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Djeyes absent (*Djeya*) controls prototypic planarian eye regeneration by cooperating with the transcription factor *Djsix-1*

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Abstract

A conserved network of nuclear proteins is crucial to eye formation in both vertebrates and invertebrates. The finding that freshwater planarians can regenerate eyes without the contribution of Pax6 suggests that alternative combinations of regulatory elements may control the morphogenesis of the prototypic planarian eye. To further dissect the molecular events controlling eye regeneration in planarians, we investigated the role of *eyes absent* (*Djeya*) and *six-1* (*Djsix-1*) genes in *Dugesia japonica*. These genes are expressed in both regenerating eyes and in differentiated photoreceptors of intact adults. Through RNAi studies, we show that *Djsix-1* and *Djeya* are both critical for the regeneration of normal eyes in planarians and genetically cooperate in vivo to establish correct eye cell differentiation. We further demonstrate that the genetic interaction is mediated by physical interaction between the evolutionarily conserved domains of these two proteins. These data indicate that planarians use cooperatively Djsix-1 and Djeya for the proper specification of photoreceptors, implicating that the mechanism involving their evolutionarily conserved domains can be very ancient. Finally, both *Djsix-1* and *Djeya* double-stranded RNA are substantially more effective at producing no-eye phenotypes in the second round of regeneration. This is probably due to the significant plasticity of the planarian model system, based on the presence of a stable population of totipotent stem cells, which ensure the rapid cell turnover of all differentiated cell types.

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Keywords: Planarians; Regeneration; Eye; Central nervous system; Eyes absent; six-1; RNAi; In situ hybridization

Introduction

Progress in understanding the molecular mechanisms that promote eye formation is mainly due to studies carried out on *Drosophila*. The combined activity of Notch and EGF Receptor signaling pathways (Kumar and Moses, 2001a) and a complex network of nuclear proteins, including seven top regulatory factors, namely twin of eyeless (toy), eyeless (ey), eye gone (eyg), sine oculis (so), optix (opt), eyes absent (eya) and dachshund (dac) are critical for the specification of the eye disc primordium (Treisman, 1999). These proteins form feedback loops and probably behave as an eye-specific multimeric complex (Heberlein and Treisman, 2000; Kumar and Moses, 2001b).

Extensive studies on eye development revealed the conserved use of similar mechanisms and molecules to control eye morphogenesis in *Drosophila* and vertebrates (Gehring and Ikeo, 1999; Treisman, 1999). These fascinating aspects of homology, recently integrated by a flux of data from a variety of metazoan taxa, have suggested that the different types of eye present in the animal kingdom share a common ancestor (Gehring and Ikeo, 1999). A pivotal conservatism through these developmental regulatory similarities is the use of the master gene *Pax6*, which is essential for eye formation in all species where it has been studied (Pichaud and Desplan, 2002). However, interesting exceptions have also come to light. For example, the two *Pax6* homologues *toy* and *ey* are dispensable for *Drosophila* larval light-sensing system (Bolwing organ) development (Suzuki and Saigo, 2000). Moreover, *Drosophila ey* is not required to form

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normal ocelli (Gehring, 2002). No *Pax6* expression has been detected in developing ganglionic photoreceptors of *Amphioxus* (Glardon et al., 1998) or during the adult eye development of the polychaete *Platynereis dumerlii* (Arendt et al., 2002). In the squid (Cephalopoda, Mollusca), *Pax6* expression covers the early eye anlagen, but it is not detected in the differentiating retina (Tomarev et al., 1997). Furthermore, RNA interference (RNAi) studies have demonstrated that planarians can regenerate eyes without *Pax6* (Pineda et al., 2002).

Planarian flatworms are free-living members of Platyhelminthes. The eyes of these organisms are simple in structure, and are considered similar to a hypothetical ancestral type (reviewed by Saló et al., 2002). The extensive capability of planarians to regenerate—a process based on the presence of totipotent stem cells (neoblasts)—provides a good opportunity to study the genetic pathway that leads to eye morphogenesis during regeneration. Here we report the isolation of two planarian genes, *Djsix-1* and *Djeyes absent* (*Djeya*), in *Dugesia japonica* and describe their expression pattern and loss of function by RNAi during cephalic regeneration. These genes were chosen to further analyze the genetic pathway responsible for early visual system formation during planarian regeneration. In the planarian *Girardia tigrina*, *six-1* is expressed in the eyes and is essential for their regeneration (Pineda et al., 2000). In contrast, *Gtsix3*, a planarian homologue of *Drosophila optix*, is expressed in brain branches, but not in eye cells (Pineda and Saló, 2002). *Djeya* belongs to the *eya* gene family, encoding nuclear proteins involved in the morphogenesis of a variety of structures, including eyes, both in *Drosophila* and vertebrates (Bonini et al., 1993, 1998; David et al., 2001; Xu et al., 2002). Biochemical analyses have demonstrated that the evolutionarily conserved eya domain (ED) does not bind DNA, but forms a protein complex with sine oculis and/or the retinal determination protein dachshund (Chen et al., 1997; Ikeda et al., 2002; Pignoni et al., 1997) that has been found to be able to directly interact with DNA (Kim et al., 2002). We show that both *Djsix-1* and *Djeya* are critical for the regeneration of normal eyes in planarians, since their RNAi-mediated loss of function generates small eyes or abolishes the formation of these structures. Interestingly, both *Djsix-1* and *Djeya* double-stranded RNA (dsRNA) were substantially more effective at producing mutant phenotypes in the second round of regeneration. Our results also demonstrate that Djsix-1 and Djeya physically interact in vitro through their evolutionarily conserved domains.

Materials and methods

Animals

Planarians used in this work belong to the asexual strain GI of the species *D. japonica* (Orii et al., 1993). Animals were cultured in autoclaved stream water at 18°C and starved for 1 or 2 weeks before being used in the experiments. Fragments regenerating a head were obtained by transverse amputation at the pre-pharyngeal level.

Isolation of Djsix-1 and Djeya

To obtain *Djsix-1* and *Djeya* from *D. japonica*, a homology-based cloning strategy was used. A *Djsix-1* cDNA fragment of 162 bp was amplified with degenerated primers corresponding to the conserved homeodomain regions WDGEE and NWFKNR. PCR was carried out for 30 cycles at an annealing temperature of 40°C. A *Djeya* fragment of 335 bp was similarly obtained using degenerated primers from the conserved regions WDLDET and WMRKLAF, under annealing conditions of 46°C, for 30 cycles. The SMART 5′/3′ RACE cDNA amplification kit (Clontech) was used to obtain the full-length sequence of both genes. The following sequence-specific primers were used:

Djsix-1 5′ RACE reverse 5′-CTTGGGGATGGATATGGATTATGT-3′.
Djsix-1 3′ RACE forward 5′-CCATATCCATCCCCAAGAGAAAAG-3′.
Djeya 5′ RACE reverse 5′-AAATAAGCTCTTCCATTCTTAAGCC-3′.
Djeya 3′ RACE forward 5′-ATGCCACCAACGAGTGAAGACAA-3′.

The PCR products were TA-cloned using pGEM-T easy vector (Promega). The clone *Djeya*-1411 contains the region from nucleotide 370 to nucleotide 1781 of *Djeya*. The clone *Djsix-1*-505, containing the region from nucleotide 718 to nucleotide 1223 of *Djsix-1*, was cloned in pBluescript SK(−). All clones were sequenced by automated fluorescent cycle sequencing (ABI).

Sequence analysis

We used BLAST search (Altschul et al., 1990) to identify related sequences. Sequence comparison between *G. tigrina* six-1 (Pineda et al., 2000) and *D. japonica* six-1 (accession no. AJ557022) was performed using the GAP program of the GCG package. Sequences corresponding to the eya domain were aligned with CLUSTALW. Evolutionary distances were calculated using Kimura's equation (Nei and Koehn, 1983) and used for the phylogenetic tree construction by the Neighbor-joining method. Sequences were obtained from the EMBL/GenBank.

In situ hybridization

Whole-mount in situ hybridization was carried out on intact and regenerating planarians following Agata et al. (1998). Sense and antisense digoxigenin-labeled RNA probes used for the experiments were obtained using the RNA in vitro labeling kit (Roche). The following clones

were used to obtain sense and antisense riboprobes: *D. japonica opsin* (*Djops*) 480 bp (accession no. AJ421264); *D. japonica synaptotagmin* (*DjSyt*) (Tazaki et al., 1999), *Djeya*-1411, *Djsix-1*-505.

Synthesis of dsRNA and microinjection

Double-stranded RNA was synthesized as described by Sánchez Alvarado and Newmark (1999). *Djsix-1*-505 was digested with *Cla*I and *Xba*I to obtain sense and antisense RNA, respectively. *Djeya*-1411 was digested with *Apa*I and *Sac*I to obtain sense and antisense RNA, respectively. Planarians were injected with $10^{10}–10^{11}$ molecules of each dsRNA preparation or an equimolar mixture of *Djeya* and *Djsix-1* dsRNA. Control injections were performed with water or *β-Gal* dsRNA. A Drummond Scientific (Broomall, PA) nanoject injector was used. Regenerating planarians were maintained in a not crowded state to avoid any perturbation of eye development (Sakai et al., 2000). After the first injection, performed before amputation (6–24 h), the regenerants were daily injected for 3 days. Additional injections were done at 6 and 9 days from amputation. At 10 days of regeneration, the injected specimens were again subjected to head amputation and allowed to regenerate. Regenerating fragments were monitored for eye regeneration for periods between 6 and 14 days. For a more detailed study of the phenotypes obtained by RNAi, some injected specimens were processed by whole-mount in situ hybridization with *Djops* to mark the presence of photoreceptor cells, and with *Djsyt* to monitor cephalic ganglia regeneration. A sample of *Djsix-1* dsRNA-injected specimens was hybridized with *Djeya*-1411 riboprobe. Some *Djeya* dsRNA-injected specimens were hybridized with *Djsix-1*-505 riboprobe.

Fig. 1. Sequence analysis of Djeya. (A) Nucleotide sequence of *Djeya* cDNA and its predicted amino acid sequence. The region corresponding to the eya domain (ED) is boxed in grey. Amino acid residues that are potential casein kinase II (CK2) sites are underlined. Amino acids corresponding to putative kinase C sites are double-underlined. EMBL/Genbank accession no. for *Djeya* cDNA is AJ557023. (B) Sequence comparison of Djeya ED to ED of eya proteins in different species. Identical amino acids are indicated by dots. DJ-EYA, *D. japonica* eya; DM-EYA, *Drosophila melanogaster* eya; AG-EYA, *Anopheles gambiae* eya; DR-EYA1, *Danio rerio* eya1; OL-EYA1, *Oryzias latipes* eya1; XL-EYA1, *Xenopus laevis* eya1; HS-EYA1, *Homo sapiens* eya1; MM-EYA1, *Mus musculus* eya1; HS-EYA2, *H. sapiens* eya2; MM-EYA2, *M. musculus* eya2; GG-EYA2, *Gallus gallus* eya2; HS-EYA3, *H. sapiens* eya3; MM-EYA3, *M. musculus* eya3; HS-EYA4, *H. sapiens* eya4; MM-EYA4, *M. musculus* eya4. (C) Phylogenetic tree based on the ED regions of the eya proteins shown in B. Bootstrap values (1000 replicates) are indicated at the corresponding nodes.

B

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DJ_EYA   269 ERIFVWDLDETIIIFHSLLTGIYAQRYQKDASTAVALGLRMEELIFSLADNHMFFNDLEE 328
DM_EYA   487 ..V........L....T..S.S..N..T..H.SLMTIAF....MV.NM..T.F...EI.. 546
AG_EYA   347 .....................S..G..N.NRDHQ.Q..Y.......NM..AYF....... 406
DR_EYA1  339 ..V.I........V.....T.S..N.FGR.PP.S.S.......M..N...T.F....... 398
OL_EYA1  125 ..V.I........V.....T.S..N..GR.PP.S.S.......M..N...T.L....... 184
XL_EYA1  317 ..V.I........V.....T.S..N..GR.PPSS.S.......M..N...T.L....... 376
HS_EYA1  289 ..V.I........V.....T.S..N..GR.PP.S.S.......M..N...T.L....... 348
MM_EYA1  321 ..VLL........V.....T.S..N..GGIHLLLFPWDYG...M..N...T.L....... 380
HS_EYA2  268 ..V................T.TF.S..G..TT.S.RI..M...M..N...T.L......D 327
MM_EYA2  203 ..V................T.TF.S..G..TT.S.RI..M...M..N...T.L......D 262
GG_EYA2  264 ..V................T.TF.S..G..TT.S.RI..M...M..N...T.L......D 323
HS_EYA3  177 ..L.L..............T.S...K.G..PTVVIGS..T...M..EV..T.L....... 236
MM_EYA3  240 ..V.L..............T.S...K.G..PTAVIGS..T...M..EV..T.L....... 299
HS_EYA4  369 ..V..........V.....T.S...K.G..PPM..T.......M..N...T.L....... 428
MM_EYA4  346 ..V..........V.....T.S...K.G..PPM..T.......M..N...T.L....... 405

DJ_EYA   329 CDQVHIDDVSSDDNGQDLSNYNFLVDGFAGPASICNGVSTVIQGGPLGLPGNTSVRGGVD 388
DM_EYA   547 ...................A...AT...H---------TNTPP.A.PN.CLP.G...... 597
AG_EYA   407 ...I.....A........N....AA...H---------TATP..A.PNVCLP.G...... 457
DR_EYA1  399 ...................T...ST...HA------------AATSAN.CLA.G...... 446
OL_EYA1  185 ...................T...SA...HA------------AATSAN.CLA.G...... 232
XL_EYA1  377 ...................T...GT...PA------------AATSAN.CLA.G...... 424
HS_EYA1  349 ...................T...GT...PA------------AATSAN.CLA.G...... 396
MM_EYA1  381 ...................T...GR...PA------------AATSAN.CLA.G...... 428
HS_EYA2  328 ...I.V.............T...SA...HS------------SAPGAN.CLGSG.H.... 375
MM_EYA2  263 ...I.V.............T...ST...HS------------TAPGAS.CLG.G.H.... 310
GG_EYA2  324 ...I...............T...SA...HS------------SAASAN.CLGSG.H.... 371
HS_EYA3  237 .....VE..A...........S.ST...S.------------S..SGSHGSSVG.Q.... 284
MM_EYA3  300 .....VE..A...........S.ST...S.------------S..SGSHGSSVG.Q.... 347
HS_EYA4  429 ...................T.S.AT...HA------------AASSAN.CLP.G...... 476
MM_EYA4  406 ...................T.S.AT...HA------------AASSAN.CLP.G...... 453

DJ_EYA   389 WMRKLAFRYRRIKELYNSYRNNVGGLLGTQKREHWLQLRQDIETLTDNWLTLALKALTLI 448
DM_EYA   598 ..........K..DI.....G...T...PG...A...I.SE..VA....A.....C.SM. 657
AG_EYA   458 ..........K..DT..T..........P...D....V.S...HE..S.HS.T..C.NM. 517
DR_EYA1  447 ...........V..I.TT.K........PA...A.....AE..A...S............ 506
OL_EYA1  233 ...........V..I.TT.K........PA...A.....AE..A...S............ 292
XL_EYA1  425 ...........V..I..T.K........PA...A.....AE..A...S............ 484
HS_EYA1  397 ...........V..I..T.K........PA...A.....AE..A...S.........S.. 456
MM_EYA1  429 ...........V..I..T.K.K......PA...A.....AE..A...S.........S.. 488
HS_EYA2  376 ...........V..M..T.K......I..P...T.....AEL.A...L...HS....N.. 435
MM_EYA2  311 .........C.V..M..T........I.AP...T.....AEL.A...L...HS....N.. 370
GG_EYA2  372 ...........V..M..T.K......I.AP...T.....AEL.A...L...H.....N.. 431
HS_EYA3  285 ..........KVR.I.DKHKS......SP.RK.ALQR..AE..V...S..GT...S.L.. 344
MM_EYA3  348 ..........KVR.I.DKHKS......SP.RK.ALQR..AE..V...S..GT...S.L.. 407
HS_EYA4  477 ...........V.....T.K........PA..DA.....AE..G...S...N...S.SI. 536
MM_EYA4  454 ...........V.....T.K........PA..DA.....AE..G...S...N...S.SI. 513

DJ_EYA   449 SQRNNCVNVLVTTTQLVPALAKVLLYGLGGVFQIENIYSANKIGKESCFERISSRFGRKS 508
DM_EYA   658 ...E........S...A........F....I.N.......H...H.T.Y...VT...... 717
AG_EYA   518 A..E..................I......Q..PV..V.........Q.....VT...... 577
DR_EYA1  507 HS.S....I.......I............V..P.......T..........VIQ.....V 566
OL_EYA1  293 HS.S....I.......I............V..P.......T..........VIQ.....V 352
XL_EYA1  485 HS.T....I.......I............I..P.......T...........IQ.....V 544
HS_EYA1  457 HS.T....I.......I............I..P.......T...........IQ.....V 516
MM_EYA1  489 HS.T....I.......S............I..P.......T...........IQ.....V 548
HS_EYA2  436 NS.P............I............S..P.......T.T.........MQ.....A 495
MM_EYA2  371 NS.P............I............S..P.......T.T.........MQ.....A 430
GG_EYA2  432 HS.P............I............T..P.......T.T.........MQ.....A 491
HS_EYA3  345 QS.K.....PI..................EI.P.......T...........V....K.V 394
MM_EYA3  408 QS.K..A...I..................EI.P.......T...........V....K.V 467
HS_EYA4  537 .T.S..I.........I.........S...A.P.......T...........MQ.....V 596
MM_EYA4  514 .T.S............I.........S...A.P.......T...........V....TNI 573

DJ_EYA   509 TYVVVGDGKDEETAARQVNWPFWRISSHSDIVALHHALSLGYL 551
DM_EYA   718 ....I...NE.....KAM.F......A....R..YT..DM.F. 760
AG_EYA   578 ........Q...N..KNL.F...........RS..T..EM.F. 620
DR_EYA1  567 V.......VE..QGSKKH.M..........LM......D.E.. 609
OL_EYA1  353 V.I.....VE..QGSKKH.M..........LM......N.E.. 395

XL_EYA1  545 V.......AE..QG.KKHSM.....T....LM......D.E.. 587
HS_EYA1  517 V...I...VE..QG.KKHAM..........LM......E.E.. 559
MM_EYA1  549 V.LLI...VE..QG.KKHAM....V.....LM......E.E.. 591
HS_EYA2  496 V...I...VE..QG.KKH.M......C.A.LE..R...E.E.. 538
MM_EYA2  431 V.I.I...VE..QG.KKH.M......C.A.LE..R...E.E.. 473
GG_EYA2  492 V.I.I...VE..QG.KKH.M......C.A.LE..R...E.E.. 534
HS_EYA3  395 ....I...R...I..K.H.M.....TN.G.L.S..Q..E.DF. 437
MM_EYA3  468 ....I...R...I..K.H.M.....TN.G.L.S..Q..E.DF. 510
HS_EYA4  597 V...I...VE..Q..KKH.M..........LL...Q..E.E.. 639
MM_EYA4  574 ....I...R...H..N.H.M..........LL...Q..E.E.. 616
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Fig. 1 (*continued*).

Analysis of endogenous transcripts by RT-PCR

Total RNA was extracted from four regions along the antero-posterior body axis of intact planarians and from injected fragments, at various regeneration stages, using the NucleoSpin RNAII kit (Macherey-Nagel). cDNA was generated from 1 μg of total RNA using Superscript First Strand Synthesis System for RT-PCR (Invitrogen). Control reactions were performed in the absence of reverse transcriptase. In all experiments, the constitutively expressed elongation factor gene *DjEF2* was amplified as an internal control using forward 5′ TTAATGATGGGAAGATATGTTG 3′ and reverse 5′ GTACCATAGGATCTGATTTTGC 3′ primers. The comparative PCR experiments, carried out along the antero-posterior body axis of adult planarians, were performed using the following primers: *Djeya* forward 5′

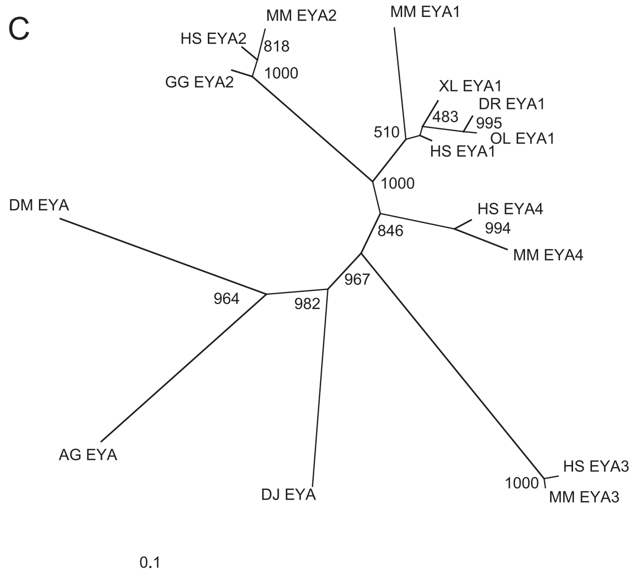

Fig. 1 (*continued*).

CCTTGAATTACTGTTGATACACC 3′; *Djeya* reverse 5′ TATTTTCAGTTTGGCCGGTAATC 3′. Specific oligonucleotides from *Djsix-1* and *Djeya*, designed from two regions, one internal and the other external to the sequence used for dsRNA synthesis, were used to assess the reduction of *Djsix-1* and *Djeya* endogenous transcripts in injected specimens. The primers used were: *Djsix-1* forward 5′ GTTAGCCCATTTAGTACAAG 3′, *Djsix-1* reverse 5′ ATTTGGCGTTTGATCTGTTG 3′; *Djeya* forward 5′ GGCTTAAGAATGGAAGAGCTTATT 3′, *Djeya* reverse 5′ TCGCTTTTGAGTTCCCAATAATCC 3′.The concentration of cDNA and the number of cycles used were optimized for each PCR reaction to observe a quantifiable signal within the linear range of amplification, according to both the putative abundance of each mRNA amplified and the size of the corresponding PCR product. The analysis was performed in duplicate with RNA extracted from at least two experiments.

Glutathione-S-transferase (GST) pull-down assay

To prepare the (GST)-SD + HD fusion protein, a *Djsix-1* cDNA fragment (amino acid region 31–230) was amplified from the full-length *Djsix-1* clone, using the forward primer *six s1*, (5′ TCGCGGATCCGGTTTTACACAAGAACAAGTTGCTT 3′), containing a *Bam*HI restriction site and the reverse primer *six as1* (5′ GCCGCTCGAGCTTTGATATCCTTTAAAGATTCACTGT 3′), containing an *Xho*I restriction site. To prepare the (GST)-SD fusion protein, a cDNA fragment corresponding to the amino acid region 31–151 was amplified from the same clone using the forward primer *six s1* and the reverse primer *six as2* (5′ GCCGCTCGAGTCTCTTCACCATCCCATATTGTTCT 3′) containing an *Xho*I restriction site. The (GST)-HD fusion protein was prepared by amplification of a cDNA fragment corresponding to the amino acid region 151–230 with the forward primer *six s2* (5′ TCGCGGATCCGATGGTGAAGAGACGAGTTATTGTT 3′) containing a *Bam*HI restriction site and the reverse primer *six as1*. Following *Bam*HI/*Xho*I digestion, the amplified fragments were cloned into pGEX-4T3 (Pharmacia) in frame with the GST sequence. Recombinant plasmids and pGEX-4T3 (to produce GST) were introduced into BL21 cells (Novagen). (GST)-SD + HD was produced by inducing cell cultures with 100 μM IPTG, for 2 h at 28°C. (GST)-SD and (GST)-HD proteins were similarly obtained by inducing the cell culture for 3 h. Approximately 100 ml of each growing culture was pelleted and proteins were extracted with the B-PER reagent (Pierce), following the manufacturer's recommendations. Lysates were incubated with the glutathione resin (Pierce) for 1 h at 4°C. GST fusion proteins bound to glutathione resin were washed several times with PBS containing 0.8% Triton X-100 and resuspended in protein binding buffer (20 mM HEPES KOH pH 7.7, 0.1% NP-40, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 50 μg/ml BSA, 10% glycerol) containing 150, 200 or 500 mM NaCl. The cDNA for *Djeya* ED (amino acid region 269–551) was amplified from *Djeya*-1411 clone using the forward primer *eya s1* (5′ TCGCGGATCCACCATGGAAAGAATCTTTGTTTGGGATTTA 3′) containing a *Bam*HI restriction site at the 5′ end and a Kozak sequence (Kozak, 1987) with the initiator codon in frame with the *Djeya* sequence. The reverse primer *Djeya as1* (5′ CCATCGATGGCCAAGACTGAGA 3′) contained a *Cla*I restriction site at the 5′ end. After *Bam*HI/*Cla*I digestion, the amplification product was *Bam*HI/*Cla*I-cloned in pBluescript SK(−). Capped sense RNA of the ED was produced with the mMessage mMachine kit (Celbio) and the ED ^{35}S-radiolabeled protein was obtained by in vitro translation using the Retic lysate IVT kit (Celbio) following the manufacturer's recommendations. On the basis of SDS-PAGE Coomassie staining, similar amounts of sepharose-coupled GST proteins were incubated in 400 μl of protein binding buffer with 10 μl of translation product, for 2 h at 4°C with gentle rocking. The resin was washed three times (30 min each) in protein binding buffer and twice in PBS. Bound ^{35}S-radiolabeled proteins were extracted with SDS-PAGE sample buffer by boiling, subjected to SDS-PAGE and visualized by autoradiography.

Results

Isolation of Djsix-1 and Djeya in D. japonica

To investigate the role of eye regulatory genes during planarian eye regeneration, we cloned two genes in *D. japonica*: *Djsix-1*, a gene that encodes a Six/so protein nearly identical (over 90% identity) to Gtsix-1, previously isolated in *G. tigrina* (Pineda et al., 2000, 2001), and *Djeya*. *Djeya* encodes a predicted polypeptide of 551 amino acids, and is characterized by a proline-serine-threonine-rich (PST) N-

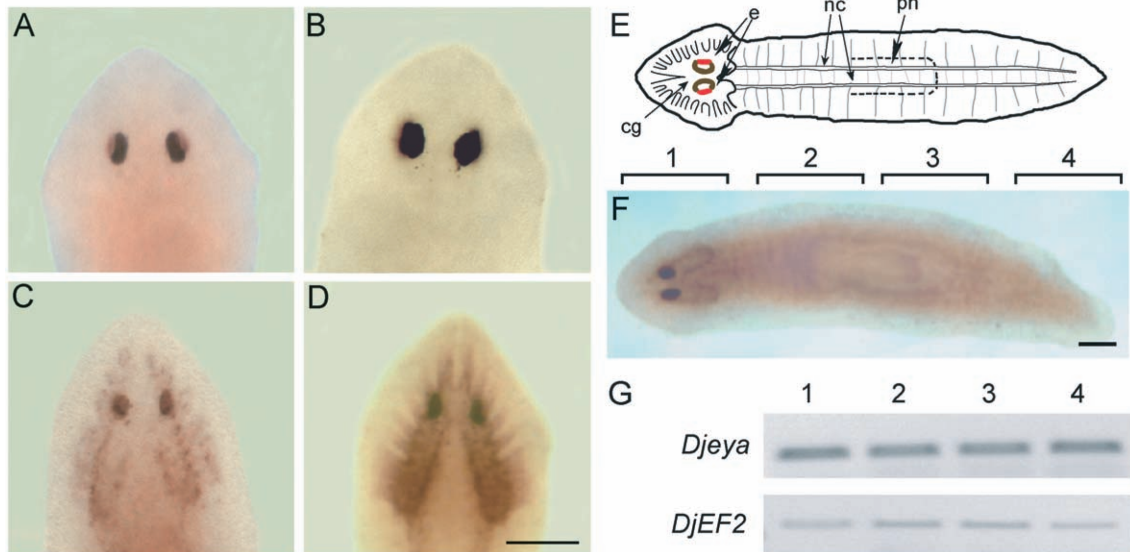

Fig. 2. Expression of *Djsix-1*, *Djops*, *Djeya* and *Djsyt* in intact *D. japonica*. (A,B) Dorsal view of a planarian head after whole-mount in situ hybridization with *Djsix-1* (A) and *Djops* (B). (C,D) Ventral view of a head after whole-mount in situ hybridization with *Djeya* (C) and *Djsyt* (D). (E) Schematic drawing of a planarian oriented with anterior to the left. Abbreviations—cg: cephalic ganglia; e: eyes (red: photoreceptors; brown: pigment cells); nc: nerve cords; ph: pharynx. (F) Dorsal view of a planarian after whole-mount in situ hybridization with *Djeya*. *Djeya* expression is detectable not only in the eyes and at the CNS level, but also in the parenchyma. (G) Visualization of the presence of *Djeya* transcripts by comparative RT-PCR experiments, performed along regions (1–4 in F and G) of the antero-posterior axis of the body. The constitutively transcribed elongation factor gene *DjEF2* was used as an internal amplification control. Scale bars: 500 μm.

terminal region and a highly conserved C-terminal ED of approximately 270 amino acids. Although no MAPK phosphorylation sites are present in the Djeya sequence, the presence of seven potential casein kinase II (CK2) sites and two kinase C sites suggests that the activity of this protein is modulated by phosphorylation (Fig. 1A). Comparison with eya proteins in the GeneBank and EMBL databases indicates that, although the Djeya N-terminal sequence shows little conservation, Djeya ED shares high amino acid similarity with the ED of eya proteins of various species (Fig. 1B). Based on the conserved ED domain, the evolutionary analysis between Djeya and eya proteins of other organisms supports the similarity of the planarian protein to invertebrate eya members (Fig. 1C).

Expression pattern of Djsix-1 and Djeya in intact and regenerating planarians

Expression of *Djsix-1* and *Djeya* was analyzed by whole-mount in situ hybridization in intact planarians. While *Djsix-1* was detected only in the photoreceptors (Fig. 2A), which were identified by the expression of *Djops* (Fig. 2B), *Djeya* showed an elaborate expression pattern (Fig. 2C). In addition to the eye cells, *Djeya* expression

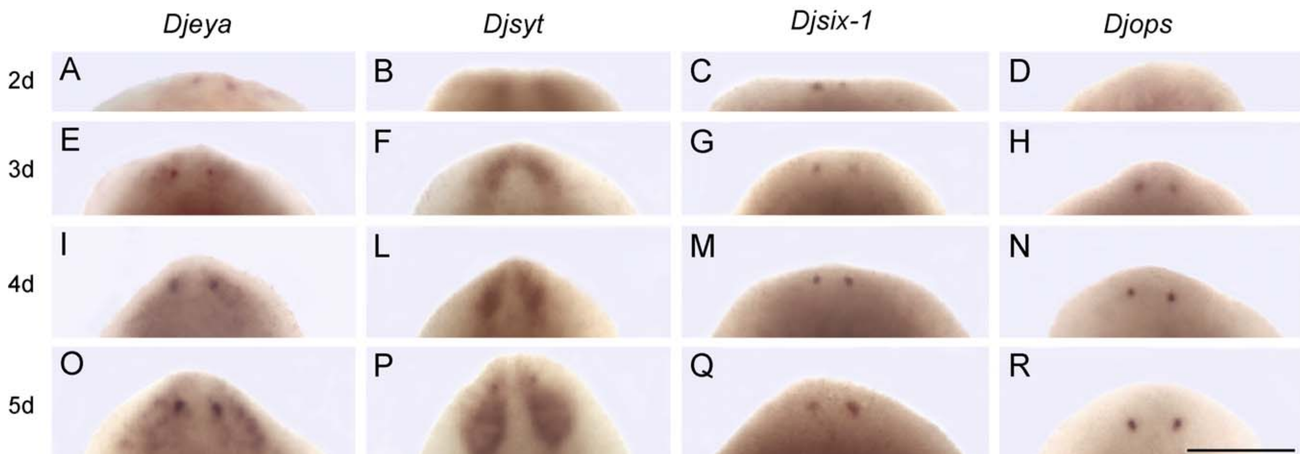

Fig. 3. Expression of *Djeya*, *Djsyt, Djsix-1* and *Djops* during head regeneration in *D. japonica*. (A–R) Distinct regeneration stages of a planarian visualized by whole-mount in situ hybridization. *Djeya* (A, E, I, O). *Djsyt* (B, F, L, P). *Djsix-1* (C, G, M, Q). *Djops* (D, H, N, R). 2d, 3d, 4d, 5d: 2, 3, 4, 5 days after amputation. Scale bar: 500 μm.

also extended to groups of cells distributed at the level of the medial and lateral region of the cephalic ganglia. The organization of the cephalic ganglia—the planarian brain including nine branches that connect to the sensory organs (Figs. 2D,E)—was identified by the expression of the pan-neural marker *Djsyt* in Fig. 2D. However, we do not know for certain whether *Djeya* transcripts are present in the same cells that express *Djsyt*. Outside the brain regions, the presence of *Djeya* transcripts was detected throughout the parenchyma (Fig. 2F). The specificity of *Djeya* parenchymal expression was confirmed by comparative RT-PCR experiments (Fig. 2G). Given the essential role of *six-1* in

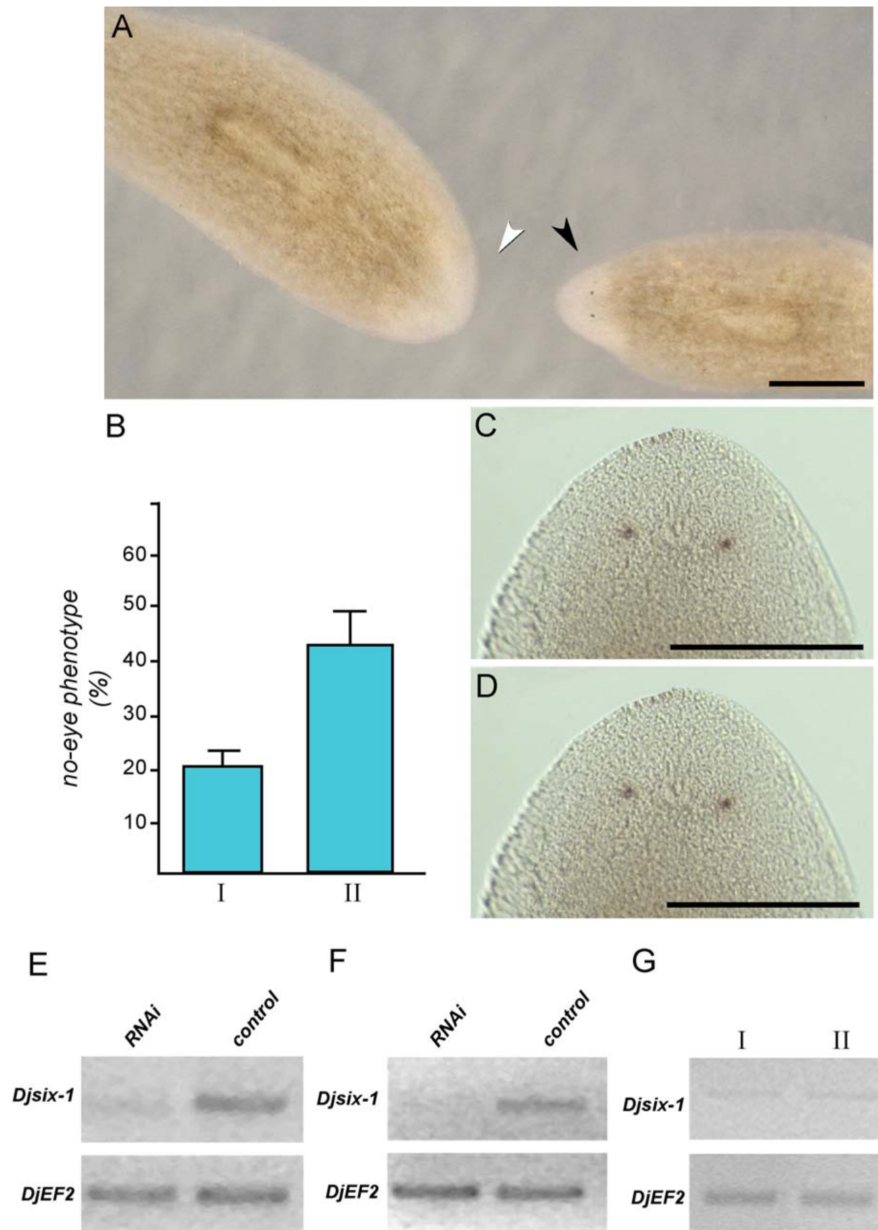

Fig. 4. Effect of *Djsix-1* dsRNA injection during head regeneration in *D. japonica*. (A) Bright-field image of a *Djsix-1* dsRNA-injected planarian (white arrowhead) and a water-injected control (black arrowhead). The organisms are in dorsal view, 8 days after amputation. Disruption of *Djsix-1* RNA produces a no-eye phenotype in the regenerating head. Two small differentiating eyes are visible in the regenerating head of the control. (B) *Djsix-1* RNAi mediated loss of function after the first amputation (I) and after the second amputation (II): the histogram compares the percentage of no-eye phenotypes. The percentage is presented as the mean ± SEM of three independent RNAi assays. The number of animals used in each RNAi experiment varied from 30 to 50. A corresponding number of water-injected controls were used. (C) Whole-mount in situ hybridization with *Djops* in a *Djsix-1* dsRNA-injected planarian, at 10 days of regeneration. Two small spots of hybridization signal are visible. (D) Whole-mount in situ hybridization with *Djops* performed in a water-injected control at 10 days of cephalic regeneration is shown for comparison. (E–G) Visualization of the comparative RT-PCR experiments. *DjEF2* is used as an internal amplification control. (E,F) The relative levels of *Djsix-1* endogenous transcripts in *Djsix-1* dsRNA-injected and in water-injected planarians (control) are shown. (E) Ten days of regeneration after the first amputation. (F) Ten days of regeneration after the second amputation. (G) The levels of *Djsix-1* endogenous transcripts in *Djsix-1* dsRNA-injected planarians are compared, at 10 days of regeneration after the first (I) and second (II) amputation. Scale bars: 500 μm.

planarian eye regeneration (Pineda et al., 2000), we examined the correlation between *Djeya* and *Djsix-1* by comparing their expression patterns during cephalic regeneration. The expression of synaptotagmin (*Djsyt*) and opsin (*Djops*) allowed us to monitor brain and eye regeneration, respectively (Figs. 3A–R). At day 2 after amputation, *Djeya* and *Djsix-1* transcripts were identified as two small hybridization spots in the dorsal region of the regeneration blastema, indicating that both genes are early coexpressed in the eye precursor cells (Figs. 3A,C). At that time, no regenerating ganglia or differentiated photoreceptors were detectable in the blastema by expression of *Djsyt* and *Djops*

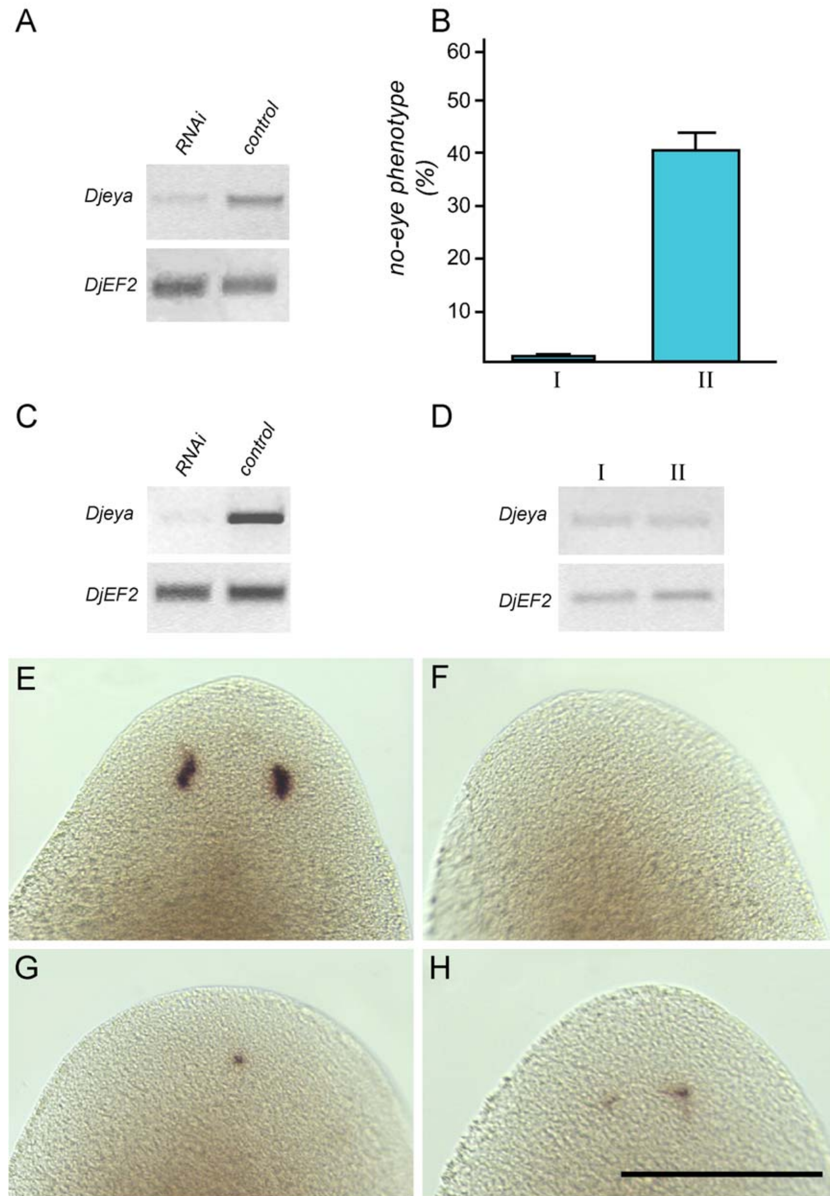

Fig. 5. Effect of *Djeya* dsRNA injection during head regeneration in *D. japonica*. (A) Visualization of a comparative RT-PCR experiment showing the relative levels of *Djeya* endogenous transcripts in *Djeya* dsRNA-injected planarians and in water-injected controls at 10 days of regeneration after the first amputation. Amplification of *DjEF2* is shown as an internal control. (B) *Djeya* RNAi-mediated loss of function after the first amputation (I) and after the second amputation (II): the histogram compares the percentage of no-eye phenotypes. The percentage is presented as the mean ± SEM of two independent RNAi assays. The number of animals used in each RNAi experiment varied from 50 to 55. A corresponding number of water-injected controls was used. (C) Visualization of a comparative RT-PCR experiment showing the relative levels of *Djeya* endogenous transcripts in *Djeya* dsRNA-injected planarians and in water-injected controls at 10 days of regeneration after the second amputation. Amplification of *DjEF2* is shown as an internal control. (D) Visualization of an RT-PCR experiment comparing the relative levels of endogenous transcripts in *Djeya* dsRNA-injected planarians at 10 days of regeneration after the first (I) and second (II) amputation. (E–H) Whole-mount in situ hybridization with *Djops*. (E) A water-injected control after 6 days from the second amputation. (F) A *Djeya* dsRNA-injected planarian regenerating a no-eye phenotype after 6 days from the second amputation. In some *Djeya* dsRNA-injected planarians at the same regenerative stage shown in E and F, one (G) or two (H) very small spots of hybridization signals can be observed, indicating differentiation of a small number of photoreceptor cells. Scale bar: 500 μm.

(Figs. 3B,D). In our experimental conditions, blastemal expression of *Djsyt* and *Djops* started at day 3 of regeneration (Figs. 3F,H), when *Djeya* was expressed in the photoreceptors and in a region underneath the regenerating eyes (Fig. 3E). Successively, *Djeya* transcripts were found in subsets of cells distributed at the level of the regenerating ganglia (Figs. 3I,O), which were visualized by *Djsyt* (Figs. 3L,P). The expression of *Djsix-1*, *Djops* and *Djeya* remained high in the eye cells throughout regeneration (Figs. 3G,I,M,N,O,Q,R).

Knocking down the expression of Djeya and Djsix-1 by RNAi

We injected planarians with *Djeya* dsRNA and used *Djsix-1* dsRNA to assess the effects of the orthologue of *Gtsix-1* on the eye regeneration of *D. japonica*. To monitor normal regeneration, a similar number of individuals were injected with water or *β-Gal* dsRNA. As expected, *Djsix-1* dsRNA injections produced a no-eye phenotype, thus confirming that the transcription factor *six-1* is essential to regenerate eyes in planarians (Fig. 4A). We observed that, unlike the consistent effects observed in *G. tigrina*, in which a single *Gtsix-1* dsRNA injection is sufficient to produce a long-lasting, no-eye phenotype in all treated fragments (Pineda et al., 2000), only 20–25% of the specimens regenerated a no-eye phenotype after successive injections in *D. japonica* (Fig. 4B). In addition, whole-mount in situ hybridization with *Djops* revealed that some of the no-eye phenotypes had two small spots of hybridization signal in the eye region, suggesting that a reduced number of photoreceptors had differentiated, compared with controls (Figs. 4C,D). Interestingly, dsRNA was more effective at producing interference in the successive regeneration, since about half of the treated animals showed a true no-eye phenotype when subjected to a second round of cephalic regeneration (Fig. 4B). In all *Djsix-1* dsRNA-injected planarians, the cephalic ganglia appeared normally differentiated after whole-mount in situ hybridization with *Djsyt* (data not shown). We also compared, by RT-PCR, the level of endogenous *Djsix-1* RNA in *Djsix-1* dsRNA-injected animals and water-injected controls, during the first and second regeneration (Figs. 4E,F). The reduction in endogenous *Djsix-1* RNA from the first and second regeneration was also compared (Fig. 4G). RNAi drastically decreased endogenous *Djsix-1* RNA to a similar extent during the first and second regeneration. We also investigated the potential of this technique to interfere with the function of *Djeya,* by using a similar experimental protocol. At 10 days of regeneration after the first amputation, comparative RT-PCR demonstrated a substantial reduction in endogenous *Djeya* RNA (Fig. 5A). *Djeya* dsRNA-injected animals regenerated normal heads, including eyes and brain. The effects of the interference were detectable only in the worms that were again decapitated and allowed to regenerate for the second time. While controls regenerated normal heads, we observed that about 40–45% of *Djeya* dsRNA-injected animals regenerated heads without eyes (Fig. 5B). Comparative RT-PCR experiments again demonstrated that these specimens had severely reduced endogenous *Djeya* transcripts (Fig. 5C). During the first and the second regeneration, the decrease of *Djeya* endogenous RNA in *Djeya* dsRNA-injected animals was comparable (Fig. 5D). To test whether *Djeya* depletion by RNAi caused the complete loss of eye cells or a significant reduction in their number, we screened a representative sample of no-eye phenotypes by in situ hybridization with *Djops*. Compared with controls, only some specimens were devoid of hybridization signal (Figs. 5E,F), one or two small spots of

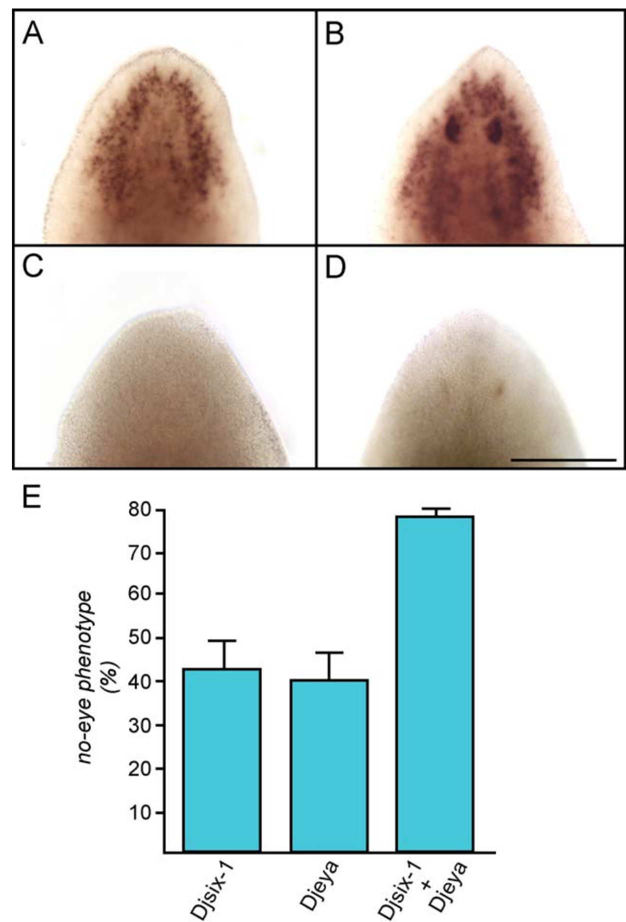

Fig. 6. Cooperation between Djeya and Djsix-1 in eye formation. (A,B) Effect of *Djsix-1* RNAi on the expression pattern of *Djeya*, visualized by whole-mount in situ hybridization. (A) A *Djsix-1* dsRNA-injected planarian regenerating a no-eye phenotype, after 10 days from the second amputation. (B) A water-injected control at the same regenerative stage shown in A. (C,D) Effect of *Djeya* RNAi on the expression pattern of *Djsix-1*, visualized by whole-mount in situ hybridization. (C) A *Djeya* dsRNA-injected planarian regenerating a no-eye phenotype, after 6 days from the second amputation. No hybridization signal is visible. (D) At the same regenerative stage as shown in C, presence of *Djsix-1* hybridization signal can be observed in a *Djeya* dsRNA-injected fragment, in which small eye spots were regenerating. (E) The histogram compares the effects of *Djeya*, *Djsix-1* and *Djeya* + *Djsix-1* dsRNA injection, observed after the second amputation. The percentage of no-eye phenotypes is presented as the mean ± SEM of two independent RNAi assays. The number of animals used in each RNAi experiment varied from 30 to 40. Scale bar: 500 μm.

hybridization signal being visible in the eye region of other regenerating fragments (Figs. 5G,H). No gross defects in the brain morphology were observed after in situ hybridization with *Djsyt* (data not shown). To test the effect of *Djsix-1* RNAi on *Djeya* expression, planarians regenerating a no-eye phenotype owing to *Djsix-1*dsRNA injection were analyzed by in situ hybridization with *Djeya*. *Djeya* hybridization signal was not detected in the blastema at the level of eye presumptive region (Fig. 6A). In contrast, *Djeya* was strongly expressed in the regenerating eyes of water-injected planarians (Fig. 6B). At the brain level, as well as along the antero-posterior axis in the parenchyma, *Djeya* expression was unaffected by *Djsix-1* RNAi and the hybridization pattern was similar to that found in the controls (Figs. 6A,B). Planarians regenerating a no-eye phenotype because of *Djeya* RNAi were also hybridized with *Djsix-1*. While no hybridization signal was found in planarians regenerating a no-eye phenotype (Fig. 6C), *Djsix-1* transcripts were detected when *Djeya* dsRNA-injected animals regenerated small eye spots (Fig. 6D). Interestingly, coinjection of an equimolar mixture of *Djeya* and *Djsix-1* dsRNA dramatically augmented the number of planarian fragments regenerating a no-eye phenotype during the second regeneration (78%). These results suggest that both genes function by direct regulatory interaction (Fig. 6E).

Djsix-1 and Djeya interact through their evolutionarily conserved domains

Drosophila eya and so are thought to form a functional heterodimer essential for the transcriptional regulation of specific genes involved in eye development (Pignoni et al., 1997). Since both *Djsix-1* and *Djeya* RNAi suppress the eye phenotype, we performed an in vitro binding analysis to assess the hypothesis of physical interaction between Djsix-1 and Djeya through their evolutionarily conserved domains (Fig. 7A). We tested whether the planarian ED is associated with the Djsix-1 six domain (SD), the Djsix-1 homeodomain

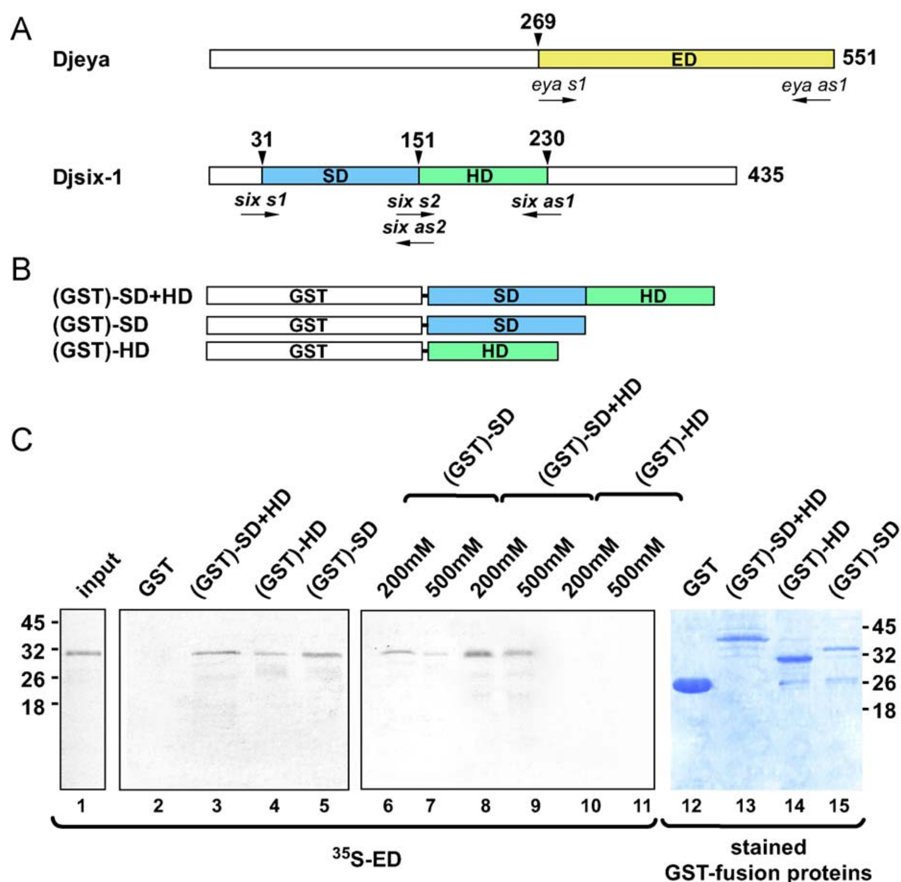

Fig. 7. In vitro analysis of the interactions between *Djeya* and *Djsix-1*. (A) Schematic representation of *Djeya* and *Djsix-1*. Abbreviations: ED, eya domain; SD, six domain; HD, homeodomain. Arrowheads with the relative numeric value indicate the amino acid position. Numbers on the right refer to the amino acid residues of each protein. Primers utilized for the isolation of the different domains are indicated under each diagram. (B) Diagrams of the GST fusion proteins utilized for the in vitro binding experiments. (C) ^{35}S-labeled ED (lane 1: input). In the presence of 150 mM NaCl, ^{35}S-labeled ED binds to (GST)-SD + HD (lane 3), (GST)-HD (lane 4) and (GST)-SD (lane 5), but not to GST (lane 2). In the presence of 200 or 500 mM NaCl, only (GST)-SD and (GST)-SD + HD are still capable to bind ED (lanes 6, 7 and 8, 9, respectively). (GST)-HD (lanes 10 and 11) does not bind ED in these conditions. SDS-PAGE Coomassie-stained GST (lane 12) and the GST fusion proteins (lanes 13–15) used to bind in vitro-translated ^{35}S-labeled ED. Molecular weight markers are shown on the left (for lanes 1–11) and on the right (for lanes 12–15).

(HD) or both (SD + HD). The interaction between the ^{35}S-labeled ED and the GST-fusion proteins containing SD, HD or SD + HD was examined in vitro by a conventional GST pull-down assay. In the presence of 150 mM NaCl, the ^{35}S-labeled ED bound efficiently to the evolutionarily conserved Djsix-1 domains, but did not bind to GST (Figs. 7B,C). By increasing the ionic strength to 200 or 500 mM NaCl, the peptide containing SD or SD + HD still bound ED, while the peptide containing HD did not (Fig. 7C). This suggests that the region that mediates the interaction with Djeya maps to the SD.

Discussion

The planarian eye

> The simplest organ which can be called an eye, consists of an optic nerve, surrounded by pigment cells and covered by translucent skin... (Darwin, 1882)

Planarian flatworms are not capable of image formation, but they perceive and react to light by simple eyes composed by two cell types: pigmented cells and rhabdomeric photoreceptors. The pigmented cells form a cup-shaped structure, which surrounds the rhabdomeres in all directions with only one opening for light entry (reviewed by Saló et al., 2002). The axons of retinal cells project onto a dorsal-medial brain region defined by the expression of *OtxA,* an *otd/Otx*-related homeobox gene, also expressed in the visual cells (Umesono et al., 1999). This simple photoreceptive structure, which is reminiscent of the hypothetical Darwinian eye, is of particular interest in light of the proposal that all eyes originate from a common evolutionary precursor (Gehring and Ikeo, 1999). Eye regeneration is exceptionally rapid in planarians. Small regenerating eyes become clearly delineated in the cephalic blastema around 4 days after amputation, and are completed in the following week. Thus, all major eye differentiative events occur in a short time, making the planarian eye an excellent model for deciphering the genetic network that mediates the morphogenesis of a structure during regeneration.

Djeya and Djsix-1 genes from D. japonica

Although some genes involved in the eye formation of other organisms have now been isolated in planarians (see Saló et al., 2002), the only gene that was found essential for eye regeneration is *Gtsix-1* (Pineda et al., 2000). *Gtsix-1* is the orthologous of the *so* gene, which is required for the development of the entire visual system in *Drosophila* (Cheyette et al., 1994; Serikaku and O'Tousa, 1994). In the fly, *so* participates in the eye regulatory network by physical interaction with another nuclear factor encoded by *eya* to form a complex that feeds back on *ey* expression (Bonini et al., 1993, 1997; Pignoni et al., 1997). Since no defect on eye regeneration has been reported for the targeted inactivation of the *ey* homologues in planarians (Pineda et al., 2002; Saló et al., 2002), we wondered to what extent this circuit of gene regulation is conserved to regenerate the planarian eye. We thus cloned and investigated the role of *Djeya*, the planarian homologue of *Drosophila eya*, in *D. japonica*. This gene is expressed strongly in the eye of adult planarians, as well as in other tissues. To test the hypothesis that planarian and *Drosophila eya* genes are involved in analogous processes during eye specification, we also identified *Djsix-1*, a member of the *Six/so* gene family, in *D. japonica*. *Djsix-1* shows an astonishing identity with its *G. tigrina* counterpart, and is also exclusively expressed in photoreceptor cells, suggesting a conservation of function as well.

Djeya and Djsix-1 are early coexpressed in eye precursor during regeneration

In situ hybridization reveals that both *Djeya* and *Djsix-1* are expressed early in the blastema regions where eyes are regenerating. In fact, two spots of hybridization signal were observed in the dorsal-anterior region of the cephalic blastema after 2 days of regeneration at 18°C, while no *Djops* or *Djsyt* hybridization signal was detected in correspondent regenerants, in the same experimental conditions. *Djeya* and *Djsix-1* transcripts may thus colocalize early in eye precursor cells. After 3 days of regeneration, *Djeya* was also clearly expressed at the level of new brain primordium. Expression of *Djeya* and *Djsix-1* was then maintained throughout regeneration and in intact adults. Continuous expression of developmental genes in differentiated tissues is not unusual in planarians, because all differentiated cells are unceasingly replaced by substitutes generated from neoblasts (see Baguñà, 1998). Planarian eyes are therefore dynamically maintained. The regeneration eye field in the head region (Sakai et al., 2000) may play a primary signaling role in defining the correct differentiation/maintenance of these cells.

Djeya and Djsix-1 act cooperatively in reconstructing the planarian eye

A major advantage of using planarian regeneration for genetic research is the possibility to produce targeted loss of function by the use the RNAi technique (Sánchez Alvarado and Newmark, 1999). In our RNAi experiments, dsRNA was more effective in producing interference in the successive regeneration. The detection of altered phenotypes during the second regeneration in dsRNA-injected planarians is probably due to the rapid neoblast-based cell turnover, which typifies these invertebrates. A number of neoblasts fated to become eye cells, and thus containing the appropriate regulatory proteins, may be present in the parenchyma and prevent eye defects during the first regeneration. Accordingly, the percentage of no-eye phenotypes increased during the second regeneration in *Djsix-1* dsRNA-injected planarians.

In addition, while no visible *Djeya* RNAi effects were detected during the first regeneration, a rather variable phenotype, ranging from total loss of eyes to more or less reduced eyes, was obtained in the successive regeneration. The number of planarians regenerating a no-eye phenotype strongly increased by coinjection of *Djsix-1* and *Djeya* dsRNA. This suggests that *Djsix-1* and *Djeya* products form a complex that regulates the regeneration of eye cells. Our in vitro results demonstrate that the planarian six and eya interact through their conserved domains. Correct photoreceptor differentiation probably requires proper gene expression of both *Djsix-1* and *Djeya*. In planarians injected with *Djeya* dsRNA, several committed neoblasts may not find the appropriate amount of *Djeya* product. Planarian cells receiving wrong or conflicting differentiation signals may be eliminated, probably by a cell death mechanism similar to that described in *Drosophila* (Bonini and Fortini, 1999; Bonini et al., 1993). Although both six-1 and eya are nuclear proteins and present cross-regulatory interactions (Pignoni et al., 1997), only six-1 encodes a DNA-binding transcription factor (Cheyette et al., 1994; Serikaku and O'Tousa, 1994), while eya belongs to a new class of protein tyrosine phosphatases (Rayapureddi et al., 2003). Therefore, the presence of planarians with a no-eye phenotype, detected during the first regeneration in *Djsix-1* dsRNA-injected animals, may be due to the specific disruption of an essential player. In contrast, only a very low protein level, such as that resulting from prolonged *Djeya* RNAi-mediated silencing after the second amputation, may affect the formation of the Djsix/Djeya complex required to regenerate normal photoreceptors. In conclusion, our gene knockdown experiments clearly demonstrate that selective silencing of *Djsix-1* and/or *Djeya* abolishes (or reduces the number of) eye precursors, indicating that both these genes are required for the correct specification of eye cells. Moreover, in agreement with the absence of *Djsix-1* transcripts in the cephalic ganglia, depletion of *Djsix-1* did not affect *Djeya* transcription in the brain, suggesting that Djeya interacts with a different protein in that structure.

The eye morphogenetic pathway and the perspective of eye evolution

Although inductive signals for brain formation have begun to be characterized in planarians (Cebrià et al., 2002), nothing is known about the genes that respond to these signals and initiate a program to specify the regeneration field, in which new eyes differentiate in a short time. Apparently, the genetic pathway leading to the correct regeneration of a planarian eye misses some of the partners involved in the eye development both in invertebrates and vertebrates. Although *Pax6* genes are expressed in the photoreceptors of the planarian eye, no detectable interference was observed in *Pax6* dsRNA-injected specimens, also during the second round of cephalic regeneration (unpublished results), supporting the hypothesis that the genetic network of planarian eye regeneration is *Pax6* independent (Pineda et al., 2002). Conversely, our data demonstrate that the planarian *Djsix-1* and *Djeya* gene products cooperate for eye regeneration, suggesting that the eya-so regulatory cassette represents an evolutionarily conserved mechanism to form eyes.

Recent evidence in *Drosophila* indicates that other *Pax6*-related genes, such as *eye gone* (*eyg*) (Jang et al., 2003), can act cooperatively and functionally replace *ey*. The absence of *Pax6* dsRNA-mediated interference effect in planarian eye regeneration may be because of an *eyg*-related gene substitutes the planarian *Pax6* in the same pathway. Alternatively, regeneration may represent a peculiar scenario, in which some regulatory steps would be unnecessary (Saló et al., 2002). However, photoreceptors differentiating in the absence of *Pax6* have also been reported in developing eyes of other Lophotrochozoan taxa (Arendt et al., 2002; Tomarev et al., 1997), and in *Amphioxus* (Glardon et al., 1998). These examples of *Pax6*-independent eye formation could represent peculiar, derived conditions instead of an ancestral condition, even if additional molecular comparative data will be needed to shed light on this issue.

In addition, the coexistence of distinct eye types, which are defined by specific genetic pathways, makes it difficult to define evolutionary relationships between Lophotrochozoan and insect eyes. In insects, a putative cerebral extraocular photoreceptor lies close to, but separated from, the compound eye (Felisberti et al., 1997; Fleissner and Frisch, 1993; Hagberg, 1986; Schulz et al., 1984; Seifert et al., 1987). In *Drosophila*, this extraretinal photoreceptor, called "Hofbauer–Buchner eyelet", contains some cells with pigment and numerous microvilli arranged in Rhodopsin 6-positive rhabdomeres (Yasuyama and Meinertzhagen, 1999). The genetic pathway driving eyelet development has not been supported by molecular evidence so far. However, recent data demonstrate that the eyelet derives from the 12 photoreceptors of the larval light-sensing Bolwig organ (Helfrich-Förster et al., 2002). Bolwig organ formation is controlled by the combined action of Hedgehog, so and eya (Suzuki and Saigo, 2000). Conversely, *ey* is down regulated and absent during most phases of Bolwig organ development (Sheng et al., 1997). The unattended morphological and molecular similarities between the inverted cup-shaped eye of planarians and the *Drosophila* eyelet/Bolwig organ suggest homology between these structures. The isolation of new factors such as *eye gone*, as well as the use of new genetic tools, such as transgenic planarians (González-Estévez et al., 2003), will be crucial to define the relationships between these eye regulatory partners.

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