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RESEARCH ARTICLE

STEM CELLS AND REGENERATION

egr-4, a target of EGFR signaling, is required for the formation of the brain primordia and head regeneration in planarians

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ABSTRACT

During the regeneration of freshwater planarians, polarity and patterning programs play essential roles in determining whether a head or a tail regenerates at anterior or posterior-facing wounds. This decision is made very soon after amputation. The pivotal role of the Wnt/β-catenin and Hh signaling pathways in re-establishing anterior-posterior (AP) polarity has been well documented. However, the mechanisms that control the growth and differentiation of the blastema in accordance with its AP identity are less well understood. Previous studies have described a role of *Smed-egfr-3*, a planarian epidermal growth factor receptor, in blastema growth and differentiation. Here, we identify *Smed-egr-4*, a zinc-finger transcription factor belonging to the early growth response gene family, as a putative downstream target of *Smed-egfr-3*. *Smed-egr-4* is mainly expressed in the central nervous system and its silencing inhibits anterior regeneration without affecting the regeneration of posterior regions. Single and combinatorial RNA interference to target different elements of the Wnt/β-catenin pathway, together with expression analysis of brain- and anterior-specific markers, revealed that *Smed-egr-4*: (1) is expressed in two phases – an early *Smed-egfr-3*-independent phase and a late *Smed-egfr-3*-dependent phase; (2) is necessary for the differentiation of the brain primordia in the early stages of regeneration; and (3) that it appears to antagonize the activity of the Wnt/β-catenin pathway to allow head regeneration. These results suggest that a conserved EGFR/egr pathway plays an important role in cell differentiation during planarian regeneration and indicate an association between early brain differentiation and the proper progression of head regeneration.

KEY WORDS: Planarian, Early growth response genes, Patterning

INTRODUCTION

After almost any type of amputation, freshwater planarians are capable of regenerating missing regions with the appropriate polarity. Once polarity is established, the appropriate morphogenetic and patterning programs must direct the differentiation of those blastemas into either anterior or posterior regions and their corresponding tissues and organs. Thus, cephalic ganglia develop *de novo* in decapitated planarians. The instrumental role of Wnt/β-catenin signaling in defining head versus tail identity during planarian regeneration is well documented (Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008). However, less is known about the genes and/or pathways that mediate the patterning, growth and differentiation of blastemas after the re-establishment of polarity (Felix and Aboobaker, 2010).

Conserved signaling pathways play important roles in morphogenesis in all animals. One such pathway is the epidermal growth factor receptor (EGFR) pathway, which regulates multiple biological processes, including cell proliferation, differentiation, apoptosis and cell survival. We have previously shown that silencing of *Smed-egfr-3*, a planarian homologue of epidermal growth factor receptors, impairs regeneration and blastema growth in these organisms, probably by disrupting cellular differentiation (Fraguas et al., 2011). Similar effects have been reported after inhibition of ERK (MAPK extracellular signal-related kinase; Tasaki et al., 2011), a gene whose expression is regulated by EGFR signaling in many organisms. In the present study, we conducted digital gene expression (DGE) analyses to identify putative target genes of *Smed-egfr-3* in order to better characterize the function of the EGFR signaling pathway during planarian regeneration. One of the isolated candidate genes that was downregulated after *egfr-3* RNAi was *Smed-egr-4* (Wenemoser et al., 2012), henceforth *egr-4*, a member of the early growth response (*egr*) gene family. *egr* genes were first characterized by their induction by nerve growth factor (Milbrandt, 1987) and other mitogens (Sukhatme et al., 1987), and are implicated in the regulation of multiple cellular processes (Calogero et al., 2004; Cole et al., 1989; Dussmann et al., 2011; Shafarenko et al., 2005; Sukhatme et al., 1988). Members of this family of zinc-finger transcription factors are considered immediate-early genes; they are rapidly induced by many environmental signals, including growth factors, hormones and neurotransmitters (Thiel and Cibelli, 2002), and are rapidly and transiently upregulated by a variety of signaling pathways, including the EGFR and MAPK/ERK signaling pathways (Aggeli et al., 2010; Cabodi et al., 2008; Kaufmann and Thiel, 2001; Ludwig et al., 2011b; Mayer et al., 2009; Tsai et al., 2000). EGFR signaling in mice stimulates the expression of *egr2* to promote cell proliferation during bone formation (Chandra et al., 2013), while EGF induces Egr-1 expression in endothelial cells (Tsai et al., 2000).

We found that *egr-4* was mainly expressed in the central nervous system (CNS) and was rapidly induced after different types of injury. Although early *egr-4* expression after injury was independent of EGFR signaling, it became *Smed-egfr-3* dependent from the second day of regeneration. Functional analyses based on RNA interference (RNAi) revealed that *egr-4* was required for head regeneration but not for the regeneration of posterior regions. *egr-4* silencing significantly impaired the formation of anterior blastemas; these animals exhibited either small mispatterned cephalic ganglia or a total absence of new brain tissue. The differentiation of other cell types normally present in the anterior region was also altered in *egr-4(RNAi)* animals. However, the early expression of polarity determinants required for the re-establishment of anterior polarity was unaffected. Simultaneous silencing of *egr-4* and different elements of the Wnt/β-catenin pathway revealed that *egr-4* is required for the differentiation of the brain primordia. Moreover, the results of these experiments suggest

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that *egr-4* promotes head regeneration by antagonizing the inhibitory action of the Wnt/β-catenin pathway. Taken together, these findings identify *egr-4* as one of the first known genes necessary for the differentiation of the brain primordia during planarian regeneration. In view of these findings, we discuss how the failure to differentiate proper brain primordia may lead to the blockade of blastema growth and head regeneration.

RESULTS

DGE analyses identify *Smed-egr-4* as a putative target of *Smed-egfr-3*

We have previously demonstrated that silencing of *Smed-egfr-3*, a planarian homologue of the epidermal growth factor receptor (EGFR) family, blocks regeneration, probably by disrupting cellular differentiation (Fraguas et al., 2011). To better characterize the functions of the EGFR pathway during planarian regeneration, we constructed and sequenced DGE (Digital Gene Expression) libraries to compare the transcriptomic profiles of control and *egfr-3(RNAi)* regenerating animals (supplementary material Table S1). Genes predicted to undergo up- or downregulation after *Smed-egfr-3* RNAi on days 1 and 3 post-amputation are shown in supplementary material Table S2. After applying the cut-off criteria, 680 genes were upregulated on day 1 and 2403 on day 3 in *Smed-egfr-3(RNAi)* animals. A total of 655 genes were downregulated on day 1 and 800 on day 3 in *Smed-egfr-3(RNAi)* animals (supplementary material Fig. S1). All genes that were up- and downregulated on day 3 post-amputation (2949 and 921, respectively) were annotated and assigned Gene Ontology (GO) categories (supplementary material Fig. S2). Because EGFR signaling regulates multiple downstream targets and pathways, the annotated genes fell into a wide range of GO categories. Overrepresented categories included 'signal transduction', 'cell differentiation', 'response to stress', 'catabolic process', 'nucleotide binding' and 'protein kinase activity' (supplementary material Fig. S2). To identify putative target genes of *Smed-egfr-3* that may be linked to the phenotypic defects observed after its silencing (Fraguas et al., 2011), we selected a group of genes for further characterization, based either on their proposed involvement in the EGFR pathway in other models or their role in processes such as neurogenesis, cell proliferation and differentiation, cancer and tumorigenesis, apoptosis and cell survival, and inflammation and the immune response (supplementary material Fig. S3).

Among the candidate targets that exhibited interesting RNAi phenotypes and merited further characterization was a gene displaying sequence similarity to the *early growth response* (*egr*) gene family of transcription factors (Contig_2669_90e), a well-known family of genes that are regulated by EGFR signaling in other models (Cabodi et al., 2008; Kaufmann and Thiel, 2001; Mayer et al., 2009; Tsai et al., 2000). Although several members of this family have been described in planarians (Onal et al., 2012; Sandmann et al., 2011; Wenemoser et al., 2012; Wagner et al., 2012), Contig_2669_90e was the only *egr* gene downregulated after *Smed-egfr-3* RNAi. Sequence analyses revealed that this gene corresponded to the previously annotated *egr-4* (Wenemoser et al., 2012) and phylogenetic analyses confirmed that *egr-4* belongs to this gene family (supplementary material Fig. S4).

Whole-mount *in situ* hybridization in intact planarians revealed *egr-4* expression mainly in the cephalic ganglia, with weak expression in the ventral nerve cord (vnc) and the mesenchyme (Fig. 1A). We have previously reported *Smed-egfr-3* expression in neoblasts and in the CNS (Fraguas et al., 2011). Here, we better characterized the expression of *Smed-egfr-3* by fluorescent *in situ* hybridization (FISH) (Fig. 1B). Although we did not succeed in performing double FISH of *egr-4* and *Smed-egfr-3*, their similar expression patterns (compare Fig. 1A with 1B) suggest that these genes are probably co-expressed in the CNS. Moreover, *egr-4* expression in the cephalic ganglia was strongly reduced after *Smed-egfr-3* silencing, further supporting the DGE data (Fig. 1C). To investigate whether *egr-4* expression in the mesenchyme corresponded to neoblasts we performed *in situ* hybridization on irradiated animals at different time points (supplementary material Fig. S5). Whereas *Smed-egfr-3* expression in the mesenchyme disappears 1 day after irradiation (Fraguas et al., 2011), no change in the pattern of *egr-4* expression was observed, even at 7 days after neoblast elimination (supplementary material Fig. S5), suggesting an absence of *egr-4* expression in stem cells. During anterior regeneration, *egr-4* was upregulated in the wound region from day 1 of regeneration (Fig. 1D). As regeneration progressed, *egr-4* expression became restricted mainly to the differentiating cephalic ganglia (arrowheads in Fig. 1D). By contrast, no upregulation of *egr-4* was observed during posterior regeneration at later time points (Fig. 1D). However, strong expression at earlier stages (1 h and 3 h post-amputation) was observed in both anterior and posterior blastemas. *egr-4* has been recently categorized as a W1 (wound-induced class 1) gene (Wenemoser et al., 2012). *In situ* hybridization revealed rapid upregulation of *egr-4* after a small incision (Fig. 1E), in agreement with previous reports for other planarian *egr* genes (Wenemoser et al., 2012; Sandmann et al., 2011). This strong upregulation of *egr-4* was not dependent on neoblasts (Fig. 1E′) or on *Smed-egfr-3* (Fig. 1E″), as no differences were observed in irradiated or *Smed-egfr-3(RNAi)* animals.

Finally, to better characterize the dependence of *egr-4* expression on *Smed-egfr-3* during regeneration we carried out a detailed time-course analysis of *egr-4* expression after *Smed-egfr-3(RNAi)* (Fig. 1F). Normal *egr-4* expression in the wound region was observed up to day 1 of regeneration. However, by day 2, *egr-4* expression was downregulated (Fig. 1F), suggesting that *egr-4* expression becomes *Smed-egfr-3* dependent once its expression becomes restricted to the regenerating cephalic ganglia.

egr-4 is necessary for proper head regeneration and neoblast differentiation

We conducted RNAi experiments to characterize the role of *egr-4* in regeneration (see Materials and Methods). Both trunk and tail fragments regenerating a new head exhibited blastemas that were either very small or abnormally differentiated. After 8 days of regeneration, about half of the anteriorly regenerating trunks (n=18/34) formed normal-sized blastemas but with cyclopic eyes, and very few (n=3/34) regenerated small blastemas without eyes (Fig. 2A). By contrast, at the same time point, most tails (n=30/34) failed to properly regenerate a head. Of these 30 tails, some developed very small blastemas (n=12/30), whereas others appeared to only heal the wound (n=18/30) (Fig. 2A). These results indicate that the severity of *egr-4* RNAi varied along the anterior-posterior (AP) axis. Posterior regeneration proceeded normally (Fig. 2A), indicating that *egr-4* RNAi specifically blocked anterior regeneration.

Although *egr-4* was not expressed in neoblasts, we investigated whether these defects in regeneration were due to a non-cell-autonomous effect of *egr-4* RNAi on neoblast maintenance and/or proliferation. However, no differences in neoblast number or distribution were observed after immunostaining and *in situ* hybridization for the neoblast-specific markers anti-SMEDWI-1 antibody and *Smed-histone-2B*, respectively (Fig. 2B) (Reddien et al., 2005; Guo et al., 2006; Solana et al., 2012). Thus, neoblasts were normally found below the wound epithelium on day 3. Eight

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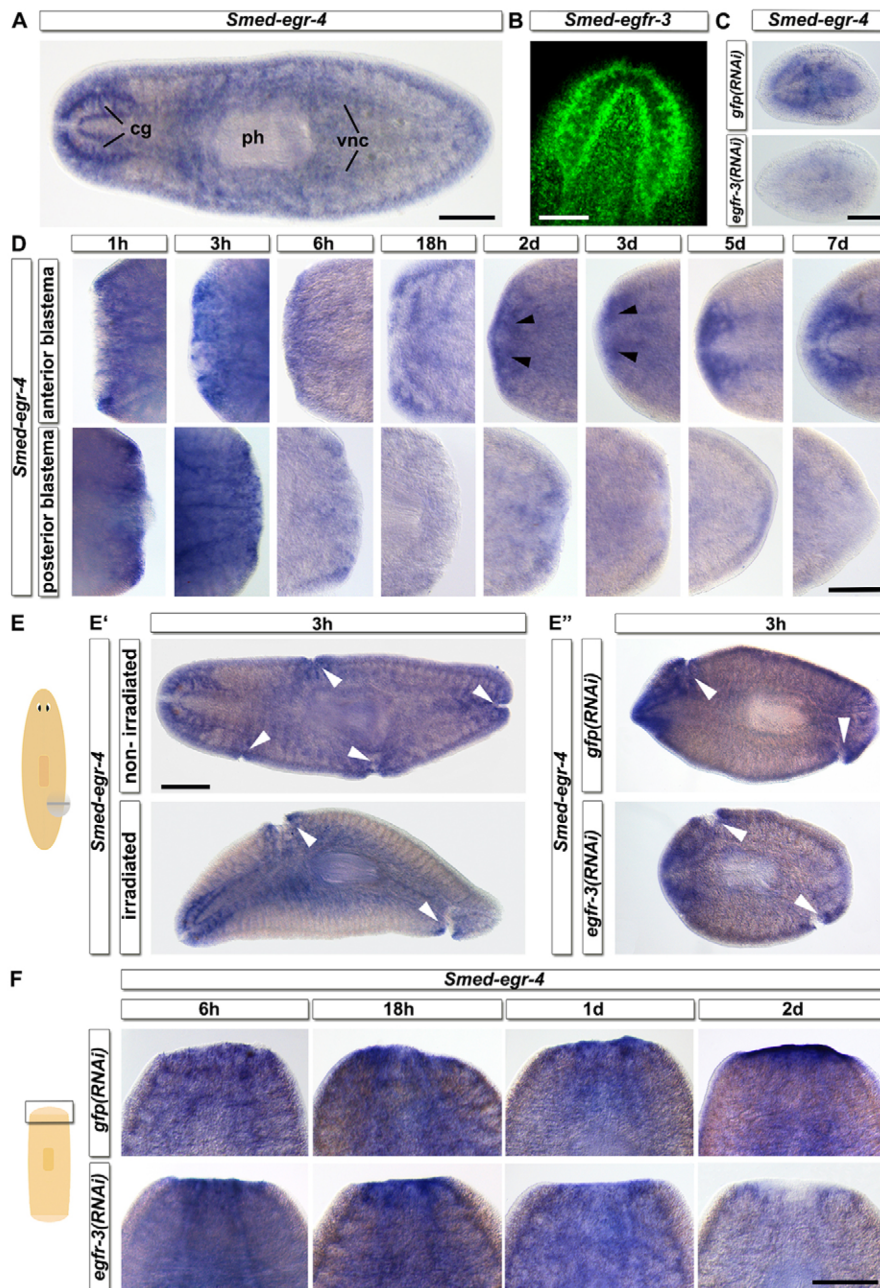

Fig. 1. *egr-4* expression pattern in intact and regenerating planarians. (A) In intact animals, *egr-4* was expressed in the cephalic ganglia (cg), ventral nerve cord (vnc) and mesenchyme. (B) *Smed-egfr*-3 expression in the cephalic ganglia. (C) *egr-4* expression was downregulated after *Smed-egfr-3* silencing. These samples correspond to regenerating head fragments after 10 days of regeneration. (D) Expression of *egr-4* in anteriorly and posteriorly regenerating bipolar trunks. Arrowheads indicate the brain primordia. (E) *egr-4* was rapidly upregulated after injury. Small incisions induced *egr-4* expression after 3 h (arrowheads). This early expression was not dependent on either neoblasts (E′) or *Smed-egfr-3* (E″). (F) *Smed-egfr-3* silencing resulted in the downregulation of *egr-4* expression from day 2 of regeneration. (A,C-E) Anterior towards the left. (B,F) Anterior at the top. Scale bars: 300 µm in A,D,F; 200 µm in B,C,E.

days after amputation, few *Smed-histone-2B*-positive cells were detected in the new head region of controls. Similarly, despite impaired blastema growth and regeneration, few *Smed-histone-2B*-expressing cells were observed after *egr-4(RNAi)*, indicating that neoblast dynamics (in terms of distribution) was unaffected.

We next used an anti-phospho-histone H3 antibody to characterize the mitotic response after *egr-4(RNAi)* in both anterior and posterior regenerating fragments (Fig. 2C). In most cases, the first mitotic peak, which is associated with injury and occurs 6 h post-amputation, was significantly attenuated when compared with controls. Interestingly, the second mitotic peak at 48 h post-amputation, which is associated with tissue loss (Wenemoser and Reddien, 2010), was significantly attenuated in anterior, but not posterior, blastemas. However, despite this decrease in the rate of proliferation, a significant number of mitoses were observed at all stages after *egr-4* RNAi (supplementary material Fig. S6), suggesting that the severe impairment of blastema growth and regeneration in these animals was not exclusively due to defective mitosis. Finally, we characterized neoblast progeny at different stages of differentiation using the lineage markers *Smed-NB21.11e* and *Smed-AGAT-1*, which are specific to early and late neoblast postmitotic progeny, respectively (Eisenhoffer et al., 2008). Although the early neoblast progeny were unaffected, the number of late progeny was significantly decreased after *egr-4* RNAi (Fig. 2D). Taken together, these results indicate that *egr-4* RNAi impairs anterior regeneration probably by affecting cell differentiation rather than neoblast pool maintenance or proliferation.

egr-4 RNAi impairs tissue and organ differentiation

To further characterize these defects in regeneration, we first used the pan-neural marker anti-SYNORF-1 (Cebrià, 2008) to study CNS regeneration. Both control and *egr-4(RNAi)* head fragments regenerated normal pharynges and ventral nerve cords grew into the newly developed tails (Fig. 3A). By contrast, in anteriorly regenerating trunks, *egr-4* RNAi generally resulted in the formation

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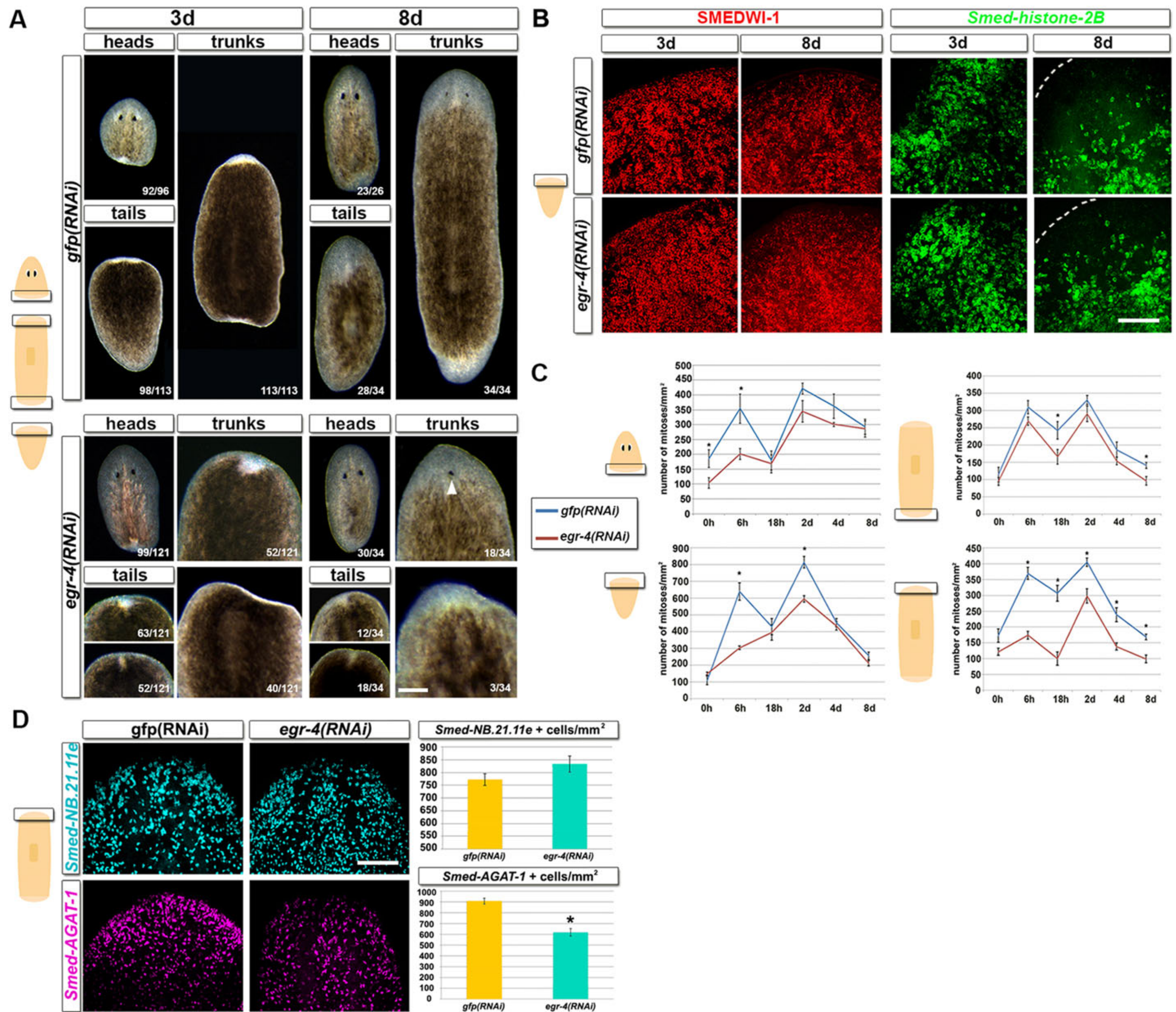

Fig. 2. Effects of *egr-4* RNAi on head regeneration and neoblast dynamics. (A) Effects of *egr-4* RNAi after 3 and 8 days (d) of regeneration in head, bipolar trunk and tail fragments. Although heads regenerated normally, most tail fragments were unable to regenerate a proper head after *egr-4* silencing. Anteriorly regenerating *egr-4(RNAi)* trunks exhibited a milder phenotype when compared with corresponding tail fragments, displaying defects mainly in head morphogenesis, as evidenced by the development of cyclopic eyes (arrowhead). (B) The impairment of head regeneration was not due to neoblast loss, as demonstrated by using anti-SMEDWI-1 immunostaining and *Smed-histone-2B in situ* hybridization. Dashed lines indicate the border of the anterior head. (C) Quantification of mitotic cells after *egr-4 RNAi*, detected by anti-phospho-histone H3 immunostaining in head, bipolar trunk and tail fragments at different time points. Values represent the mean±s.e.m. (Student's *t*-test, $*P<0.05$) of an average of 10 samples per time point and amputation level. (D) Although early neoblast progeny (*Smed-NB21.11e*-positive cells) were unaffected by *egr-4* RNAi, a significant decrease in the number of late neoblast progeny (*Smed-AGAT-1*-positive cells) was observed. Samples correspond to trunk sections after 8 days of regeneration. Values represent the mean±s.e.m. (Student's *t*-test, $*P<0.05$) of an average of 10 samples per time point. All panels are oriented with the anterior towards the top. Scale bars: 200 µm for *egr-4 (RNAi)* trunks and 300 µm for all other panels in A; 200 µm in B,D.

of blastemas with truncated ventral nerve cords and little cephalic ganglia differentiation, whereas normal cephalic ganglia regeneration was observed in controls (Fig. 3A). We next used additional neural (Fig. 3B) and anterior (Fig. 3C) markers to better characterize the *egr-4(RNAi)* phenotype. After 5-7 days of regeneration, most *egr-4 (RNAi)* trunk fragments displayed significantly smaller or near-absent cephalic ganglia after *in situ* hybridization for *Smed-gpas*, a gene specific to the brain lateral branches (Iglesias et al., 2011). Moreover, the expression of markers of distinct neuronal populations such as *Smed-tbh* (octopaminergic neurons; Fraguas et al., 2012; Nishimura et al., 2008) and *Smed-th* (dopaminergic neurons; Fraguas et al., 2011; Nishimura et al., 2007) was significantly reduced (Fig. 3B). These markers revealed that *egr-4 (RNAi)* animals developed small and aberrant cephalic ganglia. To study the patterning of these cephalic ganglia, we analyzed expression of the otd/Otx family gene *Smed-otxA* and the homeobox-containing gene *orthopedia Smed-otp* (Umesono et al., 1997, 1999; Iglesias et al., 2011) (Fig. 3B). *otp* is normally expressed in the most lateral region of the cephalic ganglia, whereas *otxA* is expressed more medially (Umesono et al., 1999; Iglesias et al., 2011). Although *egr-4(RNAi)* animals often displayed

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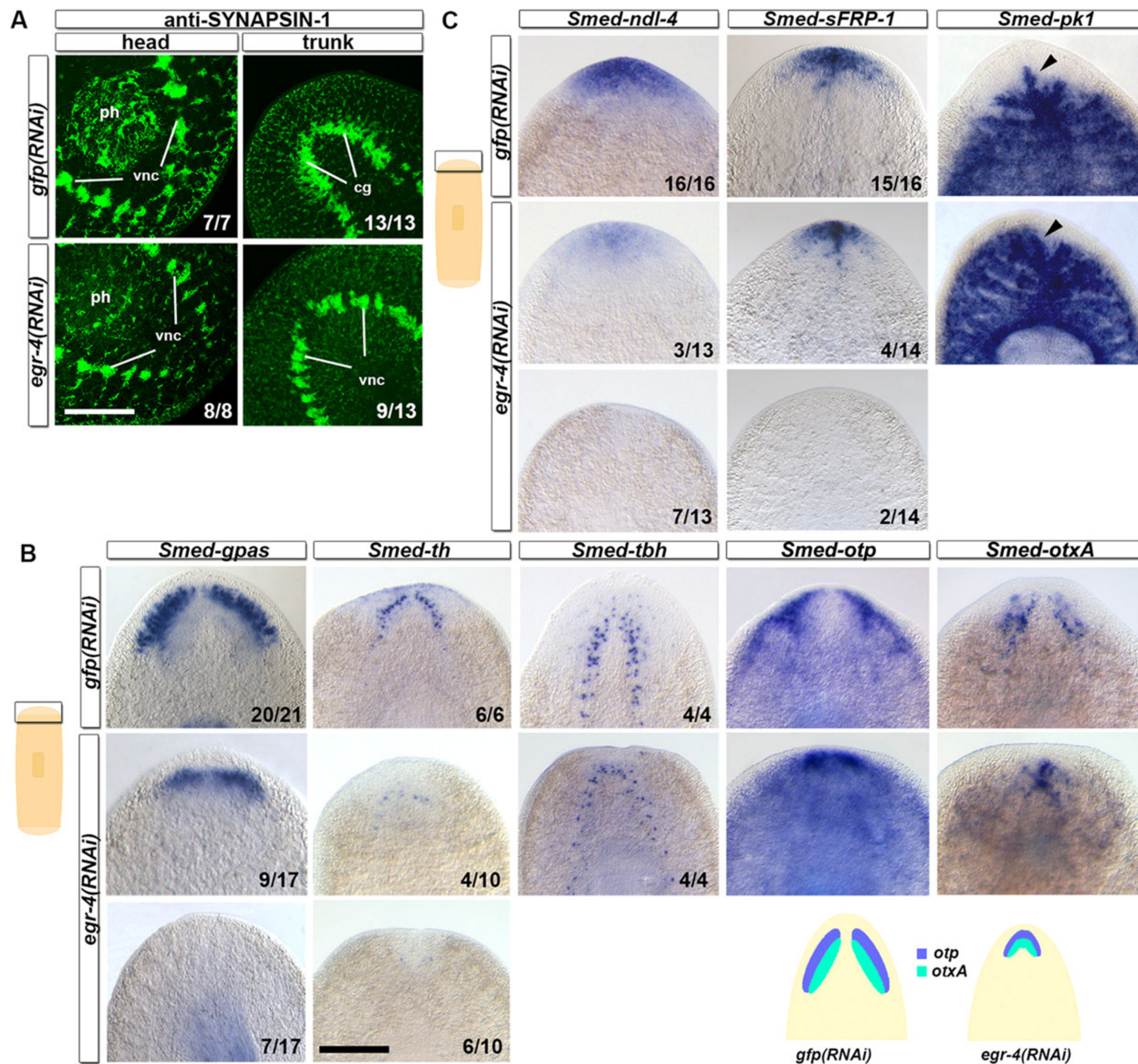

Fig. 3. Loss of brain and anterior markers after *egr-4* RNAi. (A) Whole-mount immunostaining with anti-SYNORF1 in head fragments revealed correct pharynges with typical nervous plexus and ventral nerve cords in both control and *egr-4(RNAi)* animals. The majority of anteriorly regenerating *egr-4(RNAi)* trunks exhibited truncated ventral nerve cords with no cephalic ganglia differentiation, in contrast to the normal regeneration seen in control animals. (B) The expression of several markers of specific brain subpopulations was reduced in *egr-4(RNAi)* animals. Most of these animals regenerated small cephalic ganglia instead of normal bilateral ganglia, as evidenced by the expression of *Smed-gpas*, *Smed-th* and *Smed-tbh*. In some cases, the expression of these markers was completely abolished. However, the patterning of those small abnormal cephalic ganglia appeared not to be affected after *in situ* hybridization with the mediolateral patterning genes *Smed-otp* and *Smed-otxA*. (C) Expression of the anterior marker *ndl-4* was also significantly attenuated. By contrast, the expression of the anterior marker *sFRP-1* was normal in most *egr-4(RNAi)* animals (n=8/14). Finally, *egr-4(RNAi)* animals failed to correctly regenerate the anterior gut branch (arrowheads). cg, cephalic ganglia; vnc, ventral nerve cords; ph, pharynx. In A all panels are oriented with the anterior towards the top left. In B and C all panels are oriented with the anterior towards the top. Samples correspond to trunks after 5-8 days of regeneration. Scale bars: 150 μm in head and 200 μm in trunk fragments in A; 300 μm in B,C.

abnormal cephalic ganglia, these distinct domains along the mediolateral axis were clearly distinguishable (Fig. 3B). Taken together, our data indicate that *egr-4* RNAi results in the development of small but well-patterned cephalic ganglia.

In addition to the aforementioned CNS defects, *egr-4(RNAi)* animals failed to properly regenerate the anterior gut branch into the blastema (Fig. 3C). Moreover, the expression of the anterior marker *Smed-ndl-4*, a FGF-receptor-like protein of the *nou-darake* family (Cebrià et al., 2002a; Rink et al., 2009), was either significantly reduced or completely absent in the majority of *egr-4(RNAi)* animals (Fig. 3C). The expression of *Smed-sFRP-1*, another anterior marker (Gurley et al., 2008; Petersen and Reddien, 2008), was not as strongly affected. Most *egr-4(RNAi)* animals (n=8/14) exhibited normal *Smed-sFRP-1* expression; some (n=4/14) showed a very slight decrease (probably due to the smaller size of the regenerated anterior region) and the marker was completely absent in only 2 of the 14 animals (Fig. 3C). These results indicate that not all genes previously proposed as candidate anterior patterning genes (*ndl-4* and *sFRP-1*; Chen et al., 2013) respond equally to *egr-4* RNAi.

Taken together, our data confirm that *egr-4* silencing impairs the proper differentiation of several cell types and organs (CNS and gut) during anterior regeneration. However, no defects were observed after *egr-4* silencing in intact non-regenerating planarians; all CNS- and anterior-specific markers were normally expressed (supplementary

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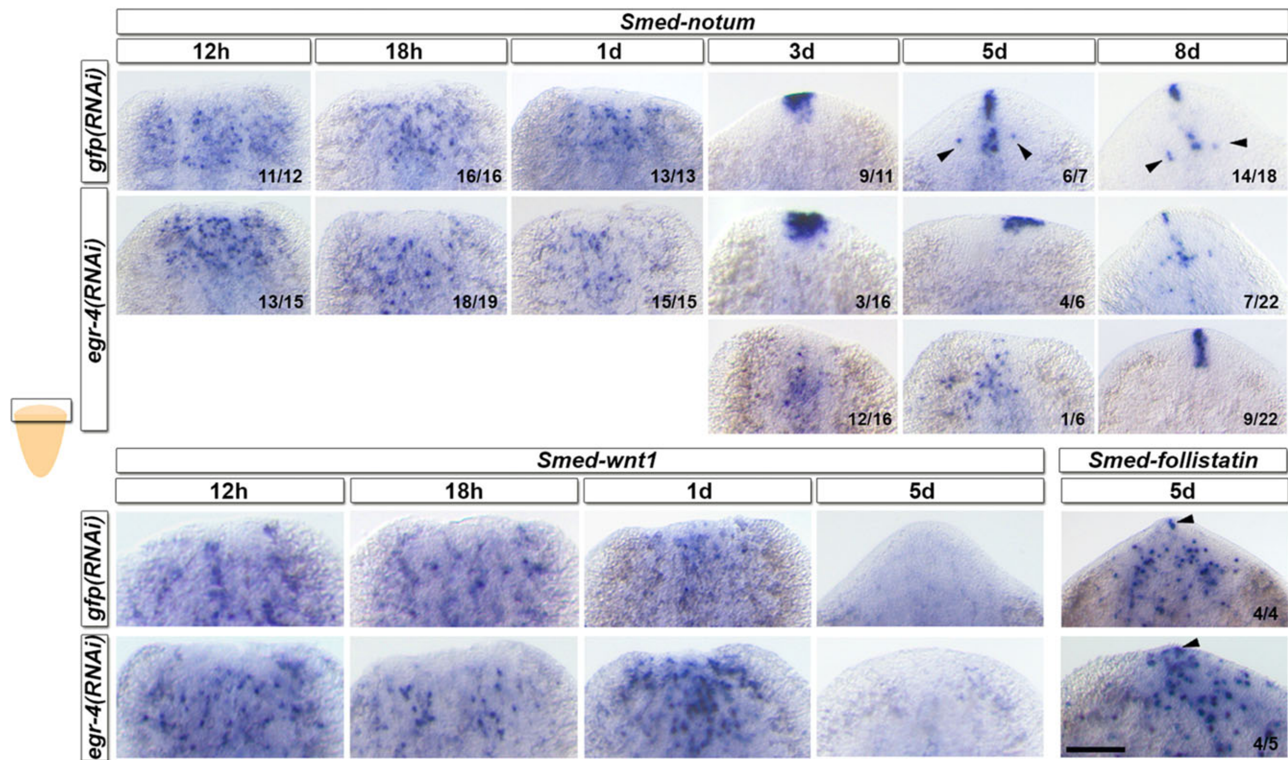

Fig. 4. The early expression of AP polarity determinants is unaffected by *egr-4* silencing. *In situ* hybridization with *Smed-notum*, *Smed-wnt1* and *Smed-follistatin* after *egr-4* RNAi in regenerating tail fragments at different time points. Although *egr-4* silencing had no effect on *Smed-notum* expression at early time points, a delay in the temporal expression of this gene was observed beginning on day 3. Arrowheads indicate *Smed-notum*-positive bilateral cells adjacent to photoreceptors. The dynamics of *Smed-wnt1* expression were unaffected by *egr-4* RNAi. After 5 days of regeneration, normal *Smed-follistatin* expression was detected in the anterior-most tip of the regenerating head (arrowheads). All panels are oriented with anterior towards the top. Scale bar: 200 μm.

material Fig. S7A). Moreover, neoblast proliferation was unaltered in intact *egr-4(RNAi)* animals when compared with corresponding controls (supplementary material Fig. S7B).

egr-4 RNAi disrupts head regeneration without impairing the establishment of anterior polarity

To determine whether the impaired head regeneration observed after *egr-4* RNAi was due to defects in the establishment of anterior polarity, we analyzed the expression of the anterior polarity determinants *Smed-notum*, *Smed-wnt1* and *Smed-follistatin* (Adell et al., 2009; Gurley et al., 2010; Petersen and Reddien, 2009, 2011; Yazawa et al., 2009; Roberts-Galbraith and Newmark, 2013). Control animals exhibited three typical phases of *Smed-notum* expression (Fig. 4). At 12 h post-amputation, *Smed-notum* expression was strongly upregulated in the wound region, giving rise to a dotted pattern of discrete cells. Between 18 h and 1 day after amputation, the number of *Smed-notum*-positive cells decreased (12 h to 1 day, phase I). After 3 days of regeneration, *Smed-notum*-positive cells coalesced at the tip of the anterior blastema (phase II). Finally, between 5 and 8 days post-amputation, the expression of *Smed-notum* became restricted to a small number of cells at the tip of the new head and along the midline, and to two bilateral groups of cells (arrowheads in Fig. 4; phase III), a pattern similar to that observed in intact planarians. No differences in the pattern of *Smed-notum* expression were observed between *egr-4(RNAi)* animals and controls up to 3 days post-amputation (Fig. 4). From this stage on, however, the progression of *Smed-notum* expression appeared to be delayed or arrested in *egr-4(RNAi)* animals (Fig. 4); after 3 days of regeneration, the clustering of *Smed-notum*-positive cells at the tip of the blastema seen in control animals was observed in only 3 out of 16 *egr-4(RNAi)* animals. By day 5 of regeneration, this clustering of *Smed-notum*-positive cells was observed in most *egr-4(RNAi)* animals. Normal *Smed-notum* expression was detected in several *egr-4(RNAi)* animals (*n*=7/22) by day 8; in others (*n*=9/22) the pattern of *Smed-notum*-positive cells was somewhere between that of phase II and III, while the remaining *egr-4(RNAi)* animals displayed no *notum* expression (*n*=6/22).

We observed no significant differences in the expression pattern of *Smed-wnt1* between controls and *egr-4(RNAi)* animals (Fig. 4). Between 12 h and 1 day post-amputation, a dotted distribution of discrete *Smed-wnt1*-expressing cells was observed in the anterior wound. By 2 days post-amputation this anterior expression had been completely lost in both controls and *egr-4(RNAi)* animals, indicating that the lack of head regeneration after *egr-4* silencing was not due to blastema posteriorization (Fig. 4). *Smed-wnt1* expression in the tip of the tail was normal in all these samples (data not shown).

Smed-follistatin and *Smed-notum* co-expression was recently demonstrated in planarians in a small cluster of cells at the tip of the head (Roberts-Galbraith and Newmark, 2013). Functional analyses suggest that *Smed-follistatin* inhibits the Activin/ActR-1/Smad2/3 signaling pathway, which represses anterior regeneration. It has been proposed that *Smed-follistatin* and *Smed-notum* cooperate to promote anterior identity, thus acting as an anterior signaling center (Roberts-Galbraith and Newmark, 2013). We found that *Smed-follistatin* expression at the tip of the regenerating head re-appeared after *egr-4* RNAi, although slightly later than in controls, as also observed for *Smed-notum* (data not shown). However, after 5 days

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of regeneration most animals displayed normal *Smed-follistatin* expression at their anterior-most tips (Fig. 4).

Overall, these results suggest that the polarity determinants *Smed-notum*, *Smed-wnt1* and *Smed-follistatin* are normally expressed in the early regenerative stages during which AP polarity is established (Petersen and Reddien, 2009, 2011) and that *egr-4* silencing impairs normal head regeneration without disrupting the establishment of anterior polarity.

egr-4 is required for the formation of the brain primordia

As *egr-4* was highly expressed in the mature and differentiating cephalic ganglia from very early stages of regeneration, we investigated its role in the regeneration of the cephalic ganglia by simultaneously silencing *egr-4* and *Smed-apc-1*, an inhibitor of the Wnt/β-catenin pathway. *apc-1* silencing results in the regeneration of a tail, rather than a head, from anterior wounds (Gurley et al., 2008). Remarkably, despite this polarity reversal, two small neuronal clusters known as 'brain primordia' differentiate within these posteriorized anterior blastemas (Evans et al., 2011; Iglesias et al., 2011). These brain primordia are equivalent to what it has also been termed 'brain rudiments' and correspond to the initial neuronal clusters that will differentiate into the new cephalic ganglia (Cebrià et al., 2002b; Kobayashi et al., 2007; Agata and Umesono, 2008).

As previously shown, *egr-4* silencing inhibited the differentiation of normal cephalic ganglia (Fig. 5A). After *apc-1* RNAi, approximately one-third of the tails regenerated small brain primordia with a similar morphology to that described previously (Evans et al., 2011; Iglesias et al., 2011). By contrast, no brain tissues were detected in most of the double *egr-4(RNAi);apc-1(RNAi)* regenerating tails, although in very few cases the differentiation of very reduced brain primordia was observed (Fig. 5A,B). Although *in situ* hybridization revealed no brain primordia in over half of the *apc-1(RNAi)* tails, differentiation of a pharynx was observed in most cases, indicating that the regenerative process had been somehow moved forward. By contrast, in *egr-4(RNAi);apc-1(RNAi)* animals, a greater number of tails failed to regenerate any brain primordia, when compared with either

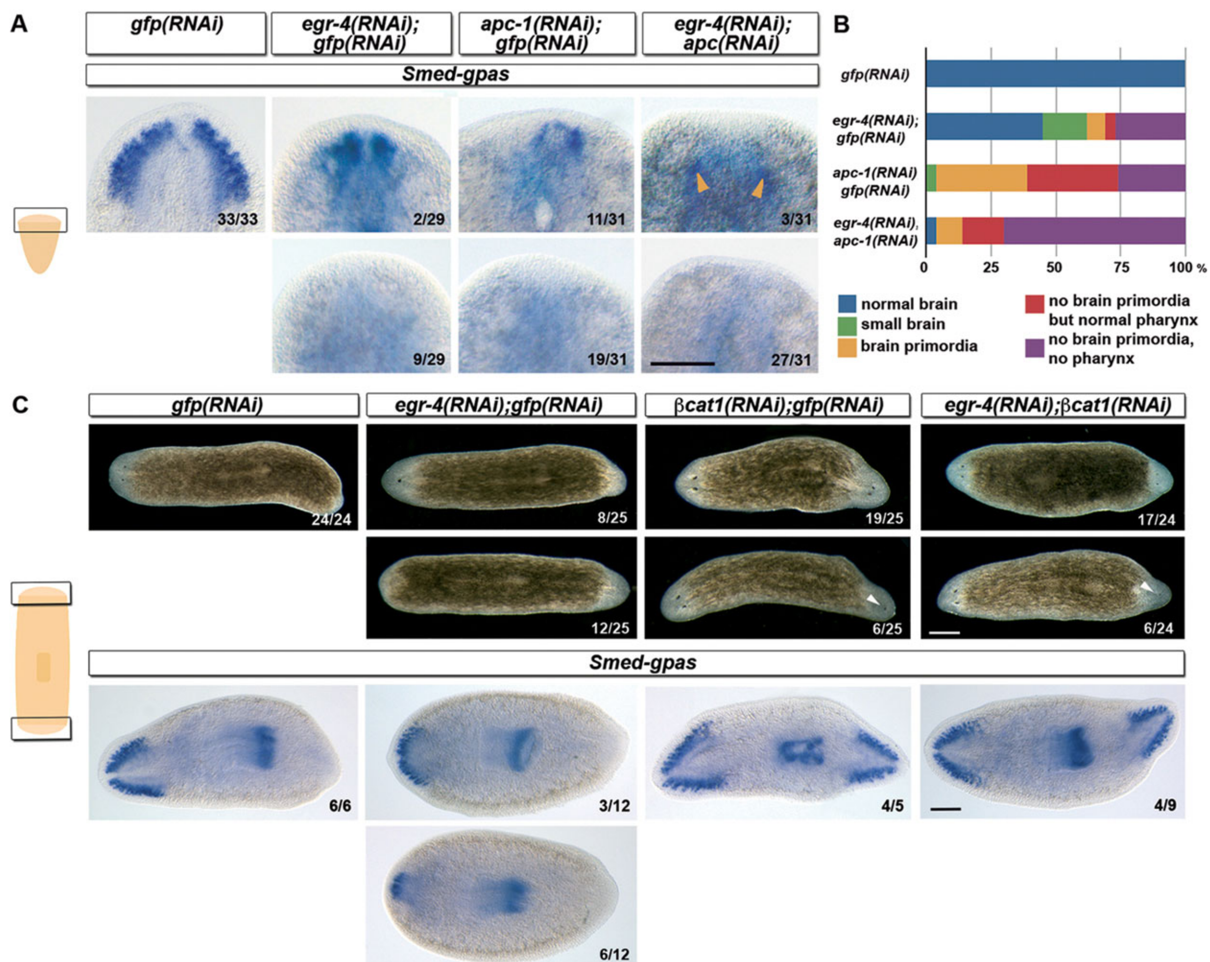

Fig. 5. *egr-4* is required for the differentiation of the brain primordia and the effects of its silencing are reversed by *βcat1* RNAi. (A) *In situ* hybridization with the brain marker *Smed-gpas* in tail fragments after 7-9 days of regeneration. After *egr-4;apc-1* RNAi, most animals failed to differentiate any brain or developed very small brain primordia (arrowheads) when compared with *apc-1(RNAi)* samples. (B) Quantification of the different phenotypes obtained after individual and simultaneous silencing of *egr-4* and *apc-1*. In all cases in which a normal brain (blue) or some brain tissue (yellow and green) differentiated, a pharynx was also observed. (C) Double *egr-4;βcat1* RNAi rescued *egr-4(RNAi)* phenotypes. In live images of bipolar trunks after 7 days of regeneration following two rounds of RNAi and amputation, *egr-4* knockdown resulted in animals with cyclopic eyes or small blastemas without eyes. *βcat1(RNAi)* animals displayed the typical anteriorization of the posterior blastema; some samples displayed cyclopia in the posterior head (arrowhead). Double *egr-4 (RNAi);βcat1(RNAi)* animals displayed the same phenotype as *βcat1(RNAi)* animals, with a similar percentage of cyclopic posterior heads (arrowhead). *In situ* hybridization with *Smed-gpas* revealed that most *egr-4(RNAi)* animals regenerated smaller, mispatterned brains when compared with controls. All *βcat1(RNAi)* and *egr-4(RNAi);βcat1(RNAi)* animals regenerated normal anterior brains and most regenerated normal posterior brains. All panels are oriented with the anterior towards the left. Scale bars: 200 μm in A; 300 μm in C.

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single *apc-1* or *egr-4* RNAi counterparts, and most of these failed to develop a normal pharynx (Fig. 5B). These results indicate that the differentiation of the brain primordia after *apc-1* RNAi requires *egr-4*.

Given that *egr-4* RNAi inhibits only anterior regeneration and the Wnt/β-catenin pathway mediates the specification of head versus tail regeneration, we further investigated the relationship between *egr-4* and the Wnt/β-catenin pathway suggested by our data. Although most single *egr-4(RNAi)*-treated bipolar trunks displayed either smaller anterior blastemas with cyclopia or without eyes, treatment with double *egr-4(RNAi);βcat1(RNAi)* resulted in two-headed animals with normal anterior heads and two bilateral eyes, as also observed after *βcat1* RNAi alone (Fig. 5C). The ability of *βcat1* RNAi to rescue head regeneration in *egr-4(RNAi)* animals suggests that the *egr-4(RNAi)* phenotype requires *βcat1*, and indicates that in normal physiological situations *egr-4* may antagonize β-catenin activity to allow head regeneration.

egr-4(RNAi)-induced defects in regeneration depend on the timing of silencing and are rescued by the presence of brain tissue

We next sought to delimit the period during which *egr-4* is required for head regeneration. *egr-4* was silenced by dsRNA injections 24 h and 48 h after amputation. Most of the animals injected 48 h post-amputation developed normal heads and only small number displayed defects in eye morphogenesis (Fig. 6A). By contrast, most of the animals injected 24 h post-amputation exhibited defects in the regenerated eyes (including ectopic and fused eyes; Fig. 6A). However, all of these animals regenerated normal-sized heads and brains (data not shown), in contrast to the severe impairment in head regeneration observed when *egr-4* was silenced prior to amputation (Figs 2-5). Although it is unclear exactly how long after injection RNAi begins to exert its effect, these results suggest that *egr-4* function is necessary during the first 2-3 days post-amputation, after which it may not be essential for proper head regeneration.

As this time window coincides with the development of the brain primordia (Cebrià et al., 2002b), we speculated that no phenotype is observed when *egr-4* is silenced 48 h post-amputation because the brain primordia are already formed at this stage. We thus investigated whether *egr-4* RNAi impaired head regeneration even when some brain tissue remained after amputation. Animals were injected with *egr-4* dsRNA, amputated and allowed to regenerate. As expected, no defects in head regeneration were observed after this first round of amputation (see Materials and Methods). These animals were then re-injected (beginning 5 days post-amputation) and re-amputated at two different levels: level 1, immediately posterior to the newly regenerated eyes, leaving some of the regenerated brain tissue in the stump; and level 2, which involved decapitation and the removal of all brain tissue (Fig. 6B). Animals

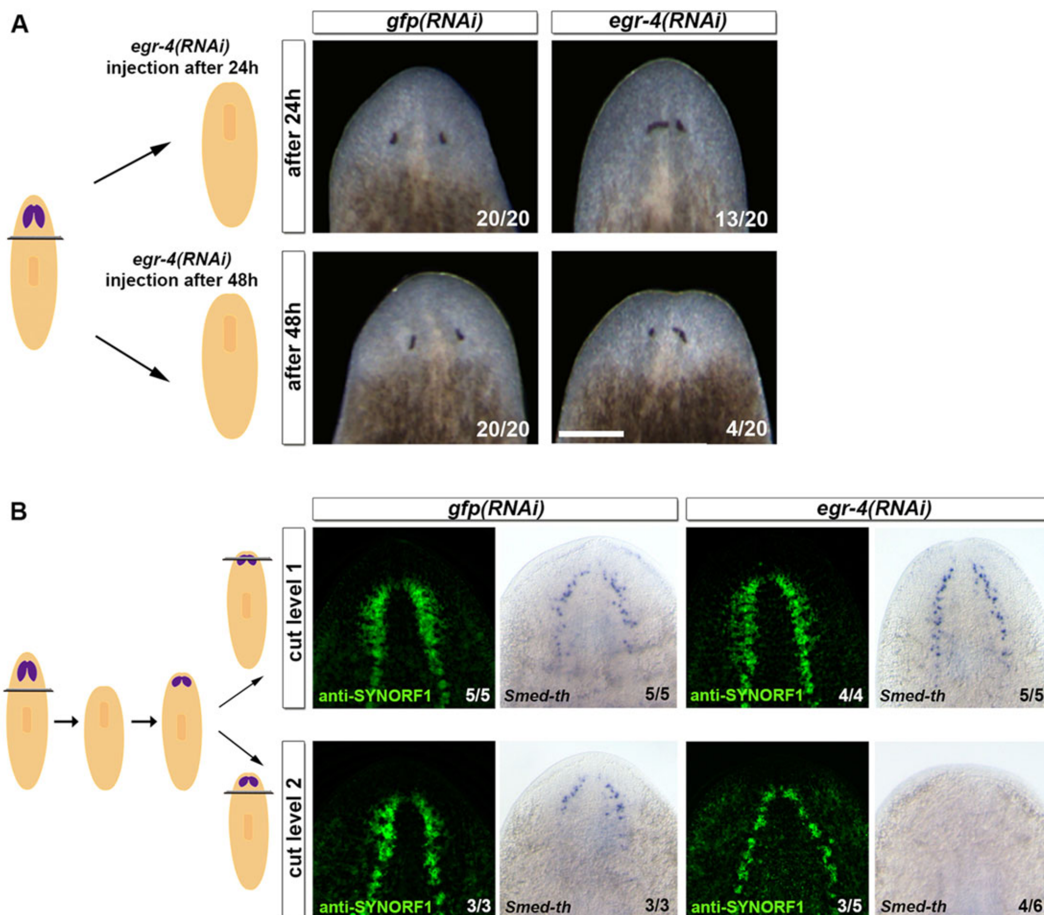

Fig. 6. The effects of *egr-4* RNAi depend on the time of the silencing and the presence of brain tissue. (A) Injection of the first dose of *egr-4* dsRNA after 24 h of regeneration resulted in defects in the newly regenerated eyes of most animals. By contrast, most animals regenerated normally when the first injection was delivered 48 h after amputation. (B) After the first round of injection and amputation, regenerating animals were re-injected and re-amputated, leaving a portion of the newly regenerated small brain (level 1) or removing all brain tissue (level 2). Analysis of the expression of the CNS markers anti-SYNORF1 and *Smed-th* revealed that the presence of brain tissue rescued head regeneration after *egr-4* RNAi. Scale bar: 200 µm.

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amputated at level 1 regenerated normally and differentiated normal brains as evidenced by anti-SYNORF-1 immunostaining and *in situ* hybridization with *Smed-th* (Fig. 6B). However, most of the decapitated fragments from level 2 amputations failed to regenerate proper heads or cephalic ganglia (Fig. 6B). Similarly, *egr-4(RNAi)* animals that were amputated sagittally along the midline regenerated properly (supplementary material Fig. S8). These results suggest that the presence of brain tissue is sufficient to rescue the blockade of head regeneration following *egr-4* RNAi.

DISCUSSION

egr-4, a downstream target of EGFR signaling, regulates cell differentiation in anterior blastemas

EGFR signaling plays important roles in many biological processes by activating or inhibiting many downstream pathways, including PI3K/AKT, MAPK (mitogen-activated protein kinase), PLCγ and JAK/STAT (signal transducer and activator of transcription) (Haley and Gullick, 2009). Previous reports suggest an important role for the EGFR signaling pathway in cell differentiation during planarian regeneration and homeostasis (Fraguas et al., 2011; Rink et al., 2011). *Smed-egfr-1* regulates eye-pigment cell differentiation; *Smed-egfr-3* seems to be necessary for blastema growth and cell differentiation (Fraguas et al., 2011); and *Smed-egfr-5* is required for flame cell maintenance and regeneration, and for guiding branch extension in protonephridia (Rink et al., 2011). These observations are consistent with the demonstrated role of the EGFR signaling pathway in cell differentiation in other organisms (Jones et al., 2009; Lejard et al., 2011; Harris and Horvitz, 2011); for example, EGFR is required for the differentiation of mammary epithelial cells (Mukhopadhyay et al., 2013) and human neural progenitors (Lemcke and Kuznetsov, 2013). Here, we identify *egr-4* as a putative target of *Smed-egfr-3* (Fig. 1) that is required for anterior regeneration, likely through its regulation of neoblast differentiation.

Although *Smed-egfr-3* is expressed in neoblasts and in the CNS of planarians (Fraguas et al., 2011; Fig. 1B), we found that *egr-4* was mainly expressed in the CNS and in the mesenchyme in irradiation-insensitive cells, suggesting that *egr-4* and *Smed-egfr-3* are co-expressed in the CNS (compare Fig. 1A and 1B). Indeed, silencing of *Smed-egfr-3* resulted in marked downregulation of *egr-4* expression in the cephalic ganglia (Fig. 1C).

Like other planarian *egr* homologues, *egr-4* expression was rapidly and locally upregulated after any small incision or during regeneration. Remarkably, this effect was independent of *Smed-egfr-3* (Fig. 1E″,F). However, after 2 days of regeneration, the expression of *egr-4* in the blastema became *Smed-egfr-3* dependent (Fig. 1F). These results suggest that *egr-4* is expressed in two distinct phases during regeneration, an early, *Smed-egfr-3*-independent, phase, and a subsequent phase that is controlled by EGFR signaling. These findings are supported by previous studies demonstrating that the expression of *egr* genes is regulated by the EGFR pathway in different organisms and contexts (Kaufmann and Thiel, 2002; Tsai et al., 2000, Lindzen et al., 2012).

The silencing of *egr-4* impaired head regeneration, resulting in the development of either extremely reduced blastemas or small blastemas with aberrant photoreceptors and cephalic ganglia (Figs 2 and 3). Planarian regeneration is dependent on pluripotent stem cells known as neoblasts. Specific anti-SMEDWI-1 and *Smed-histone-2B* markers revealed no differences between control and *egr-4(RNAi)* animals (Fig. 2B). We did, however, observe differences in neoblast mitotic activity between these two groups. In *egr-4(RNAi)* animals, the first, wound-related mitotic peak (at 6 h) was markedly attenuated in anterior stumps and in posterior stumps from head sections, when compared with controls. This decrease may reflect a role of early *egr-4* expression in this initial proliferative response. Interestingly, the second mitotic peak (associated with tissue loss and appearing at 48 h) was significantly attenuated only in anterior blastemas. This decrease may be a consequence, at least in part, of the marked attenuation of the first mitotic peak. However, because the decrease in the second peak was specific to anterior regeneration, we cannot rule out a potential role of later *egr-4* expression in regulating neoblast proliferation (directly or indirectly) at this stage. A normal bimodal proliferative response was observed after *egr-4* RNAi. Together with the slight attenuation of neoblast proliferation seen during posterior regeneration and the presence of a large number of mitotic cells in anterior blastemas at all stages (supplementary material Fig. S6), these findings suggest that the severe phenotypes observed after *egr-4* silencing are not solely due to defects in proliferation. Although *egr-4* silencing had no effect on early neoblast differentiation (normal anti-SMEDWI-1 and *Smed-NB21.11e* expression was observed), it resulted in a significant decrease in late neoblast progeny (Fig. 2D), suggesting that *egr-4* RNAi impairs late neoblast differentiation.

Taken together, these results suggest that *egr-4* is expressed in two distinct phases during regeneration. Early *egr-4* expression may participate in the initial (injury-induced) proliferative response, whereas the late expression, mediated by EGFR signaling, appears to regulate cell differentiation during anterior regeneration. These data provide the first evidence of a conserved EGFR/egr pathway in planarians. Further experiments will be necessary to better understand the relationship between *egr-4* and *Smed-egfr-3*, and to elucidate their specific roles during anterior and posterior regeneration.

egr-4 is required for early differentiation of the cephalic ganglia downstream of polarity determinants

egr genes have been implicated in cell proliferation, differentiation, inflammation, apoptosis, wound healing and liver regeneration (Thiel and Cibelli, 2002; Dussmann et al., 2011). Several *egr* genes have been identified in planarians (Sandmann et al., 2011; Wagner et al., 2012; Wenemoser et al., 2012).

Many of these genes, including *egr-4*, are upregulated immediately after injury or amputation and are barely expressed (if at all) in intact planarians (Sandmann et al., 2011; Wenemoser et al., 2012). To date, no functional characterization of the injury-induced expression of planarian *egr* genes has been reported. However, given the decrease reported here in the first mitotic peak after *egr-4* RNAi and the demonstrated role of several *egr* genes in wound healing, tissue fibrosis and inflammatory responses in other models (Schmidt et al., 2008; Wu et al., 2009; Chen et al., 2006), it seems plausible that early *egr* genes expression in planarians is involved in the initial stages of regeneration.

egr-4 was also expressed in the mature and differentiating CNS (Fig. 1). Given the conserved function of *egr* genes in neural development (reviewed by O'Donovan et al., 1999; Pérez-Cadahía et al., 2011) and the severe phenotypes observed in the regenerating CNS after *egr-4* RNAi, we investigated whether this gene is required for the regeneration of cephalic ganglia in planarians. The results of double RNAi of *egr-4* and *Smed-apc-1* (Fig. 5) suggest that *egr-4* is necessary for the early differentiation of the brain primordia. However, *egr-4* was not required for the maintenance of the CNS either in intact planarians (supplementary material Fig. S7) or in regenerating sections in which brain tissue remained after amputation (Fig. 6). These findings identify *egr-4* as the first gene

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known to be involved in the initial stages of neural regeneration in planarians. This role in neural differentiation is in agreement with functions attributed to *egr* genes in other organisms. For example, *egr-1* regulates astrocyte growth through via activation of the ERK signaling cascade triggered by EGF receptors (Mayer et al., 2009; Biesiada et al., 1996). In cultured PC12 cells, p35 is essential for neurite outgrowth and is induced via the *egr-1*-mediated activation of ERK (Harada et al., 2001). Similarly, *egr-1* regulates neurite extension during development in *Xenopus* (Anelli et al., 2013), and ERK-dependent *egr-4* expression is required for the maturation of GABAergic neurons (Ludwig et al., 2011a).

The silencing of different genes required for the respecification of anterior polarity or pole formation produces phenotypes similar to those described here after *egr-4* RNAi (Petersen and Reddien, 2009, 2011; Almuedo-Castillo et al., 2012; Roberts-Galbraith and Newmark, 2013; Blassberg et al., 2013; Chen et al., 2013). It is thus possible that the regeneration defects observed after *egr-4* RNAi are due to dysregulation of the respecification of polarity, given that double *egr-4(RNAi)/β-catenin(RNAi)* rescues proper head regeneration. However, although the knockdown of polarity or pole determinants completely silences *Smed-notum* (Roberts-Galbraith and Newmark, 2013; Blassberg et al., 2013; Chen et al., 2013), the expression of *Smed-notum* and *Smed-follistatin*, genes that are required for anterior polarity (Roberts-Galbraith and Newmark, 2013; Petersen and Reddien, 2011), is upregulated and largely maintained in regenerating *egr-4(RNAi)* animals. After normal upregulation of *Smed-notum* in *egr-4(RNAi)* animals during the first 24 h of regeneration (neoblast-independent upregulation; Chen et al., 2013), the recovery of the normal expression pattern in the days that follow is delayed when compared with controls. This delay may be associated with the attenuation of the second mitotic peak induced by *egr-4(RNAi)*. By 5-8 days of regeneration, most animals exhibited *Smed-notum* expression at the tip of the blastema. Moreover, *egr-4(RNAi)* did not affect the normal expression of *Smed-follistatin* at the blastema tip. We cannot rule out the possibility that the delay in restoring *Smed-notum* and *Smed-follistatin* expression at the tip of the blastema in *egr-4* knockdowns in turn influences polarity respecification (and/or maintenance), thus giving rise to the observed defects. Nonetheless, our findings suggest that the impaired head regeneration observed after *egr-4* RNAi is caused by disruption of *egr-4*-mediated differentiation of the cephalic ganglia.

The results of the double *egr-4(RNAi)/β-catenin(RNAi)* suggest that the impairment of head and cephalic ganglia regeneration after *egr-4* silencing is β-catenin dependent. Thus, in wild-type planarians, *egr-4* may inhibit β-catenin activity to allow head regeneration. In support of this hypothesis, defects in brain regeneration were more severe in *egr-4(RNAi);apc-1(RNAi)* animals, in which β-catenin activity is augmented. These results suggest that the effect of *egr-4* on cephalic ganglia regeneration is at least partially mediated by antagonism of the Wnt/β-catenin pathway. Although the Wnt/β-catenin pathway has been primarily associated with the establishment of AP polarity (Iglesias et al., 2008; Gurley et al., 2008; Petersen and Reddien, 2008), other studies suggest that this pathway may regulate the differentiation of cephalic ganglia independently of axial polarity (Iglesias et al., 2011; Blassberg et al., 2013).

Overall, our data point to *egr-4* as a unique factor that plays a key role in the differentiation of the brain primordia by antagonizing β-catenin function downstream of the polarity determinants *Smed-notum* and *Smed-wnt1*. Further experiments will be required to unravel the exact relationship between *egr-4* and Wnt/β-catenin signaling.

The role of the cephalic ganglia in head regeneration

If *egr-4* is required for the early development of the cephalic ganglia, how does the inhibition of CNS differentiation block head regeneration? Previous studies have shown that the inhibition of *Smed-egfr-3* (Fraguas et al., 2011) and ERK signaling (Tasaki et al., 2011) yields phenotypes similar to those obtained after *egr-4* RNAi: blastema growth is severely affected due to impaired cell differentiation. Tasaki and co-workers (2011) have suggested that ERK activation within the blastema is necessary for the early differentiation of an initial cohort of cells that is subsequently required to induce neoblast proliferation in the stump, and is probably necessary for the migration of neoblast progeny into the blastema, ultimately promoting blastema growth. We propose that this cohort of cells that is necessary to maintain blastema growth might be the initial brain primordia, the differentiation of which is dependent on *egr-4*. Under normal physiological situations, the brain primordia could emit some form of signal to promote neoblast proliferation in the stump and/or the migration of neoblast progeny cells into the blastema, where they subsequently differentiate. However, this mechanism may be dysregulated in *egr-4(RNAi)* animals, in which differentiation of the brain primordia is impaired (Fig. 7). Future experiments will be required to investigate this hypothesis. Further suggesting that brain cells are required for blastema growth, regenerating *egr-4(RNAi)* planarians in which some brain tissues was retained following amputation (Fig. 6; supplementary material Fig. S8) were capable of regenerating

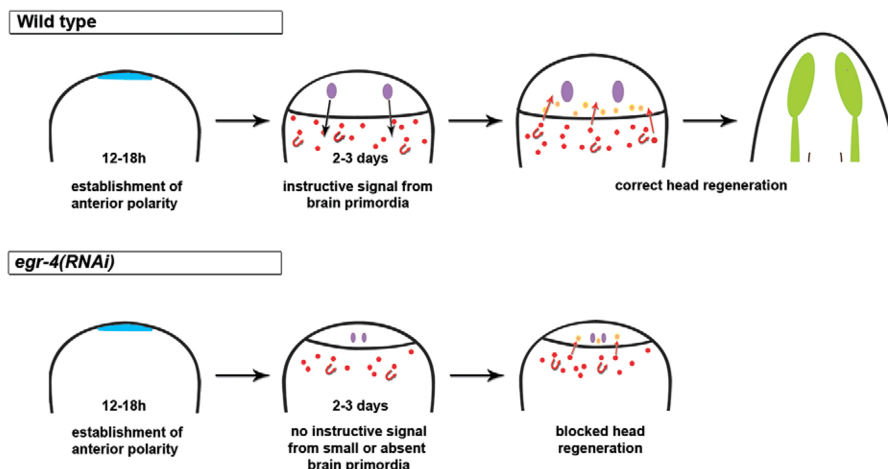

Fig. 7. Proposed model illustrating the requirement of brain primordia for head regeneration in planarians. In wild type the brain primordia would send some signal to the stump to promote the proliferation, migration or differentiation of the neoblasts to allow blastema growth and head regeneration. In the absence of a proper brain primordia after *egr-4* RNAi, the lack of such putative inducing signal would explain the inhibition of head regeneration. Blue, anterior pole; red dots, proliferating neoblasts; orange dots, differentiating neoblasts; purple dots, brain primordia; green, mature CNS.

normal heads with proper brains. Given that EGFR/ERK signaling regulates *egr* genes in several other contexts (Mayer et al., 2009; Harada et al., 2001; Tarcic et al., 2012; Mukhopadhyay et al., 2013), planarians provide a unique opportunity with which to study the function of this conserved pathway during regeneration.

A recent study proposed that during planarian regeneration neoblasts follow a default program, triggered by ERK activation, to differentiate into head tissues (Umesono et al., 2013). These authors suggest that in posterior regions the Wnt/β-catenin pathway inhibits this ERK activation, resulting in regeneration of a tail. It will thus be of interest to analyze the relationship between *egr-4* and ERK in future studies to determine to what extent *egr-4* mediates the differentiation of head tissues after ERK activation.

Finally, the requirement of the brain primordia for blastema growth supports an evolutionarily conserved role of the nervous system in animal regeneration (Kumar et al., 2007; Kumar and Brockes, 2012; Miljkovic-Licina et al., 2007; Singer and Craven, 1948). Although several planarian studies have proposed a role of the nervous system in regeneration (Baguñà et al., 1989; Cebrià and Newmark, 2007; Oviedo et al., 2010; Stéphan-Dubois and Lender, 1956), the underlying molecular basis remains unknown. Accordingly, *egr-4* may be the first identified gene linking the differentiation of the brain primordia with head regeneration in planarians.

MATERIALS AND METHODS

Animals

Schmidtea mediterranea from the BCN-10 clonal line were used in all experiments. Planarians were starved for at least 1 week prior to experiments. Genes and RNAi experiments were named using the nomenclature proposed by Reddien et al. (2008).

Construction, sequencing and analysis of the DGE libraries

For the DGE experiment, total RNA from control (GFP) and *Smed-egr-3 (RNAi)* planarians was extracted 1 day and 3 days after amputation using TRIzol (Invitrogen). Libraries were generated by the Skuldtech transcriptomic service (Montpellier, France). Sequence tag preparation was performed using Illumina's Digital Gene Expression Tag Profiling Kit according to the manufacturer's instructions (version 2.1B). Cluster generation was performed after applying 4 pM of each sample to the individual lanes of the Illumina 1G flowcell according to the manufacturer's instructions. Image analysis and base calling were performed using the Illumina Pipeline, from which sequence tags were obtained after purity filtering. This was followed by sorting and counting the unique tags.

Four libraries were obtained from control and *Smed-egfr-3(RNAi)* animals on days 1 and 3 post-amputation (henceforth referred to as C1, C3, R1 and R3) (supplementary material Table S1). For comparison between libraries, tags with a sum of occurrences below 10 in the two compared sets or with a P>0.005 were discarded. P-value determination was performed as previously described (Piquemal et al., 2002). Tags were mapped using the short sequence mapping tools SeqMap (Jiang and Wong, 2008) and MPscan (Rivals et al., 2009) against the Smed454_90edb transcriptome dataset (Abril et al., 2010). Annotation of the sequences and assignation of Gene Ontology (GO) categories was performed using the Blast2GO suite (Conesa et al., 2005).

RNAi experiments

Double-stranded RNAs (dsRNA) were delivered into the planarian digestive system for three consecutive days as previously described (Sánchez-Alvarado and Newmark, 1999). All controls were injected with GFP dsRNA. After one round of dsRNA injections and amputation all *egr-4(RNAi)* planarians regenerated well. Defective phenotypes were identified after two rounds of injections and amputation. To increase the penetrance of the phenotypes the animals were injected with dsRNA for 3 consecutive days, cultured un-cut for 5 more days, re-injected for 3 consecutive days and then amputated pre- and post-pharyngeally. All experiments were performed following this protocol unless otherwise specified. In all figures showing a quantification of the RNAi phenotypes when the fractions do not add up to 100% the missing animals are normal, unless specified in the main text.

In situ hybridization

Gene expression analysis was carried out by whole-mount *in situ* hybridization, as previously described (Molina et al., 2007; Umesono et al., 1997; Cebrià et al., 2007; Pearson et al., 2009). All samples were observed through a Leica MZ16F stereomicroscope and images from representative organisms of each experiment were captured with a ProgRes C3 camera from Jenoptik. Confocal laser scanning microscopy was performed with a Leica SP2. A lethal dose of 100 Gy was used in irradiation experiments, and animals were fixed and hybridized at the indicated time points.

Immunohistochemistry

Immunostaining was carried out as described previously (Cebrià and Newmark, 2005). The following antibodies were used: anti-SYNORF-1, a monoclonal antibody specific for synapsin, which was used as a pan-neural marker (Cebrià, 2008) (1:10; Developmental Studies Hybridoma Bank); anti-SMEDWI-1 (1:1500; Guo et al., 2006; März et al., 2013); and anti-phospho-histone H3 (H3P), which was used to detect mitotic cells (1:300; Cell Signaling Technology). Alexa 488-conjugated goat anti-mouse (1:400) and Alexa 568-conjugated goat anti-rabbit (1:1000; Molecular Probes) were used as secondary antibodies. Confocal laser scanning microscopy was performed using a Leica TCS-SPE and a Leica SP2. Confocal stacks from representative organisms in each experimental condition are shown.

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Competing interests

The authors declare no competing financial interests.

Author contributions

F.C., S.F., M.I. and S.B. conceived and designed the experiments. S.F., S.B. and M.I. performed the experiments. G.R.-E. and F.C. analyzed the DGE data. S.F. and F.C. wrote the paper.

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Supplementary material

Supplementary material available online at
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References

Abril, J. F., Cebrià, F., Rodriguez-Esteban, G., Horn, T., Fraguas, S., Calvo, B., Bartscherer, K. and Saló, E. (2010). Smed454 dataset: unravelling the transcriptome of *Schmidtea mediterranea*. *BMC Genomics* **11**, 731.

Adell, T., Saló, E., Boutros, M. and Bartscherer, K. (2009). Smed-Evi/Wntless is required for beta-catenin-dependent and -independent processes during planarian regeneration. *Development* **136**, 905-910.

Agata, K. and Umesono, Y. (2008). Brain regeneration from pluripotent stem cells in planarian. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **363**, 2071-2078.

Aggeli, I. K., Beis, I. and Gaitanaki, C. (2010). ERKs and JNKs mediate hydrogen peroxide-induced Egr-1 expression and nuclear accumulation in H9c2 cells. *Physiol. Res.* **59**, 443-454.

Almuedo-Castillo, M., Sureda-Gómez, M. and Adell, T. (2012). Wnt signaling in planarians: new answers to old questions. *Int. J. Dev. Biol.* **56**, 53-65.

Anelli, T., Cardarelli, S., Ori, M., Nardi, I., Biagioni, S. and Poiana, G. (2013). 5-Hydroxytryptamine 1A and 2B serotonin receptors in neurite outgrowth: involvement of early growth response protein 1. *Dev. Neurosci.* **35**, 450-460.

Baguñà, J., Saló, E. and Romero, R. (1989). Effects of activators and antagonists of the neuropeptides substance P and substance K on cell proliferation in planarians. *Int. J. Dev. Biol.* **33**, 261-266.
Biesiada, E., Razandi, M. and Levin, E. R. (1996). Egr-1 activates basic fibroblast growth factor transcription. Mechanistic implications for astrocyte proliferation. *J. Biol. Chem.* **271**, 18576-18581.
Blassberg, R. A., Felix, D. A., Tejada-Romero, B. and Aboobaker, A. A. (2013). PBX/extradenticle is required to re-establish axial structures and polarity during planarian regeneration. *Development* **140**, 730-739.
Cabodi, S., Morello, V., Masi, A., Cicchi, R., Broggio, C., Distefano, P., Brunelli, E., Silengo, L., Pavone, F., Arcangeli, A. et al. (2008). Convergence of integrins and EGF receptor signaling via PI3K/Akt/FoxO pathway in early gene egr-1 expression. *J. Cell. Physiol.* **218**, 294-303.
Calogero, A., Lombari, V., De Gregorio, G., Porcellini, A., Ucci, S., Arcella, A., Caruso, R., Gagliardi, F. M., Gulino, A., Lanzetta, G. et al. (2004). Inhibition of cell growth by EGR-1 in human primary cultures from malignant glioma. *Cancer Cell Int.* **4**, 1.
Cebrià, F. (2008). Organitzation of the nervous system in the model planarian *Schmidtea mediterranea*: an immunocytochemical study. *Neurosci. Res.* **61**, 375-384.
Cebrià, F. and Newmark, P. A. (2005). Planarian homologs of netrin and netrin receptor are required for proper regeneration of the central nervous system and the maintenance of nervous system architecture. *Development* **132**, 3691-3703.
Cebrià, F. and Newmark, P. A. (2007). Morphogenesis defects are associated with abnormal nervous system regeneration following roboA RNAi in planarians. *Development* **134**, 833-837.
Cebrià, F., Kobayashi, C., Umesono, Y., Nakazawa, M., Mineta, K., Ikeo, K., Gojobori, T., Itoh, M., Taira, M., Sánchez Alvarado, A. et al. (2002a). FGFR-related gene nou-darake restricts brain tissues to the head region of planarians. *Nature* **419**, 620-624.
Cebrià, F., Nakazawa, M., Mineta, K., Ikeo, K., Gojobori, T. and Agata, K. (2002b). Dissecting planarian central nervous system regeneration by the expression of neural-specific genes. *Dev. Growth Differ.* **44**, 135-146.
Cebrià, F., Guo, T., Jopek, J. and Newmark, P. A. (2007). Regeneration and maintenance of the planarian midline is regulated by a slit orthologue. *Dev. Biol.* **307**, 394-406.
Chandra, A., Lan, S., Zhu, J., Siclari, V. A. and Qin, L. (2013). Epidermal growth factor receptor (EGFR) signaling promotes proliferation and survival in osteoprogenitors by increasing early growth response 2 (EGR2) expression. *J. Biol. Chem.* **288**, 20488-20498.
Chen, S.-J., Ning, H., Ishida, W., Sodin-Semrl, S., Takagawa, S., Mori, Y. and Varga, J. (2006). The early-immediate gene EGR-1 is induced by transforming growth factor-beta and mediates stimulation of collagen gene expression. *J. Biol. Chem.* **281**, 21183-21197.
Chen, C.-C. G., Wang, I. E. and Reddien, P. W. (2013). pbx is required for pole and eye regeneration in planarians. *Development* **140**, 719-729.
Cole, A. J., Saffen, D. W., Baraban, J. M. and Worley, P. F. (1989). Rapid increase of an immediate early gene messenger RNA in hippocampal neurons by synaptic NMDA receptor activation. *Nature* **340**, 474-476.
Conesa, A., Götz, S., García-Gómez, J. M., Terol, J., Talón, M. and Robles, M. (2005). Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* **21**, 3674-3676.
Dussmann, P., Pagel, J. I., Vogel, S., Magnusson, T., Zimmermann, R., Wagner, E., Schaper, W., Ogris, M. and Deindl, E. (2011). Live in vivo imaging of Egr-1 promoter activity during neonatal development, liver regeneration and wound healing. *BMC Dev. Biol.* **11**, 28.
Eisenhoffer, G. T., Kang, H. and Sánchez Alvarado, A. (2008). Molecular analysis of stem cells and their descendants during cell turnover and regeneration in the planarian Schmidtea mediterranea. *Cell Stem Cell* **3**, 327-339.
Evans, D. J., Owlarn, S., Tejada-Romero, B., Chen, C. and Aboobaker, A. A. (2011). Combining classical and molecular approaches elaborates on the complexity of mechanisms underpinning anterior regeneration. *PLoS ONE* **6**, e27927.
Felix, D. A. and Aboobaker, A. A. (2010). The TALE class homeobox gene Smed-prep defines the anterior compartment for head regeneration. *PLoS Genet.* **6**, e1000915.
Fraguas, S., Barberán, S. and Cebrià, F. (2011). EGFR signaling regulates cell proliferation, differentiation and morphogenesis during planarian regeneration and homeostasis. *Dev. Biol.* **354**, 87-101.
Fraguas, S., Barberán, S., Ibarra, B., Stöger, L. and Cebrià, F. (2012). Regeneration of neuronal cell types in Schmidtea mediterranea: an immunohistochemical and expression study. *Int. J. Dev. Biol.* **56**, 143-153.
Guo, T., Peters, A. H. F. M. and Newmark, P. A. (2006). A Bruno-like gene is required for stem cell maintenance in planarians. *Dev. Cell* **11**, 159-169.
Gurley, K. A., Rink, J. C. and Sánchez Alvarado, A. (2008). Beta-catenin defines head versus tail identity during planarian regeneration and homeostasis. *Science* **319**, 323-327.
Gurley, K. A., Elliott, S. A., Simakov, O., Schmidt, H. A., Holstein, T. W. and Sánchez Alvarado, A. (2010). Expression of secreted Wnt pathway components reveals unexpected complexity of the planarian amputation response. *Dev. Biol.* **347**, 24-39.
Haley, J. D. and Gullick, W. J. (2009). EGFR Signaling Networks in Cancer Therapy. *New York, USA*: Humana Press.
Harada, T., Morooka, T., Ogawa, S. and Nishida, E. (2001). ERK induces p35, a neuron-specific activator of Cdk5, through induction of Egr1. *Nat. Cell Biol.* **3**, 453-459.
Harris, D. T. and Horvitz, H. R. (2011). MAB-10/NAB acts with LIN-29/EGR to regulate terminal differentiation and the transition from larva to adult in C. elegans. *Development* **138**, 4051-4062.
Iglesias, M., Gomez-Skarmeta, J. L., Saló, E. and Adell, T. (2008). Silencing of Smed-betacatenin1 generates radial-like hypercephalized planarians. *Development* **135**, 1215-1221.
Iglesias, M., Almuedo-Castillo, M., Aboobaker, A. A. and Saló, E. (2011). Early planarian brain regeneration is independent of blastema polarity mediated by the Wnt/β-catenin pathway. *Dev. Biol.* **358**, 68-78.
Jiang, H. and Wong, W. H. (2008). SeqMap: mapping massive amount of oligonucleotides to the genome. *Bioinformatics* **24**, 2395-2396.
Jones, J. E., Wang, L., Kropf, P. L., Duan, R. and Johnson, D. E. (2009). Src family kinase gene targets during myeloid differentiation: identification of the *EGR-1* gene as a direct target. *Leukemia* **23**, 1933-1935.
Kaufmann, K. and Thiel, G. (2001). Epidermal growth factor and platelet-derived growth factor induce expression of Egr-1, a zinc finger transcription factor, in human malignant glioma cells. *J. Neurol. Sci.* **189**, 83-91.
Kaufmann, K. and Thiel, G. (2002). Epidermal growth factor and thrombin induced proliferation of immortalized human keratinocytes is coupled to the synthesis of Egr-1, a zinc finger transcriptional regulator. *J. Cell. Biochem.* **85**, 381-391.
Kobayashi, C., Saito, Y., Ogawa, K. and Agata, K. (2007). Wnt signaling is required for antero-posterior patterning of the planarian brain. *Dev. Biol.* **306**, 714-724.
Kumar, A. and Brockes, J. P. (2012). Nerve dependence in tissue, organ, and appendage regeneration. *Trends Neurosci.* **35**, 691-699.
Kumar, A., Godwin, J. W., Gates, P. B., Garza-Garcia, A. A. and Brockes, J. P. (2007). Molecular basis for the nerve dependence of limb regeneration in an adult vertebrate. *Science* **318**, 772-777.
Lejard, V., Blais, F., Guerquin, M.-J., Bonnet, A., Bonnin, M.-A., Havis, E., Malbouyres, M., Bidaud, C. B., Maro, G., Gilardi-Hebenstreit, P. et al. (2011). EGR1 and EGR2 involvement in vertebrate tendon differentiation. *J. Biol. Chem.* **286**, 5855-5867.
Lemcke, H. and Kuznetsov, S. A. (2013). Involvement of connexin43 in the EGF/EGFR signalling during self-renewal and differentiation of neural progenitor cells. *Cell. Signal.* **25**, 2676-2684.
Lindzen, M., Carvalho, S., Starr, A., Ben-Chetrit, N., Pradeep, C.-R., Köstler, W. J., Rabinkov, A., Lavi, S., Bacus, S. S. and Yarden, Y. (2012). A recombinant decoy comprising EGFR and ErbB-4 inhibits tumor growth and metastasis. *Oncogene* **31**, 3505-3515.
Ludwig, A., Uvarov, P., Soni, S., Thomas-Crusells, J., Airaksinen, M. S. and Rivera, C. (2011a). Early growth response 4 mediates BDNF induction of potassium chloride cotransporter 2 transcription. *J. Neurosci.* **31**, 644-649.
Ludwig, A., Uvarov, P., Pellegrino, C., Thomas-Crusells, J., Schuchmann, S., Saarma, M., Airaksinen, M. S. and Rivera, C. (2011b). Neurturin evokes MAPK-dependent upregulation of Egr4 and KCC2 in developing neurons. *Neural Plast.* **2011**, 1-8.
März, M., Seebeck, F. and Bartscherer, K. (2013). A Pitx transcription factor controls the establishment and maintenance of the serotonergic lineage in planarians. *Development* **140**, 4499-4509.
Mayer, S. I., Rössler, O. G., Endo, T., Charnay, P. and Thiel, G. (2009). Epidermal-growth-factor-induced proliferation of astrocytes requires Egr transcription factors. *J. Cell Sci.* **122**, 3340-3350.
Milbrandt, J. (1987). A nerve growth factor-induced gene encodes a possible transcriptional regulatory factor. *Science* **238**, 797-799.
Miljkovic-Licina, M., Chera, S., Ghila, L. and Galliot, B. (2007). Head regeneration in wild-type hydra requires de novo neurogenesis. *Development* **134**, 1191-1201.
Molina, M. D., Saló, E. and Cebrià, F. (2007). The BMP pathway is essential for re-specification and maintenance of the dorsoventral axis in regenerating and intact planarians. *Dev. Biol.* **311**, 79-94.
Mukhopadhyay, C., Zhao, X., Maroni, D., Band, V. and Naramura, M. (2013). Distinct effects of EGFR ligands on human mammary epithelial cell differentiation. *PLoS ONE* **8**, e75907.
Nishimura, K., Kitamura, Y., Inoue, T., Umesono, Y., Sano, S., Yoshimoto, K., Inden, M., Takata, K., Taniguchi, T., Shimohama, S. et al. (2007). Reconstruction of dopaminergic neural network and locomotion function in planarian regenerates. *Dev. Neurobiol.* **67**, 1059-1078.
Nishimura, K., Kitamura, Y., Inoue, T., Umesono, Y., Yoshimoto, K., Taniguchi, T. and Agata, K. (2008). Characterization of tyramine beta-hydroxylase in planarian Dugesia japonica: cloning and expression. *Neurochem. Int.* **53**, 184-192.
O'Donovan, K. J., Tourtellotte, W. G., Millbrandt, J. and Baraban, J. M. (1999). The EGR family of transcription-regulatory factors: progress at the interface of molecular and systems neuroscience. *Trends Neurosci.* **22**, 167-173.

Önal, P., Grün, D., Adamidi, C., Rybak, A., Solana, J., Mastrobuoni, G., Wang, Y., Rahn, H.-P., Chen, W., Kempa, S. et al. (2012). Gene expression of pluripotency determinants is conserved between mammalian and planarian stem cells. *EMBO J.* **31**, 2755-2769.

Oviedo, N. J., Morokuma, J., Walentek, P., Kema, I. P., Gu, M. B., Ahn, J.-M., Hwang, J. S., Gojobori, T. and Levin, M. (2010). Long-range neural and gap junction protein-mediated cues control polarity during planarian regeneration. *Dev. Biol.* **339**, 188-199.

Pearson, B. J., Eisenhoffer, G. T., Gurley, K. A., Rink, J. C., Miller, D. E. and Sánchez Alvarado, A. (2009). Formaldehyde-based whole-mount in situ hybridization method for planarians. *Dev. Dyn.* **238**, 443-450.

Pérez-Cadahía, B., Drobic, B. and Davie, J. R. (2011). Activation and function of immediate-early genes in the nervous system. *Biochem. Cell Biol.* **89**, 61-73.

Petersen, C. P. and Reddien, P. W. (2008). Smed-betacatenin-1 is required for anteroposterior blastema polarity in planarian regeneration. *Science* **319**, 327-330.

Petersen, C. P. and Reddien, P. W. (2009). A wound-induced Wnt expression program controls planarian regeneration polarity. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 17061-17066.

Petersen, C. P. and Reddien, P. W. (2011). Polarized notum activation at wounds inhibits Wnt function to promote planarian head regeneration. *Science* **332**, 852-855.

Piquemal, D., Commes, T., Manchon, L., Lejeune, M., Ferraz, C., Pugnère, D., Demaille, J., Elalouf, J.-M. and Marti, J. (2002). Transcriptome analysis of monocytic leukemia cell differentiation. *Genomics* **80**, 361-371.

Reddien, P. W., Bermange, A. L., Murfitt, K. J., Jennings, J. R. and Sánchez Alvarado, A. (2005). Identification of genes needed for regeneration, stem cell function, and tissue homeostasis by systematic gene perturbation in planaria. *Dev. Cell* **8**, 635-649.

Reddien, P. W., Newmark, P. A. and Sánchez-Alvarado, A. (2008). Gene nomenclature guidelines for the planarian *Schmidtea mediterranea*. *Dev. Dyn.* **237**, 3099-3101.

Rink, J. C., Gurley, K. A., Elliott, S. A. and Sánchez Alvarado, A. (2009). Planarian Hh signaling regulates regeneration polarity and links Hh pathway evolution to cilia. *Science* **326**, 1406-1410.

Rink, J. C., Vu, H. T.-K. and Sánchez Alvarado, A. (2011). The maintenance and regeneration of the planarian excretory system are regulated by EGFR signaling. *Development* **138**, 3769-3780.

Rivals, E., Salmela, L., Kiiskinen, P., Kalsi, P. and Tarhio, J. (2009). MPSCAN: fast localisation of multiple reads in genomes. *Lect. Notes BioInform.* **5724**, 246-260.

Roberts-Galbraith, R. H. and Newmark, P. A. (2013). Follistatin antagonizes activin signaling and acts with notum to direct planarian head regeneration. *Proc. Natl. Acad. Sci. U.S.A.* **110**, 1363-1368.

Sánchez-Alvarado, A. and Newmark, P. A. (1999). Double-stranded RNA specifically disrupts gene expression during planarian regeneration. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 5049-5054.

Sandmann, T., Vogg, M. C., Owlarn, S., Boutros, M. and Bartscherer, K. (2011). The head-regeneration transcriptome of the planarian *Schmidtea mediterranea*. *Genome Biol.* **12**, R76.

Schmidt, J., Stoffels, B., Moore, B. A., Chanthaphavong, R. S., Mazie, A. R., Buchholz, B. M. and Bauer, A. J. (2008). Proinflammatory role of leukocyte-derived Egr-1 in the development of murine postoperative ileus. *Gastroenterology* **135**, 926-936.

Shafarenko, M., Liebermann, D. A. and Hoffman, B. (2005). *Egr*-1 abrogates the block imparted by c-Myc on terminal M1 myeloid differentiation. *Blood* **106**, 871-878.

Singer, M. and Craven, L. (1948). The growth and morphogenesis of the regenerating forelimb of adult Triturus following denervation at various stages of development. *J. Exp. Zool.* **108**, 279-308.

Solana, J., Kao, D., Mihaylova, Y., Jaber-Hijazi, F., Malla, S., Wilson, R. and Aboobaker, A. (2012). Defining the molecular profile of planarian pluripotent stem cells using a combinatorial RNA-seq, RNA interference and irradiation approach. *Genome Biol.* **13**, R19.

Stéphan-Dubois, F. and Lender, T. (1956). Corrélations humorales dans la régénération de planaires paludicoles. *Ann. des Sc. Nat. Zool.* **18**, 223-230.

Sukhatme, V. P., Kartha, S., Toback, F. G., Taub, R., Hoover, R. G. and Tsai-Morris, C. H. (1987). A novel early growth response gene rapidly induced by fibroblast, epithelial cell and lymphocyte mitogens. *Oncogene Res.* **1**, 343-355.

Sukhatme, V. P., Cao, X., Chang, L. C., Tsai-Morris, C.-H., Stamenkovich, D., Ferreira, P. C. P., Cohen, D. R., Edwards, S. A., Shows, T. B., Curran, T. et al. (1988). A zinc finger-encoding gene coregulated with c-fos during growth and differentiation, and after cellular depolarization. *Cell* **53**, 37-43.

Tarcic, G., Avraham, R., Pines, G., Amit, I., Shay, T., Lu, Y., Zwang, Y., Katz, M., Ben-Chetrit, N., Jacob-Hirsch, J. et al. (2012). EGR1 and the ERK-ERF axis drive mammary cell migration in response to EGF. *FASEB J.* **26**, 1582-1592.

Tasaki, J., Shibata, N., Nishimura, O., Itomi, K., Tabata, Y., Son, F., Suzuki, N., Araki, R., Abe, M., Agata, K. et al. (2011). ERK signaling controls blastema cell differentiation during planarian regeneration. *Development* **138**, 2417-2427.

Thiel, G. and Cibelli, G. (2002). Regulation of Life and Death by the zinc finger transcription factor egr-1. *J. Cell. Physiol.* **193**, 287-292.

Tsai, J. C., Liu, L., Guan, J. and Aird, W. C. (2000). The egr-1 gene is induced by epidermal growth factor in ECV304 cells and primary endothelial cells. *Am. J. Physiol. Cell Physiol.* **279**, 1414-1424.

Umesono, Y., Watanabe, K. and Agata, K. (1997). A planarian orthopedia homolog is specifically expressed in the branch region of both the mature and regenerating brain. *Dev. Growth Differ.* **39**, 723-727.

Umesono, Y., Watanabe, K. and Agata, K. (1999). Distinct structural domains in the planarian brain defined by the expression of evolutionarily conserved homeobox genes. *Dev. Genes Evol.* **209**, 31-39.

Umesono, Y., Tasaki, J., Nishimura, Y., Hrouda, M., Kawaguchi, E., Yazawa, S., Nishimura, O., Hosoda, K., Inoue, T. and Agata, K. (2013). The molecular logic for planarian regeneration along the anterior-posterior axis. *Nature* **500**, 73-76.

Wagner, D. E., Ho, J. J. and Reddien, P. W. (2012). Genetic regulators of a pluripotent adult stem cell system in planarians identified by RNAi and clonal analysis. *Cell Stem Cell.* **10**, 299-311.

Wenemoser, D. and Reddien, P. W. (2010). Planarian regeneration involves distinct stem cell responses to wounds and tissue absence. *Dev. Biol.* **344**, 979-991.

Wenemoser, D., Lapan, S. W., Wilkinson, A. W., Bell, G. W. and Reddien, P. W. (2012). A molecular wound response program associated with regeneration initiation in planarians. *Genes Dev.* **26**, 988-1002.

Wu, M., Melichian, D. S., de la Garza, M., Gruner, K., Bhattacharyya, S., Barr, L., Nair, A., Shahrara, S., Sporn, P. H. S., Mustoe, T. A. et al. (2009). Essential roles for early growth response transcription factor Egr-1 in tissue fibrosis and wound healing. *Am. J. Pathol.* **175**, 1041-1055.

Yazawa, S., Umesono, Y., Hayashi, T., Tarui, H. and Agata, K. (2009). Planarian Hedgehog/Patched establishes anterior-posterior polarity by regulating Wnt signaling. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 22329-22334.