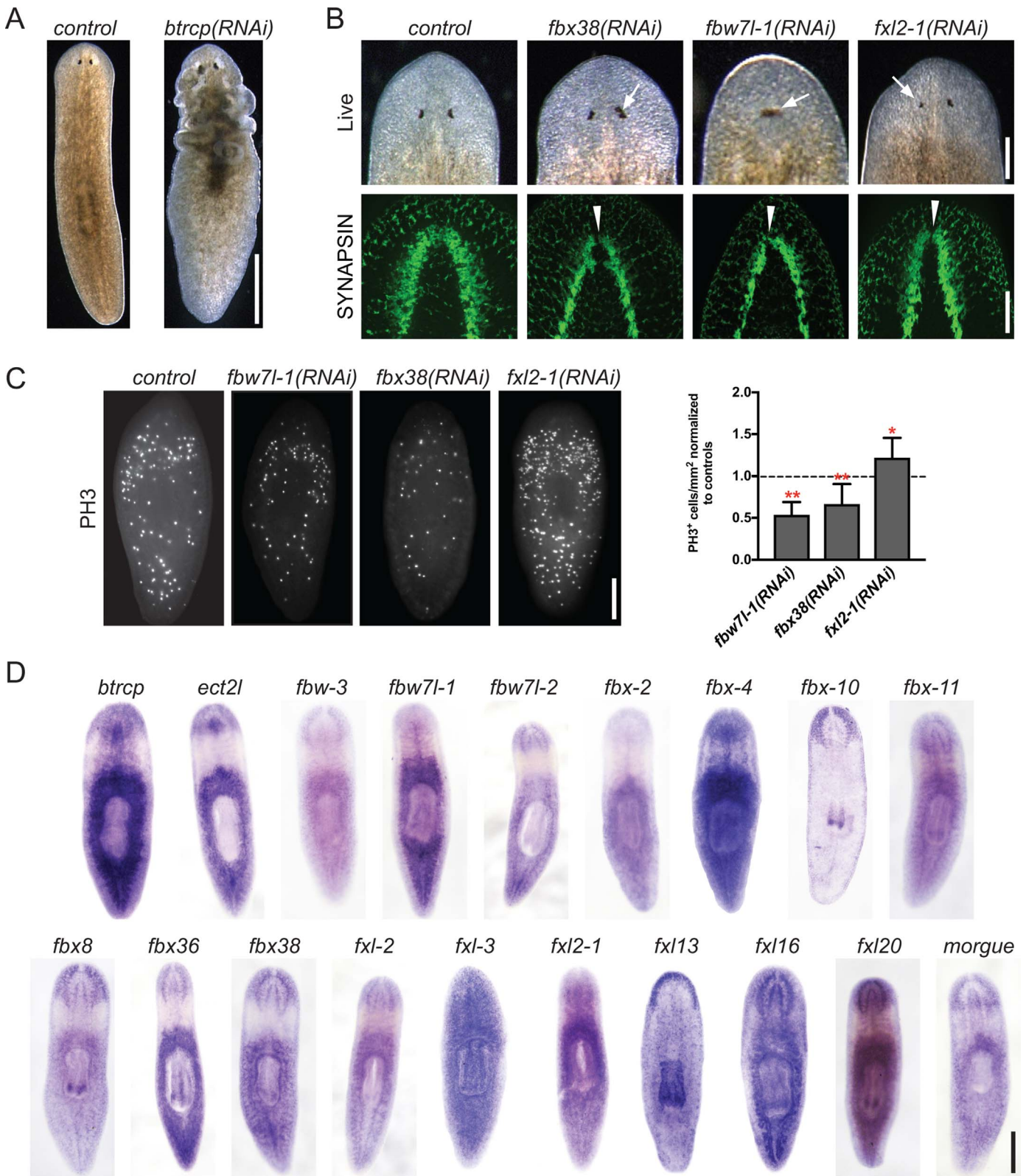

Fig. 3. RNAi and expression analysis of F-box genes in *S. mediterranea*. A) Animals were fed dsRNA 6 times over 3 weeks against *gfp* (*control*; n = 13) or *btrcp* (n = 13). B) Animals were fed dsRNA 6 times over 3 weeks against *gfp* (*control*; n = 26), *fbx38* (n = 22), *fbw7-like-1* (*fbw7l-1*; n = 14), or *fxl2-1* (n = 26), amputated pre-pharyngeally, and allowed to regenerate for 10 days. Trunk-regenerate heads of live animals were imaged. Subsequently, animals were sacrificed, fixed and stained with anti-SYNAPSIN to visualize the nervous system. The white arrows in the live animals denote asymmetric or improperly patterned eyes in F-box gene RNAi worms; arrowheads in SYNAPSIN images mark failed or reduced anterior commissures observed in RNAi planarians. C) Animals were fed dsRNA 6 times over 3 weeks against *gfp* (*control*; n = 10 for each group), *fbx38* (n = 10), *fbw7-like-1* (*fbw7l-1*; n = 10), or *fxl2-1* (n = 10), amputated pre-pharyngeally, allowed to regenerate for 10 days, and then stained with anti-phospho-Histone H3 (PH3) to visualize mitotic neoblasts. Graph shows the mean ± s.d. of values normalized to *gfp* controls. *P < 0.05 or **P < 0.01, Student's *t*-test. D) Whole-mount *in situ* hybridization to *f-box* genes that showed a phenotype following RNAi. Abbreviations: *ect2l, ect2-like*; *fbw7l-1, fbw7-like-1; fbw7l-2, fbw7-like-2*. Scale bars: A = 500 μm, B = 250 μm for live images, 100 μm for SYNAPSIN staining, C = 250 μm, D = 500 μm.

potential role for *btrcp* in canonical Wnt signaling by analyzing the *btrcp(RNAi)* tissue outgrowths or performing Western blot to *Smed-β-catenin-1* following RNAi (Stuckemann et al., 2017; Sureda-Gomez et al., 2016).

FBXW7 proteins are known tumor suppressors and regulators of stem cell differentiation (Takeishi and Nakayama, 2014). In *S. mediterranea*, two *fbxw7-like* genes (*fbw7-like-1* and *fbw7-like-2*) had roles in eye regeneration, blastema formation, mitosis, and nervous system regeneration (Fig. 3). Interestingly, loss of *Fbxw7* in mice leads to the accumulation of neural stem cells and loss of differentiated neurons (Hoeck et al., 2010; Matsumoto et al., 2011). Fbxw7 is also a known regulator of the Notch signaling pathway (Matsumoto et al., 2011), which has recently been implicated in midline formation in the regenerating planarian (Sasidharan et al., 2017). The nervous system patterning and eye regeneration phenotypes observed in *cul1*, *skp1* and *fbw7-like-1* RNAi worms are all consistent with possible defects in midline formation (Fig. 2B, 3B, S3B). Intriguing preliminary results suggest that *cul1(RNAi)* worms fail to express *slit1* (Cebrià et al., 2007) at the midline of head blastemas (data not shown). However, given the pleiotropic nature of the *cul1* phenotype, future studies on the role of *fbw7-like-1* and *−2* in planarian regeneration are a logical next step to investigate if these genes are directly regulating neurogenesis and body patterning, and what proteins are being ubiquitylated by putative $SCF^{Fbw7\text{-}l\text{-}1}$ or $SCF^{Fbw7\text{-}l\text{-}2}$ complexes.

Another F-box that our data implicated in planarian regeneration is *fxl2-1*, a homolog of the tumor suppressor *FBXL2* (B.B. Chen et al., 2012). FBXL2 targets Cyclin D3 to arrest mitotic activity in human and mouse lung epithelial cells (Chen et al., 2011). *fxl2-1* RNAi in planarians led to a significant increase in $PH3^{+}$ cells (Fig. 3C), suggesting that *fxl2-1* may have a conserved function in regulating cell division. Few gene knockdowns lead to hyperproliferation phenotypes in planarians (e.g., *Smed-p53, -smg-1,* and *-huwe1*) (González-Estévez et al., 2012; Henderson et al., 2015; Pearson and Sánchez Alvarado, 2010), yet this work has identified a putative $SCF^{Fxl2\text{-}1}$ complex as a candidate suppressor of mitosis. Further analysis of the *fxl2-1* RNAi phenotype using proteomic approaches (discussed below) could uncover other factors that regulate stem cell populations.

fbx38(RNAi) worms phenocopied several *cul1* and *skp1* RNAi phenotypes, like defects in blastema formation and nervous system regeneration, and a reduction in mitotic activity (Table 1 and S2, and Fig. 3B-C). Mutations in *FBX38* have been identified in patients with bipolar disorder and spinal muscular atrophy (Feng and Zhu, 2010; Sumner et al., 2013). Additional studies of *fbx38* in *S. mediterranea* could inform how mutations in *FBX38* cause nervous system disorders in humans.

Previous work from our laboratory revealed roles for HECT E3 ubiquitin ligases in planarian regeneration and tissue patterning (Henderson et al., 2015). Here, we demonstrate that it is possible to dissect the function of CRL complexes within the context of adult tissue regeneration, accentuating the prospect of using planarians as a model to investigate how ubiquitylation regulates regenerative processes. We found that knockdown of *cul1* or *skp1* led to several phenotypes, and that *f-box* gene knockdowns recapitulated aspects of the *cul1(RNAi)* and *skp1(RNAi)* phenotypes, suggesting we can isolate the effects of specific SCF complexes *in vivo*. Further study of *f-box* genes, and of other substrate recognition proteins in CRL complexes, will define the specific roles of these complexes in stem cell regulation. Importantly, F-box domains, either alone or in combination with other domains, are involved in protein-protein interactions that could also function in other cellular processes outside of ubiquitylation (Hermand, 2006). Future studies should expand analysis of specific *f-box* genes on the neoblast population and determine the impact of RNAi knockdowns on protein ubiquitylation in planarians, which can be measured by Western blot analysis (Henderson, 2013). Once a direct link to ubiquitin signaling is established, it should be possible to apply quantitative techniques, such as DiGly proteomics (Bennett et al., 2010; Kim et al., 2011), to identify molecular targets of SCF ubiquitin ligases involved in planarian stem cell regulation.

4. Materials and methods

4.1. Planarian Husbandry

A clonal, asexual strain of *Schmidtea mediterranea* (CIW4) was maintained as described previously (Cebrià and Newmark, 2005). Animals ranging in length from 3 to 6 mm were starved for at least one week prior to all experiments.

4.2. Informatics

TBLASTN analysis was performed using human CULLIN proteins or F-box protein domains from *S. cerevisiae*, *S. pombe*, *C. elegans*, *D. melanogaster*, *X. laevis*, *M. musculus* and *H. sapiens* against planarian transcriptomes (Adamidi et al., 2011; Brandl et al., 2016). The returned sequences were subsequently analyzed with the NCBI Conserved Domain Database Search tool (nucleotide sequences), SMART (longest ORF translation), and InterProScan (longest ORF translation) to confirm the presence of an F-box domain. Transcripts with an E-value ≤ $1e^{-03}$ from the listed domain predicting programs were considered an F-box domain-containing transcript (Table S2).

4.3. Phylogenetic analysis

S. mediterranea cullin transcripts (Table S1) were translated in Virtual Ribosome (Wernersson, 2006) and aligned to annotated Cullin protein sequences from *Caenorhabditis elegans*, *Drosophila melanogaster*, *Homo sapiens*, and *Mus musculus*, by ClustalW within MEGA7 software (Kumar et al., 2016) using default settings. Phylogenetic relationships were inferred using the Neighbor-Joining method in MEGA7. Evolutionary distances were computed using the Jones-Taylor-Thornton model with uniform mutation rates and pairwise deletion of gaps. Phylogenetic relationships were tested by Bootstrap resampling with 1000 replications and displayed rooted at the mid-point.

4.4. Cloning

Three Cullin genes and 13 F-box containing genes were obtained from a library of sequenced cDNA clones in pBluescript II SK (+) (Zayas et al., 2005) (Table S3). The remaining three Cullin and 22 F-box genes were directionally cloned into the pJC53.2 vector using forward and reverse primers equipped with XhoII and NotII restriction sites (Collins et al., 2010) or with primers containing overhangs homologous to the pPR-T4P vector (J. Rink) using ligation-independent cloning (Aslanidis and de Jong, 1990) (Table S3). Inserts in the pBluescript II SK (+) vector were shuttled into either pPR242 or pPR244 using the Gateway system (Invitrogen) (Reddien et al., 2005b).

4.5. Whole-mount in situ hybridization

Animals were sacrificed and processed using a 5% N-Acetyl Cysteine solution prior to fixation in 4% formaldehyde. Antisense RNA probes were produced as previously described (Pearson et al., 2009). Briefly, DNA templates were PCR amplified from cDNA clones in pJC53.2, pBS, pPR244/242, and pPR-T4P plasmid vectors. Antisense riboprobes labeled with digoxigenin were synthesized at 37 °C. Probes were purified via ethanol precipitation and whole-mount *in situ* hybridization was performed in an InsituPro VS liquid handling robot (Intavis). Samples were incubated with an anti-digoxigenin AP antibody (Roche, 1:2000) and developed with NBT/BCIP as previously described (Pearson et al., 2009).

4.6. RNA interference

Plasmid templates in pJC53.2, pPR244/242, or pPR-T4P were transformed into the RNase-free cell line HT115 (DE3) for double-stranded RNA production by using IPTG inducible promoters (Collins et al., 2010; Reddien et al., 2005b). Bacterially-expressed *gfp* dsRNA was used as a control in all experiments. Animals were fed a dsRNA bacterial pellet mixed with an approximate 3:1 ratio of liver:water paste. Five to six RNAi feedings were performed over a period of three weeks and animals were cut pre-pharyngeally 24–48 h after the last feed. Planarians were fixed following 10 days of regeneration. All experiments were performed in duplicate at a minimum.

4.7. Immunostaining

Fixation and immunostaining with anti-phospho-Histone H3 (S10) (1:2000, Cell Signaling) and anti-Synapsin (1:400, Hybridoma bank) were performed as described in Cowles et al. (2013). Anti-phospho-Histone H3 (S10) was visualized with Cy3-Tyramide following incubation with goat anti-rabbit-HRP secondary antibodies (1:2000); anti-Synapsin was visualized with goat anti-mouse Alexa Fluor 488 (Thermo Fisher Scientific, A11029; 1:1000, Waltham, MA).

4.8. Image acquisition and processing

Fluorescent labeled images were acquired via an Axiocam MRm camera mounted on a Zeiss Axio Observer. Z1 equipped with ApoTome or a Zeiss SteREO Lumar V.12 for whole body images. Live RNAi and in situ images were taken with a Leica DFC290 or DFC450 camera on a Leica M205 microscope. All images are of the dorsal worm with the anterior at the top. Images were processed for brightness and the figures organized using the Adobe Creative Suite.

4.9. Statistics

Quantification of anti-phospho-Histone $H3^{+}$ cells was done by manually counting cells on ImageJ. Cell counts were normalized to the area. Student's *t*-tests were performed and graphs made on GraphPad Prism Version 6 (GraphPad Software, San Diego, CA). All graphs show mean with error bars displaying standard deviation (mean ± s.d.). The number of animals per group is indicated in the appropriate figure legend.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2017.10.011.

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