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Stem cell-dependent formation of a functional anterior regeneration pole in planarians requires Zic and Forkhead transcription factors

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ABSTRACT

Planarians can regenerate their head within days. This process depends on the direction of adult stem cells to wound sites and the orchestration of their progenitors to commit to appropriate lineages and to arrange into patterned tissues. We identified a zinc finger transcription factor, Smed-ZicA, as a downstream target of Smed-FoxD, a Forkhead transcription factor required for head regeneration. *Smed-zicA* and *Smed-FoxD* are co-expressed with the Wnt inhibitor *notum* and the Activin inhibitor *follistatin* in a cluster of cells at the anterior-most tip of the regenerating head – the anterior regeneration pole – and in surrounding stem cell progeny. Depletion of *Smed-zicA* and *Smed-FoxD* by RNAi abolishes *notum* and *follistatin* expression at the pole and inhibits head formation downstream of initial polarity decisions. We suggest a model in which ZicA and FoxD transcription factors synergize to control the formation of Notum- and Follistatin-producing anterior pole cells. Pole formation might constitute an early step in regeneration, resulting in a signaling center that orchestrates cellular events in the growing tissue.

Introduction

Secreted signaling molecules are essential components of organizing regions that regulate tissue morphogenesis in a non-autonomous manner during the development of metazoans. These signaling molecules often form activity gradients with the highest activity close to the site of secretion, thus informing cells about their relative position within a tissue (Perrimon et al., 2012).

In planarians, flatworms with enormous regenerative potential (Gentile et al., 2011), the family of secreted Wnt proteins plays a crucial role during regeneration (Adell et al., 2009; Petersen and Reddien, 2009). When depleted of *Smed-wnt1*, a *wnt* gene initially expressed in cells at both anterior and posterior wounds, planarians regenerate heads instead of tails (Adell et al., 2009; Petersen and Reddien, 2009). In contrast, the secreted Wnt-inhibitor Notum is induced asymmetrically, mainly at anterior-facing wounds, and its knockdown generates two-tailed animals (Petersen and Reddien, 2011). This suggests that Wnt activity is required for tail regeneration in the posterior, while in the anterior, the Wnt-inhibiting activity of Notum allows head regeneration.

Planarian regeneration is based on adult stem cells, called neoblasts. Neoblasts are distributed in the parenchyma of intact planarians and make up 25–30% of all cells (Baguna et al., 1989). They are characterized by their morphology, their ability to proliferate, and by the distinctive expression of markers, such as the PIWI family member *smedwi-1* (reviewed in (Reddien, 2013)). At least some neoblasts are pluripotent, as they can give rise to cells of different germ layers and rescue stem cell-depleted animals (Wagner et al., 2011).

Neoblasts respond to tissue loss through migration to the wound site and local proliferation (Wenemoser and Reddien, 2010). Their descendants form the regeneration blastema, a tissue that develops into the highly organized structures of an intact animal (Eisenhoffer et al., 2008). After polarity decisions are made, anterior and posterior blastemas produce tissues of the corresponding identity. It is currently unknown how cellular events in the blastema are orchestrated and whether organizing regions that control tissue growth exist, similarly to vertebrate developmental organizing regions. Recently, it was shown that the Wnt inhibitor Notum, the Activin inhibitor Follistatin and the transcription factor Smed-FoxD are expressed at the poles of anterior regeneration blastemas and that these genes are required

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for head regeneration (Gavino et al., 2013; Petersen and Reddien, 2011; Roberts-Galbraith and Newmark, 2013). How the anterior pole forms during the head regeneration process is currently not known.

In this study, we identified a Zic transcription factor gene, *Smed-zicA*, as a putative downstream target of *Smed-FoxD* (*zicA* and *FoxD* for simplicity), and demonstrate its requirement for head regeneration and for the formation of a functional anterior regeneration pole. Both transcription factors are co-expressed with *notum* and *follistatin* in anterior pole cells and are essential for their expression. We show that *zicA* and *FoxD* are induced in stem cells and stem cell progeny in close proximity to the anterior regeneration pole and propose a model in which *zicA* and *FoxD* are required for the differentiation of anterior pole cells once polarity has been re-established. This pole might in turn regulate head formation through local production of secreted modulators of signaling molecules, such as inhibitors of Wnt and TGF-β family members.

Materials and methods

Planarian culture and RNA interference

Asexual animals of the species *Schmidtea mediterranea* (clonal line BCN-10) were starved 1 week prior to experiments. RNA interference (RNAi) was induced by injection of dsRNAs as previously described (Sandmann et al., 2011). For homeostatic experiments, 3×32.2nl of a 2–2.3μg/μl *gfp* (control), *FoxD* or *zicA* dsRNA solution was injected as indicated in the schemes. Primers used for RNAi probes are shown in Supplementary Table 1.

Irradiation

Intact planarians were lethally γ-irradiated at 60 Gy with a Gammacell-40 Exactor (MDS Nordion). Animals were kept intact or cut 1 day after irradiation for regeneration experiments.

In situ hybridization and immunostaining

Whole mount in situ hybridization (WISH) was carried out as previously described (Nogi and Levin, 2005; Umesono et al., 1999). For fluorescent in situ hybridization (FISH) and double FISH, animals were treated as described (Marz et al., 2013; Pearson et al., 2009). Primers used for in situ probes are shown in Supplementary Table 1. Animals for immunostainings were processed as previously described (Sandmann et al., 2011). Primary antibodies used were mouse anti-Synapsin (3C-11, Developmental Studies Hybridoma Bank) at 1:100, and rabbit anti-SMEDWI-1 (Guo et al., 2006; Marz et al., 2013) at 1:1000. Secondary antibodies used were Alexa 488 goat anti-mouse and Alexa 647 goat anti-rabbit (Molecular Probes) at 1:400. DNA staining was performed with Hoechst 33342 (Life Technologies).

Microscopy

Live images were taken with a Leica M80 microscope. Images for WISH were acquired with a Leica M165 FC microscope and FISH images were captured with a Zeiss LSM700 confocal laser-scanning microscope. For quantification of *FoxD*- and *zicA*-expressing cells, cells within 60–70 μm confocal stacks of at least three different animals were counted in the indicated areas.

Sample collection and preparation for FoxD RNAi transcriptome analysis

Total RNA was extracted from regenerating control and *FoxD* RNAi tail stumps at 0 and 3 days post amputation (dpa), followed by RNA-sequencing on the HiScanSQ System (Illumina). Sequencing libraries were prepared from 1 μg total RNA following the TruSeq RNA Sample Prep v2 LS protocol. RNAi animals went through two rounds of regeneration.

Sequencing library pre-processing and differential expression analysis

Reads were split into barcoded sets using Casava [CASAVA 1.8.2], followed by mapping to SRG12 (Boser et al., 2013), an in-house assembled transcriptome, using bowtie2 with default options [bowtie2-align version 2.1.0]. SRG12 reads for each transcript were counted using samtools idxstats [samtools Version: 0.1.18 (r982:295)]. Raw count tables for reads (one row per transcript, one column per sample) were generated using a custom R script [R version 2.15.2]. Negative binomial tests for transcripts were run using DESeq (GFP vs FoxD at 0d/3d, and GFP0d/FoxD 0d vs 3d) [DESeq 1.8.3]. Differential expression results were combined with FrameDP-generated BLASTx results (for transcript protein name) [FrameDP 1.2.0]. A matrix (variance stabilized) was generated using DESeq. The MA plot of expression difference over mean expression (GFP vs FoxD at 3d) was generated using a custom R script.

Accession numbers

GenBank IDs of *Smed-FoxD*, *Smed-zicA*, and *31119* are KC577557, KF751216, and KF581192, respectively; sequencing data was deposited at NCBI: SRP031467.

Results

zicA is a FoxD-dependent regulator of planarian head regeneration

Planarian head regeneration depends on *FoxD*, a Forkhead transcription factor gene expressed at the anterior regeneration pole (Fig. 1A–C) (Koinuma et al., 2003; Roberts-Galbraith and Newmark, 2013). To improve our understanding of *FoxD*-mediated downstream events during head regeneration, we sequenced the transcriptomes of regenerating control and *FoxD* RNAi tail fragments at 3 days post amputation (dpa) (Fig. 1D), when the accumulation of *FoxD*-positive cells at the anterior pole was most prominent (Fig. 1A). Among the top down regulated hits (Fig. 1E; Supplementary Table 2) were the known anterior signaling molecules *secreted frizzled-related protein 1* (*sFRP-1*) (Gurley et al., 2008), *noggin-like 1* (*ngl-1*) (Molina et al., 2007), *netrin* (Cebria and Newmark, 2005), *notum* (Petersen and Reddien, 2011), the FGF receptor related gene *nou-darake* (*ndk*) (Cebria et al., 2002) and a zinc finger transcription factor, which we named *Smed-zicA* (*zicA* for simplicity), according to a phylogenetic study on Zic transcription factors from diverse species including planarians (Aruga et al., 2006). Interestingly, *zicA* was expressed at the anterior pole of intact and regenerating animals (Fig. 1F), similar to *FoxD* (Fig. 1A), *follistatin* (Gavino et al., 2013; Roberts-Galbraith and Newmark, 2013) and *notum* (Petersen and Reddien, 2011). Reminiscent of *FoxD* RNAi animals (Fig. 1B and C), knockdown of *zicA* resulted in animals that regenerated one or no eyespot (Fig. 1G) and smaller brains (Fig. 1H).

Since *FoxD* and *zicA* showed similar expression patterns and RNAi resulted in similar phenotypes, we asked whether *FoxD* might regulate the expression of *zicA* during regeneration. Consistent with the transcriptome analysis of *FoxD* RNAi animals, the expression of *zicA* at the anterior regeneration pole was lost in *FoxD* RNAi animals at 3 dpa (Fig. 1I). Interestingly, the expression of *FoxD* was also reduced after *zicA* RNAi (Fig. 1J), suggesting that *FoxD* and *zicA* might regulate each other's expression.

Next, we investigated whether *FoxD* and *zicA* might act synergistically to regulate head formation at the pole. While *FoxD*/*zicA* RNAi animals regenerated only one or no eyespot, *FoxD*/control RNAi animals were able to regenerate two eyespots and *zicA*/control

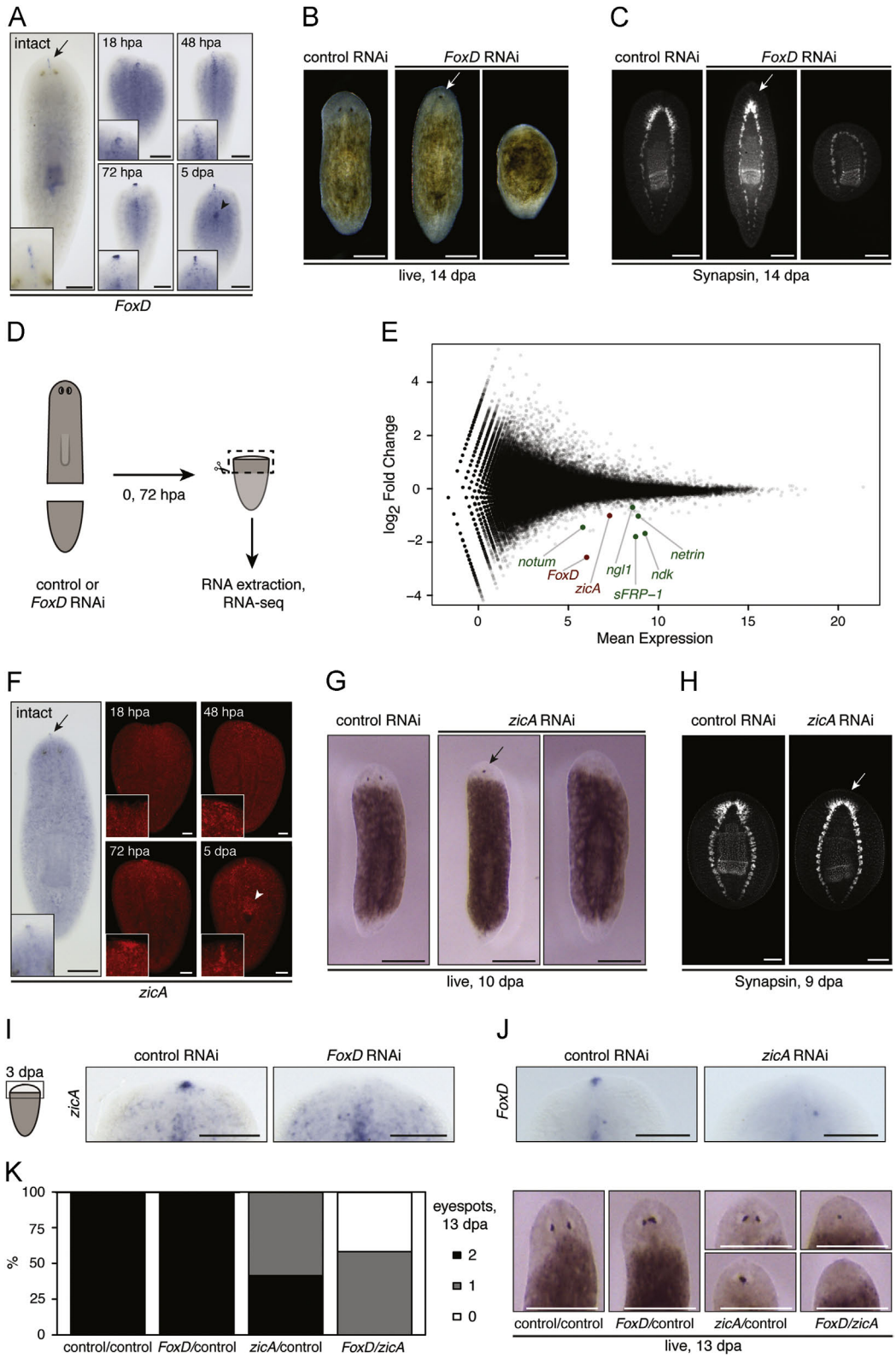

Fig. 1. *zicA is a FoxD-dependent regulator of planarian head regeneration.* (A) *FoxD* expression in intact planarians and in regenerating tail pieces at 18, 48, 72 hours post amputation (hpa) and 5 days post amputation (dpa), detected by WISH. The expression was most prominent at the anterior pole (insets and black arrow) and the pharynx (black arrowhead). (B) Live animals at 14 dpa. *FoxD* RNAi trunks regenerated one eyespot (white arrow) ($n=16/27$) or no eyespot ($n=11/27$). Control animals regenerated normally ($n=27/27$). (C) Immunostaining of the central nervous system with an antibody against Synapsin at 14 dpa. *FoxD* RNAi trunks regenerated smaller brains (white arrow) compared to control animals, or no brain tissue at all. (D) and (E) Identification of *zicA* with an RNA-seq approach. (D) Sample collection: total RNA was extracted from regenerating control and *FoxD* RNAi tail stumps at 0 and 3 dpa, followed by RNA-sequencing on the Illumina platform. (E) The expression fold change (*FoxD* 3d/control 3d) of each transcript was plotted against the number of mean normalized reads. *zicA* was found among the top down-regulated hits (FC < -0.5 and padj < 0.1). Down-regulated known anterior genes are highlighted in green. (F) *zicA* expression in intact planarians and in regenerating tail pieces at 18, 48, 72 hpa and 5 dpa, detected by WISH and FISH. The expression was most prominent at the anterior pole (insets and black arrow) and the pharynx (white arrowhead). (G) Live animals at 10 dpa. *zicA* RNAi trunks regenerated one eyespot (black arrow) ($n=8/10$) or no eyespot ($n=2/10$). Control animals regenerated normally ($n=10/10$). (H) Immunostaining of regenerating trunk pieces with an antibody against Synapsin at 9 dpa. Note that *zicA* RNAi animals had a smaller brain (white arrow) compared to control animals. (I) and (J) WISH of tail fragments from control, *FoxD* or *zicA* RNAi animals at 3 dpa. Pictured regions are indicated in the schemes. Note that *zicA* expression was lost after *FoxD* RNAi ($n=5/5$) (I) and *FoxD* expression was lost after *zicA* RNAi ($n=5/5$) (J) at the anterior pole. (K) Eyespot scoring of double RNAi trunks at 13 dpa. Concomitant *FoxD* RNAi and *zicA* RNAi enhanced the *FoxD*/control RNAi and *zicA*/control RNAi phenotypes ($n=12$ each). Animals in (B), (C), and (D) went through two rounds of regeneration. Scale bars: (A), (F), 100 μm; (B), (C) and (H)–(J), 200 μm; (G), (K), 500 μm.

RNAi animals at least one eyespot (Fig. 1K). These data suggest that double RNAi resulted in an additive effect on head regeneration and that *FoxD* might act together with *zicA* during the head formation stage.

zicA and FoxD are required for proper head formation downstream of early polarity decisions

Knockdown of *FoxD* and *zicA* resulted in animals with anterior regeneration defects (Fig. 1B, C, and G, H). Consistently, the expression of the anterior markers *ndk*, *sFRP-1,* and *prep* (Felix and Aboobaker, 2010) was greatly reduced in *FoxD* and *zicA* RNAi animals (Fig. 2A and B, Supplementary Fig. 1A and B). However, the expression of *pbx* (Blassberg et al., 2013; Chen et al., 2013) seemed unaffected after *FoxD* and *zicA* RNAi (Supplementary Fig. 1A and B). Furthermore, anterior expression of *slit*, a gene encoding a secreted repulsive axon guidance cue (Cebria et al., 2007), was reduced after *FoxD* and *zicA* RNAi (Fig. 2C). This indicates that the cyclopic phenotype of *FoxD* and *zicA* RNAi animals might be a consequence of reduced *slit* expression and a subsequent midline collapse.

Unlike the expression of anterior markers, the induction of the posterior markers *wnt11-2* and *frizzled-4* (*fz-4*) (Gurley et al., 2008, 2010) was comparable to that of control RNAi animals (Fig. 2D and E), showing that *FoxD* and *zicA* are crucial for head but not tail regeneration.

To test if *FoxD* and *zicA* are required for the early polarity decision to form a head instead of a tail, we analyzed the expression of the secreted Wnt inhibitor *notum*, which is the earliest gene known to be asymmetrically expressed mainly at anterior-facing wound sites. There, it is thought to antagonize wound-induced *wnt1* expression, and thus mediate the re-establishment of anterior polarity and the induction of anterior cell types (Petersen and Reddien, 2011). Notably, neither *wnt1* nor *notum* expression was affected in *FoxD* and *zicA* RNAi fragments at 18 hours post amputation (hpa) (Fig. 2F, Supplementary Fig. 1C and D). These data suggest that once initial polarity decisions are correctly made, *FoxD* and *zicA* are required for head regeneration downstream of wound signaling and polarity choices.

Since *FoxD* and *zicA* were expressed in regenerating pharynges (Fig. 1A and F), we tested *FoxD* and *zicA* RNAi animals for their ability to regenerate pharynges and secretory cells associated with it. *FoxD* RNAi animals regenerated pharynges and secretory cells *de novo* with no obvious defects (Supplementary Fig. 1E and F). Knockdown of *zicA* resulted in animals with normal secretory marker gene expression but smaller pharynges (Supplementary Fig. 1G and H).

Altogether, these results suggest that *FoxD* and *zicA* are required for head regeneration, and that *zicA* might play an additional role in pharynx regeneration.

zicA and FoxD are co-expressed with notum and follistatin in collagen-positive cells at the anterior regeneration pole

Since *FoxD* and *zicA* showed similar expression patterns (Fig. 1A and F), we speculated that *FoxD* might be co-expressed with *zicA*

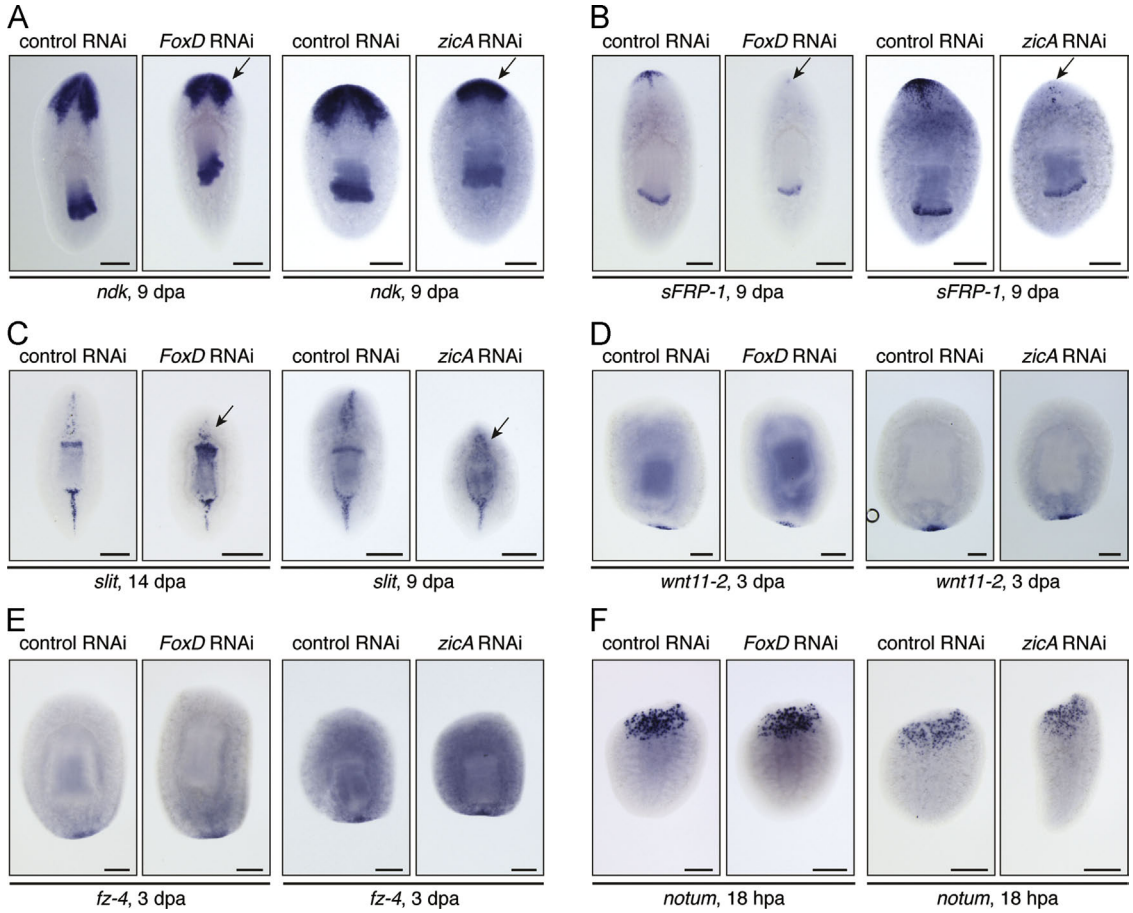

Fig. 2. *zicA and FoxD are required for head regeneration downstream of early polarity decisions.* (A)–(F) WISH of *ndk*, *sFRP-1*, *slit*, *wnt11-2*, *fz-4* and *notum* after control, *FoxD* or *zicA* RNAi. (A) and (B) *FoxD* and *zicA* RNAi trunks showed a great reduction of the anterior markers *ndk* and *sFRP-1* ($n=5/5$) at 9 days post amputation (dpa) (black arrows). (C) Anterior *slit* expression was reduced ($n=5/5$) in *FoxD* and *zicA* RNAi trunks at 14 dpa and 9 dpa, respectively (black arrows). (D) and (E) Expression of the posterior markers *wnt11-2* and *fz-4* was not affected in *FoxD* and *zicA* RNAi trunks at 3 dpa. (F) *notum* expression in tail pieces was *FoxD* and *zicA* independent at 18 hours post amputation (hpa). Scale bars: (A)–(F), 200 μm.

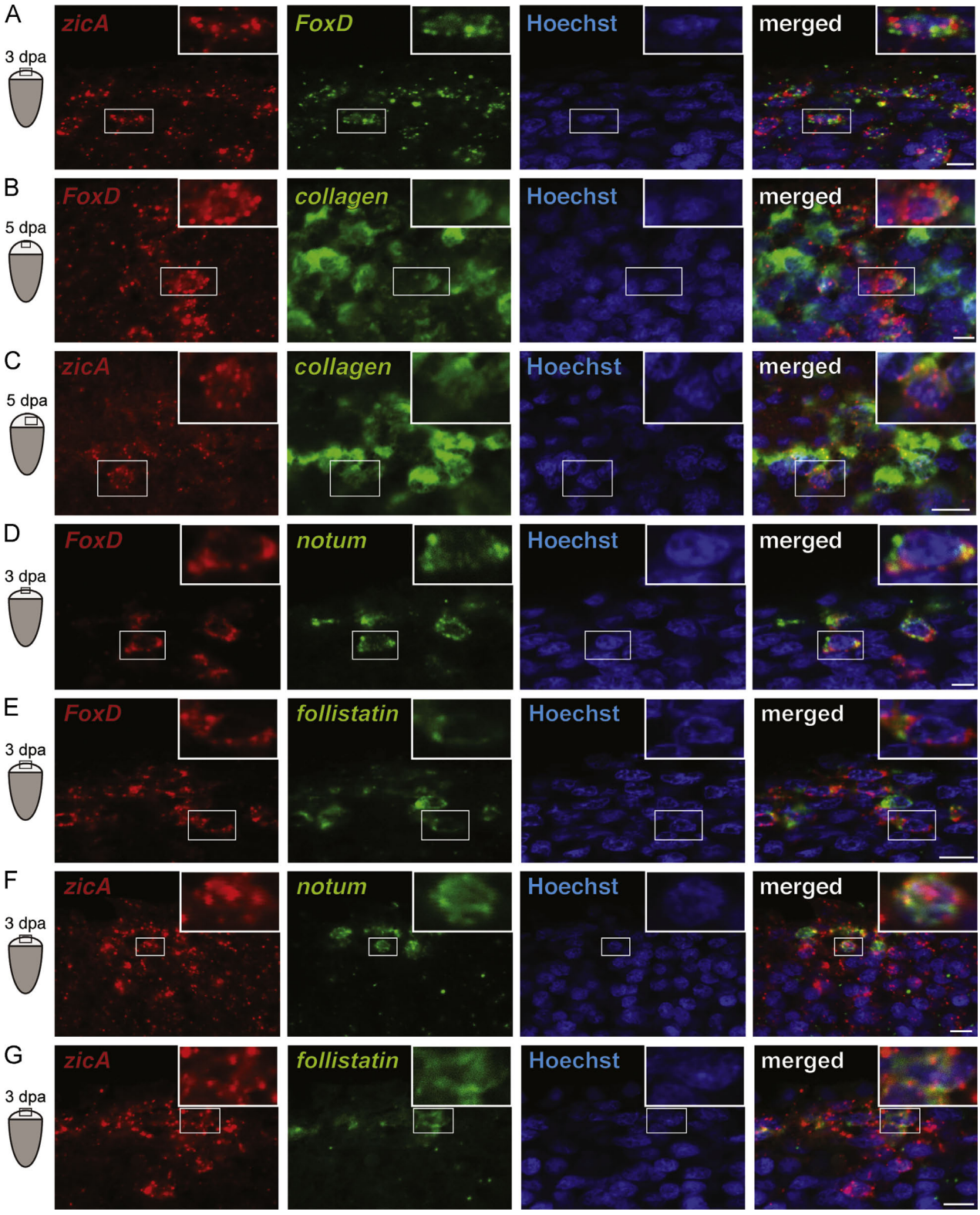

Fig. 3. *zicA is co-expressed with FoxD, notum and follistatin in collagen-positive cells at the anterior regeneration pole.* (A)–(G) Double FISH (against transcripts as indicated) in regenerating tail fragments at 3 days post amputation (dpa) (A), (D)–(G) and 5 dpa (B) and (C). Pictured regions are indicated in the schemes. Nuclei are labeled with Hoechst (blue). Single representative cells are enlarged in the upper right corners for better visibility. Percentages of *FoxD* and *zicA* expressing cells that were positive for the indicated transcripts are: (A) 96 ± 2% *FoxD* with *zicA*. (B) 72 ± 1% *FoxD* with *collagen*. (C) 69 ± 2% *zicA* with *collagen*. (D) 69 ± 7% *FoxD* with *notum*. (E) 62 ± 5% *FoxD* with *follistatin*. (F) 61 ± 8% *zicA* with *notum*. (G) 59 ± 7% *zicA* with *follistatin*. Scale bars: (A)–(G), 10 μm.

in anterior pole cells. We found that almost all *FoxD*-expressing cells were *zicA*-positive (Fig. 3A). Recently, it was shown that *notum* is expressed in *collagen*-positive sub-epidermal muscle cells (Witchley et al., 2013). As both FoxD and Zic transcription factors have been implicated in the development of mesodermal tissues in vertebrates (Fujimi et al., 2012; Inoue et al., 2007; Steiner et al.,

2006) – collagen-rich structures such as connective tissue, muscle and bones (Doss et al., 2012) – we asked whether *FoxD*- and *zicA*-expressing cells might be *collagen*-positive. Interestingly, we detected *collagen* mRNA in *FoxD*- and *zicA*-expressing cells (Fig. 3B and C), raising the possibility that these cells might be indeed of mesodermal nature. Furthermore, *FoxD* and *zicA* were co-expressed with the Wnt inhibitor *notum* as well as with *follistatin*, an antagonist of the TGF-β family member Activin (Fig. 3D–G). Hence, anterior pole cells are characterized by the co-expression of *FoxD, zicA, notum, follistatin* and *collagen*.

zicA and FoxD are required for the expression of notum and follistatin at the anterior pole in regenerating and intact planarians

FoxD and *zicA* are co-expressed with *notum* and *follistatin* at the anterior regeneration pole (Fig. 3D–G) and knockdown of either

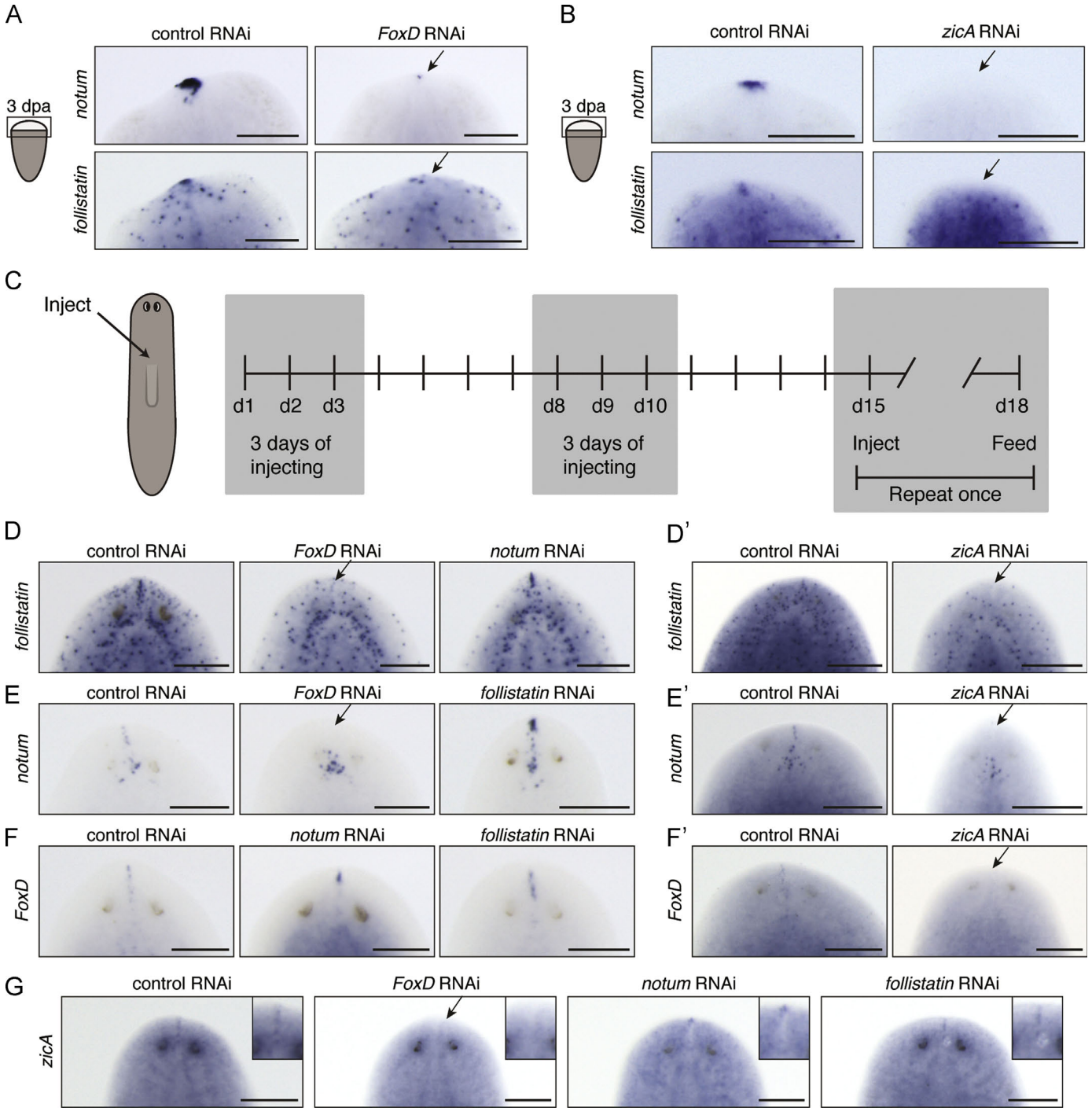

Fig. 4. *zicA and FoxD are required for the expression of notum and follistatin during regeneration and homeostasis.* (A) and (B) WISH of tail fragments from control, *zicA* or *FoxD* RNAi animals at 3 days post amputation (dpa). Pictured regions are indicated in the schemes. *notum* ($n=5/5$) and *follistatin* ($n=5/5$) expression was absent or reduced at the anterior pole in *FoxD* (A) and *zicA* (B) RNAi animals (black arrows). Note that *follistatin* expression was not affected in cells outside the anterior pole region. (C) Schematic overview of the homeostatic experiment, shown in (D)–(G). Animals were injected for three days in a row over two consecutive weeks. Animals were then injected and fed once for the subsequent weeks. The experiment was conducted for 29 days. (D)–(G) WISH of intact planarians treated with dsRNAs against the genes indicated. Shown are head regions. Arrows point at anterior poles with absent or reduced expression of *follistatin* ($n=5/5$) (D) and (D′), *notum* ($n=5/5$) (E) and (E′), *FoxD* ($n=5/5$) (F′), and *zicA* ($n=5/5$) (G). Note that *notum* and *follistatin* mRNAs were lost after *FoxD* and *zicA* RNAi, but not vice versa. Scale bars: (A) and (B), (D)–(G), 200 μm.

gene leads to regeneration defects of the whole head (Fig. 1B, C and G, H) (Gavino et al., 2013; Petersen and Reddien, 2011; Roberts-Galbraith and Newmark, 2013). Furthermore, it has been shown that the expression of *follistatin* is *FoxD*-dependent during regeneration and homeostasis (Roberts-Galbraith and Newmark, 2013). Hence, we hypothesized that *FoxD* and *zicA* might control head formation through the activation of *notum* and *follistatin*. Interestingly, the expression of *notum* and *follistatin* was greatly reduced in *FoxD* (Fig. 4A) and *zicA* RNAi tail fragments at 3 dpa (Fig. 4B), demonstrating that *FoxD* and *zicA* RNAi interfere with the production of secreted signaling modulators during head regeneration.

Since *FoxD* and *zicA* RNAi inhibited head regeneration, it was unclear whether loss of *notum* and *follistatin* expression was a direct effect of *FoxD* and *zicA* knockdown or an indirect consequence due to defective upstream events that might result in the absence of anterior tissues. To overcome this limitation we decided to analyze the *FoxD/zicA/notum/follistatin* relationship in a setting in which anterior tissues are present and properly patterned. Hence, we performed gene expression analysis in intact animals at day 29 after *FoxD*, *zicA*, *notum* and *follistatin* dsRNA injection (Fig. 4C–G). We found that *follistatin* expression at the anterior pole was unaffected in *notum* RNAi animals but was absent after knockdown of *FoxD* and *zicA* (Fig. 4D and D′). Similarly, *notum* expression at the anterior stripe was undetectable in homeostatic *FoxD* and *zicA* RNAi animals but persisted after *follistatin* RNAi (Fig. 4E and E′). Neither the expression of *notum* nor *follistatin* outside of the anterior pole, in between the eyes or throughout the body, was affected by *FoxD* and *zicA* RNAi (Fig. 4D and E). As *FoxD* and *zicA* transcripts were still present after both *notum* and *follistatin* RNAi (Fig. 4F and G), our data suggest that the transcription factors FoxD and ZicA act upstream of the secreted signaling modulators Notum and Follistatin at the anterior pole. Interestingly, the expression of *FoxD* was lost after *zicA* RNAi and vice versa (Fig. 4F′ and G). Given that double RNAi against both genes resulted in an enhanced phenotype (Fig. 1K), it is likely that *FoxD* and *zicA* act in the same pathway, possibly in a positive feedback loop, to regulate the expression of *notum* and *follistatin* at the anterior pole of intact and regenerating animals.

Stem cell-based turnover is not the major cause of notum and follistatin loss in homeostatic animals after FoxD and zicA RNAi

The expression of *notum* and *follistatin* was lost in homeostatic *FoxD* and *zicA* RNAi animals, but not vice versa (Fig. 4D–G). To investigate the time frame of the loss of *notum* and *follistatin* expression in more detail, we performed expression analysis at different time points after *FoxD* and *zicA* dsRNA injection (Fig. 5A). Interestingly, the expression of *notum* and *follistatin* at the anterior pole was already greatly reduced between 3 and 7 days post injection (dpinj) (Fig. 5B and C). A role of *FoxD* and *zicA* in the differentiation of *notum*- and *follistatin*-expressing cells, combined with a fast turnover of anterior pole cells, might be a reason for the rapid loss of gene expression in these cells. To test for this hypothesis, we depleted stem cells and their progeny by γ-irradiation (Eisenhoffer et al., 2008) and monitored the expression of *smedwi-1,* a stem cell marker (Reddien et al., 2005b), and *notum* for 8 days, until the first animals displayed head regression due to stem cell loss (Reddien et al., 2005a). The expression of *smedwi-1* was lost 1-day post irradiation (dpirr), indicating that stem cell depletion by γ-irradiation was efficient (Fig. 5D). Interestingly, unlike the expression of *smedwi-1*, the expression of *notum* persisted until at least 8 dpirr (Fig. 5D), a time when stem cell progeny markers have long disappeared (Eisenhoffer et al., 2008). These results suggest that stem cell-based turnover is not the major cause of *notum* and *follistatin* loss and indicate that *FoxD* and *zicA* are required for the regulation of *notum* and *follistatin* expression in anterior pole cells.

zicA and FoxD are expressed in stem cells and stem cell progeny during anterior pole regeneration

Next, we tested whether *FoxD* and *zicA* might be expressed in differentiating anterior pole cells. Recently, it has been shown that polarity re-establishment during the first 24 h after amputation requires the expression of *notum* in differentiated, irradiation-insensitive, cells (Witchley et al., 2013). We speculated that, unlike polarity re-establishment, anterior pole formation might require irradiation-sensitive stem cells. Hence, we depleted stem cells and their progeny by γ-irradiation (Eisenhoffer et al., 2008) and performed FISH experiments. Notably, in γ-irradiated animals *zicA*, *FoxD*, *notum* and *follistatin* expression was lost at the anterior regeneration pole after 3 dpa (Fig. 6A), suggesting that these genes depend on stem cells and might be expressed in stem cells and/or their progeny. To further test this hypothesis, we used the stem cell marker *smedwi-1* whose expression is switched off upon stem cell differentiation (Eisenhoffer et al., 2008). SMEDWI-1 protein, in contrast, can still be present in stem cell descendants that are positive for differentiation markers, even after *smedwi-1* transcription has been switched off (Wenemoser and Reddien, 2010). Fully differentiated cells are considered to be both *smedwi-1* mRNA- and protein-negative (Wenemoser and Reddien, 2010). Consistently, we found *FoxD* and *zicA* transcripts in *smedwi-1*- (Fig. 6B and C) and SMEDWI-1-positive cells (Fig. 6D and E) in close proximity to the anterior pole at 3 dpa. We tested for co-expression with the commonly used stem cell progeny markers *NB.32.1g* and *AGAT-1* (Eisenhoffer et al., 2008), but did not detect any obvious overlap (Fig. 7A and D). Therefore, it is likely that *FoxD*- and *zicA*-expressing cells define an anterior pole cell lineage, which might differentiate independently of *NB.32.1g* and *AGAT-1*.

FoxD-expressing cells cluster very densely at the anterior pole at 3 dpa. Hence, we took a closer look at tail fragments at 5 dpa, when anterior pole cells were more spread out along the regenerating anterior midline. We detected SMEDWI-1-positive cells expressing *FoxD* either without (Fig. 8A and B) or with *follistatin* or notum (Fig. 8C and D) close to the newly forming anterior pole at 5 dpa. In contrast, at the anterior-most tip of the regenerating pole, we found only fully differentiated pole cells, as they were *FoxD*/*follistatin*/*notum*-positive but negative for SMEDWI-1 (Fig. 8E and F). Given the fact that almost all *FoxD*-expressing cells were *zicA*-positive, these data suggest that fully differentiated anterior pole cells arise from a stem cell lineage in the blastema that is characterized by the expression of *FoxD* and *zicA* (Fig. 9A).

Discussion

Polarity versus head formation

Planarians can regenerate their heads at an enormous speed and several genes have been described to interfere with this process, yet little is known about the sequence of events that govern head regeneration after amputation. Anterior polarity re-establishment (the decision to regenerate a head rather than a tail) is marked by expression of *wnt1* at all wounds and polarized expression of the Wnt inhibitor *notum* at anterior facing wound sites at 18 hpa (Petersen and Reddien, 2009, 2011). Neither expression is affected after knockdown of *FoxD* or *zicA* (Fig. 2F, Supplementary Fig. 1C and D), suggesting that polarity decisions are independent of these transcription factors. They are, however, required for the following stage of head regeneration, as RNAi animals display defects in the induction of anterior genes, such as the TALE-class homeodomain gene *prep* (Felix and Aboobaker, 2010) (Fig. 9B, Supplementary Fig. 1A and B). As a result, head patterning and formation are severely impaired in these animals.

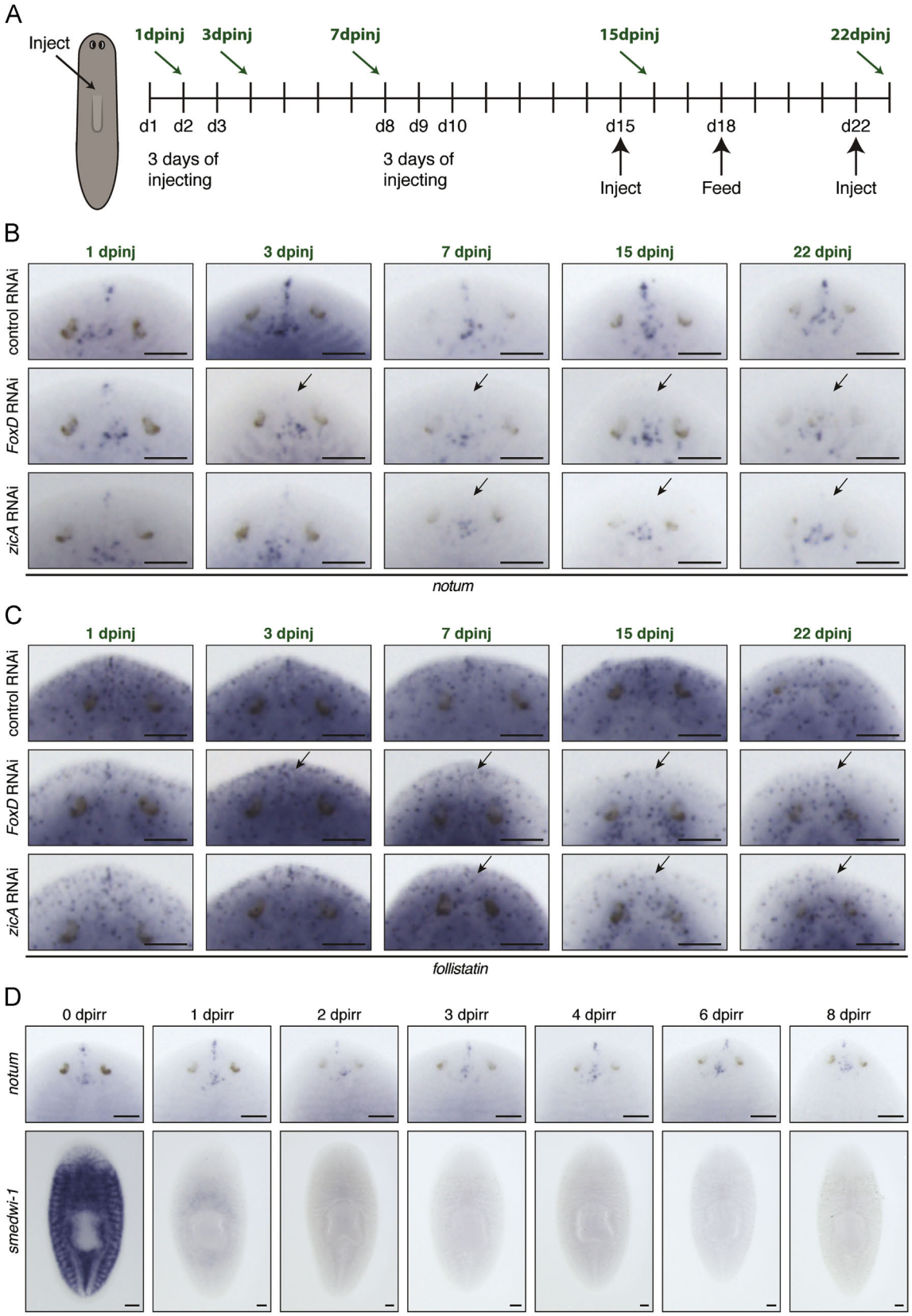

Fig. 5. *Defective stem cell-based turnover is not the major cause of notum and follistatin loss after FoxD and zicA RNAi.* (A) Schematic overview of the homeostatic experiment shown in (B) and (C). Animals were injected and fed as indicated in the scheme. dpinj: days after the first injection. (B) and (C) WISH of intact planarians treated with dsRNAs against the genes indicated. Shown are head regions. Arrows point at anterior poles with absent or reduced expression of *notum* ($n=5/5$ for each time point) (B) and *follistatin* ($n=5/5$) (C). (D) WISH of γ-irradiated homeostatic animals from 0 to 8 days post irradiation (dpirr). Note that the expression of *notum* persisted throughout the time course, whereas the expression of *smedwi-1* was lost 1 dpirr. Scale bars: (B)–(D), 200 μm.

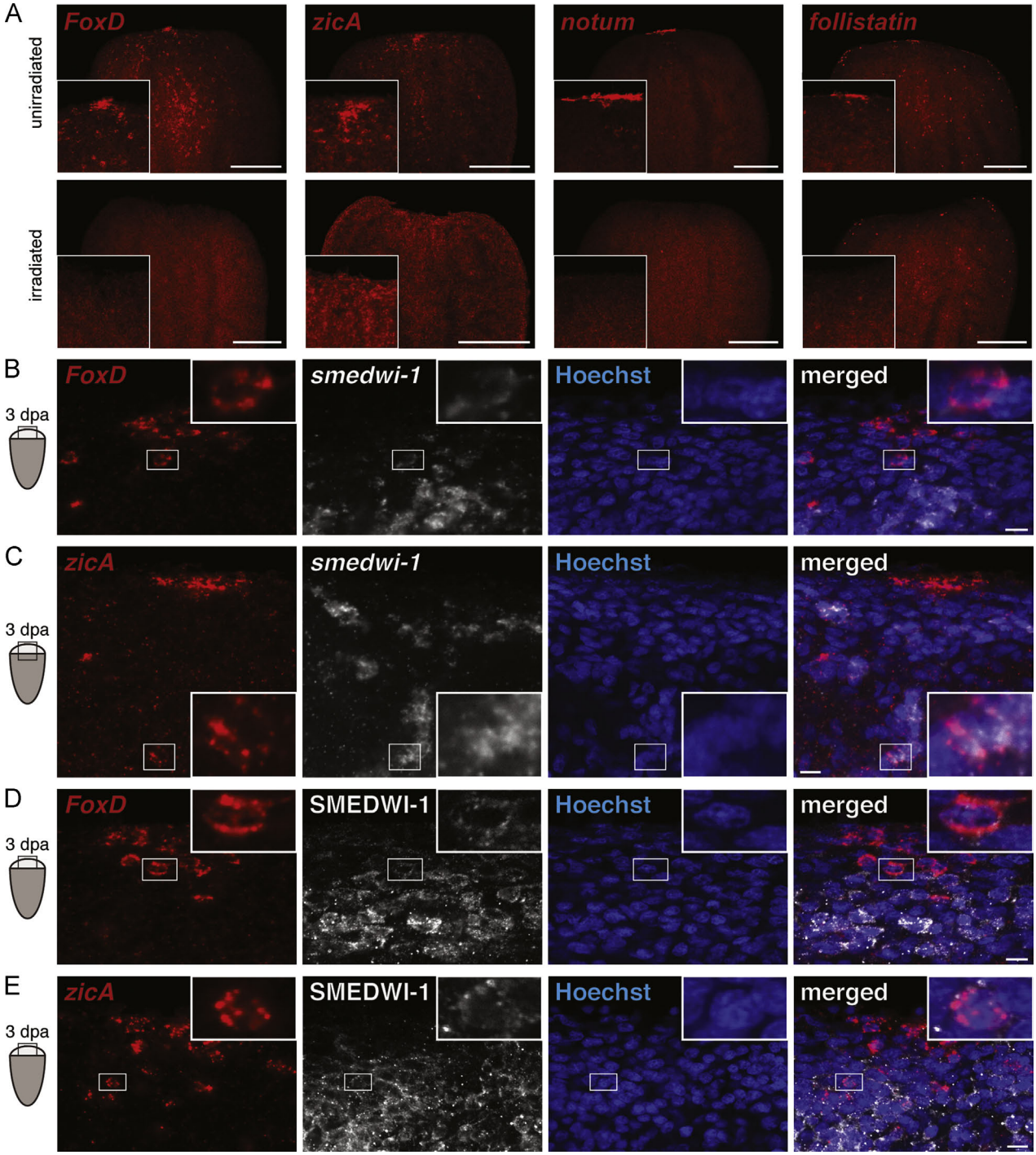

Fig. 6. *zicA and FoxD are expressed in stem cells and stem cell progeny*. (A) *FoxD*, *zicA*, *notum* and *follistatin* expression in irradiated and unirradiated tail fragments at 3 days post amputation (dpa) analyzed by FISH. Note that the expression of *FoxD, zicA, notum* and *follistatin* was lost at the anterior regeneration pole after irradiation. Animals were cut one day after irradiation. (B)–(E) FISH against transcripts as indicated in regenerating tail fragments at 3 dpa. An antibody was used to visualize SMEDWI-1 protein in (D) and (E). Pictured regions are indicated in the schemes. Single representative cells are enlarged in the corners for better visibility. Note that *FoxD*+/*smedwi-1*+ (B), *zicA*+/*smedwi-1*+ (C), *FoxD*+/SMEDWI-1+ (D), and *zicA*+/SMEDWI-1+ (E) cells were found in proximity to the anterior pole. Percentages of *FoxD* and *zicA* expressing cells positive for the indicated markers are: (B) 6 ± 2% *FoxD* with *smedwi-1*. (C) 5 ± 2% *zicA* with *smedwi-1*. (D) 48 ± 12% *FoxD* with SMEDWI-1. (E) 43 ± 3% *zicA* with SMEDWI-1. Scale bars: (A) 200 μm; (B)–(E) 10 μm.

The fact that early *notum* is induced in differentiated cells (Witchley et al., 2013), while late *notum* expression occurs in irradiation-sensitive SMEDWI-1-positive stem cell progeny (Fig. 6A, Fig. 8D), is consistent with a scenario in which polarity decisions and head formation are temporally and spatially separable from each other. These data are in line with the regulation of the polarity gene *wnt1* at the posterior regeneration pole, where the LIM-homeobox gene *islet* and the homeobox/pituitary homeobox gene *pitx* have been implicated in *wnt1*-mediated tail formation, but not in *wnt1*-dependent posterior polarity re-establishment (Currie and Pearson, 2013; Hayashi et al., 2011; Marz et al., 2013). The induction of polarity genes after wounding and their subsequent involvement in the morphogenesis of regenerating structures, two distinct processes that could require a different set of transcription factors, might therefore be a common theme of regenerative growth.

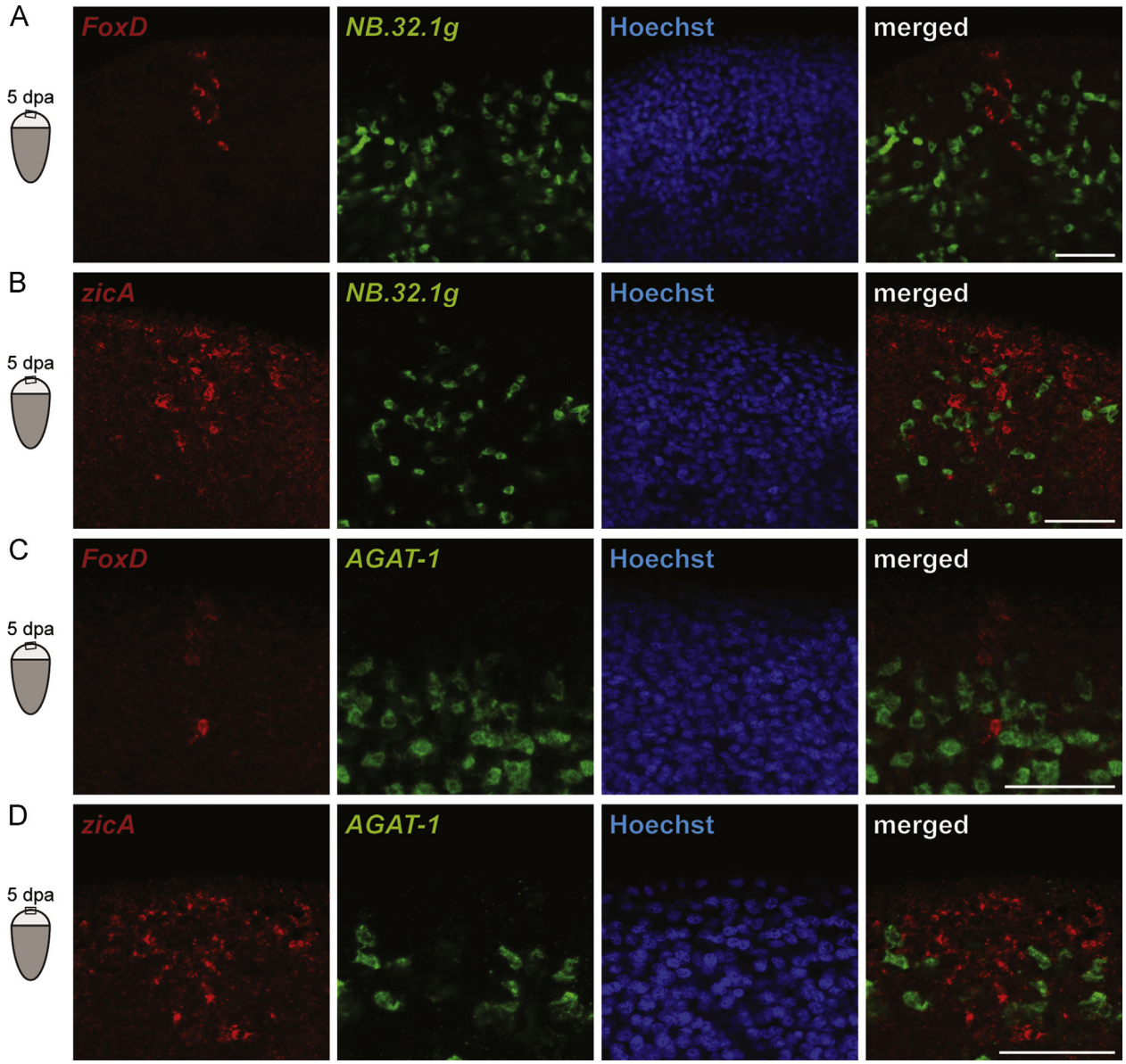

Fig. 7. *zicA and FoxD are not co-expressed with commonly used stem cell progeny markers.* (A) and (B) Double FISH of *FoxD*/*zicA* (red) and *NB.32.1g* (green) combined with Hoechst at 5 days post amputation (dpa). (C) and (D) Double FISH of *FoxD*/*zicA* (red) and *AGAT-1* (green) combined with Hoechst at 5 dpa. Pictured regions are indicated in the schemes. *FoxD* and *zicA* were neither co-expressed with *NB.32.1g* nor *AGAT-1.* Scale bars: 50 μm.

A model for anterior pole formation

What might be the role of *FoxD* and *zicA* in the head formation phase? Several lines of evidence suggest that *FoxD* and *zicA* might be involved in anterior pole cell differentiation (Fig. 9A). First, *FoxD* is co-expressed with *zicA* in almost all cells in and around the anterior regeneration pole (Fig. 3A), and this expression is induced in *smedwi-1*-positive stem cells (Fig. 6B and C). Second, stem cell descendants producing *follistatin* or *notum* transcripts are also positive for *FoxD,* and hence for *zicA* (Fig. 8C and D). Third, terminally differentiated cells (*FoxD*/*zicA*/*notum*/*follistatin*-positive; SMEDWI-1-negative) are found exclusively at the anterior-most tip of the pole (Fig. 8E and F, Fig. 6E), while cells further away are SMEDWI-1-positive (Fig. 8C and D, Fig. 6E). This raises the possibility that stem cell progeny acquire terminal differentiation character the closer they get to their final destination (Fig. 9A), a concept similar to what has been proposed for the differentiation of planarian eye progenitor cells (Lapan and Reddien, 2011).

Alternatively, differentiation of anterior pole cells might be independent of *FoxD* and *zicA*. Our irradiation experiments in intact planarians (Fig. 5D) show that *notum* and *follistatin* expression depends on *FoxD* and *zicA* in already mature anterior pole cells. *notum* and *follistatin* transcripts disappear after *FoxD*/*zicA* RNAi long before anterior pole cells are replaced by stem cell-dependent pole cell turnover (Fig. 5B and C). This suggests that *notum* and *follistatin* might be direct or indirect transcriptional targets of *FoxD* and/or *zicA* in anterior pole cells (Fig. 9C), yet does not exclude the possibility that other FoxD/ZicA target genes might be involved in the differentiation process.

Whatever scenario is true, a functional anterior regeneration pole is not formed in the absence of *FoxD* and *zicA*.

Interdependence of ZicA and FoxD during head regeneration

Similar expression patterns during regeneration and homeostasis, co-localization in cells around the anterior regeneration

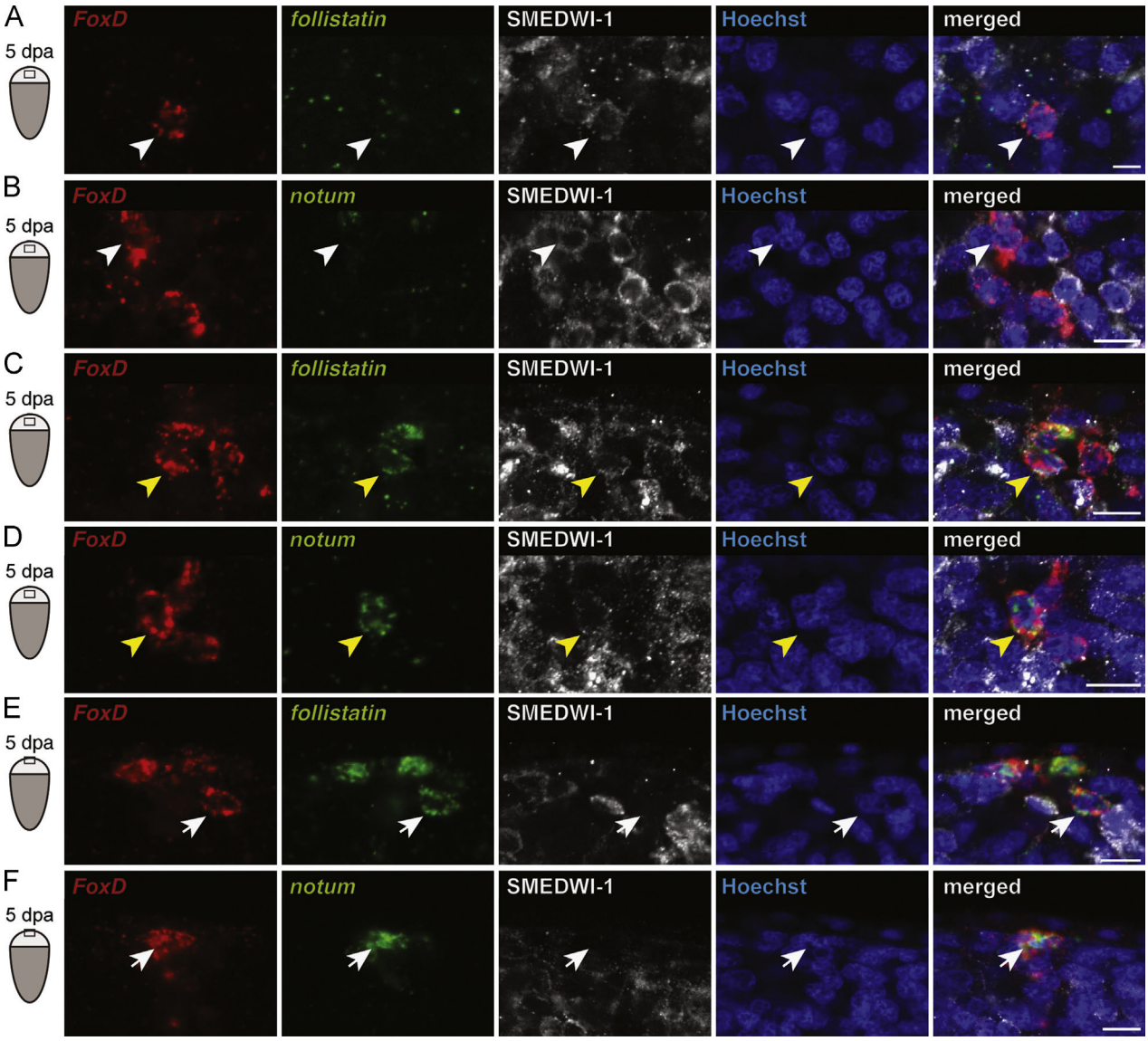

Fig. 8. *notum and follistatin are induced in FoxD-positive stem cell progeny.* (A)–(F) Double FISH (against transcripts as indicated) in regenerating tail fragments at 5 days post amputation (dpa). An antibody was used to detect SMEDWI-1 protein. Pictured regions are indicated in the schemes. White arrowheads point to a *FoxD*+/*follistatin*-/SMEDWI-1+ cell (26 ± 4% of all *FoxD*-expressing cells) in (A) and to a *FoxD*+/*notum*-/SMEDWI-1+ cell (21 ± 8% of all *FoxD*-expressing cells) in (B). Yellow arrowheads point to *FoxD*+/*follistatin*+/SMEDWI-1+ cell (20 ± 5% of all *FoxD*-expressing cells) in (C) and to a *FoxD*+/*notum*+/SMEDWI-1+ cell (16 ± 2% of all *FoxD*-expressing cells) in (D). White arrows point to a *FoxD*+/*follistatin*+/SMEDWI-1- cell (54 ± 1% of all *FoxD*-expressing cells) in (E) and to a *FoxD*+/*notum*+/SMEDWI-1- cell (63 ± 8% of all *FoxD*-expressing cells) in (F). Scale bars: 10 μm.

pole and similar RNAi phenotypes suggest that *zicA* and *FoxD* might interact in the same molecular program, steering the expression of *notum* and *follistatin*, anterior pole cell differentiation and subsequent head formation. We found *zicA* expression reduced after *FoxD* RNAi (Fig. 1E and I, Fig. 4G) providing the possibility that *zicA* might be a transcriptional target downstream of FoxD. Notably, a similar experiment analyzing *FoxD* expression in *zicA* RNAi animals (Fig. 1J, Fig. 4F′) indicates that *FoxD* could be also a downstream target of ZicA. These results are consistent with data from Xenopus, which show that FoxD mRNA can induce Zic factors and vice versa (Sasai et al., 2001). Based on these observations two scenarios are possible: both genes act in the same pathway, possibly in a positive feedback loop, or in two separate pathways, both required for the expression of *notum* and *follistatin* and for anterior pole cell differentiation (Fig. 9A and C). Furthermore, Forkhead and Zic transcription factors are important transcriptional regulators of differentiation during the development of vertebrates (Brewster et al., 1998; Lister et al., 2006; Nakata et al., 1997, 1998), which is in line with the possibility that FoxD and ZicA contribute to the differentiation of anterior pole cells and thereby to the formation of a functional anterior regeneration pole.

Requirement for an anterior signaling center

What might be the function of the anterior pole during regeneration? Notum and Follistatin are conserved secreted inhibitors of Wnt and TGF-β family members, signaling molecules that control fate decisions and differentiation in a variety of organisms (Fainsod et al., 1997; Flowers et al., 2012; Gerlitz and Basler, 2002; Giraldez et al., 2002; Han et al., 2005; Hashimoto et al., 1992; Kreuger et al., 2004; Nakamura et al., 1990). Consistently, the expression of *FoxD* and *zicA* has been associated with several signaling centers in vertebrates (Aruga, 2004; Odenthal and Nusslein-Volhard, 1998; Pohl and Knochel, 2001; Sasai et al., 2001; Steiner et al., 2006; Yamagata and Noda, 1998). As *FoxD*, *zicA*, *notum* and *follistatin* are also expressed in the anterior

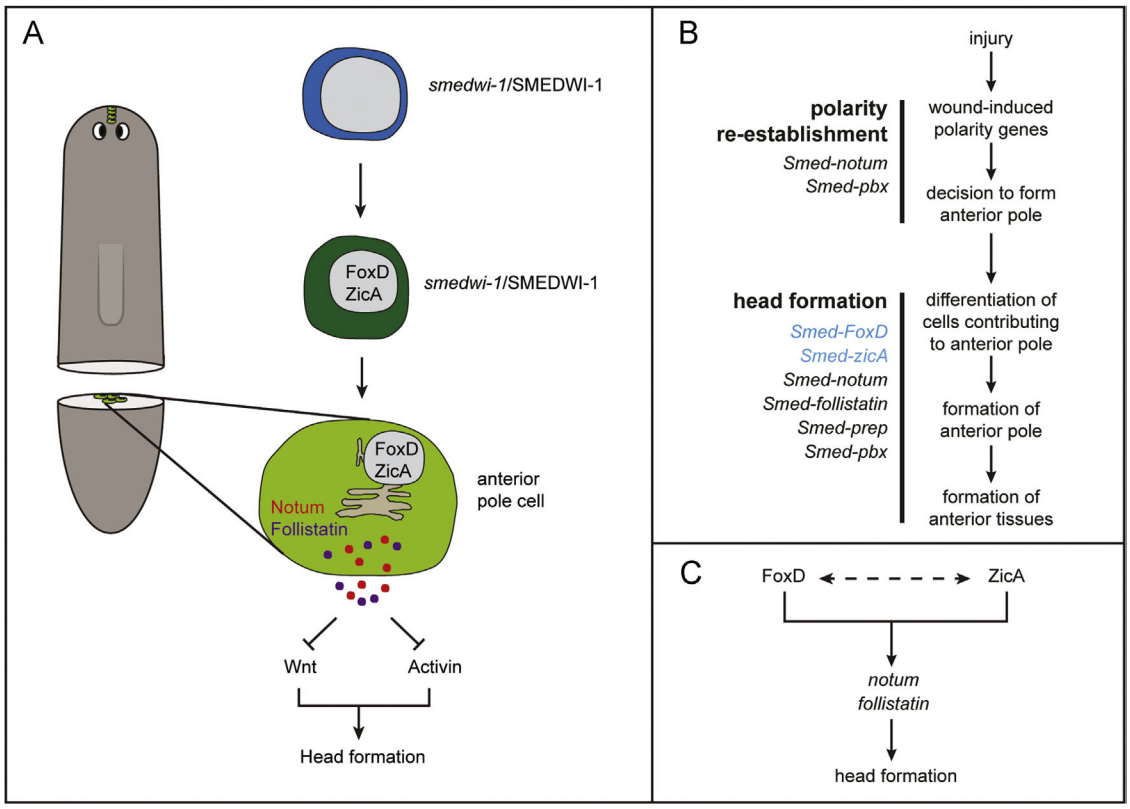

Fig. 9. *Model of FoxD/zicA function and stem cell-dependent anterior pole formation.* (A) *FoxD+/zicA+* cells mark a stem cell lineage that results in the generation of *notum*- and *follistatin*-expressing anterior pole cells. Secreted Notum and Follistatin inhibit Wnt and Activin pathways in the surrounding tissue, enabling head formation. *FoxD/zicA*-dependent expression of *notum* and *follistatin* is maintained at the anterior pole of intact animals for proper homeostatic tissue turnover. (B) *FoxD* and *zicA* in the context of head regeneration: injury induces the expression of anterior polarity determinants such as *notum* (Petersen and Reddien, 2011) and *pbx* (Blassberg et al., 2013); anterior polarity is required for the induction of anterior genes such as *prep* (Felix and Aboobaker, 2010), *pbx* (Blassberg et al., 2013; Chen et al., 2013), *follistatin* (Gavino et al., 2013; Roberts-Galbraith and Newmark, 2013), *notum* (Petersen and Reddien, 2011), *FoxD* (Roberts-Galbraith and Newmark, 2013), and *zicA*. (C) *FoxD* and *zicA* are mutually required for their expression, and for the production of *notum* and *follistatin* mRNAs at the anterior pole.

regeneration pole in planarians, where they are required for head formation ((Gavino et al., 2013; Petersen and Reddien, 2011; Roberts-Galbraith and Newmark, 2013), and this study), it is intriguing to speculate that this anterior pole might constitute a localized, non-autonomous signaling center similar to vertebrate developmental organizing regions. From there, secreted molecules might spread to inform blastema cells about their relative position within the tissue, allowing tissue outgrowth and proper patterning.

In summary, ZicA and FoxD regulate anterior regeneration through controlling the formation and maintenance of a functional anterior regeneration pole. The enormous impact that *FoxD* and *zicA* loss of function have on head regeneration, despite being expressed in only a few cells, is most likely due to the loss of Notum, Follistatin and possibly other secreted proteins, demonstrating the importance of non-autonomous signaling in the orchestration of regeneration processes.

Note added in proof:

While this manuscript was under review a similar study by Scimone et al. was published, which also confirms the role of planarian FoxD in anterior pole formation.

Scimone, M.L., Lapan, S.W., Reddien, P.W., 2014. A forkhead transcription factor is wound-induced at the planarian midline and required for anterior pole regeneration. PLoS Genet 10, e1003999.

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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.2014.03.016.

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