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Two *msh/msx*-related genes, *Djmsh1* and *Djmsh2*, contribute to the early blastema growth during planarian head regeneration

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ABSTRACT Regeneration in planarians is an intriguing phenomenon, based on the presence of pluripotent stem cells, known as neoblasts. Following amputation, these cells activate mitotic divisions, migrate distally and undergo differentiation, giving rise to the regeneration blastema. We have identified two *msh*/*msx*-related genes, *Djmsh1* and *Djmsh2,* which are expressed in distinct cell populations of the planarian *Dugesia japonica* and activated, with different patterns, during head regeneration. We demonstrate that RNA interference of *Djmsh1* or *Djmsh2* generates a delay in the growth of cephalic blastema, interfering with the dynamics of mitoses during its initial formation. Our data also reveal that the activity of the two planarian *msh* genes is required to regulate *Djbmp* expression during head regeneration. This study identifies, for the first time, a functional association between muscle segment homeobox (MSH) homeoproteins and BMP signaling during stem cell-based regeneration of the planarian head and provides a functional analysis of how *msh* genes may regulate *in vivo* the regenerative response of planarian stem cells.

KEY WORDS: *planarian, regeneration, msh/msx, RNAi, bmp*

Introduction

MSX homeoproteins related to the *Drosophila muscle-segment homeobox* (*msh*) gene product, represent key regulatory factors in the control of cell differentiation during embryogenesis (Davidson, 1995). Several studies demonstrate that vertebrate MSX proteins function as transcriptional repressors in pathways that integrate proliferative, apoptotic and differentiation signals to generate organs of correct shape and size (Bendall and Abate-Shen, 2000; Hu *et al*., 2001; Kwang *et al*., 2002; Lee *et al*., 2004). Mechanisms are emerging for how MSX proteins exert their effects. For example it has been found in mouse that MSX1 inhibits the differentiation of multiple mesenchymal and epithelial cell types by maintaining high levels of *cyclin D1* expression (Hu *et al*., 2001). In addition MSX1 prevents myogenic differentiation by direct repression of regulatory genes (Kuwajima *et al*., 2004; Lee *et al*., 2004; Brunelli and Cossu, 2005; Lee *et al*., 2006). MSH/MSX-related transcriptional repression is also implicated in neural development of flies and vertebrates (Ramos and Robert, 2005). In *Drosophila*, MSH is involved in dorso-ventral patterning of the neural progenitors, being mainly required for proper division of some lateral neuroblasts and for their ability to migrate correctly (Ramos and Robert, 2005). Conversely, the multiple *msx* genes of vertebrates appear to control neural tube formation with distinct activities, including control of programmed cell death, regulation of proliferation or inhibition of neural differentiation by repressing regulatory genes (Tribulo *et al*., 2003; Wu *et al*., 2003; Liu *et al*., 2004). Recent studies on the development of neural crests also support the possibility that MSX homeoproteins are essential components of a core of transcription factors that could integrate multiple signals setting the 'context', i.e. the stage-dependent specificity of regulatory pathways (Raible, 2006).

A number of organisms in the animal kingdom show the fascinating property to regenerate lost body parts, based on the ability to dedifferentiate specialized cell types and/or activate

Abbreviations used in this paper: msh, muscle segment homeobox.

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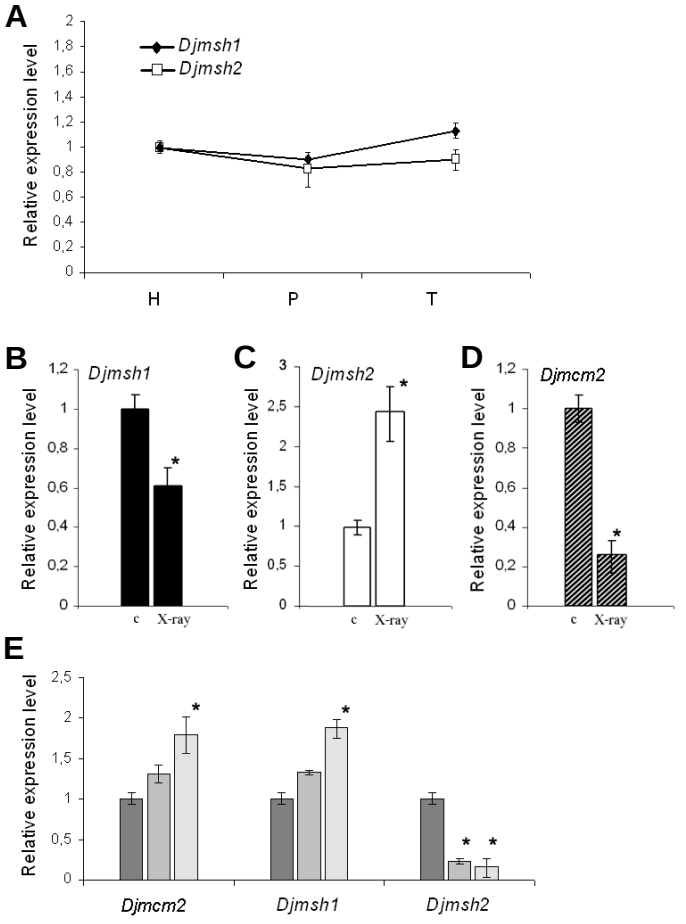

Fig. 1. Real time RT-PCR analysis of *Djmsh1* and *Djmsh2* expression in intact planarians. (A) *Expression level of* Djmsh1 *and* Djmsh2 *in different body regions. H, head; P, pharynx; T, tail. Expression levels are indicated in relative units assuming as unitary the value of the head region. Each value is the mean ± standard deviation of three independent samples, performed in duplicate.* **(B-D)** *Expression level of* Djmsh1 **(B)**, Djmsh2 **(C)** *and* Djmcm2 *(neoblast marker)* **(D)***, 2 days after X-ray irradiation. Expression levels are indicated in relative units, assuming the value of untreated specimens (c: control) as unitary. Each value is the mean ± standard deviation of three independent samples, analyzed in duplicate. The expression values of* Djmsh1, Djmsh2 *and* Djmcm2 *at 2 days after X-ray irradiation were compared with those of control using the unpaired t-test. *P< 0.05.* **(E)** *Expression levels of* Djmcm2, Djmsh1 *and* Djmsh2 *in cell fractions obtained by filtration through nylon meshes of 50 μm (dark gray), 20 μm (gray) and 8 μm (light gray) pore size. Expression levels are indicated in relative units, assuming the value of 50 μm nylon meshes as unitary. Each value is the mean ± standard deviation of three independent samples, analyzed in duplicate. The expression values of 8 μm and 20 μm nylon meshe samples were compared with those of 50 μm nylon meshe samples using the unpaired t-test. *P< 0.05.*

endogenous stem cell reservoirs to form a regeneration blastema (reviewed in Sánchez Alvarado and Tsonis, 2006). Several studies in vertebrate model systems suggest that the activity of *msx* genes is essential for regeneration (Akimenko *et al.*, 1995; Reginelli *et al.*, 1995; Koshiba *et al.*, 1998; Echeverri and Tanaka, 2002; Beck *et al.*, 2003; Han *et al.*, 2003; Thummel *et al.*, 2006). It has been recently demonstrated that morpholino-induced down-regulation of *msxb* inhibits blastema growth during fin regeneration in zebrafish, suggesting that MSXB controls the rate of blastemal cell proliferation (Thummel *et al.*, 2006). MSX1 represents a pivotal regulator of the regenerative cellular plasticity in urodeles, activating de-differentiation and mitotic division of cells that can then be reprogrammed to differentiate (Kumar *et al.*, 2004; Odelberg, 2004). A hyperactive form of MSX1 can restore regenerative abilities during refractory period in tails of transgenic *Xenopus* tadpoles (Beck *et al.*, 2003), while null mutations in its mammalian ortholog arrest digit tip regeneration of fetal mice (Han *et al.*, 2003). Although several functional and expression studies highlight the importance of *msh/msx* genes in a variety of regenerative processes, that make extensive use of dedifferentiation/transdifferentiation strategy, the functional relevance of these genes in a regenerative process that employs stem cells, such as that of planarians, is completely unknown.

Planarian flatworms (Platyhelminthes, Lophotrochozoa) are unique for their stem cell-based ability to regenerate complete organisms from small fragments of the body (recently reviewed in Agata *et al.*, 2006; Saló, 2006; Sánchez Alvarado, 2006). Their exceptional regenerative capability is in fact due to neoblasts, stem cells located in the mesenchymal tissue (parenchyma), with the potential to differentiate into all specialized cell types. After amputation, epithelial-parenchymal interaction at wound epithelium and signals from the neighbouring damaged tissues (Kato *et al.*, 2001) activate proliferation of neoblasts in the stump region about 0-300 μm beneath the wound (postblastema), allowing regeneration to proceed. Cells produced by the mitotic activity migrate and progressively accumulate under the wound epithelium, forming a regenerative blastema. Blastemal cells undergo differentiation into new tissues and organs, while the stump is drastically remodeled. Although functional characterization of an increasing collection of genes begins to molecularly dissect the regeneration process in this model system, surprisingly little is understood regarding to how the blastema forms.

In this work we report the characterization of two *msh/msx*-related genes, *Djmsh1* and *Djmsh2,* whose expression appears specifically activated, with distinct patterns, during head regeneration in the planarian *Dugesia japonica*. We demonstrate by RNA interference (RNAi) that down-regulation of these two genes produces a delay in the growth of cephalic blastema, interfering on the dynamics of mitoses during its initial formation. We also provide functional data that implicate DjMSH1 and DjMSH2 in the regulation of the expression of *Djbmp*, a member of the BMP/transforming growth factor-β _TGF-β_ gene family, critical not only for the dorso-ventral (DV) body patterning (Orii *et al.*, 1998; Orii and Watanabe, 2007), but also for the induction of regeneration in these organisms (Sánchez Alvarado and Tsonis 2006; Molina *et al.*, 2007). These studies provide the first functional analysis of how *msh* genes may regulate *in vivo* the regenerative response of planarian stem cells.

Results

Cloning of planarian msh *genes*

Using a 3'RACE method and primers corresponding to some conserved parts of the homeodomain, several gene fragments containing the 3' end of the homeodomain were isolated in *D. japonica*. Two sequences encoding a portion of an MSH/MSX-type homeodomain were then completed and named *Djmsh1* and

Djmsh2. Djmsh1 and *Djmsh2* cDNAs revealed an open reading frame of 663 bp and 666 bp, respectively. DjMSH1 showed the closest similarity to *Branchiostoma floridae* MSX (e-value $1e^{-32}$), while DjMSH2 was similar to *Mus musculus* MSX1 (e-value $3e^{-30}$). Although the residue-specific signatures in the homeodomain and flanking regions appeared to be well conserved between the two genes, the remaining part of the sequence displayed a significant divergence. In fact, DjMSH1 and DjMSH2 shared only 36% overall amino acid identity, while 97% identity was found in the homeodomain plus the flanking regions. In addition, the residues considered as essential for mediating TATA binding protein (TBP) interaction and transcriptional repression (Zhang *et al.,* 1996) and a sequence related to the eh1 Engrailed repressive motif appeared well conserved in the N-terminal region of both sequences, suggesting that DjMSH1 and DjMSH2 function as transcriptional repressors (Supplementary Fig. S1A; see doi: 10.1387/ijdb.072476lm). An *in silico* search in the genome of the planarian *Schmidtea mediterranea* enabled us to isolate two putative orthologs of DjMSH1 and DjMSH2: SmedMSH1 (similarity to DjMSH1: 87.4%) and SmedMSH2 (similarity to DjMSH2: 82.6%). A comparison of the homeodomain and the flanking regions of the planarian MSH1 and MSH2 with those of published MSH/MSX proteins from different taxa is shown (Supplementary Fig. S1B).

Djmsh1 *and* Djmsh2 *expression in intact planarians*

As our attempts to unambiguously visualize *Djmsh1* and *Djmsh2* expression in intact animals by *in situ* hybridization were unsuccessful under our experimental conditions, we used real time RT-PCR to analyze their expression levels in various body regions (head, pharynx and tail) and observed that both genes were expressed, at similar levels, throughout the planarian body (Fig. 1A). We also performed real time RT-PCR in intact animals sacrificed two days after a lethal dose of X-rays, a treatment that destroys neoblasts (Salvetti *et al.,* 2000; Reddien *et al.,* 2005b; Hayashi *et al.,* 2006; Rossi *et al.,* 2007), and noted that irradiation produced a significant down-regulation of *Djmsh1*, while *Djmsh2* expression level increased (Fig. 1B,C). Comparison of the expression level of the neoblast marker *Djmcm2* before and after irradiation was shown as a control (Fig. 1D). These results may be explained by the fact that *Djmsh1* is mainly expressed in X-ray-sensitive cells (cells that rapidly disappear following X-ray irradiation). On the other hand, *Djmsh2* expression may have been induced, as a stress response, in some irradiation-insensitive cells (cells that survive X-ray irradiation). To assess whether *Djmsh2* is a stress-responsive gene we compared *Djmsh1* and *Djmsh2* expression levels in planarians after a heat shock (28°C o/n), and observed that this treatment activated *Djmsh2,* but did not produce any significant variation in the expression level of *Djmsh1* (Supplementary Fig. S2). To extend our knowledge on the cells that express the planarian *msh* genes, we performed real time RT-PCR using RNA obtained from dissociated cell fractions, enriched in neoblasts by progressive filtering (Salvetti *et al.,* 2005; Rossi *et al.,* 2007). We found that, as for the neoblast marker *Djmcm2, Djmsh1* expression was preferentially detected in the fractions enriched in small cells, while *Djmsh2* transcripts were concentrated in the fraction that included large cells (Fig. 1E).

Djmsh1 *and* Djmsh2 *expression is activated during head regeneration*

To investigate the expression profile of the two *msh* genes during regeneration, RNA obtained from a small region including blastema and postblastema of head and tail fragments was analyzed by real time RT-PCR (Fig. 2A). The results demonstrated that both genes were strongly activated in fragments regenerating a new head (anterior regeneration), whereas no significant activation was observed in fragments regenerating a tail (posterior regeneration) (Fig. 2B). Specific activation of *Djmsh1* and *Djmsh2* expression during head regeneration was also confirmed by whole mount *in situ* hybridization (Fig. 3). *Djmsh1* transcripts were first observed at day 2 of regeneration in a central region of the dorsal side, at the boundary of the old tissue and the blastema (Fig. 3A). As regeneration proceeded, *Djmsh1* expression extended dorsally along the midline in the blastema (Fig. 3B,C), however the level of the hybridization signal became progressively fainter and was no longer detectable when regeneration was completed. *Djmsh2* expression was clearly visualized

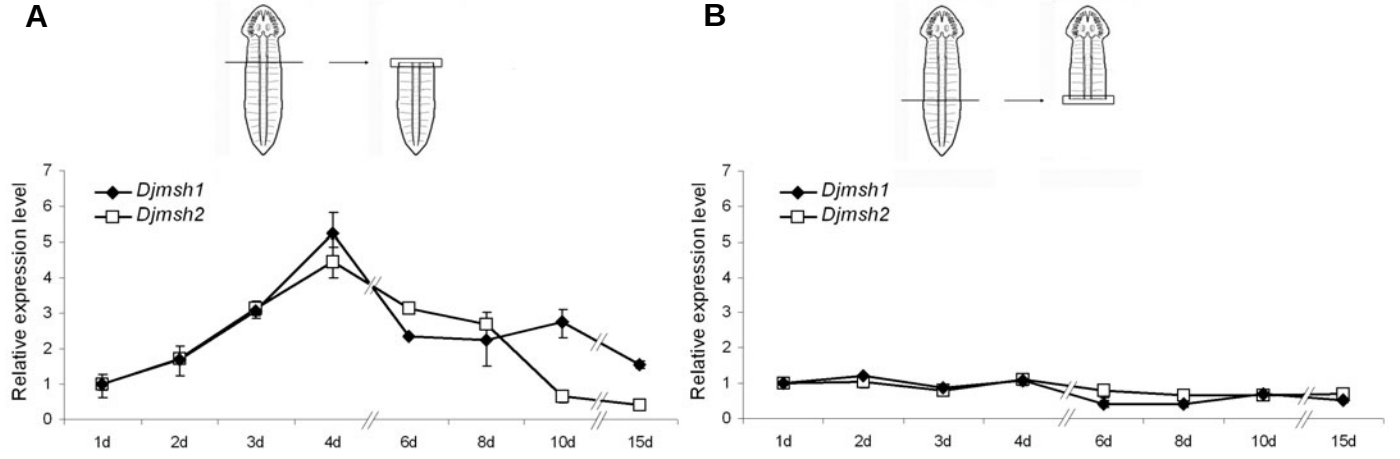

Fig. 2. Real time RT-PCR analysis of *Djmsh1* and *Djmsh2* expression in regenerating planarians. Djmsh1 *and* Djmsh2 *expression levels during head* **(A)** *or tail regeneration* **(B)**. *d, days of regeneration. For both experiments, the expression levels are indicated in relative units, assuming the value of one day-regenerating fragments as unitary. Each value is the mean ± s.d. of two independent samples, analyzed in duplicate.*

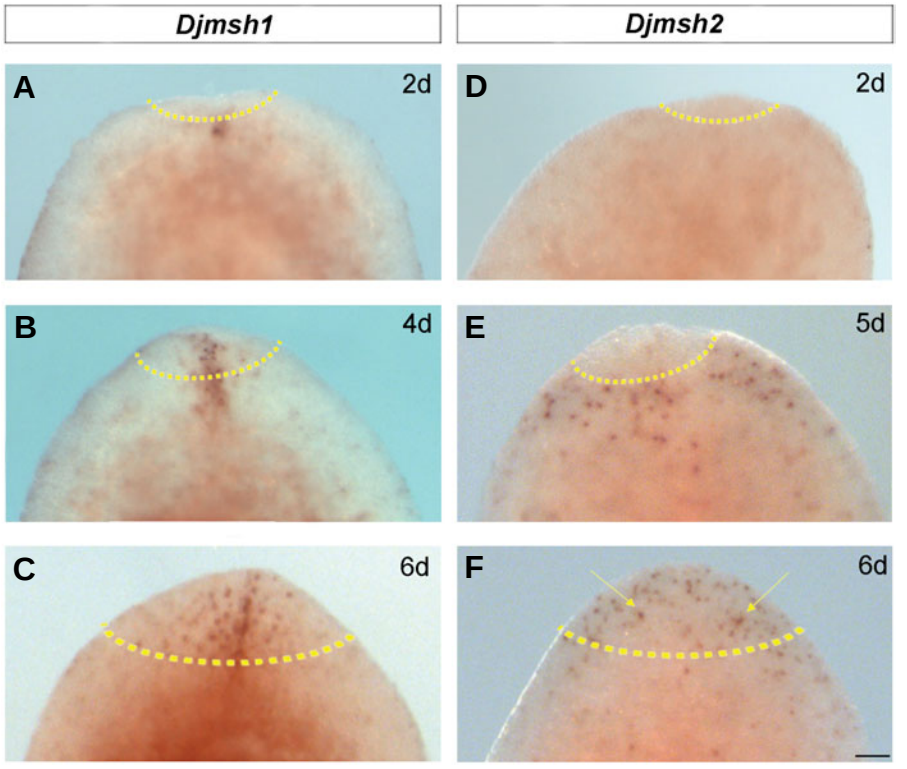

Fig. 3. Expression of *Djmsh1* and *Djmsh2* during head regeneration. *Dorsal view of the regenerating fragments visualized by whole mount* in situ *hybridization with Djmsh1* **(A-C)** *and Djmsh2* **(D-F)**. *d, days of regeneration. Anterior is to the top. The dashed yellow line indicates the border between the regenerating region and the stump. Yellow arrows indicate regenerating eyes in (F). Scale bar, 50 μm.*

around days 4-5 of regeneration in cells that were spread beneath the blastema (Fig. 3D,E). Around day 6 *Djmsh2*-expressing cells were mainly scattered in the regenerating area. At this stage of regeneration, *Djmsh2* hybridization signal was also observed in the regenerating eyes of some specimens, suggesting a temporary requirement for this gene during the formation of these structures (Fig. 3F). In the following days of regeneration, *Djmsh2* expression declined at undetectable levels and was no longer observed when regeneration was completed.

Silencing of the two msh *planarian genes delays the growth of the cephalic blastema*

To establish how *Djmsh1* and *Djmsh2* function, we evaluated the effects of their silencing by RNAi on intact animals and regenerating fragments. RNAi of both *Djmsh1* and *Djmsh2* caused no abnormal phenotypes in intact specimens or in fragments regenerating a tail (Fig. 4A-E), but produced a significant delay in the growth of the blastema in fragments that regenerated a head *(Djmsh1* RNAi: 87/145; *Djmsh2* RNAi: 74/120). These phenotypes were observed better within the first 5-6 days of regeneration (Fig. 4F-J; Supplementary Fig. S3A,B). An analysis of the ratio between the blastema and the stump area provided quantitative evidence that RNAi-mediated down-regulation of these genes led to a very significant ($p<10^{-6}$) reduction in the relative size of the cephalic blastema (Fig. 4J). A visualization of the central nervous system by *Djsyt* expression (Salvetti *et al.,* 2000) in the injected specimens also confirmed a reduction in the size of the regenerating brain (Fig. S4). As the regeneration proceeded, the small blastemas returned to a normal size and the missing parts differentiated normally. No substantial difference in type and percentage of the phenotypes was found when RNAi was triggered by dsRNA generated by a different sequence region lying outside of the homeobox of both *Djmsh1* (1 to 258 bp) and *Djmsh2* (2 to 238 bp) or by coinjection of an equimolar mixture of *Djmsh1* + *Djmsh2* dsRNA. In addition, no significant change in the expression level of the cognate gene was observed when we analyzed, using real time RT-PCR, *Djmsh1* in *Djmsh2* RNAi animals, as well as *Djmsh2* in *Djmsh1* RNAi animals. As the blastema size depends on the mitotic activity of neoblasts, we explored the possibility that RNAi of the two *msh* genes might affect the dynamics of mitoses during head regeneration, by mitotic index analysis. We counted the mitotic metaphases in wild type regenerants (controls), and in regenerants after *Djmsh1* or *Djmsh2* RNAi, and compared the percentage of mitoses at different times of regeneration (Fig. 4K-M). Under our experimental conditions, a rapid increase in mitotic activity was observed in controls during the first day of regeneration. After a sharp reduction (Fig. 4K: 30h), a second and higher maximum of mitotic activity was observed at 54 hours of regeneration (Fig. 4K: 54h). As regeneration proceeded, no further significant peak of mitoses was visualized. This bimodal dynamics of mitotic activity, which is supported by previous studies (Baguñá, 1976; Saló and Baguñá, 1984), appeared modified in planarians that regenerated a head after RNAi treatment. We noted that, after *Djmsh1* RNAi, the fragments did not activate the first burst of proliferation (Fig. 4L: 6h-22h). An increase in the percentage of mitotic cells was only observed after the first day of regeneration and prolonged until the day 5 (Fig. 4L: 22h-5d). At later stages, the percentage of mitoses was again comparable to that of the controls (Fig. 4L: 6d-15d). A different behavior was seen in regenerants after *Djmsh2* RNAi treatment. At the onset of regeneration, we detected a transient activation of mitotic activity (Fig. 4M, 6h), which rapidly decreased to the level found in intact animals (Fig. 4M, 6h-18h). As regeneration proceeded, the percentage of mitoses remained low in these fragments. Interestingly, when we analyzed the number of mitoses in intact planarians, we did not observe any significant difference in the mitotic activity of animals injected with *Djmsh1* or *Djmsh2* dsRNA, with respect to the controls (Fig. S5A). To further support the possibility that inhibition of the function of *Djmsh1* or *Djmsh2* resulted in a reduction in cell proliferation and, consequently, in a reduced number of neoblasts involved in regeneration, we also analyzed by real time RT-PCR the expression level of the neoblast marker *Djmcm2* (Salvetti *et al.,* 2000) in controls and in RNAi-treated planarians, after 6 days of regeneration. The results provided evidence that a substantial reduction in the level of *Djmcm2* transcripts occurred both in *Djmsh1*- and *Djmsh2* RNAi regenerating animals (Fig. 4N).

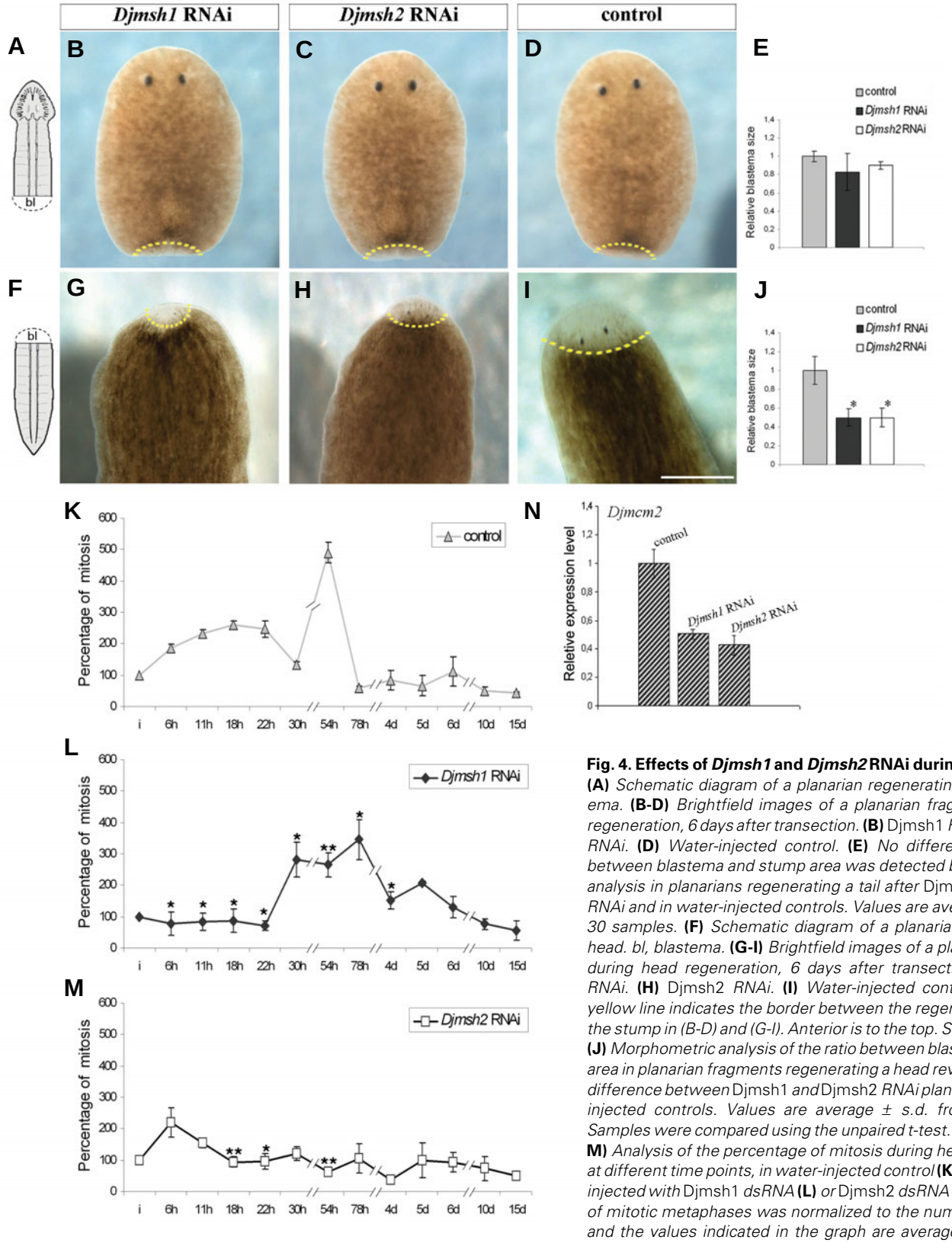

Fig. 4. Effects of *Djmsh1* and *Djmsh2* RNAi during regeneration. **(A)** *Schematic diagram of a planarian regenerating a tail. bl, blastema.* **(B-D)** *Brightfield images of a planarian fragment during tail regeneration, 6 days after transection.* **(B)** Djmsh1 *RNAi.* **(C)** Djmsh2 *RNAi.* **(D)** *Water-injected control.* **(E)** *No difference in the ratio between blastema and stump area was detected by morphometric analysis in planarians regenerating a tail after* Djmsh1 *and* Djmsh2 *RNAi and in water-injected controls. Values are average ± s.d. from 30 samples.* **(F)** *Schematic diagram of a planarian regenerating a head. bl, blastema.* **(G-I)** *Brightfield images of a planarian fragment during head regeneration, 6 days after transection.* **(G)** Djmsh1 *RNAi.* **(H)** Djmsh2 *RNAi.* **(I)** *Water-injected control. The dashed yellow line indicates the border between the regenerating area and the stump in (B-D) and (G-I). Anterior is to the top. Scale bar, 250 μm.* **(J)** *Morphometric analysis of the ratio between blastema and stump area in planarian fragments regenerating a head reveals a significant difference between* Djmsh1 *and* Djmsh2 *RNAi planarians and water-injected controls. Values are average ± s.d. from 30 samples. Samples were compared using the unpaired t-test. *P<0.00001.* **(K-M)** *Analysis of the percentage of mitosis during head regeneration, at different time points, in water-injected control* **(K)**, *and in samples injected with* Djmsh1 *dsRNA* **(L)** *or* Djmsh2 *dsRNA* **(M)**. *The number of mitotic metaphases was normalized to the number of total cells and the values indicated in the graph are average ± s.d. of three independent samples, assuming as 100% the value of intact planarians. Samples were compared using the unpaired t-test. *P<0.05, **P< 0.001. i, intact planarian; h, hours of regeneration; d, days of regeneration.* **(N)** *Expression level of* Djmcm2, *analyzed by real time RT-PCR in water-injected controls and in* Djmsh1 *and* Djmsh2 *RNAi planarians. In all experiments, the expression levels are indicated in relative units, assuming as unitary the value of the controls. Each value is the mean ± s.d. of two independent RNAi experiments, carried out in duplicate.*

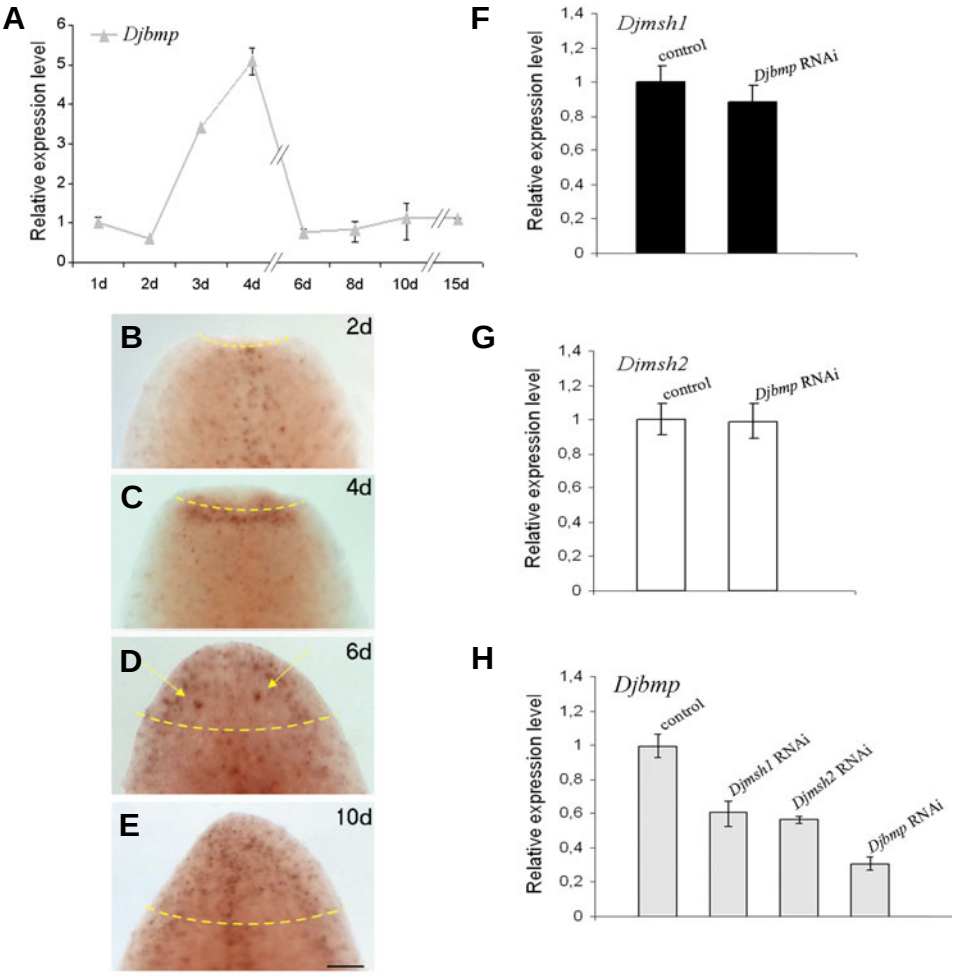

Fig. 5. Relationship between planarian *msh* genes and *Djbmp*. (A) *Real time RT-PCR analysis of* Djbmp *expression during head regeneration, d: days of regeneration. The expression level is indicated in relative units, assuming the value of one day-regenerating fragments as unitary. Each value is the mean ± s.d. of two independent samples, analyzed in duplicate.* **(B-E)** *Dorsal view of the regenerating fragments visualized by whole mount* in situ *hybridization with* Djbmp*. Anterior is to the top. The dashed yellow line indicates the border between the regenerating region and the stump in (B-E). Yellow arrows indicate the regenerating eyes in (D). Scale bar, 100 μm.* **(F-H)** *Real time RT-PCR analysis.* **(F)** Djmsh1 *expression level in water-injected controls and in* Djbmp *RNAi planarians, 6 days after transection.* **(G)** Djmsh2 *expression level in water-injected controls and in* Djbmp *RNAi planarians, 6 days after transection.* **(H)** Djbmp *expression level in water-injected controls and in* Djmsh1, Djmsh2, *or* Djbmp *RNAi planarians, 6 days after transection. In all experiments, the expression level is indicated in relative units, assuming as unitary the value of the water-injected controls. Each value is the mean ± s.d. of two independent RNAi experiments, carried out in duplicate.*

No significant variation in the *Djmcm2* expression level was observed in intact animals after *Djmsh1* or *Djmsh2* RNAi (Fig. S5B).

Relationship between the planarian msh *genes and* Djbmp

As some evidence links MSX function and BMP signaling as inducing regenerative responses in some vertebrate species (Beck *et al.,* 2003; Han *et al.,* 2003; Thummel *et al.,* 2006), we explored the possibility that the genetic regulatory network in which *Djmsh1* and *Djmsh2* are involved during cephalic regeneration also involves BMP signaling. A *bmp2/4*-related member (*Djbmp*) has been characterized in *D. japonica* (Orii *et al.,* 1998). DjBMP signaling is essential for the regeneration and maintenance of the dorso-ventral (DV) axis and its transcripts are preferentially accumulated at the planarian dorsal midline (Orii and Watanabe, 2007; Fig. S6 and Supplementary material). We analyzed *Djbmp* expression further during head regeneration. Both real time RT-PCR and whole mount *in situ* hybridization provided evidence that *Djbmp* is upregulated during head regeneration (Fig. 5A-E). The earliest activation of *Djbmp* expression was visualized at day 2 of regeneration in a central region of the dorsal side at the boundary between blastema and postblastema (Fig. 5B). At day 4, accumulation of *Djbmp* transcripts was mainly detected in the postblastema area (Fig. 5C). As regeneration proceeded, *Djbmp* expression extended to the regenerating head and, around day 6, *Djbmp*-expressing cells could be detected in the dorso-lateral regions. The original *Djbmp* pattern found in intact animals was then progressively restored (Orii *et al.,* 1998). At day 6 of regeneration, in some specimens *Djbmp* hybridization signal was also observed in the regions where small eyes were forming (Fig. 5D, arrows). *Djbmp* expression at the eye level appeared to be transient, because it was no longer observed at later regenerative stages (Fig. 5E). This pattern, which is reminiscent of *Djmsh2* expression transiently observed at the eye level (Fig. 3F), suggests an interplay between the two genes in the regeneration of the planarian visual system, an observation that deserves further investigation. Using a combination of RNAi and real time RT-PCR we demonstrated that *Djbmp* downregulation had no significant effect on *Djmsh1* and *Djmsh2* expression levels (Fig. 5F,G). On the other hand, DjMSH1 and DjMSH2 appeared to regulate *Djbmp* expression during head regeneration. In fact, a substantial decrease in the level of *Djbmp* transcripts was observed by real time RT-PCR in *Djmsh1*- and *Djmsh2* RNAi animals, when compared to water-injected controls (Fig. 5H; Fig. S7). However, the level of *Djbmp* transcripts still reached 60%, indicating that only a partial downregulation occurred with respect to that obtained by direct *Djbmp* RNAi-mediated silencing (Fig. 5H).

Discussion

Two members of the msh/msx *gene family characterize the planarian genome*

Our data identified two different members of the *msh/msx* gene family in two planarian species, *D. japonica* and *S. mediterranea*.

The presence of two *msh* genes in the planarian genome seems unusual, as previously only a single *msh*-related gene has been found in all invertebrate taxa examined (cf. Takahashi *et al.,* 2008). On the other hand, accumulation of duplicated *msx* genes, with a consequent acquisition of different roles, is a common trait in vertebrates. For example, mice have three *msx* members, and only the function of *msx1* appears to be essential for digit tip regeneration (Han *et al.,* 2003). At least five *msx* genes have been found in zebrafish (Ekker *et al.,* 1997), two of which play a role in the regenerating fin (Akimenko *et al.,* 1995; Poss *et al.,* 2003). The reasons for *msh* gene duplication in planarians are unknown, but this duplication may indicate that the two *msh* paralogs have distinct roles. Real time RT-PCR demonstrated that *Djmsh1* and *Djmsh2* are expressed in distinct cell populations and respond differently to stress conditions. *Djmsh1* transcripts were enriched in neoblast-like cells, while *Djmsh2* expression was found predominantly in the cell fraction including large cells and was activated after X-ray irradiation or heat-shock.

Djmsh1 *and* Djmsh2 *mediate the regenerative program leading to head formation*

The regeneration of complex structure with multiple cell types represents a spectacular response of planarians to a stressful event, such as the amputation of body parts. Our data, which show activation of *Djmsh1* and *Djmsh2* during head regeneration, suggest that these genes play an instructive role in the specific regenerative program leading to head formation. To evaluate how *Djmsh1* and *Djmsh2* exert their effects we focused on the formation of the cephalic blastema. When we performed RNAi experiments we clearly observed that, whereas posterior regeneration proceeded normally, functional ablation of *Djmsh1* and *Djmsh2* produced a cephalic blastema that appeared to be smaller. As the initial blastema size in planarians depends on the number of neoblasts that proliferate in a restricted stump region under the wound epidermis, we further investigated the effects of RNAi both at early and late regenerative stages, and calculated the percentage of mitotic cells in *Djmsh1*- and *Djmsh2*-RNAi animals and in water-injected controls. Our data, summarized in the diagrams in Fig. 4K-M, provided evidence that a) both *Djmsh1* and *Djmsh2* exert a control on the proliferative activity of neoblasts at a very early regenerative stage, when no specific activation can still be observed; b) these genes interfere, with different modalities, on the dynamics of mitoses. Although the molecular mechanisms that underlie the function of these genes are unknown, the finding that *Djmsh1* or *Djmsh2* RNAi did not cause a substantial diminution in the percentage of mitoses in intact planarians further supports the possibility that these genes are key players in the regeneration process. *In situ* hybridization provided evidence that these genes are activated following a specific pattern. Interestingly, the localization of *Djmsh2* transcripts in the region beneath the blastema area supports the possibility that this gene is involved in the adaptive response of some cells exposed to stress originating from the wound environment. We observed that the first detectable *Djmsh1*-positive cells demarcated a central region at the boundary between the old tissue and the blastema and then localized in the blastema midline. As in planarians the blastema is produced by accumulation of committed neoblasts that stop proliferation and undergo differentiation, how can the expression and functional data be linked? An attractive scenario that emerges from our results is that low levels - not detectable by *in situ* hybridization - of *Djmsh1* and *Djmsh2* transcripts play a role in sustaining neoblast proliferation under the wound epidermis, probably by cell-autonomous *Djmsh1*-mediated activation of neoblast proliferation and also by not cell-autonomous *Djmsh2*-mediated regulatory effects. Concomitantly with the early activation of proliferation, committed cells become accumulated in the blastema. The higher levels of *Djmsh1* and *Djmsh2* transcripts, derived by activation of these genes during head regeneration, may play a role in the transition towards a committed, non-proliferative state of these cells, as well as in patterning/remodeling events in the regenerating area. A dual role of *msx* genes in inducing cells to an undifferentiated, pluripotent state and in enhancing their cellular plasticity has been observed both *in vitro* and *in vivo* during regeneration in other organisms (Odelberg *et al.,* 2000; Poss *et al.,* 2003; Kumar *et al.,* 2004). In addition, the pattern and expression of *msxb*, an *msx* gene essential to blastema growth during fin regeneration in zebrafish, have been found to change during the transition between the blastema formation and the regenerative outgrowth. *msxb* expression was first detected in dedifferentiated and actively proliferating cells and then higher levels of transcripts were found in an area of the distal blastema that was devoid of proliferating cells (Thummel *et al.,* 2006). Although the lack of cellular analysis does not rule out the possibility that distinct subpopulations express *Djmsh1* and *Djmsh2* in homeostatic and regenerative contexts, our findings support the possibility that distinct, but spatially and timely coordinated functions of *Djmsh1* and *Djmsh2* orchestrate the early mitotic activation of planarian stem cells, as well as their subsequent transition towards a non-proliferative state.

A link between the Djmsh1/Djmsh2 and Djbmp signaling pathways in the control of cephalic blastema growth

Signaling by members of the BMP/transforming growth factor-β family regulates cell behavior in a variety of contexts by controlling hundreds of target genes in different cells (reviewed in Shi and Massague, 2003; Feng and Derynck, 2005). Functional evidence recently showed that in planarians the BMP pathway is required for correct DV body patterning (Orii and Watanabe, 2007; Molina *et al.,* 2007). We demonstrated that the expression of the *bmp2/4* planarian member *Djbmp* is activated in regenerating planarian heads, with a temporal dynamics that resembles that of *Djmsh1* and *Djmsh2*. In addition, using a combination of RNAi and real time RT-PCR data, we demonstrated that DjMSH1 and DjMSH2 regulate *Djbmp* expression during head regeneration, whereas no interplay of DjMSH1, DjMSH2 and DjBMP signaling was found in intact planarians. These findings suggest that the planarian *msh* genes may be upstream regulators of BMP signaling during the formation of the cephalic blastema. Considering the conserved transcriptional repressor activity of MSH/MSX proteins, it is unlikely that DjMSH1 and DjMSH2 directly regulate *Djbmp*. It is possible that these factors act indirectly by repressing unknown transcriptional repressor(s). A regulatory function of two *msh* genes on *Djbmp* is apparently at odds with the experimental observation that *Djbmp* knockdown interferes with the determination of DV body axis (Orii and Watanabe, 2007). However, the observation that RNAi-mediated *Djbmp* knockdown produced a more substantial reduction in endogenous transcripts with respect to that obtained as a consequence of *Djmsh1*-and

Djmsh2 dsRNA-mediated interference suggests that the residual *Djbmp* product escaping the down-regulation indirectly produced by *Djmsh1* and *Djmsh2* RNAi, was sufficient to avoid any visible defects in the DV axis. Recent work in *S. mediterranea* indicates the presence of a second, distinct effect of the BMP-mediated pathway (Reddien *et al.,* 2005a; Molina *et al.,* 2007). In fact, after *Smed_bmp2/4* RNAi the growth of the cephalic blastema appeared to be abnormal from the very beginning, especially in planarians regenerating a head, which showed a reduced or even absent blastema (Molina *et al.,* 2007). We hypothesize that BMP signaling, under the joint control of the two *msh* planarian genes, may play a role in blastema initiation, coordinating a TGF-β-mediated equilibrium between cell proliferation, differentiation and remodeling during head regeneration. Future analyses on the interaction with other head regeneration specific genes will be necessary to further understand how the interplay of DjMSH1 and DjMSH2 and DjBMP networks specifically mediate the response necessary for an appropriate regeneration of the planarian head.

Materials and Methods

Animals

The specimens used in this study belong to the asexual strain GI of the planarian species *Dugesia japonica* (Orii *et al.*, 1993). Animals were maintained in autoclaved stream water at 18°C and starved for ten days before being used in the experiments. Regenerating fragments were obtained by transverse amputation.

X-ray irradiation

Intact planarians were exposed to a lethal dose (30 Gy) of hard X-rays (200 KeV, 1 Gy/min), using a Stabilipan 250/1 instrument (Siemens, Gorla-Siama, Milan, Italy) equipped with a Radiation Monitor 9010 dosimeter (Radcal Corporation, Monrovia, CA, USA). The animals were sacrificed 2 days after irradiation for real time RT-PCR.

RNA extraction

Total RNA was extracted with the NucleoSpin RNAII kit (Macherey-Nagel) from intact planarians, regenerating fragments or dissociated cell samples prepared according to the serial filtration protocol described by Salvetti *et al.* (2005). RNA was reverse-transcribed using Superscript First Strand Synthesis System (Invitrogen). Each extraction was tested for the absence of genomic DNA by control RT-PCR reactions performed in the absence of reverse transcriptase.

Cloning of Djmsh1 *and* Djmsh2 *and gene expression analysis*

A 3'RACE-based strategy using an oligo dT anchor primer (5'/3' RACE kit, Roche Applied Science) and degenerate primers, designed from the most conserved parts of the third helix of the homeodomain was used to isolate cDNA clones representing the 3' end and the flanking 3' region of different homeobox genes (Fig. S8 and Supplementary material). The SMART 5'RACE cDNA amplification kit (Clontech) was used to complete two *msh*-related clones, named *Djmsh1* and *Djmsh2* (sequence-specific primers used: *Djmsh1* 5'RACE rev: 5'-AATCTTTGGATAAATGGAACAGAGCAG-3'; *Djmsh2*5'RACE rev: 5'-AACCCTGGTATTGATGTTGTCGATG-3'). The homologs of *Djmsh1* and *Djmsh2* in *S. mediterranea* were isolated with specific primers, taking advantage from *in silico* analysis of the ongoing *S. mediterranea* Genome project.

Whole mount *in situ* hybridization was performed according to Umesono *et al.* (1997), with minor modifications (Nogi and Levin, 2005). *Djmsh1-564* (1 to 564bp of *Djmsh1*), *Djmsh2-626* (2 to 626 bp of *Djmsh2*), *Djops* (Mannini *et al.*, 2004), *Djsyt* (Salvetti *et al.*, 2000) and *Djbmp* (Orii *et al.*, 1998) antisense probes were labeled with digoxigenin (DIG) and used in the hybridizations (10 to 50 ng/ml).

RNA interference analysis

Double-stranded RNA (dsRNA) was synthesized as described by Sánchez Alvarado and Newmark (1999). Djmsh1 dsRNA was obtained from Djmsh1-258 (1 to 258bp of Djmsh1) and Djmsh1-564; Djmsh2 dsRNA was obtained from Djmsh2-238 (2 to 238bp of Djmsh2) and Djmsh2-626; Djbmp dsRNA was obtained from Djbmp-611 (89 to 611bp of Djbmp; Orii *et al.,* 1998). The injection schedule was as described by Mannini *et al.* (2004). All samples were analyzed during the second round of regeneration. The reduction of the endogenous transcripts after RNAi was assessed by real time RT-PCR. For morphometric analysis, the areas of the blastema and the stump were determined in regenerating heads and tails of Djmsh1, Djmsh2 dsRNA-injected planarians and water-injected controls, 5 days after the second transection. The animals were photographed at the same magnification with a stereomicroscope after ethanol-fixation. The areas of the blastema and the stump were measured using Nikon ACT-2U imaging software. A number of 30 regenerating fragments were analyzed for each experimental condition. For mitotic index analysis, fragments regenerating a head from Djmsh1, Djmsh2 dsRNA-injected planarians and water-injected controls were incubated for 6 hours in 3‰ colchicine (w/v) (Sigma) in stream water at 0, 5, 12, 16, 24 hours and 2, 3, 4, 5, 6, 10, 15 days after the second transection. The animals were dissociated into single cells according to Baguñà and Romero (1981). A 20μl aliquot of the cell suspension was dropped onto glass slides, air-dried and stained with a 1μg/mL Hoechst 33342. The number of mitotic metaphases was counted under a fluorescence microscope. Three specimens were analyzed for each time; two slides for each sample were examined for a total of about 80,000 cells. The number of cells contained in the 20μl aliquot was counted by using a hemocytometer.

Real time RT-PCR

Quantitative real time PCR amplification was carried out with the ABI Prism 7000 Sequence Detection System (Applied Biosystems) to analyze the relative expression level of Djmsh1, Djmsh2, Djbmp and Djmcm2 (Table SI in the Supplementary Material at doi: 10.1387/ijdb.072476lm). DjEF2 was used as a normalizing gene to eliminate variation in cDNA concentration between the samples (Rossi *et al.,* 2007). PCR reactions were carried out using 20 ng cDNA and TaqMan Universal PCR Master Mix (Applied Biosystems), following the manufacturer's protocol. Reactions were run in duplicate and 2 to 3 independent cDNA samples per experimental condition were used. Each cDNA sample was obtained from three intact or regenerating planarians. In some experiments each cDNA sample derived from four small body portions including blastema and postblastema. Relative quantification of each single-gene expression was performed using the comparative CT method as described in the ABI Prism 7700 Sequence Detection System User Bulletin No. 2.

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