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RESEARCH ARTICLE

STEM CELLS AND REGENERATION

Planarian *yorkie/YAP* functions to integrate adult stem cell proliferation, organ homeostasis and maintenance of axial patterning

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ABSTRACT

During adult homeostasis and regeneration, the freshwater planarian must accomplish a constant balance between cell proliferation and cell death, while also maintaining proper tissue and organ size and patterning. How these ordered processes are precisely modulated remains relatively unknown. Here we show that planarians use the downstream effector of the Hippo signaling cascade, *yorkie* (*yki*; *YAP* in vertebrates) to control a diverse set of pleiotropic processes in organ homeostasis, stem cell regulation, regeneration and axial patterning. We show that *yki* functions to maintain the homeostasis of the planarian excretory (protonephridial) system and to limit stem cell proliferation, but does not affect the differentiation process or cell death. Finally, we show that Yki acts synergistically with WNT/β-catenin signaling to repress head determination by limiting the expression domains of posterior WNT genes and that of the WNT-inhibitor *notum*. Together, our data show that *yki* is a key gene in planarians that integrates stem cell proliferation control, organ homeostasis, and the spatial patterning of tissues.

KEY WORDS: Hippo, Yorkie, YAP, Regeneration, Stem cells, Differentiation, Tumor suppression, WNT, β-catenin, Lophotrochozoan, Planarians, *Schmidtea mediterranea*

INTRODUCTION

In the field of developmental biology, much effort has been put towards understanding how stem and progenitor cells in an animal embryo produce the appropriate differentiated cell types in time and space to produce a functional organ. In order to develop tissues *de novo*, proliferation tends to be unrestricted, while cell death is minimized. However, once development is complete, adult stem cells (ASCs) must perform different roles such as tightly limiting proliferation to replace the exact number and type of differentiated cells lost to physiological turnover (homeostasis) or injury (regeneration). Despite a constant influx-efflux of cells, the correct spatial patterning of a tissue must be maintained or, in the case of regeneration, completely remade. The mechanisms by which ASCs accomplish this to precisely balance proliferation, cell death, regeneration, and spatial patterning in adult tissues remain relatively unknown.

The freshwater planarian flatworm *Schmidtea mediterranea* is a powerful model organism to study ASC biology, tissue homeostasis, regeneration, and spatial patterning in adult organisms *in vivo* (Newmark and Sánchez Alvarado, 2002; Reddien et al., 2005a). Approximately 10% of all planarian cells are ASCs and at least some of those are pluripotent (Reddien et al., 2005b; Wagner et al., 2011). This large population of ASCs allow for high levels of tissue homeostasis and an ability to regenerate from virtually any injury (Morgan, 1898). Functional RNAi screens have demonstrated that planarians require evolutionarily conserved regulators of stem cell maintenance and regeneration including *p53*, *Retinoblastoma* and *TOR*, among many others (Labbé et al., 2012; Pearson and Sánchez Alvarado, 2010; Tu et al., 2012; Wagner et al., 2012; Zhu and Pearson, 2013). Similarly, distinct sets of signaling pathways constantly maintain organ and axial patterning and are also required for proper regeneration. For example, the WNT/β-catenin and BMP signaling pathways specify the anterior-posterior and dorsal-ventral axes, respectively, during both homeostasis and regeneration (Gaviño and Reddien, 2011; Gurley et al., 2008; Petersen and Reddien, 2008; Reddien et al., 2007). However, despite the molecular advances in understanding planarian spatial patterning and stem cell regulation, it remains to be elucidated how planarian ASCs achieve homeostatic balance and integrate newly differentiated progeny in order to maintain properly sized and patterned organs.

The transcriptional co-factor Yorkie (Yki; YAP in vertebrates) is the canonical effector of the Hippo signaling pathway and has been implicated in stem cell regulation, cell death, growth control and organ patterning during development in flies and vertebrates (Dong et al., 2007; Halder and Johnson, 2011; Huang et al., 2005; Lian et al., 2010; Pan, 2010; Udan et al., 2003; Wu et al., 2003). Although Yki is typically regulated by a core cassette of kinases composed of Hippo (Hpo; Mst1/2 in vertebrates) and Warts (Wts; Lats1/2 in vertebrates), little is known about the downstream targets (Harvey et al., 2003; Huang et al., 2005; Oh and Irvine, 2008; Udan et al., 2003; Wu et al., 2003; Zhang et al., 2008a). In flies and vertebrates, the constitutive activation of Yki/YAP, which results in overgrown organs, is primarily mediated by its interaction with the transcription factor Scalloped (Sd; TEAD1-4 in vertebrates) (Goulev et al., 2008; Wu et al., 2008; Zhang et al., 2008b; Zhao et al., 2008). Therefore, the prevailing view is that active Yki restricts organ growth and cell division. Interestingly, this ability to control proliferation and organ size may be attributed to high levels of YAP in many tissue-specific stem cells (Ramos and Camargo, 2012). More recent studies have also described roles for Yki/YAP outside growth control, which include acute regenerative responses, embryonic kidney morphogenesis, cell fate specification in the early mammalian embryo, and the sensing of mechanical stresses (Dupont et al., 2011; Karpowicz et al., 2010; Nishioka et al., 2009; Reginensi et al., 2013; Shaw et al., 2010). These pleiotropic roles are in part the result of the many Yki/YAP binding partners, and the many cooperating signaling pathways and upstream regulators specific to a given

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tissue (Bennett and Harvey, 2006; Grzeschik et al., 2010; Ling et al., 2010; Silva et al., 2006; Yu et al., 2012; Zhao et al., 2011). Therefore, because of the multifaceted roles of Yki/YAP in stem cell maintenance, growth control and organ patterning, it is an attractive molecule to study in order to understand the link between planarian stem cell maintenance and final organ size and tissue patterning during adult homeostasis and regeneration.

Here, we show that *S. mediterranea* has a single ortholog of Yki/YAP (*Smed-yki*; herein referred to as *yki*) with pleiotropic functions. Using RNAi to knockdown *yki* function [*yki(RNAi)*], *yki* was found to be required in the homeostatic maintenance and patterning of the protonephridial (excretory) system, where it functions to maintain the tubular ultrastructure, yet it is not required for differentiation or regeneration of the organ. We further show that although *yki* is not highly expressed in the stem cell compartment, it is required to restrict the size of the stem cell compartment and the amount of cell proliferation. However, *yki* is not required for cell death or differentiation. In addition, we demonstrate that both the protonephridial and stem cell proliferation phenotypes are mediated by two planarian paralogs of the Yki binding partner Scalloped (*Smed-sd-1* and *Smed-sd-2*). Finally, we found that *yki* is also required for proper axial patterning during homeostasis and regeneration where *yki* acts synergistically with *β-catenin* to suppress 'head' identity through regulation of the WNT components *fz4*, *wnt1*, *wnt11-2* and *notum*. Together, the dysregulation of these WNT signals results in ectopic head duplications in the posterior. From these data, we conclude that planarian *yki* functions pleiotropically and our analyses support the hypothesis that *yki* homologs had ancestral roles in the maintenance of excretory nephridial systems, restriction of WNT signaling, and proliferation control of stem cell populations. These functional characterizations of *Smed-yki* demonstrate that it is a crucial factor that integrates stem cell regulation and tissue patterning in planarians.

RESULTS

Planarians have a single YKI ortholog that is ubiquitously expressed in uninjured animals

Hippo signaling is an evolutionarily conserved pathway from flies to vertebrates, which consists of a kinase cascade that impinges on the Yki transcriptional cofactor (supplementary material Fig. S1A) (Halder and Johnson, 2011; Pan, 2010). We have identified and cloned all of the planarian homologs of the core Hippo signaling cascade (supplementary material Fig. S1A). Analysis of the predicted amino acid sequence for SMED-YKI showed that it had 23% and 31% identity to *Drosophila* Yki and mouse YAP, respectively. Interestingly, the highly conserved Warts/Lats phosphorylation motif, HxRxxS, had an R to K mutation, which may exempt SMED-YKI from this key step in its regulation (supplementary material Fig. S1B) (Mah et al., 2005; Oh and Irvine, 2009; Zhao et al., 2007). It should be noted that only the closely related parasitic flatworm, *Schistosoma mansoni*, had this same substitution, whereas *Macrostomum lignano*, a more distantly related flatworm, had the canonical consensus motif (supplementary material Fig. S1B; Fig. S2).

Previous studies have demonstrated that the ability for Yki/YAP to control organ growth may reside at the level of stem cells (Ramos and Camargo, 2012). In mammalian organs, such as the skin, liver, small intestine and brain, tissue-specific stem cells are enriched in YAP compared with their respective progeny populations (Cai et al., 2010; Camargo et al., 2007; Cao et al., 2008; Dong et al., 2007; Fernandez-L et al., 2009; Lu et al., 2010; Schlegelmilch et al., 2011; Zhang et al., 2011). In addition, a recent study showed that in the basal flatworm, *M. lignano*, *Mac-YAP* was strongly expressed in the stem cells (Demircan and Berezikov, 2013). To determine whether *yki* showed similar patterns, whole-mount RNA *in situ* hybridization (WISH) was performed. In uninjured animals, we observed a ubiquitous expression pattern with no localization to specific tissue compartments or organs (supplementary material Fig. S1C). Using previous RNaseq data from purified planarian stem cells, *yki* levels were no higher in stem cells or stem cell progeny than in the rest of the tissues in the animal, and by contrast, its expression was ~20-fold higher in differentiated tissues (supplementary material Fig. S1D) (Labbé et al., 2012). Interestingly, during regeneration, *Mac-YAP* is predominantly detected in the newly regenerated blastema tissue (Demircan and Berezikov, 2013). Planarian *yki* did not show blastema-specific WISH staining, but was predominantly expressed in the *de novo* regenerating pharynx (supplementary material Fig. S1C, arrows). Even though planarian *yki* did not share these compartmentalized expression patterns, it may still have roles in stem cell maintenance, growth control or regeneration. Thus, we next tested the function of *yki* by RNAi knockdown.

yki is required for excretory system structural homeostasis

RNAi was administered to planarians and two assays were performed to measure knockdown efficiency. Both WISH at 3 days after 3 feeds (3fd3; supplementary material Fig. S1C) and RNaseq at 3fd6 (supplementary material Fig. S1E; see Materials and Methods) following RNAi showed potent downregulation of *yki*. Following *yki(RNAi)*, we observed that 98% of animals had fluid-filled edemas by 3fd9, which was indicative of a defect in protonephridial (excretory) maintenance (Fig. 1A-C) (Nogi and Levin, 2005; Rink et al., 2009; Rink et al., 2011; Scimone et al., 2011). Thus, we next tested whether the loss of osmotic regulation in *yki(RNAi)* animals was the result of a loss of protonephridia.

The planarian protonephridial system is a network of dead-end tubules required for osmoregulation and excretion and is analogous to the vertebrate kidney (Bartolomaeus and Ax, 1992; Ruppert and Smith, 1988; Scimone et al., 2011). Several previously characterized markers for different spatial domains can be used to measure the clustering and tubule architecture of protonephridia, which should be evenly distributed across the dorsal surface of the animal (Rink et al., 2011; Scimone et al., 2011). In *yki(RNAi)* worms, we observed significantly decreased nephridial clusters, expressing *carbonic anhyrdrase VII* (*CA*), *innexin10* (*inx10*) and *pou2/3*, compared with *control(RNAi)* worms (Fig. 1C,D; supplementary material Fig. S3A; $P<0.001$). Furthermore, anti-acetylated-α-tubulin (Ac-TUB) labels a highly branched tubule network that ends in ciliated flame cells (Glazer et al., 2010). In *yki(RNAi)* worms we observed that the tubule structure was thinner and the number of flame cells was reduced, which suggested that the network was collapsed and lost (Fig. 1C; supplementary material Fig. S3B). To verify that the *yki(RNAi)* protonephridial defect was not attributed to defects in ciliogenesis, which can also cause an edema phenotype, we confirmed that the dorsal midline cilia and the ciliated ventral epithelium, as determined by normal locomotion, were not affected (supplementary material Fig. S3A; supplementary material Movies 1, 2) (Glazer et al., 2010; Rink et al., 2009; Vij et al., 2012).

Although *yki(RNAi)* resulted in fewer nephridial clusters, the relative expression of all known anatomical markers and regulatory genes were unchanged at 3fd6, as determined by RNaseq analyses (Fig. 1E). To resolve this discrepancy, we examined the ultrastructure at a high magnification and uncovered that *yki(RNAi)* animals had more *CA*+ cells at each nephridial cluster and were larger (supplementary material Fig. S3B; Fig. 1C). This suggested that *yki*

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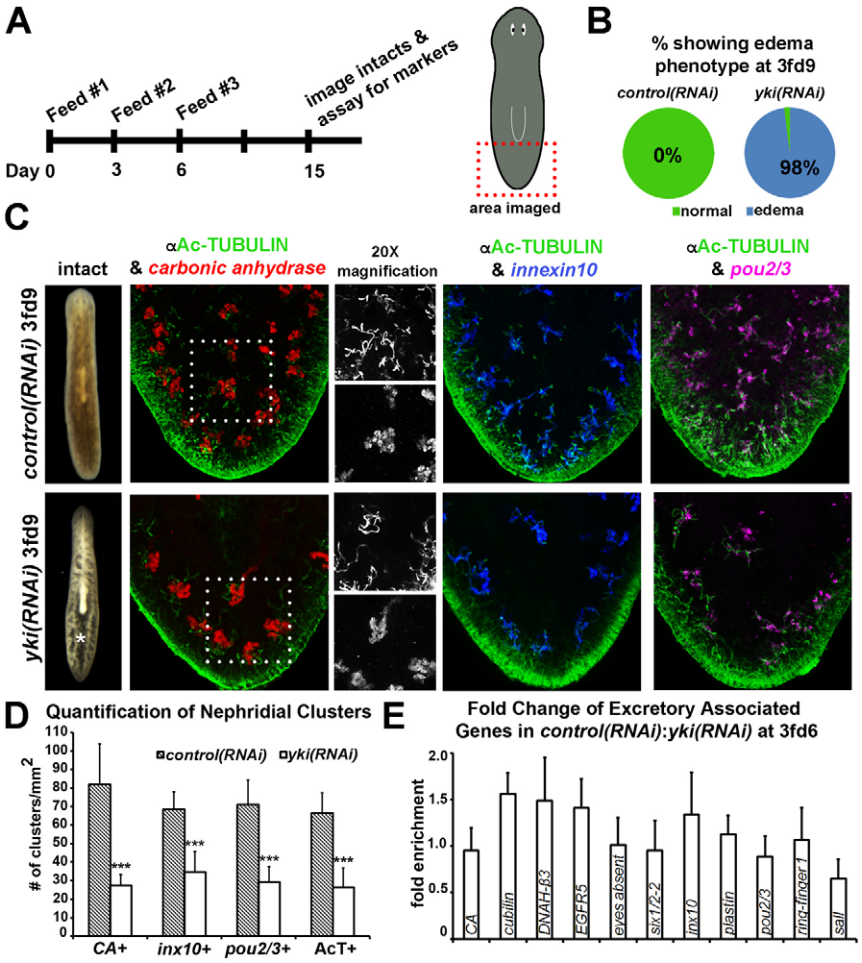

Fig. 1. Yorkie is required for excretory system homeostasis. (A) Animals were assayed at 3fd9 and their tail regions imaged. (B) Graphical representation of the percentage of animals showing an edema phenotype at 3fd9. (C) Fluorescence *in situ* hybridization (FISH) of intact worms stained for protonephridial (excretory) markers: *carbonic anhydrase VII* (*CA*), *innexin10* (*inx10*), *pou2/3*, and anti-acetylated tubulin (αAc-TUBULIN). The white asterisk denotes fluid accumulation observed in live imaged worms. The dotted white boxes indicate the areas shown in the 20× magnification panel. (D) *yki(RNAi)* causes a significant decrease in the number of excretory-related gene clusters. (E) Relative expression of excretory-related genes in *control(RNAi):yki(RNAi)* at 3fd6. Error bars indicate s.d. ***$P<0.001$.

was not required to maintain the existing protonephridial cell types, but was crucial in the overall patterning of the organ. Indeed, in the absence of stem cells, *yki(RNAi)* animals still exhibited an edema and a collapse of the network, which was also evident during regeneration at 14 days post amputation (dpa; supplementary material Figs S4, S5). Taken together, these data showed that *yki* was essential in maintaining proper patterning and ultrastructure of the excretory system without any observable effects on transcription levels of known markers or loss of differentiated protonephridial cell types.

yki limits proliferation and the size of the stem cell compartment but is not required for differentiation

Owing to the fact that *yki* homologs function in stem cell compartments in *Drosophila*, *Macrostomum* and mouse, we next tested whether planarian *yki* also affects stem cell regulation (Demircan and Berezikov, 2013; Halder and Johnson, 2011; Karpowicz et al., 2010; Lian et al., 2010; Pan, 2010; Shaw et al., 2010; Tremblay and Camargo, 2012). To this end, *yki(RNAi)* worms were assayed for known stem cell and lineage markers at various time points following RNAi (Eisenhoffer et al., 2008; Reddien et al., 2005b). Following *yki(RNAi)* treatment, the stem cell population marked by *piwi-1* (*smedwi-1*) and *histone 2B* (*H2B*) was expanded at 3fd12 and 3fd6, respectively (Fig. 2A,C,D). Correspondingly, the mitotic marker phosphorylated histone 3 (H3P) was also significantly increased compared with controls, which was evident at 3fd3 and sustained at least until 3fd20 (Fig. 2B). The expansion in the stem cell compartment was also verified by flow cytometry, which was 1.38-fold larger in *yki(RNAi)* animals compared with *control(RNAi)* animals at 3fd6 (supplementary material Fig. S6B-D). Although RNaseq analyses revealed no significant fold change of previously characterized stem cell related genes in *yki(RNAi)*:*control(RNAi)* at 3fd6, all the fold changes were >1.12 (supplementary material Fig. S6A). This fold change was in accordance with all the cell counts of *piwi-1*, *H2B*, H3P, and the FACS plots, which also showed a similar fold increase in the stem cell population in *yki(RNAi)* compared with *control(RNAi)* animals.

This hyper-proliferation phenotype was unexpected because with the exception of the mammalian intestine (Barry et al., 2013), the loss of *yki* or *YAP* in other model systems typically results in an undergrowth phenotype with decreased cell proliferation and increased cell death (Dong et al., 2007; Huang et al., 2005). Therefore, to confirm the increase in proliferation, two non-overlapping fragments of the *yki* transcript were generated for RNAi. Both split fragments recapitulated the full-length *yki(RNAi)* phenotypes, supporting the specificity of the result (supplementary material Fig. S7). It should be noted that the loss of protonephridial maintenance is not likely to be a contributing factor to changes in stem cells because previous studies have demonstrated that edema phenotypes do not trigger an injury response or changes in the number of $H3P^+$ cells (Reddien et al., 2005a) (supplementary material Fig. S8). Finally, we tested whether cell death was disrupted in *yki(RNAi)* animals by TUNEL staining. We did not detect any changes in cell death, and more importantly, cell death was clearly not inhibited (supplementary material Fig. S7).

In planarians, several genes that are required for proliferation control of stem cells, such as *p53*, *EGFR1* and *Retinoblastoma*,

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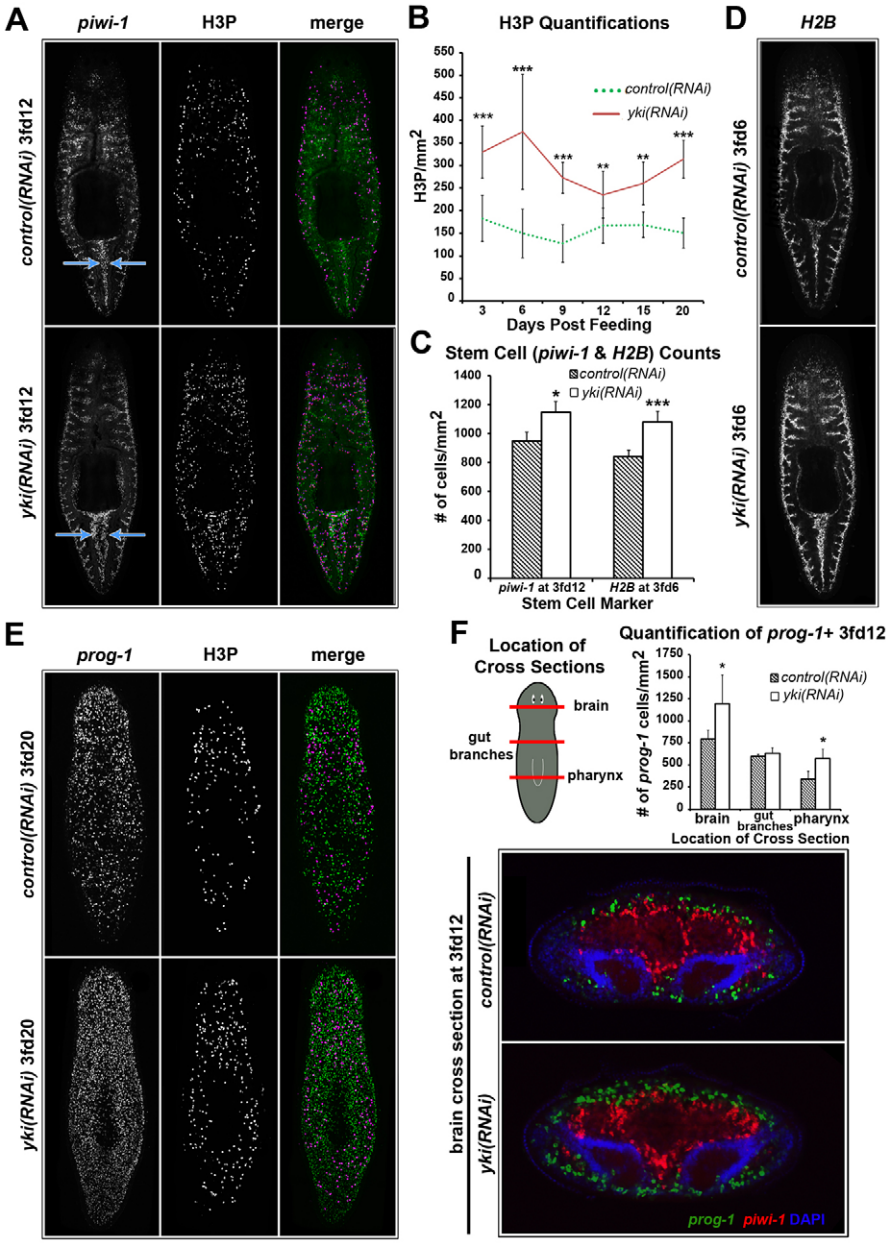

Fig. 2. Yorkie controls the size of the stem cell and *prog-1*+ progeny populations. (A) FISH of the stem cell marker *piwi-1* (green in merged panel) and the mitotic marker phosphorylated histone 3 (H3P; magenta in merged panel) at 3fd12. Blue arrows indicate the expanded *piwi-1*-expressing population. (B) *yki(RNAi)* causes a significant and prolonged increase in the number of mitotic cells. (C) Quantification of the number of *piwi-1*+ and *H2B*+ cells in animals assayed at 3fd12 and 3fd6, respectively, which was significantly increased in *yki(RNAi)* animals. (D) Representative images of animals assayed for the stem cell marker *H2B* at 3fd6 show a similar increase in expression to that of *piwi-1*. (E) Expression of the early progeny marker *prog-1* (green in merge) and H3P (magenta) at 3fd20. (F) *yki(RNAi)* causes an increase in the number of *prog-1* cells. Animals were cross sectioned (schematic) and assayed for *prog-1* (green) and *piwi-1* (red) by double FISH (right panel), and quantified (bar graph). Error bars indicate s.d. ***P<0.001, **P<0.01, *P<0.05.

also have detrimental effects on the production of stem cell progeny (Fraguas et al., 2011; Pearson and Sánchez Alvarado, 2010; Wagner et al., 2012; Zhu and Pearson, 2013). To test whether the increase in stem cells and proliferation was at the expense of differentiating progeny, we examined whether there were any changes to the stem cell progeny using the marker *prog-1* (formerly known as *NB21.11e*) (Eisenhoffer et al., 2008). Surprisingly, at 3fd12 we detected that the *prog-1* cell population was significantly increased in *yki(RNAi)* compared with controls (Fig. 2F; P<0.05). At later time points (3fd20), *prog-1* cells were still present at high levels, which showed that *yki* was not required for differentiation and that both stem cell proliferation and differentiation proceeded at increased rates (Fig. 2E). Both the stem and progenitor cell populations in *yki(RNAi)* animals were radiation sensitive with the same kinetics as controls (supplementary material Fig. S9). By contrast, RNAi against the upstream pathway components, *merlin*, *hpo* and *wts*, did not result in any detectable changes in proliferation, stem cells or stem cell progeny, despite significant knockdown of the genes themselves (supplementary material Fig. S10). Together, these data showed that *yki* is a key regulator of the stem cell compartment, and under these knockdown conditions, it was required to limit stem cell proliferation and was disconnected from the canonical kinase cascade that regulates its function in other systems.

RNAi of two TEAD-domain paralogs recapitulates *yki(RNAi)* phenotypes in the excretory system and stem cell proliferation

Yki is a transcriptional cofactor with no ability to bind DNA on its own, and must cooperate with various other transcription factors to mediate expression of target genes (Yagi et al., 1999). In canonical Hippo signaling, Scalloped is the most well-characterized binding partner and can suppress Yki overexpression-driven tissue overgrowths (Goulev et al., 2008; Wu et al., 2008; Zhang et al., 2008b; Zhao et al., 2008). Therefore, we next tested whether the observed *yki* defects were mediated by Sd in planarians. We

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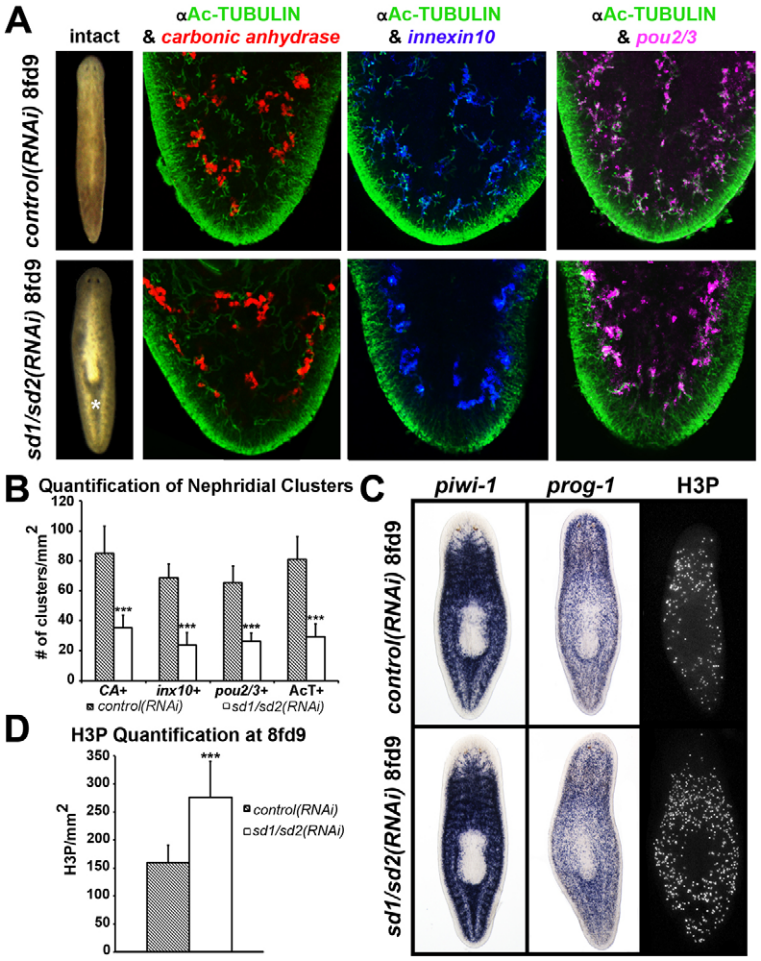

Fig. 3. *sd1/sd2(RNAi)* results in loss of excretory system maintenance and hyperproliferation. (A) Animals were assayed and imaged with previously described excretory tissue markers at 8fd9. The white asterisk indicates the edema in live imaged worms. (B) *sd1/sd2(RNAi)* causes a significant loss of nephridial clusters and associated markers. (C) WISH of uninjured intact animals stained for *piwi-1*, *prog-1*, and immunostained for H3P at 8fd9. (D) *sd1/sd2(RNAi)* also causes a significant increase in the number of mitotic cells. Error bars indicate s.d. ***P<0.001.

identified and cloned the two homologs of *sd* (*sd-1* and *sd-2*), which also displayed ubiquitous expression patterns by WISH analysis (supplementary material Fig. S11B). Interestingly, the other flatworms, *S. mansoni* and *M. lignano*, also had this duplication of the *sd* genes (supplementary material Fig. S11A). Individually, *sd-1(RNAi)* or *sd-2(RNAi)* resulted in little to no phenotypes, and therefore, both were combined to perform double RNAi. At 8fd9 of *sd1/sd2(RNAi)*, an edema phenotype was observed (Fig. 3A). Similar to *yki(RNAi)*, FISH analyses with the excretory system markers showed decreases in the number of nephridial clusters and defective ultrastructure (Fig. 3B). At 8fd9, *sd1/sd2(RNAi)* also recapitulated the significant increase in stem cell proliferation (Fig. 3C,D; P<0.01). Together, these data supported the hypothesis that in planarians, Yki functions through its canonical Sd binding partner(s) in the processes of stem cell proliferation control and protonephridial homeostasis.

yki is required for proper regeneration by restricting expression of components of the WNT signaling pathway

In the *Drosophila* midgut and mammalian intestinal crypts, Yki and YAP are crucial for regeneration following acute injury (Barry et al., 2013; Cai et al., 2010; Karpowicz et al., 2010; Shaw et al., 2010). However, these regenerative roles have only been demonstrated in one tissue type. Therefore, we also sought to test the function of *yki* in planarians, which have a much greater capacity for regenerating all three germ layers. At 3fd3 we performed transverse amputations on *yki(RNAi)* worms and assessed regeneration at 7dpa (Fig. 4A). We observed that *yki(RNAi)* animals developed edemas, but also had severely impaired regeneration (Fig. 4B,C). The severe edema defects also made subsequent long-term regeneration analyses difficult because of high worm lysis (death). To circumvent this, animals were moved to a higher salt medium, which allowed animals to escape death from osmoregulatory defects (Fig. 4A). At 3fd15 at 5dpa, a later stage of assaying regeneration, 88% of *yki(RNAi)* tail fragments still completely failed to regenerate anterior blastemas (Fig. 4D). *yki(RNAi)* trunk fragments also demonstrated this trend: 48% had severely diminished blastemas and 35% had none. Finally, albeit a lower percentage, 8% of head fragments had no posterior blastema and 72% had a smaller one (Fig. 4D,E).

Defects in regeneration are strongly associated with defects in stem cell function such as failure to self-renew or produce differentiated progeny (Labbé et al., 2012; Pearson and Sánchez Alvarado, 2010; Reddien et al., 2005a; Wagner et al., 2012). Therefore, we assayed for stem cell lineage markers and proliferation to try to identify the underlying mechanisms that contributed to the diminished and/or absent blastema. At 3fd15 5dpa, *piwi-1*$^{+}$ stem cells and *prog-1*$^{+}$ progeny were present at sites of injury, which indicated that stem cells were capable of responding to wound signals, migrating, self-renewing and differentiating (Fig. 4F,G). Furthermore, *yki(RNAi)* regenerating fragments had significantly increased numbers of mitotic cells compared with controls (Fig. 4H,I). Although hyperproliferation was observed, differentiation was not perturbed as measured by continual

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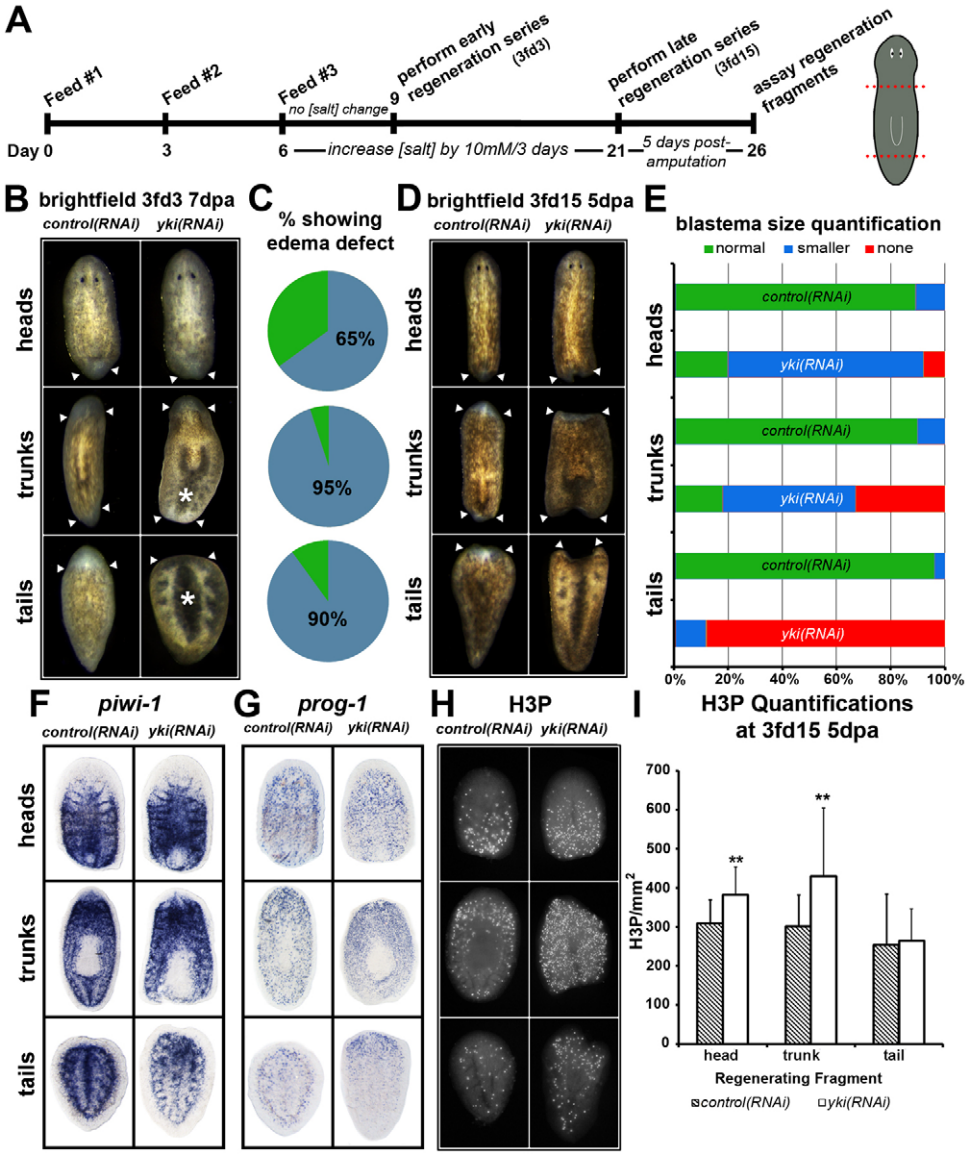

Fig. 4. The regeneration defects in *yki(RNAi)* animals are stem cell independent. (A) Regeneration experiments (schematic) were conducted at an early time point (3fd3) and assayed at 7 days post amputation (7dpa) or at a late time point (3fd15) 5dpa. For late time point experiments an increasing molarity of salts was titrated into medium to prevent excretory-related lysis. (B,C) Regenerating fragments at the early time point (3fd3 7dpa) showed an edema phenotype, indicated by the white asterisks. White arrowheads indicate plane of amputation (B,D). (D,E) At the late time point (3fd15 5dpa), regenerating fragments were 'rescued' from edema lysis by increasing the salts in the medium, but they showed a defect in blastema size. (F-H) Fragments were assayed for stem cell markers: *piwi-1* (F), *prog-1* (G) and H3P (H). (I) Similar to the defects during homeostasis, *yki(RNAi)* regenerating fragments also show a significant hyper-proliferation of the stem cells. $^{**}P<0.01$.

production of *prog-1*$^{+}$ stem cell progeny (Fig. 4G). Therefore, the *yki(RNAi)* regeneration defects were not due to lack of stem cells or their ability to divide and produce progeny.

Because stem cell self-renewal and progeny differentiation appeared to be proceeding in *yki(RNAi)* animals, we hypothesized that the lack of regenerative ability may be due to defects in re-patterning tissues during regeneration and resultant stalling of regeneration. WNT pathway genes in planarians are crucial in maintaining the anterior-posterior axis during homeostasis and are the early injury cues that define the poles during regeneration (Adell et al., 2009; Gurley et al., 2010; Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008; Petersen and Reddien, 2009; Petersen and Reddien, 2011). At 3fd15 5dpa, *yki(RNAi)* tail fragments showed a considerable upregulation of the posterior WNTs, *wnt1* and *wnt11-2*, as well as the posterior *frizzled4* (*fz4*; Fig. 5B). Conversely, in tail fragments with anterior-facing wounds, expression of neither of the anterior WNT inhibitors, *secreted frizzled related protein 7* (*sfrp*) or *notum*, was observed (Fig. 5C) (Petersen and Reddien, 2011). Although tails always showed the most severe defects, head and trunk fragments also showed expanded/disorganized *wnt1* and decreased *fz4* expression (Fig. 5B). These data supported the hypothesis that the inability to restrict the posterior WNTs and define an anterior pole is the cause of *yki(RNAi)* regeneration defects.

During regeneration, we also observed that *yki(RNAi)* tail fragments regenerated virtually no pharynx tissue whereas head fragments regenerated a significantly smaller pharynx (Fig. 5D; $P<0.001$). As expected, trunks showed a disproportionately enlarged pharynx because of the lack of regeneration and inheritance of the original-sized pharynx. Interestingly, the fragment-specific pharynx size differences may also be attributed to the fact that *yki* is normally expressed the regenerating *de novo* pharynx (supplementary material Fig. S1C). Although we could not identify the specific defect in pharynx regeneration, together our data suggested that *yki* is required during regeneration to properly rescale WNT gradients in order for proper pole and organ regeneration to proceed.

yki functions to maintain spatial patterning along the body axis during homeostasis by limiting WNT signaling components

At very late time points (e.g. later than 3fd56) in non-amputated animals, *yki(RNAi)* worms showed an axial duplication (ectopic

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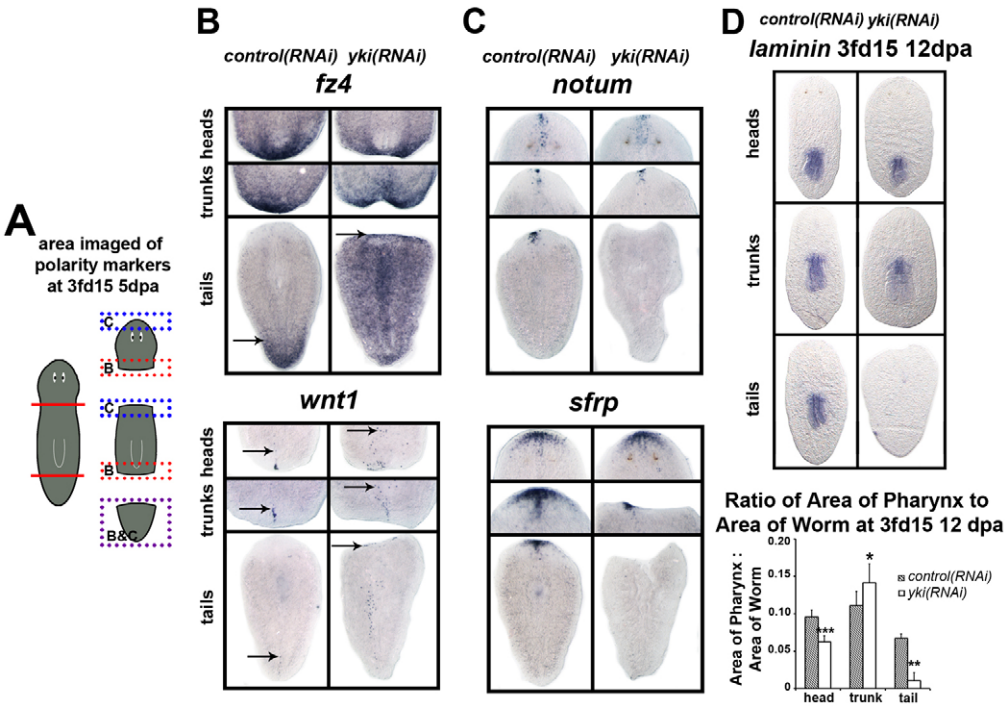

Fig. 5. Yorkie is required for proper regeneration by restricting expression of components of the WNT signaling pathway. (A) Animals were amputated at 3fd15 and assayed at 5dpa for the WNT polarity markers. (B) Posterior markers *fz4* and *wnt1* showed a strong expansion of expression in *yki(RNAi)* tail fragments (black arrows), whereas head and trunks showed disorganized and decreased expression. (C) Anterior markers *sfrp* and *notum* were not expressed in tail fragments and expression was reduced in head and trunk fragments. (D) Animals were stained for *laminin*, a pharynx-specific marker, by WISH at 3fd15 12dpa. *yki(RNAi)* causes a significantly smaller regenerated pharynx in head and tail fragments, whereas in trunk fragments, it is enlarged. Error bars indicate s.d. ***P<0.001, **P<0.01, *P<0.05.

head formation; Fig. 6A; ~10% expressivity; 7 out of 100 animals at the start). Molecular analysis of these animals showed this to be evident by the absence of posterior *fz4* expression and ectopic posterior *sfrp* expression (Fig. 6A). To identify the possible mechanisms of the axial duplication, we examined whether the WNTs were dysregulated at earlier time points, prior to the morphological phenotype. As previously noted, the early excretory phenotypes resulted in worm lysis, therefore, we used increasing salt concentrations to suppress edemas, which allowed for homeostasis assays at 3fd25.

At 3fd25, the expression patterns of *fz4*, *wnt1* and *wnt11-2* were expanded much more anteriorly (Fig. 6B). *yki(RNAi)* worms also

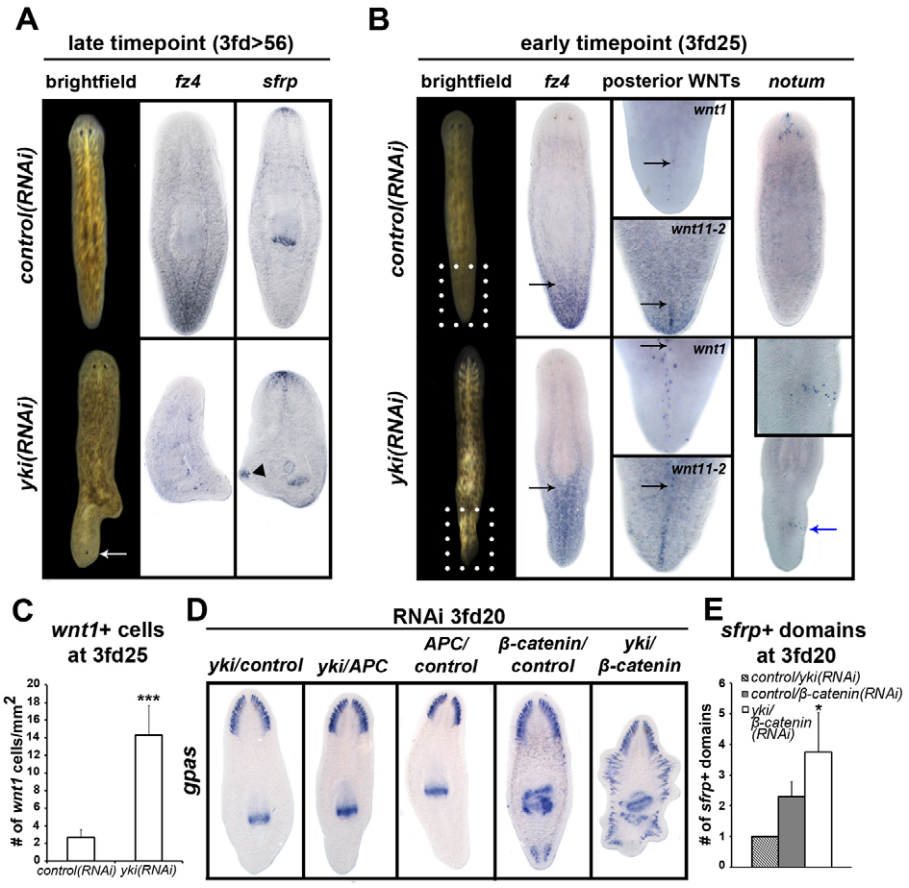

Fig. 6. Yorkie interacts with the WNT/β-catenin signaling pathway during axial patterning. (A) At late time points (3fd >56) during intact homeostasis, *yki(RNAi)* causes an axial duplication (white arrow) with ectopic *sfrp* expression in the posterior (black arrowhead). (B) At earlier time points (3fd25), animals were assayed by WISH for *fz4*, *wnt1*, *wnt11-2* and the WNT antagonist *notum*. White dotted boxes indicate the areas imaged for posteriorly expressed WNTs (*wnt1*, *wnt11-2*). Black arrows indicate the extent of expression of each respective gene. The blue arrow indicates ectopic *notum* expression (enlarged image in the inset). (C) The number of *wnt1*+ cells in *yki(RNAi)* animals at 3fd25 is significantly increased compared with controls. (D) Animals at 3fd20 were assayed for WISH with brain marker *gpas* under the following RNAi conditions: *yki/control*, *yki/APC*, *APC/control*, *β-catenin/control*, *yki/β-catenin*. (E) *yki/β-catenin(RNAi)* acted synergistically in producing a significantly increased numbed of *sfrp* expression domains compared with *yki/control(RNAi)* and *β-catenin/control(RNAi)*. Error bars indicate s.d. ***P<0.001, *P<0.05.

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had a significantly increased number of *wnt1*$^{+}$ cells compared with controls (Fig. 6C; $P<0.001$). Interestingly, *yki(RNAi)* worms also showed ectopic posterior expression of *notum,* which is normally an anteriorly expressed WNT inhibitor, necessary to define and maintain the anterior pole during regeneration (Fig. 6B) (Petersen and Reddien, 2011). Therefore, even though WNTs and WNT antagonists are dysregulated, we hypothesize that the ectopic *notum* expression in *yki(RNAi)* worms ultimately leads to an axial duplication observed at 3fd >56.

Yki acts synergistically with β-catenin signaling to suppress head identity

Previous work in planarians has demonstrated that *β-catenin(RNAi)* results in two-headed worms, which is essentially the phenotype observed for late *yki(RNAi)* animals (Gurley et al., 2008; Petersen and Reddien, 2008). In addition, a recent study demonstrated that YAP directly binds β-catenin *in vitro* (Imajo et al., 2012). Therefore, we hypothesized that these two pathways may act synergistically in suppressing head formation. To test the synergy between these two pathways in planarians, we conducted double RNAi experiments using RNAi food concentrations that normally gave no or very mild phenotypes. Worms were then analyzed for anteriorization with the brain marker *gpas* or anterior pole marker *sfrp* (Cebrià et al., 2002; Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008). At 3fd20 in uninjured animals, *yki/control(RNAi)*, *yki/APC(RNAi)* and *APC/control(RNAi)* animals all showed single brains with no axial duplication (Fig. 6D). *β-catenin/control(RNAi)* animals, as expected, showed a small ectopic brain in the tail region. However, *yki/β-catenin(RNAi)* animals showed a complete loss of the ability to suppress head identity, and a subsequent expansion of brain tissue and ectopic heads were observed across the entire periphery of the animal (Fig. 6D). The mean number of anterior poles, as defined by *sfrp* expression, was 1.0 in *yki/control(RNAi)*, 2.3 in *β-catenin/control(RNAi)* and 3.75 in *yki/β-catenin(RNAi)* worms (Fig. 6E). Together these data show that *yki* acts synergistically with β-catenin signaling, whether directly or indirectly, to suppress head formation during adult planarian homeostasis.

DISCUSSION

Planarians are a powerful model system to investigate stem cell maintenance and tissue patterning in the context of adult homeostasis and regeneration. During these processes, stem cells must constantly maintain proliferative rates but must also integrate newly differentiated progeny into properly patterned tissues (Pellettieri and Sánchez Alvarado, 2007). Here, we show that *yki* has crucial roles in both of these aspects. The removal of *yki* by RNAi results in three distinct phenotypes: loss of excretory ultrastructure; loss of anterior-posterior patterning maintenance; and stem cell hyperproliferation (Fig. 7A). Owing to the similarity of excretory and stem cell phenotypes in *yki* and *sd1/sd2* knockdown animals, we hypothesize that the canonical binding partner relationship of these molecules has been retained in planarians (Goulev et al., 2008; Wu et al., 2008; Zhang et al., 2008b; Zhao et al., 2008). In addition, *yki* functions in axial patterning by restricting the expression of posterior WNTs and by cooperating with *β-catenin* to suppress head formation (Fig. 7B). When both genes are knocked down by RNAi, ectopic heads and brain tissue occur over the entire periphery of these animals (Fig. 6D). Finally, we show that *yki* is required for proper regeneration through the re-scaling of WNT gradients (Fig. 7C). Despite these pleiotropic functions, we did not detect defects in differentiation or cell death.

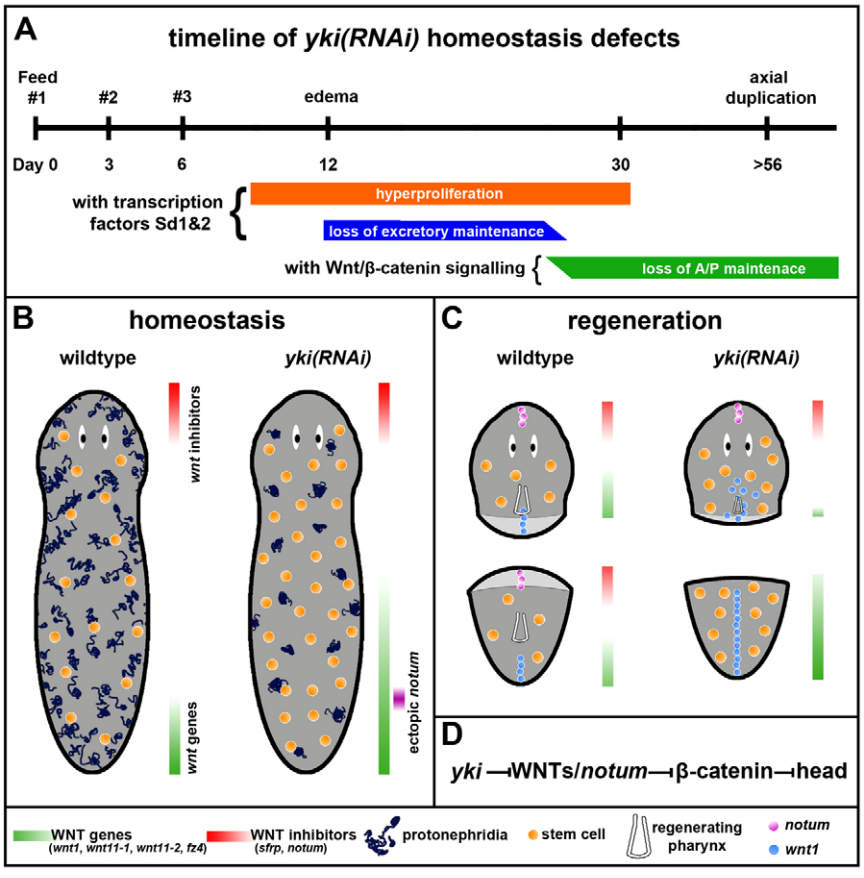

Fig. 7. A model for the pleiotropic roles of Yki during planarian homeostasis and regeneration. (A) *yki(RNAi)* causes three independent homeostatic phenotypes, which can be delineated by their onset (early to late): hyperproliferation, loss of excretory system maintenance resulting in an edema, and the loss of anterior-posterior maintenance resulting in an axial duplication. Both the maintenance of stem cell proliferation and excretory system patterning are mediated by canonical transcription factors Sd1 and 2, whereas the axial patterning defects are mediated by WNT/β-catenin signaling. (B) *yki* is required to restrict the posteriorly expressed WNTs and inhibit ectopic *notum* expression. (C) During regeneration, *yki* has similar roles in maintaining stem cell proliferation, excretory maintenance and WNT patterning. The loss of *yki* also results in a smaller or completely absent blastema and a smaller regenerated pharynx. (D) From the synergy experiments, Yki cooperates with β-catenin to suppress head identity during homeostasis. Repression 'arrows' do not suggest genetic interactions, but rather interactions of processes. We propose that *yki* functions pleiotropically in planarian homeostasis and regeneration.

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The role for *yki* in axial patterning

During adult homeostasis, *yki* is required to maintain the anterior-posterior axis by restricting the posteriorly expressed WNTs and anteriorly expressed WNT inhibitors. In *yki(RNAi)* animals, expression of *wnt1* and *fz4* is expanded anteriorly at 3fd25, which subsequently leads to an axial duplication at later time points (Fig. 6A,B). An alternative explanation to the expanded WNT gradients is that they are a consequence of edema-induced injury; however, we propose that this is not the case for several reasons. First, the expansion of *wnt1* expression is observable at 3fd6 by WISH and RNaseq analyses, which is before the onset of edema, occurring at 3fd9 (supplementary material Fig. S12A,B). Second, the loss of excretory system maintenance does not result in changes in WNT expression or produce an injury response (supplementary material Fig. S7; Fig. S13B-D) (Reddien et al., 2005a). In addition, we do not observe an axial duplication or ectopic *notum* expression in *sd1/sd2(RNAi)* or *pou2/3(RNAi)* animals even though edema occurs in both conditions (supplementary material Fig. S13A). Finally, a strong synergy between Yki and β-catenin was detected, suggesting that edema is unlikely to cause the defects in axial patterning.

The cooperation between Yki/YAP and the WNT/β-catenin signaling pathway has also been demonstrated in other animal models. One proposed *in vitro* mechanism is the direct interaction between YAP2 and β-catenin, which suppresses WNT target gene expression (Imajo et al., 2012). Interestingly, this interaction is dependent on the TEAD-binding domain in YAP2, which is also conserved in SMED-YKI (Imajo et al., 2012). Similarly, loss of YAP1 in the mouse intestinal stem cell crypts results in hypersensitive WNT signaling (Barry et al., 2013). YAP1 is also a WNT target and may act as a negative feedback to restrict WNT signaling in colorectal cancer cells (Konsavage et al., 2012). In line with these findings, *Smed-yki* has similar roles in restricting WNT signaling during homeostasis and regeneration; however, future studies are required to understand the precise biochemical interactions.

An evolutionary conserved role for *yki* in excretory systems

Recently, YAP1 has been shown to be a key regulator of mammalian embryonic kidney morphogenesis. Conditional $Yap1^{-/-}$ kidneys are smaller, but show no defects in proliferation and apoptosis, or changes in the multipotent $Six2^{+}$ nephron progenitor population. Similarly, *Smed-yki* is crucial in the patterning of the protonephridial system during adult homeostasis. *yki(RNAi)* animals show an early edema phenotype, which is due to a disorganized expression of anatomical markers, a collapse of tubule branching, and fewer ciliated flame cells (Fig. 7B; supplementary material Fig. S3). In addition, *yki(RNAi)* does not affect the relative expression of *eya*, *sall* and *osc*, which mark committed protonephridial progenitors (Fig. 1E) (Scimone et al., 2011). This suggests that *yki* acts downstream or after protonephridia are specified (supplementary material Fig. S5). Future studies will be required to understand how the downstream targets of Yki establish proper protonephridial patterning and whether these factors are cell intrinsic or extrinsic.

yki functions to restrict stem cell proliferation

In *S. mediterranea* RNAi targeted against any of the core Hippo components did not result in measurable stem cell phenotypes, despite strong gene knockdown (supplementary material Fig. S10). By contrast, the basal flatworm *M. lignano* shows the canonical Hippo phenotypes: reduced growth in *Mac-YAP(RNAi)* animals, and excess growth in knockdowns of the core Hippo pathway members (Demircan and Berezikov, 2013). One explanation that might explain the difference between planarians and *M. lignano* is that *Smed-yki* functions independently of Hippo signaling. In support of this, we noted that the consensus Warts/Lats phosphorylation motif, HxRxxS, of Yki is intact in vertebrates, flies and *M. lignano*, whereas SMED-YKI has a substitution of a lysine for an arginine (supplementary material Fig. S1B) (Zhao et al., 2007). This substitution renders the motif refractory to phosphorylation by yeast Dbf2, the homolog of Warts/Lats (Mah et al., 2005). A similar substitution in human YAP1 also decreases *in vitro* kinase activity of LATS by 50% (Hao et al., 2008); however, the consequences of this mutation have not been examined *in vivo*. Finally, not all tissues are sensitive to perturbations in Hippo signaling, including the mammalian epidermis, kidney, lung and hematopoietic stem cell population (Jansson and Larsson, 2012; Schlegelmilch et al., 2011; Song et al., 2010). Therefore, it remains a possibility that SMED-YKI is not under the regulation of upstream Hippo signaling.

In various animal models, Yki/YAP typically functions to promote growth in a stem cell and regenerative context. However, the opposite is observed in planarians. Knockdown of *yki* causes a sustained hyper-proliferation of the stem cells. Similar to planarians, YAP1 is growth restrictive in the mammalian intestinal stem cells (ISC) (Barry et al., 2013). More specifically, the loss of YAP1 during ISC regeneration causes a de-repression of WNT signaling, which promotes an expansion of the ISCs and related progenitors. Interestingly, we show that *Smed-yki* parallels these defects by restricting stem cell proliferation and WNT signaling. This supports the hypothesis that an ancestral role of Yki/YAP was to repress WNT signaling and restrict proliferation of ASCs in an adult tissue that has high rates of cell turnover and regenerative capacity, such as the adult planarian or mammalian intestine. It will be interesting to determine whether this holds true for other fast-cycling stem cell compartments in vertebrates.

Collectively, our data show that *Smed-yki* has pleiotropic roles in planarian biology and is a crucial link between two seemingly disparate regulatory programs of stem cell maintenance and tissue patterning. The multiple roles for *Smed-yki* may be attributed to its ability to bind to various transcription factors and cooperate with many key developmental signaling cascades, such as TGFβ, Hedgehog, WNT/β-catenin, and EGFR signaling (Hong and Guan, 2012; Lin et al., 2012; Reddy and Irvine, 2013; Varelas et al., 2010; Varelas and Wrana, 2012; Zhang et al., 2009). Whether these interactions and downstream targets are also conserved in planarians or work in tissue-specific contexts remains to be elucidated, and planarians are a powerful model system to dissect the multi-faceted roles of YKI signaling.

MATERIALS AND METHODS

Phylogenetics and cloning

Planarian homologs of Hippo pathway genes were found in the sequenced and assembled planarian genome and transcriptome as previously described (Currie and Pearson, 2013; Pearson and Sánchez Alvarado, 2010; Robb et al., 2008; Sánchez Alvarado et al., 2002; Sánchez Alvarado et al., 2003). Primers were designed and genes were cloned by 3′ rapid amplification of cDNA ends (RACE). *Smed-yki* and *Smed-sd-1* and *-2* homologs were converted to predicted proteins and subjected to Bayesian and Maximum Likelihood phylogenetic analyses. Protein sequences used in phylogenies were obtained from the NCBI Entrez protein database or directly from the genome sequencing projects of included organisms. *Smed-yki*, *Smed-sd-1*, *Smed-sd-2*, *Smed-hippo*, *Smed-merlin*, *Smed-warts*, *Smed-mats-1*, *Smed-mats-2* and *Smed-sav* sequences were deposited in GenBank as accession numbers: KF990477-KF990485, respectively, and can also be found in the transcriptome database associated with the NCBI GEO project: GSE47348.

Animal husbandry, exposure to γ-irradiation, RNAi and high-salt suppression of edemas

Asexual *Schmidtea mediterranea* CIW4 strain were reared as previously described (Sánchez Alvarado et al., 2002). For irradiation experiments, planarians were exposed to 60 Gy of γ-irradiation from a ^{137}Cs source (Pearson and Sánchez Alvarado, 2010). RNAi experiments were performed using previously described expression constructs and HT115 bacteria (Newmark et al., 2003). Bacteria were grown to an OD_{600} of 0.8 and induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 2 hours. Bacteria were pelleted, mixed with liver paste at a ratio of 500 µl of liver to 100 ml of original culture volume, and frozen as aliquots. The negative control '*control(RNAi)*', was the *unc22* sequence from *Caenorhabditis elegans* as previously described (Newmark et al., 2003). Experimental worms were starved for 1 week and then given 'RNAi food' every third day for a total of three feedings in *yki(RNAi)* and eight in *sd1/sd2(RNAi)* experiments. The nomenclature used for the time of analysis indicates the time of staining and number of feeds (e.g. 3fd6 is 6 days after the third feed). Amputations were performed 15 days after the final feeding unless noted otherwise, and referred to as days post amputation (dpa). All animals used for immunostaining were 2-4 mm in length and size-matched between experimental and control worms. For high-salt suppression of edema formation, the concentration of the medium was increased following RNAi addition by adding a 10 mM Instant Ocean Aquarium Salt every 3 days until 75 mM final concentration was reached.

Flow cytometry

Planarians were dissociated as previously described and sorted using a Becton Dickinson FACSAria without calcein (Reddien et al., 2005b). Three biological triplicates of at least 60,000 live-cell events were processed for each replicate in supplementary material Fig. S6.

Quantitative real-time PCR

Reverse transcription reactions were conducted on total RNA extracted from ~15 worms using a SuperScript III Reverse Transcriptase Kit (Invitrogen). Quantitative real-time PCR was performed in triplicate using a Roche LightCycler 480 Instrument II with SYBR Green PCR Master Mix (Roche) as per the manufacturer's instructions. The standard curve method was used for relative quantification. Primer pairs for ubiquitously expressed GAPDH were used as a reference (Eisenhoffer et al., 2008).

Immunolabeling, TUNEL and *in situ* hybridizations (ISH)

Whole-mount ISH (WISH), double fluorescent ISH (dFISH) and immunostainings were performed as previously described (Lauter et al., 2011; Pearson et al., 2009). Colorimetric WISH stains were imaged on a Leica M165 fluorescence dissecting microscope. dFISH and fluorescent phospho-histone H3 (rabbit monoclonal to H3ser10p, 1:500, Millipore) immunostains were imaged on a Leica DMIRE2 inverted fluorescence microscope with a Hamamatsu Back-Thinned EM-CCD camera and spinning disc confocal scan head. TUNEL was performed as previously described (Pellettieri et al., 2010). Cell counts and colocalizations were quantified using freely available ImageJ software (http://rsb.info.nih.gov/ij/). Significance was determined by a two-tailed unequal variance Student's *t*-test. For all experiments, a minimum of three biological replicates and at least five worms were used per stain and per time point. All images were processed in a similar manner using Adobe Photoshop.

Deep sequencing and statistical analysis

RNA deep sequencing (RNaseq) was performed on whole uninjured animals 6 days after RNAi feedings (3fd6) of *control(RNAi)* and *yki(RNAi)*. Experiments were performed on biological triplicate samples, sequenced to a depth of 50 million reads per sample, and multiplexed on an Illumina HiSeq2500 with 50 bp, single-end reads. Sequences were then aligned to a described planarian transcriptome (Currie and Pearson, 2013; Labbé et al., 2012). To determine significantly up or downregulated transcripts between experimental animals and controls, the freely available baySeq software was used (http://bioconductor.org/packages/release/bioc/html/baySeq.html) (Hardcastle and Kelly, 2010). Raw count data and statistical analyses are presented in supplementary material Table S1.

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Competing interests

The authors declare no competing financial interests.

Author contributions

A.Y.T.L. performed all experiments. A.Y.T.L. and B.J.P. interpreted the experimental data and wrote the manuscript.

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Supplementary material

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.101915/-/DC1

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