Int. J. Dev. Biol. 56: 155-163
doi: 10.1387/ijdb.123494ag

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Inhibitory Smads and bone morphogenetic protein (BMP) modulate anterior photoreceptor cell number during planarian eye regeneration

ALEJANDRO GONZÁLEZ-SASTRE, Mª DOLORES MOLINA*,# and EMILI SALÓ*

Departament de Genètica, Facultat de Biologia, Universitat de Barcelona and Institut de Biomedicina de la Universitat de Barcelona (IBUB), Universitat de Barcelona, Barcelona, Catalunya, Spain

ABSTRACT Planarians represent an excellent model to study the processes of body axis and organ re-specification during regeneration. Previous studies have revealed a conserved role for the bone morphogenetic protein (BMP) pathway and its intracellular mediators Smad1/5/8 and Smad4 in planarian dorsoventral (DV) axis re-establishment. In an attempt to gain further insight into the role of this signalling pathway in planarians, we have isolated and functionally characterized the inhibitory Smads (I-Smads) in *Schmidtea mediterranea*. Two I-Smad homologues have been identified: *Smed-smad6/7-1* and *Smed-smad6/7-2*. Expression of *smad6/7-1* was detected in the parenchyma, while *smad6/7-2* was found to be expressed in the central nervous system and the eyes. Neither single *smad6/7-1* and *smad6/7-2* nor double *smad6/7-1,-2* silencing gave rise to any apparent disruption of the DV axis. However, both regenerating and intact *smad6/7-2 (RNAi)* planarians showed defects in eye morphogenesis and displayed small, rounded eyes that lacked the anterior subpopulation of photoreceptor cells. The number of pigment cells was also reduced in these animals at later stages of regeneration. In contrast, after low doses of *Smed-bmp(RNAi),* planarians regenerated larger eyes in which the anterior subpopulation of photoreceptor cells was expanded. Our results suggest that *Smed-smad6/7-2* and *Smed-bmp* control the re-specification and maintenance of anterior photoreceptor cell number in *S. mediterranea*.

KEY WORDS: *planarian, regeneration, BMP pathway, eye, I-Smad*

Introduction

Planarians (order Tricladida) are free-living platyhelminths that are well known for their ability to regenerate and restore their polarity and missing organs in a short period of time (reviewed in Saló, 2006).

The light-sensing organs, the eyes, are well-defined sensory structures in planarians and can be easily recognized as two dark spots on the anterior-dorsal region of the animal (reviewed in Saló and Batistoni, 2008). Planarian eyes are composed of two cell types: photoreceptor and pigment cells. Photoreceptors are bipolar neurons. Their axons extend towards the dorsomedial side of the cephalic ganglia and form a partial optic chiasm, which integrates photosensory inputs from both sides of the animal (Okamoto *et al.*, 2005). The dendrites generally have a rhabdomeric structure, a regularly ordered microvilli assembly, where opsin protein accumulates (Orii *et al.*, 1998). The pigment cells form an eyecup which surrounds the rhabdomeres. Recently, the analysis of several prohormone genes revealed the existence of at least three different subpopulations within the photoreceptor cells. Specifically, it was shown that neuropeptide prohormone genes *eye53-1* and *npp-12* are expressed in anterior photoreceptor neurons, whereas *eye53-2* is expressed in the dorsal posterior subpopulation and *mpl-2* is expressed in both dorsal and ventral posterior subpopulation

Abbreviations used in this paper: BMP, bone morphogenetic protein; DV, dorsoventral; FISH, fluorescent *in situ* hybridization; I-Smad, inhibitory Smad; MH, Mad homology; RNAi, RNA interference.

***Address correspondence to:** Emili Saló. Departament de Genètica, Facultat de Biologia, Av. Diagonal 643, edifici annex planta 1, 08028 Barcelona, Catalunya, Spain. Tel. +34-93-403-5977. Fax +34-93-403-4420. e-mail: esalo@ub.edu - web: planarian.bio.ub.es or Mª Dolores Molina. UMR 7009 CNRS, Université Pierre et Marie Curie (Paris VI), Observatoire Océanologique de Villefranche-sur-Mer, 06230 Villefranche-sur-mer, France. Tel. +33-49-376-3785. Fax +33-49-376-3792. e-mail: loli.molina.jimenez@gmail.com - **#Present address:** Université Pierre et Marie Curie (Paris VI), UMR 7009 CNRS, Observatoire Océanologique, 06230 Villefranche-sur-mer, France.

Supplementary Material (1 figure) for this paper is available at: http://dx.doi.org/10.1387/ijdb.123494ag

Final, author-corrected PDF published online: 16 March 2012

ISSN: Online 1696-3547, Print 0214-6282

(Collins *et al*., 2010).

Planarians regenerate new eyes following head amputation (reviewed in Saló and Batistoni, 2008). Regeneration of the planarian eye employs the same basic genetic network that regulates vertebrate eye development, although the mechanism is Pax6 independent (Pineda *et al*., 2000, 2002; Mannini *et al*., 2004). Recently, several novel regulators of planarian eye regeneration have been identified (Lapan and Reddien, 2011; Fraguas *et al.,* 2011).

The Bone Morphogenetic Protein (BMP) family of secreted signalling molecules plays multiple roles during metazoan development and regeneration. For instance, BMP signalling is essential in processes such as establishment of the dorsoventral (DV) axis (reviewed in Little and Mullins, 2006) and patterning of the central nervous system (CNS; reviewed in Liu and Niswander, 2005). It also plays a key role during development and regeneration of the retina (Murali *et al*., 2004), the lens (Sjödal *et al*., 2007) and the ciliary body (Zhao *et al*., 2002) of the vertebrate eye, and enhances photoreceptor fate specification during zebrafish pineal gland determination (Quillien *et al*., 2011).

Smads are the main downstream mediators of the BMP signalling pathway (reviewed in Wrana, 2000). Three main classes of Smad proteins have been identified according to their structure and function: R-Smads or receptor-associated Smads (Smad1/5/8), co-Smads or common Smads (Smad4) and I-Smads or inhibitory Smads (Smad6/7). Smad proteins are characterized by the presence of Mad homology (MH) domains. R-Smads and co-Smad contain two MH domains: an amino terminal MH1 domain, which binds to DNA and confers the transcriptional activity, and a carboxy-terminal MH2 domain, which is involved in protein-protein interactions. I-Smads only contain the MH2 domain and, consequently, act as inhibitors of signalling (reviewed in Wrana, 2000). In addition to MH domains, R-Smads contain a C-terminal consensus sequence that is phosphorylated by the receptor.

In recent years, several studies have shown that BMP signalling is essential for correct blastema formation and specification of the planarian DV axis (reviewed in Molina *et al*., 2011b). In addition to DV phenotypes, inhibition of different elements of the pathway gives rise to abnormal eye regeneration (Reddien *et al*., 2005a, 2007; Molina *et al*., 2007, 2011a; Orii and Watanabe, 2007). For instance, aberrant projections of the visual axons are regenerated after *bmp, smad1, smad4* or *tolloid* inhibition (Reddien *et al*., 2005a, 2007; Molina *et al*., 2007), while inhibition of *bmp* and *smad4* also produces duplicated and supernumerary eyes, respectively (Reddien *et al*., 2007; Molina *et al*., 2007; Orii and Watanabe, 2007).

In an attempt to gain further insight into the role of BMP signalling in planarians, we isolated and functionally characterized two I-Smad genes in *Schmidtea mediterranea*: *Smed-smad6/7-1 and Smed-smad6/7-2.* Here we show that *smad6/7-2* silencing results in small, rounded eyes that lack the anterior subpopulation of photoreceptor cells. Remarkably, low doses of RNAi for the extracellular ligand BMP produced elongated eyes with an expanded anterior subpopulation of photoreceptor cells. Taken together, our data suggest that the BMP pathway regulates the number of anterior visual cells.

Results

Identification and isolation of S. mediterranea *inhibitory Smads*

To isolate I-Smad homologues in planarians, I-Smad proteins from several animals were used to perform *in silico* searches of *S. mediterranea* genomic (Washington University Sequencing Center, available at http://www.genome.wustl.edu) and 454 transcriptomic (Abril *et al*., 2010) databases. Two full-length homologues containing open reading frames of 340 and 182 amino acids were obtained. The predicted protein structures of both identified sequences contained the unique MH2 carboxy-terminal domain that defines I-Smads. These genes were therefore named *Smed-smad6/7-1* and *Smed-smad6/7-2*, respectively. It is important to notice, however, that *smad6/7-2* contains a significantly shorter coding region than *smad6/7-1* and homologues of I-Smads found in other organisms.

Expression pattern of *Smed-smad6/7-1* and *Smed-smad6/7-2*

Whole-mount *in situ* hybridization performed in intact and regenerating animals revealed different expression patterns for *S. mediterranea I-smads*. In intact animals, *smad6/7-1* was expressed throughout the parenchyma (Fig. 1A), in a pattern that resembles the distribution of the planarian stem cells, the neoblasts (reviewed in Handberg-Thorsager *et al*., 2008). The neoblasts are the only proliferative cells found in the animal and are specifically eliminated after X-ray irradiation (Dubois, 1949). Consequently, the expression

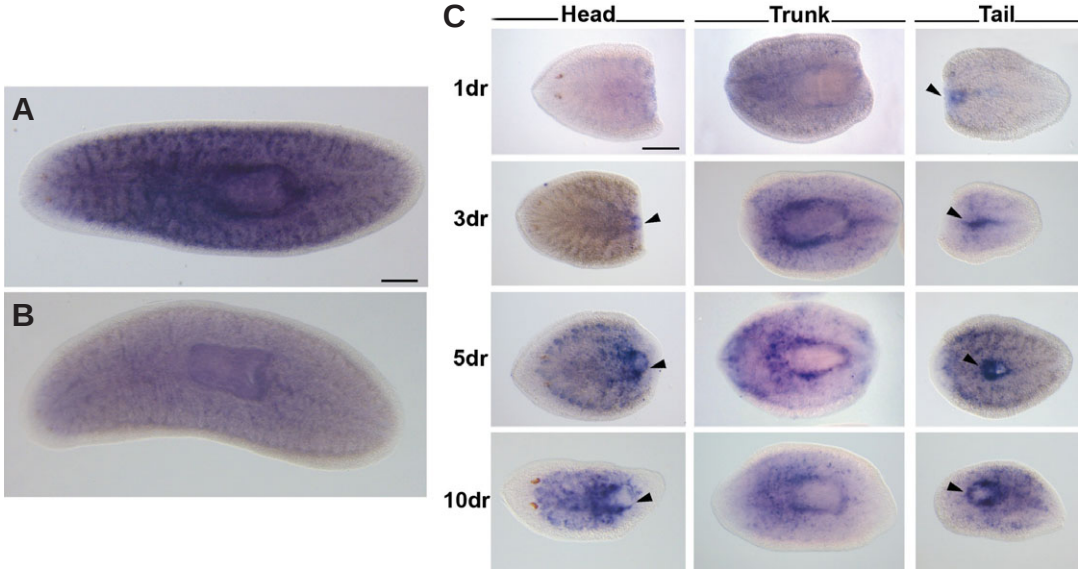


Fig. 1. Expression pattern of *Smed-smad6/7-1* in intact and regenerating planarians. (A) *Expression of* Smed-smad6/7-1 *in the parenchyma of intact planarians.* **(B)** *Down-regulation of* smad6/7-1 *expression 3 days after irradiation at 100 Gy.* **(C)** *Expression of* Smed-smad6/7-1 *during regeneration. Arrowheads point to the newly regenerated pharyngeal cavity. Anterior is to the left. dr, days of regeneration. Scale bars: 350 μm.*

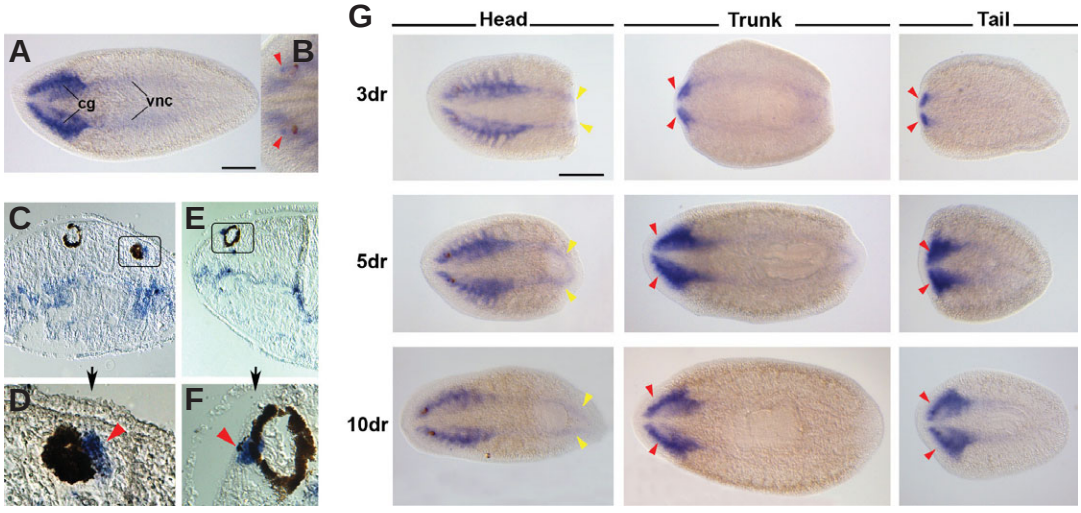


Fig. 2. Expression pattern of *Smed-smad6/7-2* in intact and regenerating planarians. (A-F) *Intact planarians.* **(A)** *Expression of* Smed-smad6/7-2 *in the cephalic ganglia (cg) and the ventral nerve cords (vnc).* **(B)** *Expression of* Smed-smad6/7-2 *in the eyes, in an anterior subpopulation of photoreceptor cells (arrowheads).* **(C-F)** *Expression of* Smed-smad6/7-2 *in the eyes (arrowheads in D,F).* **(G)** *Expression of* Smed-smad6/7-2 *during regeneration. Red arrowheads point to the newly regenerated brain. Yellow arrowheads point to the newly regenerated nerve cords in posteriorly regenerating head fragments. Ventral views. (C,D) Transverse section. (E,F) Sagittal section. Anterior is to the left in (A,B,E-G). Dorsal is to the top in (C-F). dr, days of regeneration. Scale bars: (A,G) 350 μm; (B) 175 μm; (C,E) 150 μm; (D,F) 40 μm.*

of neoblast markers is down-regulated upon irradiation (Eisenhoffer *et al*., 2008). To determine whether *smad6/7-1* is expressed in neoblasts, we performed whole-mount *in situ* hybridization in irradiated planarians. Strong down-regulation of *smad6/7-1* expression was observed 3 days after X-ray irradiation (Fig. 1B), suggesting that this gene is expressed in neoblasts. During regeneration, high levels of *smad6/7-1* expression were detected around the newly formed pharyngeal cavity (arrowheads in Fig. 1C). This expression was induced during both head and tail regeneration (Fig. 1C).

In intact animals, *smad6/7-2* was expressed in the CNS, in both the ventral nerve cords and the cephalic ganglia (Fig. 2A). Moreover, *smad6/7-2* expression was observed in the eyes, in an anterior subpopulation of photoreceptor cells (Fig. 2 B-F). During anterior regeneration, newly formed eyes started expressing *smad6/7-2* at day 5 (data not shown), whereas cephalic ganglia started expressing *smad6/7-2* at day 3 (red arrowheads in Fig. 2G). At this time, expression of *smad6/7-2* was also detected in newly regenerated nerve cords in posteriorly regenerating head fragments (yellow arrowheads in Fig. 2G).

Smad6/7-2(RNAi) *planarians have smaller rounded eyes*

To analyze the role of I-Smads during planarian regeneration, we performed RNAi knockdown experiments. Following double-stranded RNA injection, planarians were amputated pre- and post-pharyngeally and the resulting fragments were allowed to regenerate. Unless otherwise indicated, all the results presented here refer to regenerating trunk pieces that simultaneously regenerate anterior and posterior structures.

Loss of function of *smad6/7-1* did not result in any discernible morphological or molecular defects. Similar to control organisms, *smad6/7-1(RNAi)* planarians regenerated well-formed blastemas that correctly differentiated eyes, cephalic ganglia and digestive system (data not shown). On the other hand, compared to control animals, *smad6/7-2(RNAi)* planarians regenerated small, rounded eyes (Fig. 3 A-B). In both control and *smad6/7-2(RNAi)* planarians, regenerated eyes initially appeared within the anterior blastema as two dark rounded spots at 4-5 days of regeneration. Starting from day 7-8 post-amputation, however, when the pigmented spots of control organisms elongated anteroposteriorly, the eyes of *smad6/7-2*-silenced planarians remained rounded (Fig. 3 A-B).

Neither *smad6/7-1* nor *smad6/7-2* silencing resulted in apparent morphological or molecular defects related to blastema formation and DV axis re-establishment. Thus, general DV morphology of the animal seemed normal and the expression of ventral and dorsal markers did not seem affected (data not shown). This apparent lack of a DV phenotype was not caused by redundant function of I-Smads, since even after several rounds of *smad6/7-1,-2* co-silencing, planarians correctly re-specified their DV axis.

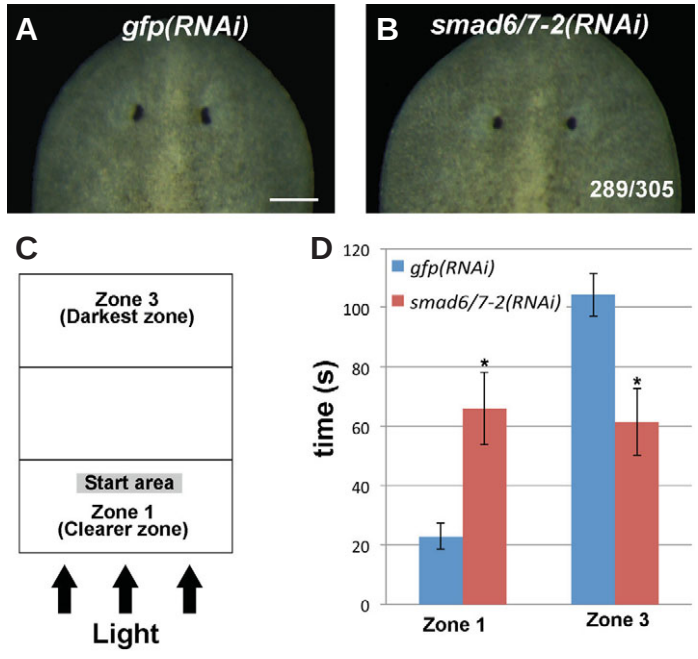


Fig. 3. Morphological and behavioural phenotypes in *Smed-smad6/7-2(RNAi)* animals. (A,B) *Live control and* smad6/7-2(RNAi) *planarians at 10 days of regeneration. Anterior is to the top.* **(C,D)** *Phototactic assay. (C) Diagram of the container. (D) Graphical representation of the time that control and* smad6/7-2(RNAi) *planarians spend in the different sectors. *p<0.01 (t test). Scale bar: 175 μm.*

smad6/7-2(RNAi) *planarians have abnormal negative phototactic behaviour*

When exposed to light, planarians display a distinctive light-avoidance behaviour known as negative phototaxis. In order to determine whether *smad6/7-2* silencing alters normal planarian negative phototactic behaviour, animals were exposed to a light gradient and their behaviour filmed and analysed. Control animals moved rapidly away from light and spent most of their time in the darkest zone (Zone 3) of the container. In contrast, *smad6/7-2(RNAi)* organisms displayed a statistically significant reduction in negative phototaxis and stayed longer in the clearest zone (Zone 1). Thus, although *smad6/7-2(RNAi)* animals moved normally, they turned more often and spent more time in the clearest zone than control animals (mean±SEM of 23.1±4.4 seconds in controls [n=15] versus 66.2±12.2 seconds in *smad6/7-2(RNAi)* animals [n=17]), while control animals spent more time in the darkest zone than *smad6/7-2(RNAi)* animals (104.5±7.2 seconds in controls [n=15] versus 61.7±11.3 seconds in *smad6/7-2(RNAi)* animals [n=15]) (Fig. 3 C-D).

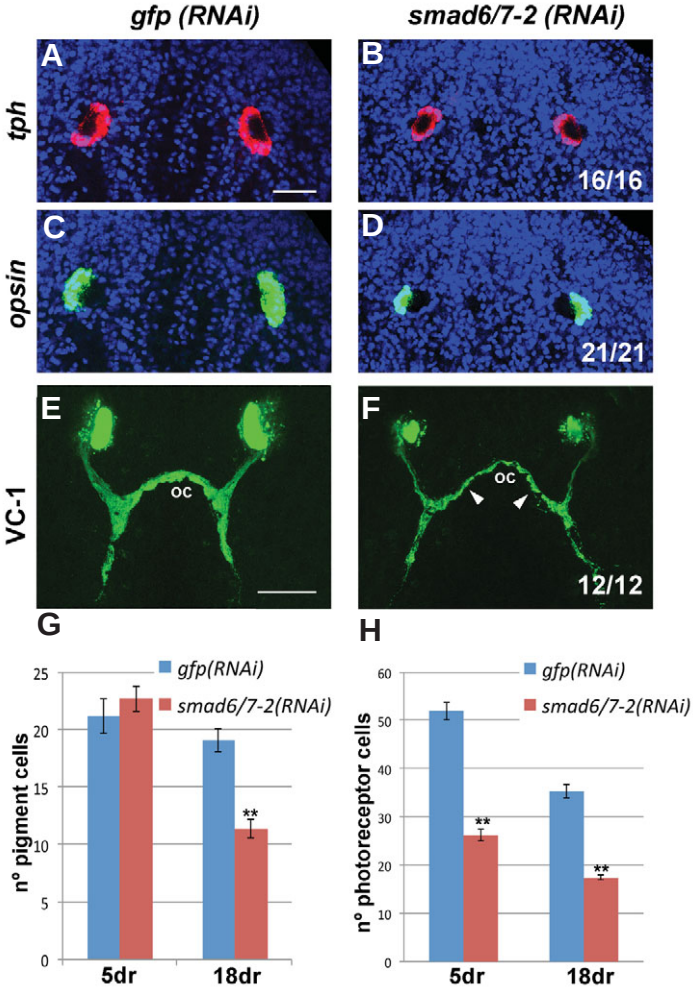


Fig. 4. Analysis of pigment and photoreceptor cells in *smad6/7-2(RNAi)* planarians. (A,B) *Fluorescent* in situ *hybridization against* tph, *a marker of pigment cells. Note the smaller expression domain in* smad6/7-2(RNAi) *animals.* **(C,D)** *Fluorescent* in situ *hybridization against* opsin, *a marker of photoreceptor cells. Note the smaller expression domain in* smad6/7-2(RNAi) *animals.* **(E,F)** *Immunofluorescence against arrestin (VC-1), labelling the photoreceptor cells and the optic chiasm (oc). Arrowheads indicate the thinner optic chiasm in* smad6/7-2(RNAi) *animals.* **(G,H)** *Graphical representation of the total number of pigment (G) and photoreceptor (H) cells at 5 and 18 days of regeneration in control and* Smed-smad6/7-2(RNAi) *planarians. **p<0.001 (t test). (A-D) 10 days of regeneration. (E,F) 18 days of regeneration. Anterior is to the top. Scale bar: 50μm.*

smad6/7-2 *silencing results in reduced numbers of eye photoreceptor and pigment cells*

To analyse the small, rounded eyes associated with *smad6/7-2* loss of function, we examined the expression pattern of specific markers of pigment and photoreceptor cells. Pigment cells were visualized and quantified by combining nuclear staining and fluorescent *in situ* hybridization (FISH) against *Smed-tph* (Fraguas *et al.*, 2011) (Fig. 4 A-B). In agreement with the normal morphological appearance of the pigmented eye cup at initial stages of regeneration, no significant differences in the number of *Smed-tph*-expressing pigment cells were observed between control and *smad6/7-2(RNAi)* planarians at 5 days (21.2±1.5 cells in controls [n=5] versus 22.7±1.1 cells in *smad6/7-2(RNAi)* eyes [n=6]) (Fig. 4G). In contrast, as regeneration proceeded, the small rounded eyecup of *smad6/7-2*-silenced animals had a significantly reduced number of pigment cells compared to control organisms (19.1±1.0 cells in controls [n=7] versus 11.4±0.8 cells in *smad6/7-2(RNAi)* eyes [n=7]) (Fig. 4G).

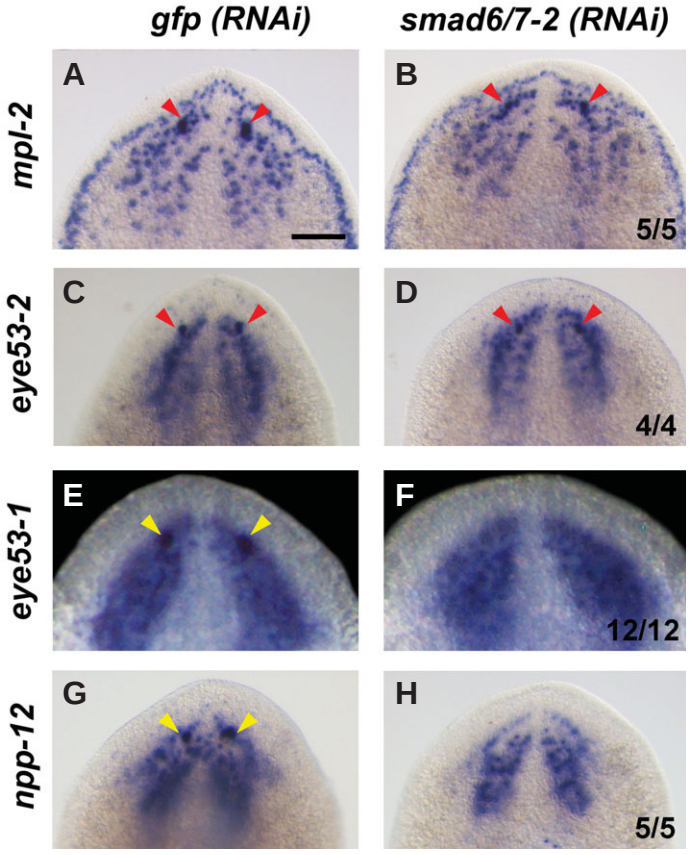
To visualize and quantify photoreceptor cells, we performed FISH against *Smed-opsin* (Sánchez Alvarado and Newmark 1999) (Fig. 4 C-D). Remarkably, *smad6/7-2*-silenced planarians already had a significant reduction in the total number of *Smed-opsin*-labelled photoreceptor cells at 5 days of regeneration (52.0±1.1 cells in controls [n=12] versus 26.3±1.2 cells in *smad6/7-2* RNAi-treated eyes [n=16]) (Fig. 4H). The reduction in the number of photoreceptor cells in *smad6/7-2(RNAi)* animals was still apparent at 18 days of regeneration (35.4±1.4 cells in controls [n=20] versus 17.5±0.5 cells in *smad6/7-2(RNAi)* eyes [n=17]) (Fig. 4H).

Photoreceptor cells are bipolar neurons that project axons towards the ipsilateral side of the cephalic ganglia or cross to the contralateral side and connect to the opposite eye and cephalic ganglia, producing an optic chiasm (Cebrià and Newmark, 2005; Okamoto *et al*., 2005, Sakai *et al*., 2000, Fig. 4E). Immunostaining with VC-1, an antibody against the arrestin protein that specifically recognizes the photoreceptor cells, allows visualization of this stereotypical pattern of axonal projections (Sakai *et al*., 2000; Okamoto *et al*., 2005). Interestingly, despite the reduced number of photoreceptor cells, *smad6/7-2*-silenced animals displayed normal stereotypical axonal projections according to VC-1 immunostaining (Fig. 4F). However, those axonal projections were thinner compared to control organisms (arrowheads in Fig. 4F).

These data indicate that, although reduced, both pigment and photoreceptor cells are present in the smaller eye obtained after *smad6/7-2* silencing. Moreover, they reveal an earlier effect on photoreceptor cells followed by a later decrease in pigment cells.

Anterior photoreceptor cells disappears after* smad6/7-2 *silencing

Several molecular markers for different populations of eye photoreceptor cells have recently been identified (Collins *et al*., 2010). To assess whether the decrease in the number of photoreceptor cells observed after *smad6/7-2* silencing differentially affects these



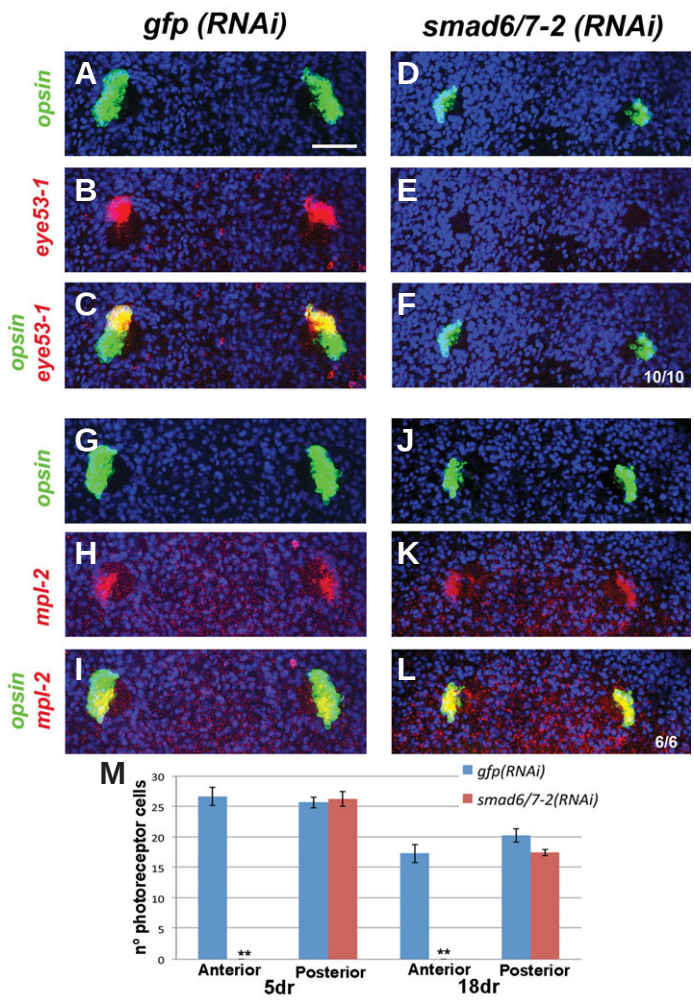


Fig. 5 (Left). Analysis of markers for anterior and posterior photoreceptor cells. (A-D) *Expression of the posterior markers* Smed-mpl-2 *(arrowheads in A,B) and* Smed-eye53-2 *(arrowheads in C,D) in control and* smad6/7-2(RNAi) *planarians.* **(E-H)** *The expression of the anterior markers* Smed-eye53-1 *(arrowheads in E) and* Smed-npp-12 *(arrowheads in G) disappears in* smad6/7-2(RNAi) *animals. Animals are shown at 12 days of regeneration. Anterior is to the top. Scale bar, 150 μm.*

Fig. 6 (Right). Anterior subpopulation of photoreceptor cells disappears after inhibition of Smed-smad6/7-2. (A-F) *Double fluorescent* in situ *hybridization against* opsin **(A,D)** *and the anterior marker* eye53-1 **(B,E)**. *Note the disappearance of the domain of expression of* eye53-1 *in* smad6/7-2(RNAi) *planarians.* **(G-L)** *Double fluorescent* in situ *hybridization against* opsin **(G,J)** *and the posterior marker* mpl-2 **(H,K)**. *Note that the expression domain of the posterior marker covers the whole expression domain of* opsin *in* smad6/7-2(RNAi) *planarians* **(L)**. **(M)** *Graphical representation of the number of anterior and posterior photoreceptor cells at 5 and 18 days of regeneration in control and* Smed-smad6/7-2(RNAi) *planarians. **p<0.001 (*t *test).* **(A-L)** *Animals are shown at 10 days of regeneration. Anterior is to the top. Scale bar: 50 μm.*

subpopulations, we analysed the expression of the anterior markers *eye53-1* and *npp-12*, and the posterior markers *eye53-2* and *mpl-2* in *smad6/7-2(RNAi)* planarians.

No differences were observed in the expression of *mpl-2* and *eye53-2* in the posterior population of photoreceptor cells compared to control organisms (red arrowheads in Fig. 5 A-D). However, in contrast, the expression of the anterior markers *eye53-1* and *npp-12* was completely absent in *smad6/7-2(RNAi)* animals (Fig. 5 E-H).

The number of anterior and posterior photoreceptor cells was quantified by combining nuclear staining and double FISH for *opsin* and the anterior marker *eye53-1* (Fig. 6 A-F) or the posterior marker *mpl-2* (Fig. 6 G-L). Anterior *eye53-1*-positive cells were absent in *smad6/7-2(RNAi)* treated planarians since initial stages of regeneration (5 days of regeneration, 26.7±1.5 cells in controls [n=7] versus 0 cells in *smad6/7-2(RNAi)* eyes [n=16]; 18 days of regeneration, 17.3±0.9 cells in controls [n=12] versus 0 cells in *smad6/7-2(RNAi)* eyes [n=17]) (Fig. 6 B,E,M). On the other hand, the total number of *mpl-2*-positive cells was normal compared to control organisms (5 days of regeneration, 25.7±1.1 cells in controls [n=7] versus 26.3±1.2 cells in *smad6/7-2(RNAi)* eyes [n=7]; 18 days of regeneration, 20.3±1.1 cells in controls versus 17.5±0.5 cells in *smad6/7-2(RNAi)* eyes [n=17]) (Fig. 6 H,K,M). Since all visual cells expressed both *opsin* and the posterior marker *mpl-2* after *smad6/7-2* silencing (Fig. 4E), these results indicate that the reduction in total number of photoreceptors was based exclusively on the lack of the anterior subpopulation of *eye53-1*-positive cells.

Similar phenotypes were obtained in intact (non-regenerating) animals (Sup. Fig. 1). Twenty days after initial treatment, the eyes of *smad6/7-2*-silenced, uncut planarians appeared rounded, and both pigment and photoreceptor cells seemed to be reduced. Also, as happened during regeneration, the anterior population of photoreceptor cells disappeared (Sup. Fig. 1). Taken together,

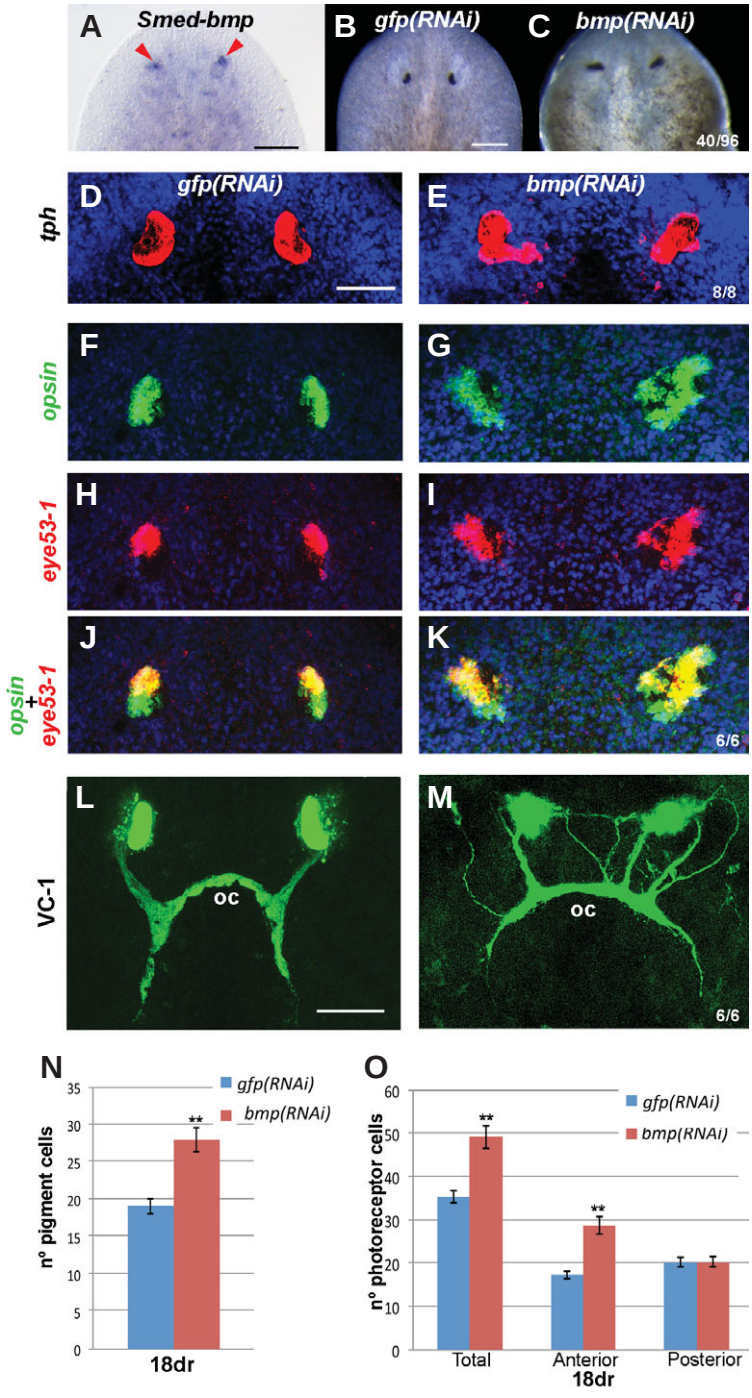


Fig. 7. Low doses of *bmp* RNAi result in expansion of pigment and anterior photoreceptor cells. (A) Smed-bmp *expression (arrowheads) in the eyes of intact planarians.* **(B,C)** *Compared to control planarians (B),* bmp(RNAi) *animals have larger, elongated eyes (C).* **(D,E)** tph *fluorescent* in situ *hybridization.* **(F-K)** *Double fluorescent* in situ *hybridization against* opsin **(F,G)** *and the anterior marker* eye53-1 **(H,I)**. *Note the expansion of the expression domain of* eye53-1 *in* bmp(RNAi) *animals.* **(L,M)** *Immunofluorescence against arrestin (VC-1) showing disorganized visual axonal projections in* bmp(RNAi) *animals.* **(N)** *Graphical representation of the number of pigment cells in control and* bmp(RNAi) *planarians.* **(O)** *Graphical representation of the number of photoreceptor cells in control and* bmp(RNAi) *planarians.* ***p<0.001 (*t *test).* **(B-M)** *Animals shown at 18 days of regeneration. Anterior is to the top. oc, optic chiasm. Scale bars, (A-C) 200 μm; (D-M) 50 μm.*

these results suggest an essential role for *Smed-smad6/7-2* activity in the specification and maintenance of the anterior subpopulation of eye photoreceptor cells.

The anterior population of photoreceptor cells expands after low doses of bmp (RNAi)

Loss of function of several elements of the BMP pathway disrupts regeneration of the planarian eyes, resulting in aberrant projections of the visual axons and supernumerary or fragmented eyes (Reddien *et al*., 2005a, 2007; Molina *et al*., 2007, 2011a; Orii and Watanabe, 2007). However, so far, only *Djbmp*, the homologue of *bmp* identified in the planarian species *Dugesia japonica*, has been found to be transiently expressed in the eyes at 6 days of regeneration (Mannini *et al*., 2008). We observed *Smed-bmp* expression in the eyes of intact *S. mediterranea* animals (Fig. 7A). Interestingly, the expression of *Smed-bmp* resembled that of *Smed-smad6/7-2* and seemed to target an anterior population of photoreceptor cells (arrowheads in Fig. 7A). Unfortunately, due to the weak expression levels of both *smad6/7-2* and *bmp* within the planarian eye, double *smad6/7-2* and *bmp* FISH did not allow us to determine whether *smad6/7-2* and *bmp* transcripts colocalize in the same cells.

To further characterize the eye phenotype associated with the loss of function of BMP signalling, we performed RNAi for *Smed-bmp*. Remarkably, we found that low doses of *bmp(RNAi)* resulted in regeneration of larger, elongated pigmentary cups (n=40/96, compare Fig. 7 B,C and D,E) that contained a larger number of pigment cells (19.1±1.0 in controls [n=7] versus 28.0±1.6 in *bmp(RNAi)* eyes [n=6]) (Fig. 7N). Moreover, *bmp(RNAi)* animals had larger numbers of photoreceptor cells (35.4±1.4 in controls [n=20] versus 49.2±2.6 in *bmp(RNAi)* eyes [n= 10]) (Fig. 7 F,G,O). Most interestingly, the increased number of photoreceptor cells after low doses of *bmp(RNAi)* was correlated with an increase in the anterior subpopulation of *eye53-1*-positive cells (17.3±0.9 in controls [n=12] versus 28.8±2.0 in *bmp(RNAi)* eyes [n=10]) (Fig.7 H-K,O), whereas there were no significant differences in the number of cells that constitute the posterior subpopulation compared to control organisms (20.3±1.1 in controls [n=12] versus 20.0±1.2 in *bmp(RNAi)* eyes [n= 10]) (Fig. 7O). Finally, as previously reported (Molina *et al*., 2007), VC-1 immunostaining after *bmp* silencing showed that axonal projections appeared disorganized compared to controls (Fig. 7 L,M).

The complementary phenotypes observed in *smad6/7-2(RNAi)* and *bmp(RNAi)* planarians suggest that the disappearance of *eye53-1*-labelled anterior photoreceptor cells after *smad6/7-2* silencing might be linked to an increase on BMP pathway activity in this cell population. Taken together, these results support an essential role of BMP signalling in specifying the number of anterior photoreceptor cells.

Discussion

I-Smads are potent antagonists of the BMP and TGFβ signalling pathways (reviewed in Wrana, 2000). Whereas two I-Smads, Smad6 and Smad7, have been described in verte-

brates, a single homologue is found in most invertebrate organisms. This study reports the isolation and functional characterization of the two I-Smad homologues in *S. mediterranea*: *Smed-smad6/7-1* and *Smed-smad6/7-2*. *Smed-smad6/7-1* and *Smed-smad6/7-2* may have arisen by internal gene duplication within the planarian lineage, as occurred in other planarian gene families (Reddien *et al*., 2005b; Palakodeti *et al*., 2008; Molina *et al.,* 2009).

Differences in the expression patterns of planarian I-Smads suggest that their functions might have diverged. The expression of s*mad6/7-1* in neoblasts, however, does not seem to be essential for stem cell survival and differentiation, as normal regeneration took place after *smad6/7-1* silencing. Similarly, although the expression of *smad6/7-2* in the CNS was especially interesting, since BMP signalling is known to act as a potent anti-neurogenic factor (reviewed in Harland, 2000), an apparently normal CNS regenerated after *smad6/7-2* silencing. Finally, in contrast to what would be expected for an antagonist of BMP signalling, no dorsalized planarians were observed after either single or double I-Smad silencing. Several rounds of RNAi treatment and regeneration are necessary to obtain partially dorsalized planarians after silencing the antagonist noggin (Molina *et al*., 2011a). In order to further evaluate the role of I-Smads in DV axis establishment, therefore, it would be interesting to determine whether combinatorial *noggins* and *smad6/7s* silencing could give rise to stronger dorsalized planarians.

The BMP pathway is essential for development and regeneration of the vertebrate eye (Haynes *et al*., 2007). In mice, different threshold levels of BMP signalling regulate distinct developmental programs (Murali *et al*., 2004). Similarly, DPP signalling triggers the retinal developmental program in *Drosophila* (reviewed in Voas and Rebay, 2004). The complementary phenotypes obtained after *smad6/7-2* silencing and low doses of *bmp* silencing support a role for this signalling pathway in planarian eye regeneration and maintenance. The anterior population of *eye53-1*-positive photoreceptor cells disappeared after upregulation of BMP signalling through RNAi of the inhibitor *smad6/7-2*, suggesting that higher levels of BMP signalling might disrupt the regeneration of this population of photoreceptor cells. In contrast, however, inhibition of the pathway by silencing the ligand *bmp* resulted in an increased number of *eye53-1*-positive photoreceptor cells. Neither *smad6/7-2* nor *bmp* silencing altered the number of posterior photoreceptor cells, suggesting that the establishment of the correct number of this cell type does not rely on BMP signalling. Taken together, our results suggest that specific levels of BMP signalling are necessary for re-specification and maintenance of the correct number of anterior photoreceptor cells in planarians. Further experiments will be necessary to determine the signalling molecules involved in specifying the number of posterior photoreceptor cells.

Previous studies in the planarian *D. japonica* have suggested that pigment and photoreceptor cells derive from common progenitor cells that express terminal differentiation markers of both cell types (Takeda *et al*., 2009). On the other hand, however, it has recently been shown that pigment and photoreceptor cell lineages can be separately traced from neoblasts in *S. mediterranea* and so they exist as distinct progenitor populations prior to terminal differentiation and aggregation in the eye (Lapan and Reddien, 2011). Although not completely contradicting the previous hypothesis, these results suggest an independent origin. Apart from this area of uncertainty, it seems clear that pigment and photoreceptor cells must interact to form and maintain the correct structure of the eye. A reduction in number of pigment cells after *egfr1-(RNAi),* for instance, is accompanied by a disorganization of photoreceptor cells (Fraguas *et al*., 2011). This disorganization, however, does not alter the number of photoreceptor cells (Fraguas *et al*., 2011). On the other hand, our data suggest that the variation in the number of photoreceptor cells could modulate the number of pigment cells. *smad6/7-2* silencing resulted in disappearance of the anterior population of *eye53-1*-positive cells and, consequently, a reduction in the total number of photoreceptor cells from early stages of regeneration. In contrast, pigment cells differentiated normally at 5 days of regeneration, but they started to diminish in number as regeneration proceeded, in parallel to the appearance of morphologically smaller and rounded pigment cups. Reciprocally, the increase in number of anterior photoreceptor cells after *bmp(RNAi)* was accompanied by a higher number of pigment cells. The delay in reduction of pigment cells observed after *smad6/7-2(RNAi)* suggest that the variation in the number of photoreceptor cells might induce the pigment cup to reorganize and adjust the cell number. Thereby, the change in the number of pigment cells could be a consequence of the variation in the number of anterior photoreceptor cells rather than a direct effect of *smad6/7-2* or *bmp* silencing. Further experiments would be necessary to understand how pigment and photoreceptor cell number are coordinated during planarian eye regeneration.

Materials and Methods

Organisms and gene nomenclature

Planarians used in these experiments belong to an asexual biotype of *S. mediterranea*, of the clonal line BCN-10 collected from an artificial spring in Montjuïc, Barcelona, Spain. The animals were maintained at 20°C in a 1:1 (v/v) mixture of distilled water and tap water treated with AquaSafe (TetraAqua, Melle, Germany). Animals were fed with organic veal liver and starved for at least a week before the experiments. Planarians 2 to 6 mm in length were used for all experiments. Genes and RNAi experiments were named using the nomenclature proposed by Reddien *et al.* (2008).

Isolation of S. mediterranea *inhibitory Smads*

I-Smad proteins from different animals were used to carry out tblastn searches on the genome assembly (v3.1, Washington University Sequencing Center, available at http://www.genome.wustl.edu) and the 454 transcriptome (Abril *et al*., 2010) of *S. mediterranea*. Sets of specific primers were designed to amplify predicted *Smed-smad6/7-1* and *Smed-smad6/7-2* homologues from cDNA made from total RNA using Superscript III (Invitrogen). The corresponding full-length transcripts were amplified by rapid amplification of cDNA ends (RACE) using the Invitrogen GeneRacer Kit (Invitrogen). GenBank accession numbers: *Smed-smad6/7-1,* JQ278719 and *Smed-smad6/7-2*, JQ278720.

RNAi analysis

Double-stranded RNAs (dsRNAs) for *Smed-smad6/7-1*, *Smed-smad6/7-2* and *Smed-bmp* were synthesized by *in vitro* transcription (Roche) as described previously (Sánchez Alvarado and Newmark, 1999). dsRNA microinjections were performed as described elsewhere (Sánchez Alvarado and Newmark, 1999) following the standard protocol of a 32 nl injection of dsRNA on three consecutive days. *Smed-smad6/7-1* and *Smed-smad6/7-2* dsRNA were injected at a concentration of 600 ng/μl, whereas dsRNA for *Smed-bmp* was injected at 250 ng/μl. Control animals were injected with dsRNA corresponding to GFP, a gene not found on the genome of *S. mediterranea*. For regeneration experiments, animals were amputated pre- and postpharyngeally 3 days after the first injection and allowed to regenerate. Unless otherwise indicated, all the results presented

refer to regenerating trunk pieces. To analyse the function of I-Smads during normal planarian homeostasis, intact uncut animals were re-injected 2 weeks after the first round of injections and analyzed 10 days after the second round of injections.

Irradiation

Intact planarians were γ-irradiated at 100 Grays (1.66 Gy/minute) with a Gammacell 1000 [Atomic Energy of Canada Limited] (Saló and Baguñà, 1985) and fixed for *in situ* hybridization 3 days after irradiation.

In situ hybridization

Whole mount *in situ* hybridizations were performed in an *In situ* Pro hybridization robot (Abimed/Intavis) as previously described (Molina *et al*., 2007, Umesono *et al*., 1997). For double FISH, animals were treated as described elsewhere (Pearson *et al*., 2009). Intact animals were processed for *in situ* hybridizations on paraffin sections as described previously (Cardona *et al*., 2005; Handberg-Thorsager and Saló, 2007). The following digoxigenin or fluorescein-labeled riboprobes were synthesized using an *in vitro* transcription kit (Roche): *Smed-smad6/7-1* and *Smed-smad6/7-2* (novel); *Smed-eye53-1* (Zayas *et al.*, 2005); *Smed-eye53-2*, *Smed-mpl-2* and *Smed-npp-12* (Collins *et al*., 2010); *Smed-bmp* (Molina *et al*., 2007); *Smed-tph* (Fraguas *et al.,* 2011) and *Smed-opsin* (Sánchez Alvarado and Newmark, 1999). Samples were observed through Leica MZ16F and Zeiss Stemi SV6 stereomicroscopes and a Zeiss Axiophot microscope; images were captured with a Nikon Coolpix E995 or Leica DFC300FX camera. Confocal laser scanning microscopy was performed with a Leica SP2.

Whole-mount immunostaining

Immunostaining was carried out essentially as described previously (Cebrià and Newmark, 2005). Anti-arrestin (VC-1) monoclonal antibody, which specifically recognized planarian photoreceptor cells, was used at dilution of 1/15,000 (Sakai *et al*., 2000). Highly cross-absorbed Alexa Fluor 488-conjugated goat anti-mouse IgG secondary antibody (Molecular Probes) was used at dilution of 1:400. Confocal laser scanning microscopy was performed with a Leica SP2.

Phototactic assay

Phototactic assay was carried out using a modified version of the method described by Inoue *et al.* (2004). Planarian behaviour was recorded for 180 seconds using an overhead digital video camera (Canon EOS550D). The behaviour analysis software SMART v.2.5.21 (Panlab, Spain) was used to quantify the time spent by the animals in each of the three virtual subdivisions of the transparent container of 60x30x10 mm, filled with 10 ml of planarian water. To obtain a light gradient, the container was protected by a black screen with a hole that allows the entrance of 500 lux of white light from one side of the container.

Acknowledgements

We would like to thank Francesc Cebrià and Marta Iglesias for critical reading of the manuscript, Dr. Hidefumi Orii and Prof. Kenji Watanabe for providing VC-1 and all members of the E. Saló, F. Cebrià, R. Romero and J.F. Abril groups for helpful discussions. We also thank Dr. Iain Patten for editorial advice. This work was supported by grant BFU2008-01544 from the Ministerio de Educación y Ciencia (MEC), Spain, and grant 2009SGR1018 (AGAUR) to E.S.; A.G-S. received a Master Fellowship from Caja España and a Beca de colaboración from the Ministerio de Educación; M.D.M. received an FPU fellowship from the Ministerio de Educación.

References

ABRIL, J.F., CEBRIÀ, F., RODRÍGUEZ-ESTEBAN, G., HORN, T., FRAGUAS, S., CALVO, B., BARTSCHERER, K., SALÓ, E. (2010). Smed454 dataset: unravelling the transcriptome of *Schmidtea mediterranea*. *BMC Genomics* 11: 731.

CARDONA, A., FERNÁNDEZ, J., SOLANA, J., ROMERO, R. (2005). An *in situ* hybridization protocol for planarian embryos: monitoring myosin heavy chain gene expression. *Dev Genes Evol* 215: 482-488.

CEBRIÀ, F., 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.

COLLINS, J.J., HOU, X., ROMANOVA, E.V., LAMBRUS, B.G., MILLER, C.M., SABERI, A., SWEEDLER, J.V., NEWMARK P.A. (2010). Genome-wide analyses reveal a role for peptide hormones in planarian germline development. *PLOS Biology* 8, e1000509.

DUBOIS, F. (1949). Contribution à l'étude de la migration des cellules de regeneration chez les planaires dulcicoles. *Bull Biol Fr Belg* 83: 213-283.

EISENHOFFER, G.T., KANG, H., 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* 11: 327-339.

FRAGUAS, S., BARBERÁN, S., CEBRIÀ, F. (2011). EGFR signalling regulates cell proliferation, differentiation and morphogenesis during planarian regeneration and homeostasis. *Dev Biol* 354: 87-101.

HANDBERG-THORSAGER, M., SALÓ, E. (2007). The planarian nanos-like gene Smednos is expressed in germline and eye precursor cells during development and regeneration. *Dev Genes Evol* 217: 403-411.

HANDBERG-THORSAGER, M., FERNÁNDEZ E., SALÓ, E. (2008). Stem cells and regeneration in planarians. *Front Biosci* 13: 6374-6394.

HARLAND, R. (2000). Neural induction. *Curr Opin Genetics Dev* 10: 357-362.

HAYNES, H., GUTIÉRREZ, C., AYCINENA, JC, TSONIS, P.A., DEL RIO-TSONIS, K. (2007). BMP signaling mediates stem/progenitor cell-induced retina regeneration. *Proc Natl Acad Sci USA* 104: 20380–20385.

INOUE, T., KUMAMOTO, H., OKAMOTO, K., UMESONO, Y., SAKAI, M., SÁNCHEZ ALVARADO A., AGATA, K. (2004). Morphological and functional recovery of the planarian photosensing system during head regeneration. *Zool Sci* 21: 275-283.

LAPAN, S.W., REDDIEN, P.W. (2011). *dlx* and *sp6-9* control optic cup regeneration in a prototypic eye. *PLOS Genetics* 7: e1002226.

LITTLE, S.C., MULLINS, M.C. (2006). Extracellular modulation of BMP activity in patterning the dorsoventral axis. *Birth Defects Research (Part C)* 78: 224-242.

LIU, A., NISWANDER, L.A. (2005) Bone morphogenetic protein signalling and vertebrate nervous system development. *Nature Rev Neurosci* 6: 945-954.

MANNINI, L., ROSSI, L., DERI, P., GREMIGNI, V., SALVETTI, A., SALÓ, E., BATISTONI, R. (2004). *Djeyes absent (Djeya)* controls prototypic planarian eye regeneration by cooperating with the transcription factor *Djsix-1*. *Dev Biol* 269: 346-359.

MANNINI, L., DERI, P., GREMIGNI, V., ROSSI, L., SALVETTI, A., BATISTONI, R. (2008). Two *msh/msx*-related genes, *Djmsh1* and *Djmsh2*, contribute to the early blastema growth during planarian head regeneration. *Int J Dev Biol* 52: 943-952.

MOLINA, M.D., SALÓ, E., 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.

MOLINA, M.D., SALÓ, E., CEBRIÀ, F. (2009). Expression pattern of the expanded noggin gene family in the planarian *Schmidtea mediterranea*. *Gene Expr Patterns* 9: 246-253.

MOLINA, M.D., NETO, A., MAESO, I., GÓMEZ-SKARMETA, J.L., SALÓ, E., CEBRIÀ, F. (2011a). Noggin and Noggin-Like genes control dorsoventral axis regeneration in planarians. *Curr Biol* 21: 300-305.

MOLINA, M.D., SALÓ, E., CEBRIÀ, F. (2011b). Organizing the DV axis during planarian regeneration. *Commun Integr Biol* 4: 498-500.

MURALI, D., YOSHIKAWA, S., CORRIGAN, R.R., PLAS, D.J., CRAIR, M.C., OLIVER, G., LYONS, K.M., MISHINA, Y., FURUTA, Y. (2004). Distinct developmental programs require different levels of Bmp signalling during mouse retinal development. *Development* 132: 913-923.

OKAMOTO, K., TAKEUCHI, K., AGATA, K. (2005). Neural projections in planarian brain revealed by fluorescent dye tracing. *Zoolog Sci* 22: 535-546.

ORII, H., KATAYAMA, T., SAKURAI, T., AGATA, K., WATANABE, K. (1998). Immunohistochemical detection of opsins in turbellarians. *Hydrobiologia* 383: 183-187.

ORII, H., WATANABE, K. (2007). Bone morphogenetic protein is required for dorsoventral patterning in the planarian *Dugesia japonica*. *Dev Growth Differ* 49: 345-349.

PALAKODETI, D., SMIELEWSKA, M., LU, Y.C., YEO, G.W., GRAVELEY, B.R. (2008). The PIWI proteins SMEDWI-2 and SMEDWI-3 are required for stem cell function and piRNA expression in planarians. *RNA* 14: 1174-1186.

PEARSON, B.J., EISENHOFFER, G.T., GURLEY, K.A., RINK, J.C., MILLER, D.E., SÁNCHEZ ALVARADO, A. (2009). Formaldehyde-based whole mount *in situ* hybridization method for planarians. *Dev Dyn* 238: 443-450.

PINEDA, D., GONZÁLEZ, J., CALLAERTS, P., IKEO, K., GEHRING, W.J., SALÓ, E. (2000). Searching for the prototypic eye genetic network: *sine oculis* is essential for eye regeneration in planarians. *Proc Natl Acad Sci USA* 97: 4525-4529.

PINEDA, E., ROSSI, L., BATISTONI, R., SALVETTI, A., MARSAL, M., GREMIGNI, V., FALLENI, A., GONZÁLEZ-LINARES, J., DERI, P., SALÓ, E. (2002). The genetic network of prototypic planarian eye regeneration is Pax6 independent. *Development* 129: 1423-1434.

QUILLIEN, A., BLANCO-SÁNCHEZ, B., HALLUIN, C., MOORE, J.C., LAWSON, N.D., BLADER, P., CAU, E. (2011). BMP signaling orchestrates photoreceptor specification in the zebrafish pineal gland in collaboration with Notch. *Development* 138: 2293-2302.

REDDIEN, P.W., BERMANGE, A. L., MURFITT, K.J., JENNINGS, J.R., SÁNCHEZ ALVARADO, A. (2005a). 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., OVIEDO, N.J., JENNINGS, J.R., SÁNCHEZ ALVARADO, A. (2005b). SMEDWI-2 is a PIWI-like protein that regulates planarian stem cells. *Science* 310: 1327-1330.

REDDIEN, P.W., BERMANGE, A. L., KICZA, A.M., SÁNCHEZ ALVARADO, A. (2007). BMP signaling regulates the dorsal planarian midline and is needed for asymmetric regeneration. *Development* 134: 4043-4051.

REDDIEN, P.W., NEWMARK P.A., SÁNCHEZ ALVARADO, A. (2008). Gene nomenclature guideline for the planarian *Schmidtea mediterranea*. *Dev Dyn* 237: 3099-3101.

SAKAI, F., AGATA, K., ORII, H., WATANABE, K. (2000). Organization and regeneration ability of spontaneous supernumerary eyes in planarians – Eye regeneration field and pathway selection by optic nerves. *Zool Sci* 17: 375-381.

SALÓ, E. BAGUÑÀ, J. (1985). Cell movement in intact and regenerating planarians. Quantitation using chromosomal, nuclear and cytoplasmic markers. *J Embryol Exp Morphol* 89: 57-70.

SALÓ, E. (2006). The power of regeneration and the stem-cell kingdom: freshwater planarians (platyhelminthes). *Bioessays* 28: 546-559.

SALÓ, E., BATISTONI, R. (2008). The planarian eye: a simple and plastic system with great regenerative capacity. In "*Animal models in eye research*" (P. A. Tsonis, Ed.), pp. 15-26) Elsevier Academic Press.

SÁNCHEZ ALVARADO, A., NEWMARK, P.A. (1999). Double-stranded RNA specifically disrupts gene expression during planarian regeneration. *Proc Natl Acad Sci USA* 96: 5049-5054.

SJÖDAL, M., EDLUND, T., GUNHAGA, L. (2007). Time of exposure to BMP signals plays a key role in the specification of the olfactory and lens placodes *ex vivo*. *Dev Cell* 13: 141-149.

TAKEDA, H., NISHIMURA, K., AGATA, K. (2009). Planarians maintain a constant ratio of different cell types during changes in body size by using the stem cell system. *Zool Sci* 26: 805-813.

UMESONO, Y., WATANABE, K., 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.

VOAS, M.G., REBAY, I. (2004). Signal integration during development: insights from the *Drosophila* eye. *Dev Dyn* 229: 162-175.

WRANA, J.L. (2000). Crossing Smads. *Sci STKE* 2000: re1.

ZAYAS, R.M., HERNÁNDEZ, A., HABERMANN, B., WANG, Y., STARY, J.M., NEWMARK, P.A. (2005).The planarian *Schmidtea mediterranea* as a model for epigenetic germ cell specification: analysis of ESTs from the hermaphroditic strain. *Proc. Natl. Acad. Sci. USA* 102: 18491-18496.

ZHAO, S., CHEN, Q., HUNG, F-C., OVERBEEK, P.A. (2002). BMP signaling is required for development of the ciliary body. *Development* 129: 4435-4442.

Further Related Reading, published previously in the *Int. J. Dev. Biol.*

Planarian regeneration: achievements and future directions after 20 years of research
Emili Saló, Josep F. Abril, Teresa Adell, Francesc Cebriá, Kay Eckelt, Enrique Fernández-Taboada, Mette Handberg-Thorsager, Marta Iglesias, M Dolores Molina and Gustavo Rodríguez-Esteban
Int. J. Dev. Biol. (2009) 53: 1317-1327

Expression of a retinal homeobox (Rx) gene during planarian regeneration
Linda Mannini, Paolo Deri, Jacopo Picchi and Renata Batistoni
Int. J. Dev. Biol. (2008) 52: 1113-1117

Two msh/msx-related genes, Djmsh1 and Djmsh2, contribute to the early blastema growth during planarian head regeneration
Linda Mannini, Paolo Deri, Vittorio Gremigni, Leonardo Rossi, Alessandra Salvetti and Renata Batistoni
Int. J. Dev. Biol. (2008) 52: 943-952

From Planarians to Mammals - the many faces of regeneration
Jerzy Moraczewski, Karolina Archacka, Edyta Brzoska, Maria-Anna Ciemerych, Iwona Grabowska, Katarzyna Janczyk-Ilach, Wladyslawa Streminska and Malgorzata Zimowska
Int. J. Dev. Biol. (2008) 52: 219-227

The genetic control of eye development and its implications for the evolution of the various eye-types
Walter J Gehring
Int. J. Dev. Biol. (2002) 46: 65-73

5 yr ISI Impact Factor (2010) = 2.961