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SUPPLEMENTARY MATERIAL

corresponding to:

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

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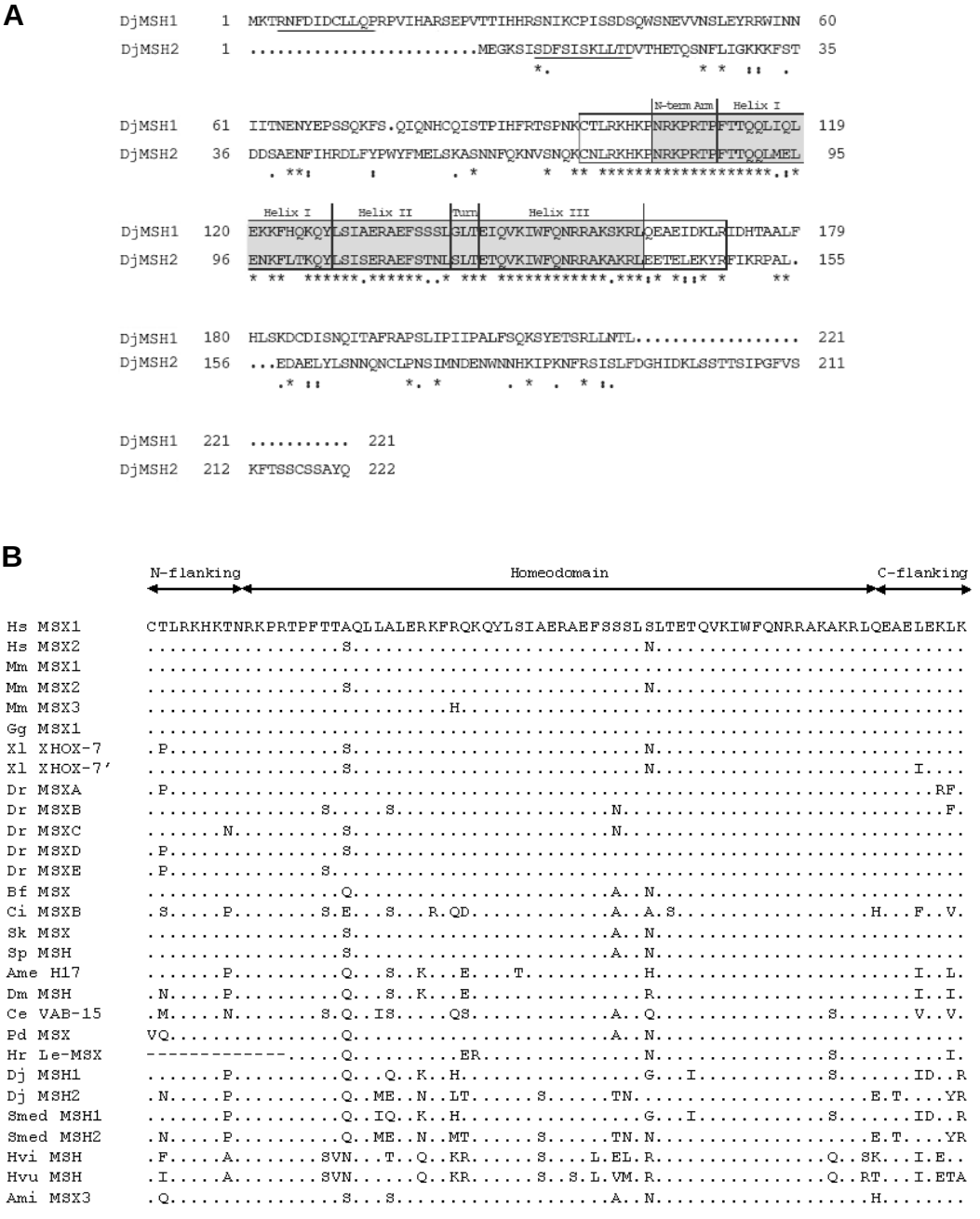


Fig. S1. *msh/msx*-related genes in planarians. (A) *Alignment of amino acid sequences of DjMSH1 and DjMSH2. Analysis of sequence similarity was performed with CLUSTAL W software. The regions flanking the homeodomain in 5' and 3' are boxed. The homeodomain is boxed and in grey. The region related to the en1 core motif, starting with a conserved Phenylalanine (position 6 and 9 in DjMSH1 and DjMSH2, respectively) is underlined.* **(B)** *Amino acid alignment of the homeodomain and flanking regions of representative MSH/MSX proteins from vertebrate and invertebrate species. Identical amino acid residues are indicated by dots with respect to the Hs MSX1 sequence; the incomplete sequence of HrLe-MSX is indicated by hyphens. Sequences were obtained from the EMBL/ GenBank. AmeH17: Apis mellifera H17 (M29491); AmiMSX3: Acropora millepora MSX3 (EF044217); BfMSX: Branchiostoma floridae MSX (AJ130766); CeVAB-15: Caenorhabditis elegans VAB-15 (AF286218); CiMSXB: Ciona intestinalis MSXB (AJ515411); DjMSH1: Dugesia japonica MSH1 (AM293348); DjMSH2: Dugesia japonica MSH2 (AM293349); DmMSH: Drosophila melanogaster MSH; DrMSXA: Danio rerio MSXA (NM131274); DrMSXB: Danio rerio MSXB (U16311); DrMSXC: Danio rerio MSXC (NM131272); DrMSXD: Danio rerio MSXD (NM131276); DrMSXE: Danio rerio MSXE (U50563); GgMSX1: Gallus gallus MSX1 (NM205488); HrLe-MSX: Helobdella robusta MSX (U61846); HsMSX1: Homo sapiens MSX1 (NM002448); HsMSX2: Homo sapiens MSX2 (NM002449); HviMSH: Hydra viridis MSH (X64629); HvuMSH: Hydra vulgaris MSH (AJ271008); MmMSX1: Mus musculus MSX1 (AF308572); MmMSX2: Mus musculus MSX2 (NM013601); MmMSX3: Mus musculus MSX3 (AF060229); PdMSX: Platynereis dumerilii MSX (AM114785); SkMSX: Saccoglossus kowalevskii (DQ431008); SmedMSH1: Schmidtea mediterranea MSH1 (AM293346); SmedMSH2: Schmidtea mediterranea MSH2 (AM293347); SpMSH: Strongylocentrotus purpuratus MSH (NM214613); XlXHOX-7: Xenopus laevis HOX-7 (P35993); XlXHOX-7': Xenopus laevis HOX-7' (Q04281).*

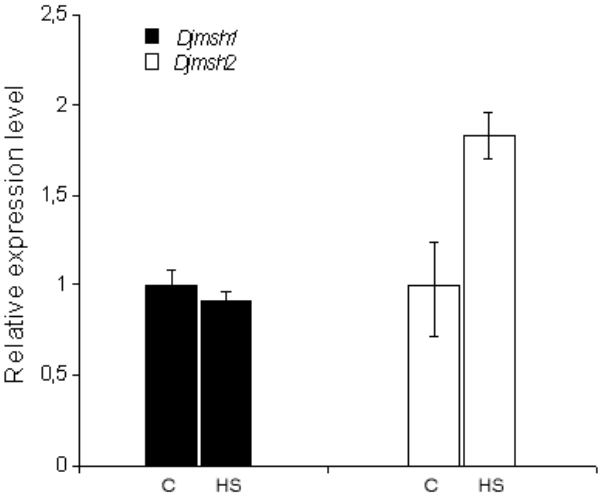


Fig. S2. Relative expression level of *Djmsh1* and *Djmsh2* in intact planarians after heat shock treatment, analyzed by real time RT-PCR. *Heat shock was performed in 30 intact planarians by overnight incubation at 28°C. The specimens were then sacrificed for RNA extraction.* Djmsh1 *expression level did not change after heat shock treatment, while* Djmsh2 *expression was strongly activated, indicating that* Djmsh2 *is a stress-responsive gene. Expression levels are indicated in relative units, assuming the value of untreated specimens (control) as unitary. Each value is the mean ± s.d. of three independent samples, analyzed in duplicate. C, control; HS, heat shock.*

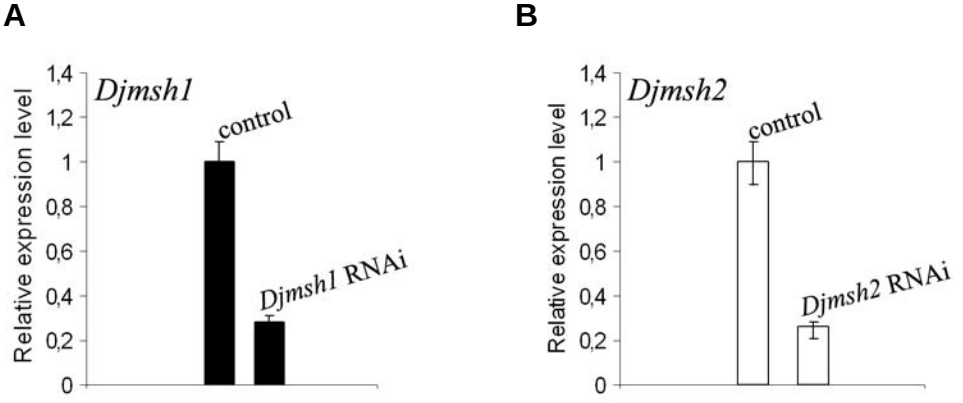


Fig. S3. RNAi-mediated downregulation of *Djmsh1* and *Djmsh2* in regenerating planarians, as visualized by real time RT-PCR. (A) *Expression level of* Djmsh1 *in water-injected controls and* Djmsh1 *RNAi animals.* **(B)** *Expression level of* Djmsh2 *in water-injected controls and* Djmsh2 *RNAi animals. In all experiments, the expression levels are indicated in relative units, assuming as unitary the value of water-injected controls. Each value is the mean ± standard deviation of two independent RNAi experiments, performed in duplicate.*

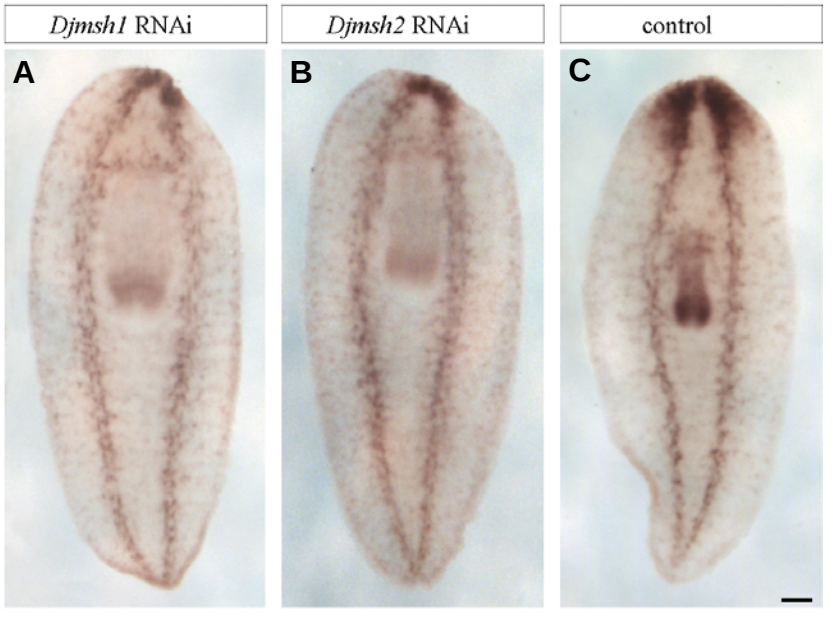


Fig. S4. Whole mount *in situ* hybridization with *Djsyt* of representative phenotypes of planarian fragments regenerating a head, 6 days after transection. (A) Djmsh1 *RNAi.* **(B)** Djmsh2 *RNAi.* **(C)** *Water-injected control. Scale bar, 100 μm.*

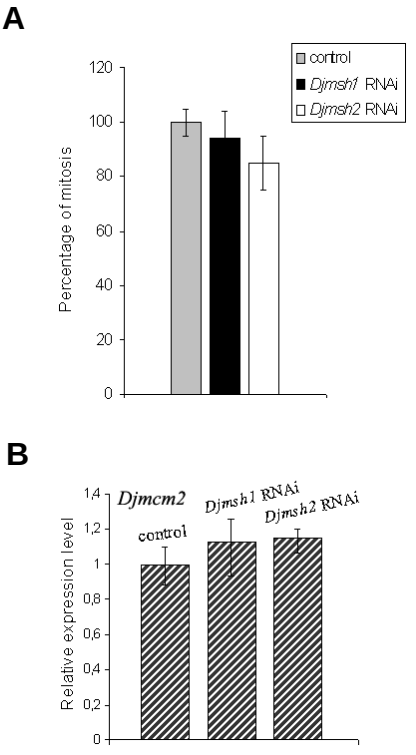


Fig. S5 (Left). Effects of *Djmsh1* and *Djmsh2* RNAi on intact planarians. (A) *Analysis of the percentage of mitoses in intact planarians injected with* Djmsh1 *or* Djmsh2 *dsRNA and in samples injected with water (control). The number of mitotic metaphases was normalized to the number of total cells and the values indicated in the graph are average ± s.d. of three independent samples, assuming as 100% the value of the control.* **(B)** *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.*

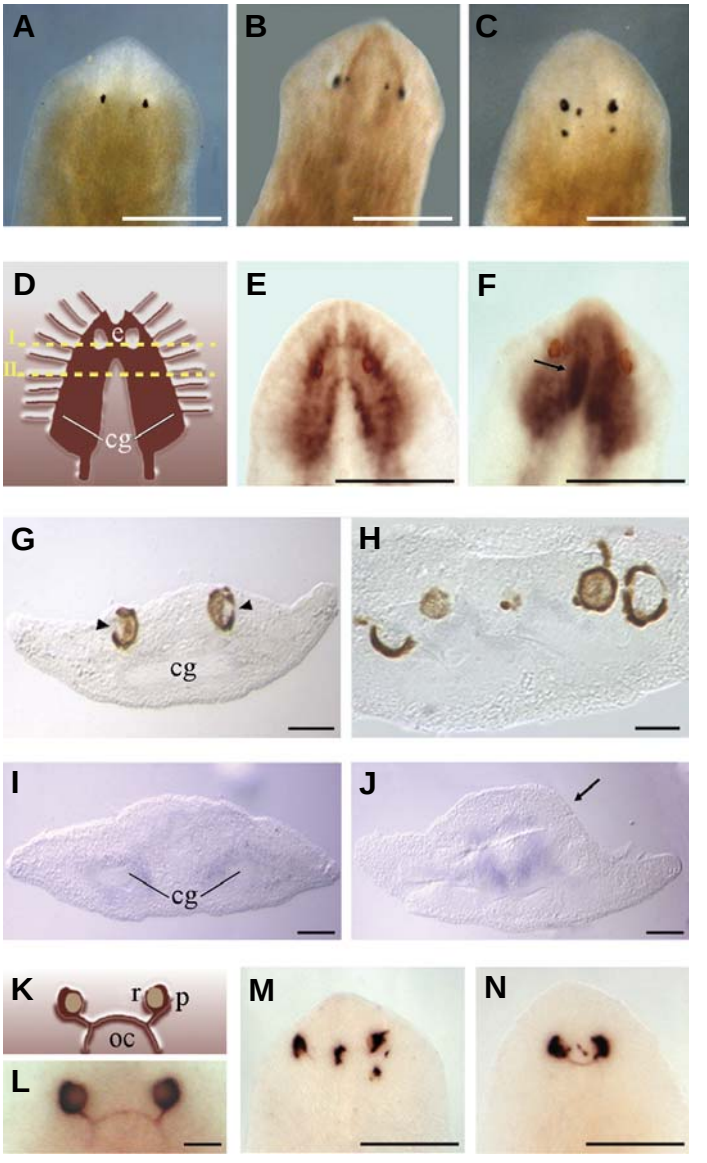
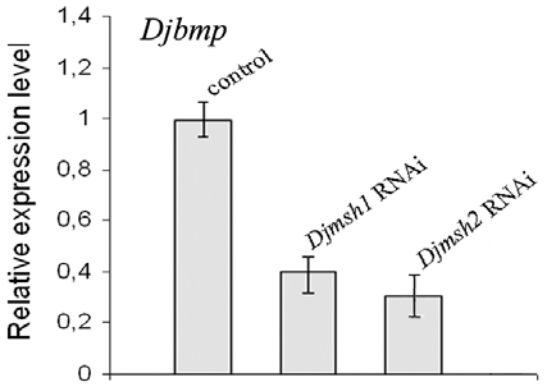


Fig. S6 (Right). Functional analysis of *Djbmp* during anterior regeneration. (A-C) *Brightfield images of a water-injected control* **(A)** *and representative* Djbmp *RNAi phenotypes* **(B,C)**, *6 days after transection.* **(D)** *Schematic drawing of the planarian brain, composed by a mass of nerve cells, the cephalic ganglia (cg), e: eyes. I and II indicate paraffin section levels.* **(E,F)** *Representative images of whole-mount* in situ *hybridization with* Djsyt *of a water-injected control* **(E)** *and a* Djbmp *dsRNA-injected planarian with abnormal cephalic ganglia (arrow)* **(F)**, *15 days after transection.* **(G-J)** *Transverse paraffin sections.* **(G)** *Control depicted in (E) (arrowheads indicate the eyes), and* **(H)** *the* Djbmp *dsRNA-injected planarian depicted in (F), at the level I.* **(I)** *Control depicted in (E) and* **(J)** *the* Djbmp *dsRNA-injected planarian depicted in (F), at the level II. Arrow shows the dorsal outgrowth.* **(K)** *Schematic drawing of the planarian visual system. p, pigmented eye cup; r, rhabdomeric photoreceptors; oc, optic chiasma.* **(L-N)** *Whole-mount* in situ *hybridization with* Djops *of a water-injected control* **(L)** *and representative* Djbmp *RNAi phenotypes with multiple eyes* **(M)** *and abnormal chiasma* **(N)**, *15 days after transection. Scale bars, 500 μm in (A-J, M,N), and 100 μm in (L). According to the* Djbmp *RNAi results recently obtained and demonstrating that* Djbmp *knockdown results in additive ventral structures in the dorsal side of regenerants (Orii and Watanabe, 2007), our functional experiments confirmed that Djbmp acts as a key signaling molecule in the dorso-ventral patterning of neural structures during head regeneration. In fact we observed that, while 20-25% of planarians died as a consequence of* Djbmp *RNAi, about 70% of the injected specimens regenerated heads with morphological defects in the dorsoventral (DV) axis and in the organization of the cephalic nervous structures. Multiple eyes were also frequently observed (B,C). A further analysis of these phenotypes by using the panneural marker* Djsyt *highlighted abnormal dorsal outgrowth of the nervous structures in the head region (E-G). Eye alterations could be better detected after* in situ *hybridization with the photoreceptor marker* Djops *that, following prolonged detection, may reveal the presence of* Djops *transcripts also in the photoreceptor axons. Using this approach, multiple eyes with altered organization of the optic cup and/or abnormal connections between eyes and brain were observed (L-N).*



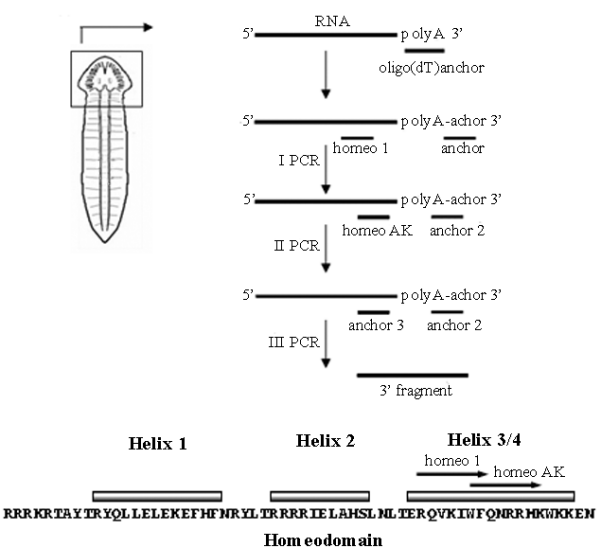


Fig. S7 (Left). *Djbmp* expression level in water-injected controls and in *Djmsh1* or *Djmsh2* RNAi planarians, 3 days after transection. In all experiments, the expression level is indicated in relative units, assuming as unitary the value of the water-injected controls. *Each value is the mean ± s.d. of two independent RNAi experiments, performed in duplicate.*

Fig. S8 (Right). RT-PCR strategy utilized to isolate *Djmsh1* and *Djmsh2* from regenerating heads. *cDNA obtained from RNA isolated from the head portion of anterior regenerants was amplified by using the anchor primer (Roche Applied Science) and a sense-strand primer, selected from the homeodomain region R(I/V)(K/R/Q)(I/V)WF (primer homeo 1: 5'-CGGRTNMRIRWITGGTT-3'), for 35 cycles at 94°C for 30 sec, 40°C for 1 min, and 72°C for 1 min. A second round of PCR was then performed with a sense-strand primer representing the homeodomain region WF(Q/K)NRRAK (primer homeo AK: 5'-TCGCGGATCCTGGTTTMARAAYMGNMGIGCAA-3'), and the primer 2 (5'-CACGCGTATCGATGTCGAC-3'). Primer homeo AK includes, at the 5' end, an adapter corresponding to primer 3 (5'-TCGCGGATCCTGGTTT-3'), the amplification reaction was conducted as described above except for the annealing temperature that was 50°C. Subsequently, a third PCR was carried out on the diluted second PCR products using the primers 2 and 3. The products of the third PCR round were ClaI/BamHI digested and directionally cloned into the ClaI/BamHI digested pBluescript SK vector (Stratagene). The clones were sequenced by automated fluorescent cycle sequencing (ABI).*

SUPPLEMENTARY TABLE S1

PROBES AND PRIMERS USED IN REAL TIME RT-PCR

Clone	1) Probe 2) Forward primer 3) Reverse primer
DjEF2	1) 5'-CCAACAAGTCCACATATGTT-3' 2) 5'-GCAATCGAAGACGTTCCATGTG-3' 3) 5'-CCAGGAAAAGTTGTTATAGTCCCAGTTT-3'
Djmsh1	1) 5'-CCAAAGATTGTGATATCTCC-3' 2) 5'-CCATACAGCTGCTCTGTTCCATTTA-3' 3) 5'-GGGCTCTGAAAGCAGTAATTTGATT-3'
Djmsh2	1) 5'-CCTGGTATTTTATGGAATTAT-3' 2) 5'-CTGATGATTCCGCTGAAAATTTCATTCA-3' 3) 5'-ATTGCACTTTTGATTTGACACATTTTTTTGAA-3'
DjBMP	1) 5'-CTTTGGCAATGAATCATTTC-3' 2) 5'-GAAAACCGAAACATTTAGGACCAGTT-3' 3) 5'-GAATACCAACCGAAGACCATGTTTG-3'
DjMCM2	1) 5'-CTCTACTGCTTCATAAGTCTG-3' 2) 5'-GGCAGGTGAAACATTGGGATCA-3' 3) 5'-GGCTACCGACATTCCTTTGGT -3'