DjPum, a homologue of *Drosophila Pumilio,* is essential to planarian stem cell maintenance

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Accepted 14 February 2005

Development 132, 1863-1874
Published by The Company of Biologists 2005
doi:10.1242/dev.01785

Summary

As stem cells are rare and difficult to study in vivo in adults, the use of classical models of regeneration to address fundamental aspects of the stem cell biology is emerging. Planarian regeneration, which is based upon totipotent stem cells present in the adult – the so-called neoblasts – provides a unique opportunity to study in vivo the molecular program that defines a stem cell. The choice of a stem cell to self-renew or differentiate involves regulatory molecules that also operate as translational repressors, such as members of PUF proteins. In this study, we identified a homologue of the *Drosophila* PUF gene *Pumilio* (*DjPum*) in the planarian *Dugesia japonica*, with an expression pattern preferentially restricted to neoblasts. Through RNA interference (RNAi), we demonstrate that gene silencing of *DjPum* dramatically reduces the number of neoblasts, thus supporting the intriguing hypothesis that stem cell maintenance may be an ancestral function of PUF proteins.

Key words: Planarians, Regeneration, Neoblasts, Stem cells, RNAi, Cell proliferation, Pumilio, PUF proteins, Confocal microscopy, TEM, Cytofluorimetry, In situ hybridization, Post-transcriptional regulation

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Introduction

Stem cells are undifferentiated cells defined by the capability to indefinitely renew themselves and to give rise to specialized cell types. The earliest stem cells in the embryo can produce all cell types. Conversely, stem cells of adult tissues usually differentiate into cells of the tissue of origin, even though evidence for adult stem cell plasticity has been claimed (see Raff, 2003). Extracellular and intracellular factors control whether a daughter cell of a stem cell division self-renews or commits to a specific pathway of differentiation. Secreted signals important to regulate embryonic development play a key role in controlling the stem cell expansion/fate (Lee et al., 2004; Maye et al., 2004; Ying et al., 2003). Furthermore, transcription factors such as Oct4, Sox2, FoxD3, Stat3 and the new recruit Nanog are crucial for regulating embryonic stem cell potency and self-renewal in mouse (see Cavaleri and Scholer, 2003). Regulatory molecules that operate as translational repressors act in addition to the transcriptional operating system in defining the intrinsic molecular program of a stem cell (Kuersten and Goodwin, 2003). Members of the evolutionary conserved PUF family of RNA-binding proteins have emerged with a pivotal role in supporting maintenance and self-renewal of stem cells in different organisms (Spassov and Jurecic, 2003a; Wickens et al., 2002). In *Drosophila*, the PUF protein Pumilio is required not only in embryonic patterning, but also in maintaining functional germline stem cells (Forbes and Lehmann, 1998; Lin and Spradling, 1997; Parisi and Lin, 1999). The *C. elegans* Pumilio homologue, FBF, is fundamental for germline stem cell self-renewal and plays a key role in sustaining mitosis by repressing *gld-1* mRNA activity (Crittenden et al., 2002). PUF proteins appear similarly involved in the maintenance of germline stem cells in mammals. Recently, mammalian *Pumilio* homologues were found to be expressed also in neural and haematopoietic stem cells. This finding suggests the interesting possibility that PUF proteins play a role in the maintenance of both germ and somatic stem cells (Spassov and Jurecic, 2003b). How PUF proteins control mRNA activity is still unclear. It has been proposed that they interact with different proteins by repressing translation or enhancing degradation of target mRNAs (Olivas and Parker, 2000; Wreden et al., 1997).

As adult stem cells are rare and difficult to study in vivo in most organisms, the use of classical models of regeneration for studying stem cell biology has been recently re-proposed (Newmark and Sánchez Alvarado, 2002; Pearson, 2001; Pennisi, 2004; Tanaka, 2003; Tsai et al., 2002; Weissman, 2000). Planarians (Platyhelminthes, Tricladida), an invertebrate group well known for the exceptional regenerative capability, retain a population of totipotent stem cells, the neoblasts, throughout their life. The unlimited capability of neoblasts for self-renewal and their ability to generate all differentiated cell types is crucial for planarian regeneration.

During this process, stem cells proliferate and accumulate beneath the wound epithelium, giving rise to the regenerative blastema, from which the missing body parts are reconstructed (Baguñà, 1998; Brønsted, 1969; Gremigni, 1981). These cells are scattered in the parenchyma with the exception of the most anterior end of the cephalic region and are preferentially accumulated in the dorsolateral body area, along the anteroposterior axis (Newmark and Sánchez Alvarado, 2000; Salvetti et al., 2000). A combination of grafting and X-ray irradiation experiments has recently demonstrated that neoblast commitment depends on the positional information signals coming from differentiated cells (Agata and Watanabe, 1999; Kato et al., 2001).

Here, we report the isolation and characterization of a planarian gene, *DjPum*, that shares significant sequence similarity with members of the PUF gene family. We demonstrate that this gene is expressed in neoblasts and its inactivation by RNA interference (RNAi) inhibits the formation of the regenerative blastema. Indeed, planarians injected with *DjPum* dsRNA are unable to regenerate and die, owing to a dramatic reduction of neoblasts. This finding demonstrates that *DjPum* plays a crucial role in neoblast maintenance and supports the intriguing possibility that PUF proteins play a key function in sustaining mitotic proliferation and self-renewal of both somatic and germline stem cells.

Materials and methods

Animals

Planarians used in this work belong to the species *Dugesia japonica*, clonal strain GI (Orii et al., 1993). Animals were kept in autoclaved stream water at 18°C and starved for 1 or 2 weeks before being used in the experiments. Regenerating fragments were obtained as described by Salvetti et al. (Salvetti et al., 1998). X-ray irradiation was performed according to Shibata et al. (Shibata et al., 1999). Planarians used in FACS analysis were kept for 2 days in kanamycine sulphate (10 μg/ml, Sigma), in order to prevent bacterial contamination.

Cloning of *DjPum* and sequence analysis

A *DjPum* cDNA fragment of 440 bp was amplified with two degenerate oligonucleotides corresponding to the amino acid sequence IQKFFEFG and IGNYVIQ directed against two conserved regions of the second and sixth repeat, respectively. The SMART RACE cDNA amplification kit (Clontech) was used to obtain the full-length *DjPum* sequence. Amplification of the 5′ region was obtained with the sequence-specific antisense primer 5′-TAATTACTGATCCCTCCAATTCACGCAC-3′. The 3′ region was amplified with the sequence-specific sense primer 5′-GTACACCAGAACAAACCGCTCCAA-3′. The PCR products were TA-cloned using pGEM-T easy vector (Promega). All clones were sequenced by automated fluorescent cycle sequencing (ABI). Sequences related to *DjPum* were identified with BLAST (Altschul et al., 1990). CLUSTALW was used to obtain the multiple alignment of the DjPum PUF repeats and the PUF repeats of human Pum 2 and *Drosophila* Pumilio. The sequences of PUF-related proteins used for the phylogenetic tree construction were obtained from the EMBL/GenBank.

In situ hybridization

Whole-mount in situ hybridization was carried out according to the protocol described by Agata et al. (Agata et al., 1998). Sense and antisense DIG-labelled RNA probes were obtained using the DIG-RNA labelling kit (Roche). The clone *DjPum 440* (Fig. 1A; 1900 bp to 2340 bp), containing the coding region from the second to the sixth PUF repeat and the clone *DjPum 550* (Fig. 1A; 2446 bp to 2996 bp), containing the seventh PUF repeat and the 3′ terminus, were used to obtain sense and antisense DIG-labelled RNA probes. The clone *DjPum 440* was also used to obtain the antisense biotin-labelled RNA probe. The clone *DjMCM2* was used to obtain the antisense DIG-labelled RNA probe (Salvetti et al., 2000). Dissociated cells were prepared as described by Hwang et al. (Hwang et al., 2004). Double fluorescent in situ hybridization was carried out using a TSA-indirect kit (NEL Life Science Products). After hybridization the biotin-labelled probe was revealed by SA-HRP and the signal was amplified using TSA-tetramethylrhodamine. DIG-labelled RNA was revealed by FITC-conjugated anti-DIG antibody (Roche). Dissociated cells were also hybridized with *DjPum 440* DIG-labelled RNA probe as described by Salvetti et al. (Salvetti et al., 2000).

RNAi experiments

DjPum 440 and *DjPum 550* were digested with *Apa*I and *Pst*I to obtain sense and antisense RNA, respectively. Sense and antisense RNA were pooled, phenol purified and denaturated at 85°C for 15 minutes. Annealing was performed incubating the reaction at 37°C for 90 minutes, at 30°C for 60 minutes and then at room temperature for 20 minutes. After ethanol precipitation, the quality and quantity of dsRNA were analyzed by agarose gel electrophoresis. RNAi was performed by injection of 10^{10}-10^{11} molecules of dsRNA, using a Drummond Scientific (Broomall, PA) nanoject injector. Negative controls were carried out by injection of water, or *β-Gal* dsRNA or *Djeya* dsRNA. *Djeya* dsRNA was obtained as described by Mannini et al. (Mannini et al., 2004). Intact specimens were injected three times, one injection every 2 days, for a week, then transected and allowed to regenerate. Regenerating planarians were injected every 3 days. In some experiments, 15-day-old regenerants were transected again and additional injections were carried out every 3 days. Regenerating fragments were monitored for blastema formation.

Analysis of endogenous transcripts by RT-PCR

Total RNA was extracted from fragments injected with *Djeya* dsRNA, *DjPum 440* dsRNA, *DjPum 550* dsRNA or water, respectively, 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). To assess the reduction of *DjPum* endogenous transcripts in the injected specimens, we used the following primers: *DjPum*, forward 5′-TCGGGAACACCTGAGCAA-3′ and *DjPum* reverse 5′-CTGGAGGAACACATTCTAC-3′.

The primers utilized to investigate the expression level of stem cell markers were: *DjMCM2*, forward 5′-CAGGCGAATTCCAGAACTTG-3′ ann reverse 5′-TTCGGAAAGAATTGGAACAAT-3′; *DjFGFR1*, forward 5′-TGAGCTATTGATACTACTTGGG-3′ and reverse 5′-TAGATTAATTGAAATTGGTGAGA-3′.

The primers utilized to investigate the expression level of differentiated cell markers were: *DjMHC-B*, forward 5′-CAACATCATCAACGTGAATTGG-3′ and reverse 5′-AGCTCATTAAGTTTATCAACGG-3′; *DjMHC-A*, forward 5′-CAAGAACGATTGCAAGATTTAG-3′ and reverse 5′-TAGATGCAGACACCGATAGAG-3′; *DjIFb*, forward 5′-CAAGTAACAAGTATTGTCAAAGG-3′ and reverse 5′-TCCGTATCCCCAATTTGATTCT-3′; *Djsix-1*: forward 5′-GTTAGCGCATTTAGTACAAG-3′ and reverse 5′-ATTTGGCGTTTGATCTGTTG-3′; *Djops*, forward 5′-ATTATCAAATCGTGAAAGCC-3′ and reverse 5′-ATATAAAGGGATTGTACATAG-3′.

The primers utilized to investigate the expression level of the apoptotic cell marker *DjClg3* were: forward, 5′-GGGAATCAGGATATTGTTGCT-3′ and reverse, 5′-CTTCCGTCAAACCCAGATCA-3′.

Control reactions were performed in the absence of reverse transcriptase. The constitutively expressed elongation factor gene *DjEF2* was amplified as an internal control using forward 5′-TTAATGATGGGAAGATATGTTG-3′ and reverse 5′-GTACCATAGGATCTGATTTTGC-3′ primers. For each PCR reaction, the concentration of

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cDNA and the number of cycles used were optimized to observe a quantifiable signal within the linear range of amplification, according to 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 independent samples.

TUNEL assay

The TUNEL assay was performed according to Hwang et al. (Hwang et al., 2004). Intact planarians were injected (as described in the RNAi experiments section) with *DjPum* dsRNA or water and sacrificed 1, 3, 5 or 7 days after the first injection.

Preparation of dissociated cell samples and FACS analysis of neoblast-enriched fractions

Five days after the second transection, planarians injected with *DjPum* dsRNA and water-injected controls were dissociated into individual cells according to Baguñà and Romero (Baguñà and Romero, 1981). Cell suspensions (50 µl), prepared from three *DjPum* dsRNA-injected planarians and from three water-injected controls were placed on glass slides, air-dried and stained with Giemsa. Three slides for each sample were examined and a total number of 100 cells for slide were analyzed. Cells morphologically referred to as neoblasts (round or pear-shaped cells of 5-8 µm in diameter, with a large nucleus and scanty cytoplasm) were counted. The experiment was repeated twice.

For FACS analysis, enriched fractions of neoblasts were obtained according to Asami et al. (Asami et al., 2002) and Baguñà et al. (Baguñà et al., 1989). Briefly, *DjPum* dsRNA-injected planarians, water-injected controls and X-ray-irradiated specimens were dissociated into single cells by gently pipetting in a Ca^{2+}/Mg^{2+}-free solution (CMF: $NaH_2PO_4.H_2O$ 2.56 mM; KCl 10.21 mM; NaCl 14.28 mM; $NaHCO_3$ 9.42) containing 30 µg/ml trypsin inhibitor type II-O (Sigma). Neoblast-enriched fractions were obtained by serial filtration through nylon meshes of decreasing pore sizes (150, 50, 20 and 8 µm, Millipore). For morphological analysis, the neoblast-enriched fraction was fixed in 4% paraformaldehyde for 30 minutes, placed on glass slides, air-dried and stained with Methylene Blue and Toluidine Blue. For FACS analysis the fractions enriched in neoblasts were fixed in 70% ethanol and incubated for 30 minutes at room temperature in PBS containing propidium iodide (PI, 50 µg/ml, Roche) to stain DNA, RNAse (6.25 µg /ml, Roche) to eliminate RNA that could contribute to the fluorescence, and IGEPAL CA-630 (0.5% v/v Sigma-Aldrich) to permeate the cells. FACS analysis was performed by using a FACScalibur cytofluorimeter (Becton Dickinson) and the data were analyzed by CELL Quest analysis software (Becton Dickinson). For comparative RT-PCR analysis, total RNA was extracted from cell fractions obtained by sequential filtration through nylon meshes of 50, 20 and 8 µm pore size.

Transmission electron microscopy

Transmission electron microscopy (TEM) was performed on either *DjPum* dsRNA- or water-injected planarians. Fragments were fixed with 2.5% glutaraldehyde solution in 0.1 M cacodylate buffer, pH 7.2, for 1 hour at 4°C and postfixed with 2% osmium tetroxide in 0.1 M cacodylate buffer for 2 hours at room temperature. After rapid dehydration in a graded series of ethanol and a final dehydration in propylene oxide, specimens were embedded in an 'Epon-Araldite' mixture. Ultra-thin sections, obtained with a diamond knife on an Ultracut Reichert-Jung ultramicrotome, were placed on Formvar-carbon coated nickel grids, stained with uranyl acetate and lead citrate and observed with a Jeol 100 SX transmission electron microscope.

Confocal microscopy

Planarians were prepared for confocal microscopy according to Newmark and Sánchez Alvarado (Newmark and Sánchez Alvarado, 2000). Polyclonal rabbit anti-phospho histone H3 antibodies (anti-H3P; Upstate Biotechnology) were used at 1:700 dilution to mark mitotic cells. For primary antibody detection, rhodamine-conjugated donkey anti-rabbit antibody was purchased from Santa Cruz Biotechnology and used at 1:200 dilution. After incubation, the specimens were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and observed under epifluorescence using a Radiance Plus confocal microscope (BioRad). The negative control was performed omitting the primary (anti-H3P) antibody.

Results

Identification of a planarian member of the *PUF* gene family

With the aim of investigating whether PUF RNA-binding proteins play a role in planarian stem cell maintenance, we carried out a PCR strategy with degenerate primers, followed by RACE, to isolate planarian homologues of *PUF* genes. We identified a full-length cDNA of 2996 bp, *DjPum*, which contains an open reading frame coding for 926 amino acids (Fig. 1A). Sequence comparison with representative members of the PUF family demonstrated that DjPum has the highest similarity with mammalian and *Drosophila* members. This conservation, which encompasses a region of 396 amino acids (561 to 957) in the C-terminal part, is remarkable at the level of a cluster of eight imperfect repeats, which constitute the evolutionarily conserved RNA-binding domain of this protein family. However, DjPum shows some additional amino acids within the eighth-repeat motif (Fig. 1B). It is of interest to note that, in *Drosophila* Pumilio, the eighth-repeat motif is essential to recruit nanos (Nos) to the *hunchback* mRNA 3′ untranslated region and any mutation in this region prevents the formation of the ternary complex (Sonoda and Wharton, 1999). Phylogenetic analysis of the RNA-binding motif of PUF-related proteins from a variety of eukaryotes clearly clusters the planarian DjPum within the group, including *Drosophila* Pumilio, *Dictyostelium* PufA, *C. elegans* PUF 8 and 9, and several vertebrate PUF members (see Wickens et al., 2002) (Fig. 1C).

Expression of *DjPum* in intact and regenerating planarians

Whole-mount in situ hybridization of intact planarians showed a complex expression pattern of *DjPum* transcripts. A detectable expression of *DjPum* was observed at the level of the cephalic ganglia (the planarian brain). In addition, *DjPum* expression was also found throughout the parenchyma, where it was preferentially arranged in anteroposterior dorsal cords (Fig. 2A,B). *DjPum* parenchymal expression resembles that of *DjMCM2*, a member of the minichromosome maintenance gene family, which represents a molecular marker to detect proliferating neoblasts in planarians (Salvetti et al., 2000). X-ray irradiation, a treatment that destroys mitotically active cells and the regenerative capability (Lange, 1968), caused a dramatic reduction in the number of *DjMCM2*-expressing neoblasts (Salvetti et al., 2000). This treatment also produced a general loss of *DjPum* hybridization signal, with the exception of that localized at the brain level (Fig. 2C). As *DjPum* expression in the brain was unaffected by irradiation, we hypothesize that, at this level, *DjPum* transcripts are present in nerve cells.

After in situ hybridization with *DjPum*, regenerating planarians showed an expression pattern, which was essentially similar to that observed in intact organisms. In addition, regenerating fragments showed a preferential accumulation of

DjPum mRNA in the parenchymal region beneath the blastema (postblastema). This accumulation was easier to detect 3 days after transection and resembled the accumulation of *DjMCM2* mRNA observed in this area at a correspondent time of regeneration (Salvetti et al., 2000) (Fig. 2D,E). During lateral regeneration, *DjPum*-positive cells, as well as *DjMCM2*-positive cells, redistributed to form a radially shaped hybridization signal orientated towards the blastema (Fig. 2F-H). Although the overall appearance of Fig. 2D,E and Fig. 2F,G is somewhat different, the expression patterns of the two genes in the parenchyma actually look similar when viewed under a light microscope by a human observer. *DjPum* hybridization signal was never detected in the posterior blastema. On the contrary, *DjPum* expression could be observed in the anterior blastema, probably because *DjPum*-positive cells are present where new cephalic ganglia are organizing (data not shown).

A

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   1 cgcgggttattagaatataccttttttgtactgtgtacatagtgtacataagggaaaaaaattgtatataatcgttcattgtcataaataaagacaaATGT  100
   1                                                                                                 M  S    2

 101 CGGATTTTCATGAAAATACAACAGAAAGAAATCCATCTGGAATAAATCCAAATGTAGCTACTTGTCAAATTGCTAATTCATCGATATATAATCCCCTTGG  200
   3  D  F  H  E  N  T  T  E  R  N  P  S  G  I  N  P  N  V  A  T  C  Q  I  A  N  S  S  I  Y  N  P  L  G   35

 201 TCAAGTATCAACTGTTATTTCGAATGTTCCTAATGGGCCTTACTATTCTTCGAGTTTTGTTTATTCAGACGATTGGTCAATGCCGGGGTTATCTTTATCA  300
  36  Q  V  S  T  V  I  S  N  V  P  N  G  P  Y  Y  S  S  S  F  V  Y  S  D  D  W  S  M  P  G  L  S  L  S    68

 301 AATACTACTGAACAATTAACAAGACAAATTTCTCAACTGAGTTATGATGACCATAGTGCTTACTGGCCACCTCATCTTAGTATGCCTTTGTCAGGTGATA  400
  69 N  T  T  E  Q  L  T  R  Q  I  S  Q  L  S  Y  D  D  H  S  A  Y  W  P  P  H  L  S  M  P  L  S  G  D  N  102

 401 ATAGAACATGGGATACTTCTGAGAATAGCATTTCACAACCGATTCCTATGCATCCAGGAAGACGAGATACTTTAGGAAACGAAAGTGGCTCTTTTATGTC  500
 103  R  T  W  D  T  S  E  N  S  I  S  Q  P  I  P  M  H  P  G  R  R  D  T  L  G  N  E  S  G  S  F  M  S   135

 501 ACCTATATCAAAGGAGATGGTTGCTTTGGGGATGCAAGTTGTAAATAAGGTTTTAAGCTCTTCTCCAGGAACTGGAGTGATGTGTAGATCGGATATTATG  600
 136  P  I  S  K  E  M  V  A  L  G  M  Q  V  V  N  K  V  L  S  S  S  P  G  T  G  V  M  C  R  S  D  I  M    168

 601 GAGAACTTCTATAATAATCCATCAAGCAATTTCGGTTATTCCAATGGAAATGCCCAACCATTTCCTCAGTCGCCACCTCACTATTATTTGCCTTCTAGCC  700
 169 E  N  F  Y  N  N  P  S  S  N  F  G  Y  S  N  G  N  A  Q  P  F  P  Q  S  P  P  H  Y  Y  L  P  S  S  Q  202

 701 AGCTTTATCAACCGTCGACTTTATGTGGTACACCAGAAAATTCTCAAGTTTTCGGTTGTAACAACCAGCAAAATCAATCAATTTCACAACAGCCTCAACA  800
 203  L  Y  Q  P  S  T  L  C  G  T  P  E  N  S  Q  V  F  G  C  N  N  Q  Q  N  Q  S  I  S  Q  Q  P  Q  Q   235

 801 ACAGAGCAGTGCCAACCAGCAGTTCTTTGACCCAGCAATTATCGCAGCTTCATTCTCGAATGCTGGCCAAGGCTATCCACCTCAACAACAAGCTCCTCAA  900
 236  Q  S  S  A  N  Q  Q  F  F  D  P  A  I  I  A  A  S  F  S  N  A  G  Q  G  Y  P  P  Q  Q  Q  A  P  Q    268

 901 GGTTTACCACCTCCACCTGGATTAGCCCCATCTCATAGTGATTATATGACTGGTGAACCCACAAGTCCGTTACATAACAAACAACCTGATGAGGAACAAA  1000
 269 G  L  P  P  P  P  G  L  A  P  S  H  S  D  Y  M  T  G  E  P  T  S  P  L  H  N  K  Q  P  D  E  E  Q  N  302

1001 ATCAATATATCTGGTCTCCCGGTACTAATAACGGTATGGTACATGATTTTATGGGCGGCAATCCGGGCCCTACTTATGGATATCATCAACAAGCGCCTCT  1100
 303  Q  Y  I  W  S  P  G  T  N  N  G  M  V  H  D  F  M  G  G  N  P  G  P  T  Y  G  Y  H  Q  Q  A  P  L   335

1101 TTATGATCGTCCGACATCAGCTTTACCAACACAACAACTATATGCTAGTCAAAACGGTACACTTCATCATGGAACACAAACATCTCAAATTCAAAATCAG  1200
 336  Y  D  R  P  T  S  A  L  P  T  Q  Q  L  Y  A  S  Q  N  G  T  L  H  H  G  T  Q  T  S  Q  I  Q  N  Q    368

1201 TCTGCTGCAGCTGCATTATTCGCACGCTCTCTATATCTGGCAGCTTTAGGTAGCGGGCATGTGCCTCCGGCAACACAACAAATGGCACCTCAACAGCAAC  1300
 369 S  A  A  A  A  L  F  A  R  S  L  Y  L  A  A  L  G  S  G  H  V  P  P  A  T  Q  Q  M  A  P  Q  Q  Q  P  402

1301 CACCAAGTTTAGTTCCAGACGCATTGCATACCTTAACCAGTATGCTGGCACATGGTTCCAATTCTTATGGTCAGCAACCGCAACAACAACCTAATTTTCC  1400
 403  P  S  L  V  P  D  A  L  H  T  L  T  S  M  L  A  H  G  S  N  S  Y  G  Q  Q  P  Q  Q  Q  P  N  F  P   435

1401 TAGTAGTCAAAATAATCTAATTGGTCAAAATCAGGCTGCTTTATCGGCTTTATCAGCAGCGATGAGTGGTTACTTTCTATCACCACAAATCGGTACTCAT  1500
 436  S  S  Q  N  N  L  I  G  Q  N  Q  A  A  L  S  A  L  S  A  A  M  S  G  Y  F  L  S  P  Q  I  G  T  H    468

1501 GCTCCTCCACCAACACAACAGTTTTCCGTAATGCCTGGAAGTAATCCTACGATGCTTGGTGCACCGCAACCGAGTCATACTTTAATGCCAGCAACCGGTT  1600
 469 A  P  P  P  T  Q  Q  F  S  V  M  P  G  S  N  P  T  M  L  G  A  P  Q  P  S  H  T  L  M  P  A  T  G  S  502

1601 CACAGCTCATCTCACACCAAGGTAATAAACCTATGCAATCAGGATACAAAGGAGTTGTAGGAAATCTTAAGGACGGAGTTACCAGGAGTCGATTATTAGA  1700
 503  Q  L  I  S  H  Q  G  N  K  P  M  Q  S  G  Y  K  G  V  V  G  N  L  K  D  G  V  T  R  S  R  L  L  E   535

1701 AGATTTTCGAGCTAATAGATTGACAACGCTCACATTACAAGATATTTCCGGCCATGTTGTAGAATTTGCTCAAGATCAACATGGCTCTCGATTCATTCAA  1800
 536  D  F  R  A  N  R  L  T  T  L  T  L  Q  D  I  S  G  H  V  V  E  F  A  Q  D  Q  H  G  S  R  F  I  Q    568

1801 CAGAAATTGCAAGAATCTAGCCATAATGAGAAAACAATGGTGTTTCGAGAAATACTTCCACAGTGCTATAGTTTAATGACGGATGTATTTGGAAATTATG  1900
 569 Q  K  L  Q  E  S  S  H  N  E  K  T  M  V  F  R  E  I  L  P  Q  C  Y  S  L  M  T  D  V  F  G  N  Y  V  602

1901 TTATTCAAAGATTTTTTGATTTGGGAACACCTGAGCAAATTCAGATTCTCGGTGATAGAATTCGTAATCAAGTCCTTCAATTATCACTTCAGATGTATGG  2000
 603  I  Q  R  F  F  D  L  G  T  P  E  Q  I  Q  I  L  G  D  R  I  R  N  Q  V  L  Q  L  S  L  Q  M  Y  G   635

2001 CTGCAGAGTTATACAGAAAGCTTTAGAAACTGTTAGTAAGGTTACTCAAATTAATATTGTGCGTGAATTGGAGGGATCAGTAATTAAATGTGTAAAAGAT  2100
 636  C  R  V  I  Q  K  A  L  E  T  V  S  K  V  T  Q  I  N  I  V  R  E  L  E  G  S  V  I  K  C  V  K  D    668

2101 CAAAATGGCAATCATGTTGTACAGAAATGTGTAGAATGTGTTCCTCCAGAACATCTCGATTTTATTATCGATGCCTTTAAAGACAACGTTTACTCTCTTT  2200
 669 Q  N  G  N  H  V  V  Q  K  C  V  E  C  V  P  P  E  H  L  D  F  I  I  D  A  F  K  D  N  V  Y  S  L  S  702

2201 CAACTCATTCGTATGGTTGTAGAGTTATACAACGCATTCTGGAACATTGTACACCAGAACAAACCGCTCCAATATTAGCCGAATTACATCATTTCACTGA  2300
 703  T  H  S  Y  G  C  R  V  I  Q  R  I  L  E  H  C  T  P  E  Q  T  A  P  I  L  A  E  L  H  H  F  T  E   735

2301 GGAATTGGTTAAGGATCAATATGGTAATTATGTTATTCAACATGTTCTTGAACACGGTAAAACCGAAGACAAAAGCAAAATTGTAAATCTCCTTAGAGGA  2400
 736  E  L  V  K  D  Q  Y  G  N  Y  V  I  Q  H  V  L  E  H  G  K  T  E  D  K  S  K  I  V  N  L  L  R  G    768

2401 CGAATAGTTGAACTCAGCATTCACAAATTTGCCAGTAATGTAGTTGAAAAAGCTGTAGCACATGCAACTCGCCAAGAACGCCAAGCATTAATAAACGAAG  2500
 769 R  I  V  E  L  S  I  H  K  F  A  S  N  V  V  E  K  A  V  A  H  A  T  R  Q  E  R  Q  A  L  I  N  E  V  802

2501 TACTTCAAGATTCCATTCCAGTTTCAGCAAGTAATGCAATAATGCGTACTGCTGATGTCAGCGGTGTAGTGTACGGATCAGAAACCGACGGTAGTGATAC  2600
 803  L  Q  D  S  I  P  V  S  A  S  N  A  I  M  R  T  A  D  V  S  G  V  V  Y  G  S  E  T  D  G  S  D  T   835

2601 CGATGGTGGTGGATCTGTACAAAGAGAATCTGTTCTTTATTGGATGATGAAAGATCAATTTGCAAATTACGTTATACAAAAAATGCTCGATGTCGCTGAA  2700
 836  D  G  G  G  S  V  Q  R  E  S  V  L  Y  W  M  M  K  D  Q  F  A  N  Y  V  I  Q  K  M  L  D  V  A  E    868

2701 CAGCCTATGAGAAAAGAGTTGATGCCAAAAATTAACCCTCATTTAGGTAGCTTAAGAAAATCCCCTTCCGGAAAACATATAATAAACAAAATGGAAAAAT  2800
 869 Q  P  M  R  K  E  L  M  P  K  I  N  P  H  L  G  S  L  R  K  S  P  S  G  K  H  I  I  N  K  M  E  K  Y  902

2801 ATTATATGAAGACCAATCAAGATCTTGGTATTTTGAGTAGTAATTCTGGTATGGCTTGTGTTGGTCATATGtgaaaacaaaattggttgatgaagatggg  2900
 903  Y  M  K  T  N  Q  D  L  G  I  L  S  S  N  S  G  M  A  C  V  G  H  M  *                              926

2901 gatgttgatgatgatgatgatgatttcattggtatttggggtatttcgattttcatatttatcgtgggtaatattacagaaatcgtttttttaccaaaaa  3000
3001 aaaaaaaaaaaaaaaaaaaaaaaaaaa  3027
```

Fig. 1. *DjPum* encodes a homologue of *Drosophila Pumilio*. (A) Nucleotide sequence and predicted protein product of the planarian *DjPum* cDNA. Grey background indicates the highly conserved PUF repeat domain and the flanking regions. *DjPum* Accession Number is AJ639658. (B) Comparison of PUF repeats and flanking regions of DjPum (Dj) with the corresponding regions of *Drosophila* Pumilio (Dm) and human Pumilio 2 (Hs2). Amino acid sequences are aligned using CLUSTAL W and visualized with BOXSHADE. Black backgrounds indicate identical residues and grey backgrounds indicate conserved residues. (C) Phylogenetic tree based on the PUF repeats and the flanking regions of PUF proteins of different species. DjPum (DJ) is circled. Accession numbers: *Caenorhabditis elegans* Ce1 (*FBF-1*), AAF39879; Ce2 (*FBF-2*), Q09312; Ce3 (*PUF-3*), CAB63369; Ce4 (*PUF-4*), T33752; Ce5 (*PUF-5*), T22634; Ce6 (*PUF-6*), T21080; Ce7 (*PUF-7*), T32528; Ce8 (*PUF-8*), T15717; Ce9 (*PUF-9*), T26218; Ce10 (*PUF-10*), AAF60691; Ce11 (*PUF-11*), AAK68592; *Drosophila melanogaster* Dm (*Pumilio*), A46221; *Leishmania major* Lm, CAB62815; *Trypanosoma brucei* Tb, AAK62674; *Saccharomyces cerevisiae* Sc1 (*JSN1*), P47135; Sc2 (*PUF2*), NP-015367; Sc3 (*PUF3*), NP-013088; Sc4 (*PUF4*), P25339; Sc5 (*PUF5/MPT5/UTH4*), P39016; Sc6 (*PUF6*), S69554; *Schizosaccharomyces pombe* Sp1, CAB60694; Sp2, Q09829; Sp3, Q92359; Sp4, Q10238; Sp5, CAA20674; Sp6, CAA18887; Sp7, CAB54870; *Neurospora crassa* Nc, T49434; *Dictyostelium discoideum* Dd (*PufA*), AAD39751; *Homo sapiens* Hs1, AAG31807; Hs2, AAG31806; *Mus musculus* Mm1, AAG42319; Mm2, AAG31805; *Xenopus laevis* Xl, BAB20864; *Arabidopsis thaliana* At1, AAC95220; At2, AAC95216; At3, AAF02808; At4, AL049480; At5, BAA97177; At6, AAF87849; At7, CAB82120; At8, AAC28191; At9, AC007727; *Oryza sativa* Os, AAK73144; *Populus tremula* × *Populus tremuloides* Pt, AAF71823.

Development

B

```
Dm   GRSRLLEDFRNQPYPNLQLRD
Hs2  GRSRLLEDFRNNRFPNLQLRD
Dj   TRSRLLEDFRANRLTTLTLQD
```

Repeat 1

```
Dm   DLANHIVEFSQDQHGSRFIQQKLERATAAEKQMVFS
Hs2  DLIGHIVEFSQDQHGSRFIQQKLERATPAERQMVFN
Dj   DISGHVVEFAQDQHGSRFIQQKLQESSHNEKTMVFR
```

Repeat 2

```
Dm   EILAAAYSLMTDVFGNYVIQKFFEFGTPEQKNTLGM
Hs2  EILQAAYQLMTDVFGNYVIQKFFEFGSLDQKLALAT
Dj   EILPQCYSLMTDVFGNYVIQRFFDLGTPEQIQILGD
```

Repeat 3

```
Dm   QVKGHVLQLALQMYGCRVIQKALESISPEQQQEIVH
Hs2  RIRGHVLPLALQMYGCRVIQKALESISSDQQSEMVK
Dj   RIRNQVLQLSLQMYGCRVIQKALETVSKVTQINIVR
```

Repeat 4

```
Dm   ELDGHVLKCVKDQNGNHVVQKCIECVDPVALQFIIN
Hs2  ELDGHVLKCVKDQNGNHVVQKCIECVQPQSLQFIID
Dj   ELEGSVIKCVKDQNGNHVVQKCVECVPPEHLDFIID
```

Repeat 5

```
Dm   AFKGQVYSLSTHPYGCRVIQRILEHCTAEQTTPILD
Hs2  AFKGQVFVLSTHPYGCRVIQRILEHCTAEQTLPILE
Dj   AFKDNVYSLSTHSYGCRVIQRILEHCTPEQTAPILA
```

Repeat 6

```
Dm   ELHEHTEQLIQDQYGNYVIQHVLEHGKQEDKSILIN
Hs2  ELHQHTEQLVQDQYGNYVIQHVLEHGRPEDKSKIVS
Dj   ELHHFTEELVKDQYGNYVIQHVLEHGKTEDKSKIVN
```

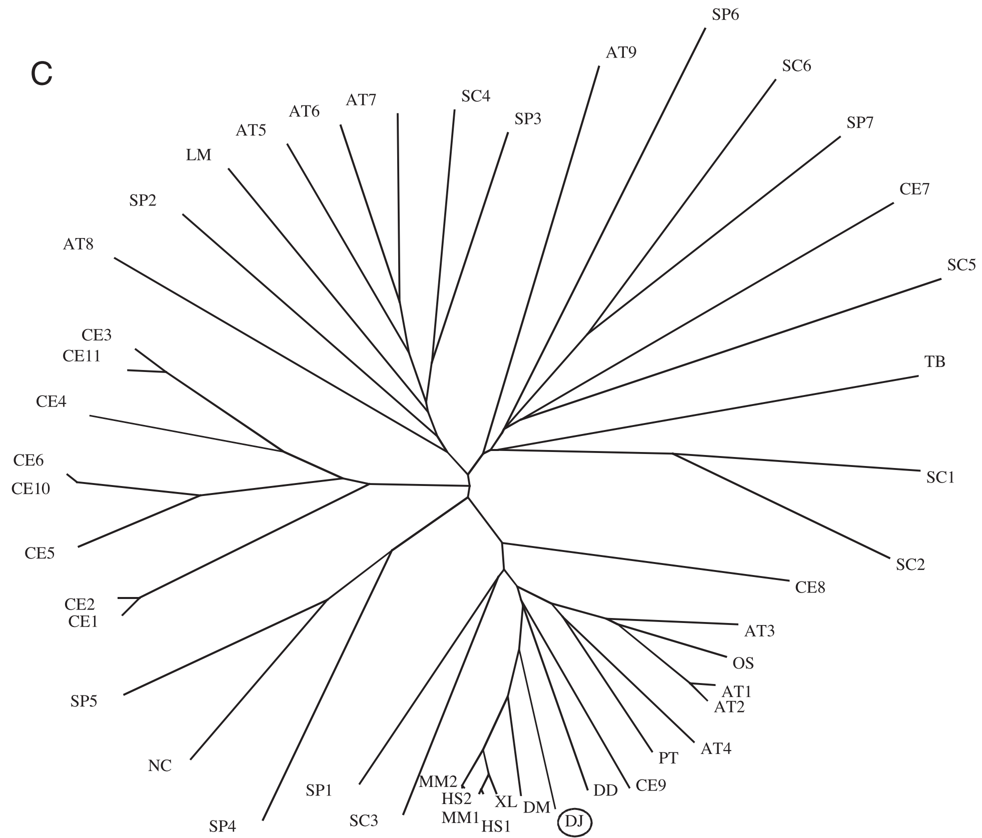
Repeat 7

```
Dm   SVRGKVLVLSQHKFASNVVEKCVTHATRGERTGLIDEVCTF
Hs2  EIRGKVLALSQHKFASNVVEKCVTHASRAERALLIDEVCCQ
Dj   LLRGRIVELSIHKFASNVVEKAVAHATRQERQALINEVLQD
```

Repeat 8

```
Dm   ND....................................NALHVMMKDQYANYVVQKMIDVSEPTQLKKLMT
Hs2  NDGP..................................HSALYTMMKDQYANYVVQKMIDMAEPAQRKIIMH
Dj   SIPVSASNAIMRTADVSGVVYGSETDGSDTDGGGSVQRESVLYWMMKDQFANYVIQKMLDVAEQPMRKELMP
```

```
Dm   KIRPHMAALRKYTYGKHINAKLEKYYMK
Hs2  KIRPHITTLRKYTYGKHILAKLEKYYLK
Dj   KINPHLGSLRKSPSGKHIINKMEKYYMK
```



In situ hybridization on dissociated cells showed the presence of *DjPum* transcripts in some neoblast-like cells (Fig. 2I). Double fluorescent in situ hybridization provided direct evidence that *DjMCM2* and *DjPum* transcripts are co-expressed in some neoblast-like cells (Fig. 2L-N). Although these results suggest that *DjMCM2*-positive cells also express *DjPum* transcripts, the faint *DjPum* expression makes difficult to unambiguously demonstrate the presence of these transcripts in all *DjMCM2*-positive cells.

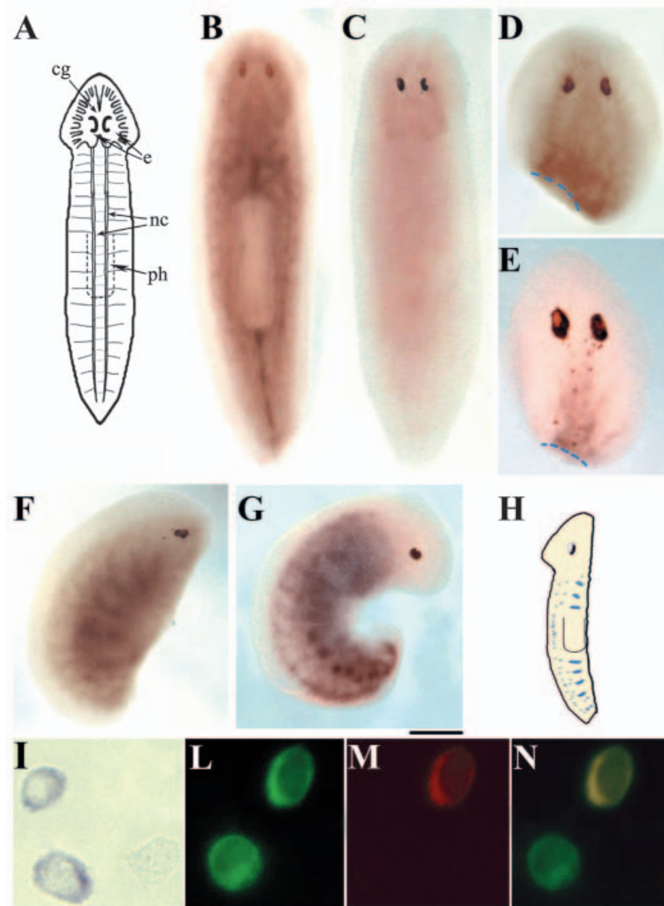


Fig. 2. Analysis of *DjPum* expression in intact and regenerating *D. japonica* by in situ hybridization. Broken blue lines indicate the blastema region, which does not contain proliferating cells. (A) Schematic drawing of a planarian (anterior towards the top). cg, cephalic ganglia; e, eyes; nc, nerve cords; ph, pharynx. (B-G) Dorsal view of whole-mount in situ hybridized planarians. (B,C) Intact specimens. (D,E) Head fragments and (F,G) lateral fragments, 3 days after transection. (B) *DjPum* expression. (C) *DjPum* expression, 10 days after X-ray irradiation. (D) *DjPum* expression. (E) Expression of *DjMCM2* is shown for comparison with the *DjPum* expression depicted in D. (F) *DjPum* expression. (G) Expression of *DjMCM2* is shown for comparison with the *DjPum* expression depicted in F. (H) Schematic drawing of a regenerating lateral fragment showing the distribution of *DjPum*- and *DjMCM2*-positive cells (blue lines). Scale bar: 400 μm. (I) Visualization of two neoblast-like cells expressing *DjPum* mRNA after in situ hybridization on dissociated cells. (L-N) Double fluorescent in situ hybridization on dissociated cells. (L) Expression of *DjMCM2* mRNA in two neoblast-like cells. (M) *DjPum* mRNA expression is detectable in one of the two cells depicted in L. (N) L and M images were combined using Adobe Photoshop 7.0. Scale bar: 10 μm.

DjPum is involved in the formation of the regenerative blastema

We analyzed the effect of RNAi-mediated gene silencing of *DjPum* during planarian regeneration. After transection, we observed that about 10% (6/57) of *DjPum* dsRNA-injected animals did not have a visible blastema and were unable to regenerate. This peculiar phenotype was seen only in anterior fragments injected with *DjPum* dsRNA. However, when the injected specimens were transected again, 95% (104/110) of them resulted devoid of blastema, independently of the level and the orientation of the cut (Fig. 3A-F). No significant difference in the type and percentage of phenotypes was found by using dsRNA obtained from two independent clones, *DjPum 440* and *DjPum 550,* which target different regions of *DjPum*. Both water or *β-Gal* dsRNA-injected fragments always regenerated a well-formed blastema (Fig. 3B-D). *DjPum* dsRNA-injected fragments did not show a blastema even 14 days after transection (Fig. 3E,F) and died within 3-4 weeks. At the same time, the water-injected controls had completely regenerated the missing body parts (data not shown). The specificity of *DjPum* RNAi was further supported by the observation that, in our experimental conditions, the RNAi-mediated inactivation of a planarian homologue of *eyes absent* (*Djeya*) never inhibited blastema formation also after the second transection. The specimens injected with *Djeya* dsRNA regenerated phenotypes devoid of eyes, as previously demonstrated by Mannini et al. (Mannini et al., 2004). As the introduction of a specific dsRNA is expected to selectively produce the degradation of cognate mRNA (Fire, 1999; Bosher and Labouesse, 2000), we analyzed the silencing of *DjPum* expression in *DjPum* dsRNA-injected animals by comparative RT-PCR. We observed that, although *DjPum* RNAi drastically decreased endogenous *DjPum* RNA, no detectable reduction in the expression level of endogenous *DjPum* mRNA was found in planarians injected with *Djeya* dsRNA or with water (Fig. 3G). TEM analysis of some *DjPum* dsRNA-injected fragments, which were unable to regenerate, confirmed the absence of unspecialized, neoblast-like cells between the wound epidermis and the stump region. In particular, RNAi-induced phenotypes devoid of a visible blastema had few neoblasts (see Morita et al., 1969), intermingled with differentiated cells (Fig. 4A). By comparison, many unspecialized, neoblast-like cells were observed in corresponding water-injected controls (Fig. 4B).

Loss of neoblasts in *DjPum* dsRNA-injected planarians

Both the similar expression pattern of *DjPum* and *DjMCM2* and the selective destruction of *DjPum* and *DjMCM2*-positive cells by X-ray irradiation prompted us to determine whether proliferating, *DjMCM2*-positive neoblasts were reduced in number after *DjPum* dsRNA injection. To address this issue, we performed in situ hybridization with *DjMCM2* on *DjPum* dsRNA-injected animals. Compared with water and *Djeya* dsRNA-injected controls, *DjPum* dsRNA-injected planarians showed a dramatic reduction of *DjMCM2* hybridization signal (Fig. 5A-G). This reduction was observed both in *DjPum* dsRNA-injected fragments that completed the first regeneration and in fragments unable to regenerate after the second transection (Fig. 5A-D). As a specificity control, we evaluated the expression of *DjMCM2* in planarians

Development

Development

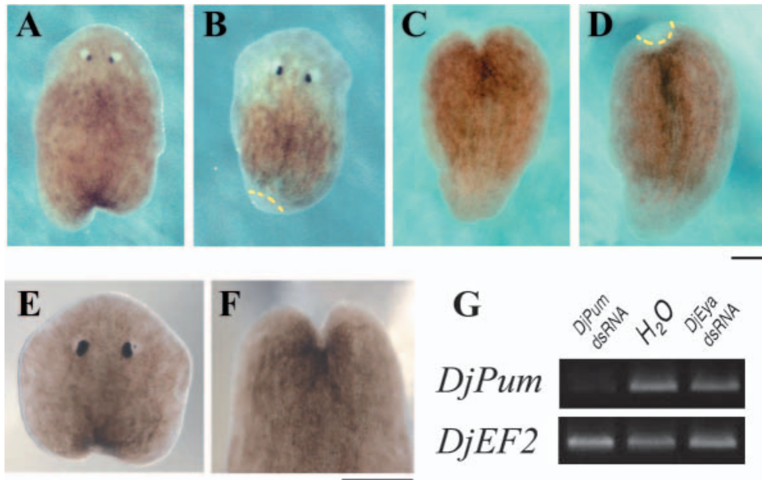


Fig. 3. Effect of *DjPum* RNAi on regeneration in *D. japonica*. Brightfield images of the injected organisms. Dorsal view, anterior is towards the top. Broken yellow lines indicate the blastema region. (A) A head fragment injected with *DjPum* dsRNA. (B) A water-injected head fragment, 4 days after the second transection. (C) A tail fragment injected with *DjPum* dsRNA, 4 days after the second transection. (D) A water-injected tail fragment, 4 days after the second transection. (E) A head fragment injected with *DjPum* dsRNA, 14 days after the second transection. (F) A tail fragment injected with *DjPum* dsRNA, 14 days after the second transection.
(G) Visualization of a comparative RT-PCR experiment in planarians injected with *DjPum* dsRNA, water and *Djeya* dsRNA. *DjEF2* is used as an internal amplification control. Scale bars: 250 μm.

regenerating heads without eyes as a consequence of *Djeya* RNAi (Mannini et al., 2004). No detectable reduction in the expression level of *DjMCM2* was observed in *Djeya* dsRNA-injected fragments (Fig. 5E,F). Comparative RT-PCR experiments were performed to assess whether *DjPum* RNAi produced variation in the expression level of stem cell markers associated (*DjMCM2*) or not associated (*DjFGFR1*) (Agata, 2003; Ogawa et al., 2002) with the cell cycle. RT-PCR experiments confirmed the strong reduction of *DjMCM2* transcripts and also showed that the mRNA level of *DjFGFR1* was significantly reduced in *DjPum* dsRNA-injected animals after the second transection (Fig. 5G). On the contrary, *DjPum* dsRNA injection did not significantly interfere with the expression of selected markers of differentiated cells [*DjMHC-A* and *DjMHC-B* (Kobayashi et al., 1998); *DjIFb* (Tazaki et al., 2002); *Djops* (Pineda et al., 2002); *Djsix-1* (Mannini et al., 2004)]. Interestingly, an activation of *DjClg3*, a caspase-like gene 3, primarily expressed in planarian apoptotic cells (Hwang et al., 2004), was observed in *DjPum* dsRNA-injected organisms sacrificed 4 days after the first injection. Successively, a substantial reduction in *DjClg3* transcript level was observed (Fig. 5G). To assess whether massive apoptotic cell death occurred during the first week, as a consequence of *DjPum* RNAi, we marked the apoptotic nuclei by using the TUNEL protocol optimized for planarians (Hwang et al., 2004). Water-injected controls showed several apoptotic cells distributed throughout the planarian body, as described by Hwang et al. (Hwang et al., 2004). However, we could observe only a slight increase in the number of apoptotic cells in *DjPum* dsRNA with respect to water-injected planarians (data not shown).

In order to confirm whether, in *DjPum*-injected animals, the reduction of *DjMCM2* transcripts corresponded to a reduction in the number of mitotic cells, we used anti-phospho histone H3 antibodies (anti-H3P), which are specific for mitotic cells (Newmark and Sánchez Alvarado, 2000; Wei et al., 1998), and confocal microscopy (Fig. 6A-D). At 15 days after transection, when regeneration was almost completed, the number of mitoses labelled with anti-H3P appeared drastically reduced in *DjPum* dsRNA-injected specimens (Fig. 6C), in comparison with that found in corresponding water-injected controls (Fig. 6B). X-ray-irradiated planarians (Fig. 6D) and the negative control, did not show any signal of immunolabeling, thus confirming the anti-H3P specificity in this planarian species. After *DjPum* RNAi, a drastic reduction of mitoses was also observed in intact planarians (data not shown).

As X-ray irradiation destroys the mitotically active stem cell population in planarians, we exploited the possibility to identify the proliferating neoblasts among the neoblast-like cells by using a combined approach of progressive filtering, X-ray treatment and FACS analysis. A population of dissociated cells enriched in neoblasts by progressive filtering was obtained from water-injected controls or irradiated planarians

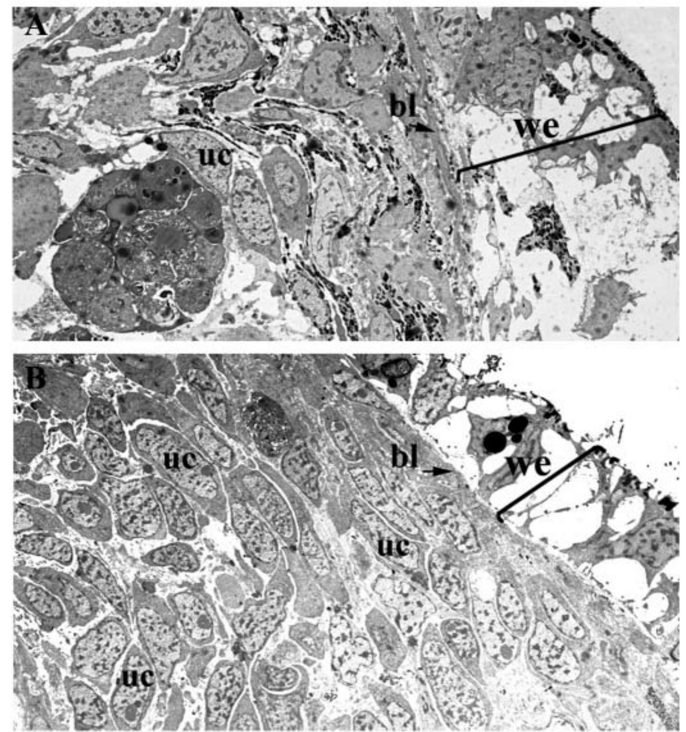


Fig. 4. Ultrastructural analysis of a phenotype lacking the blastema, as a consequence of *DjPum* RNAi in *D. japonica.* (A) Wound region of a *DjPum* dsRNA-injected fragments, 3 days after the second transection. (B) A 3-day-old blastema region from a water-injected control is shown for comparison. uc, unspecialized, neoblast-like cells; we, wound epidermis; bl, basal lamina. Scale bar: 10 μm.

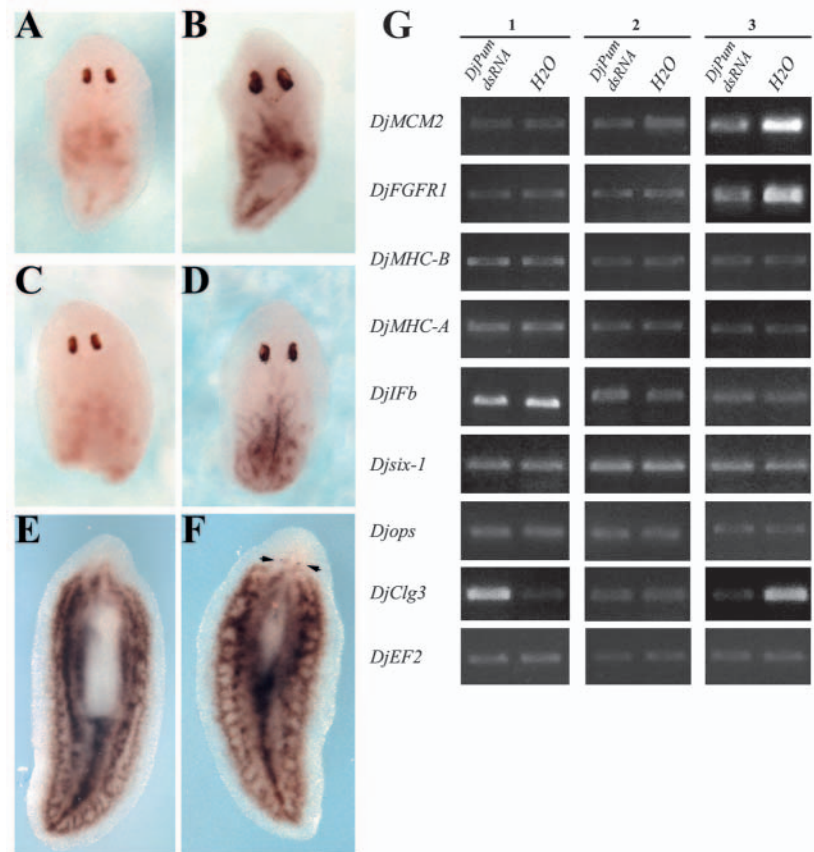


Fig. 5. Expression of representative cell markers after *DjPum* RNAi in *D. japonica.* (A-F) *DjMCM2* expression visualized by whole-mount in situ hybridization. (A) A regenerating *DjPum* dsRNA-injected head fragment, 15 days after the first transection. (B) A regenerating water-injected head fragment, 15 days after the first transection. (C) A *DjPum* dsRNA-injected head fragment lacking a visible blastema, 5 days after the second transection. (D) A regenerating water-injected head fragment, 5 days after the second transection (E) A *Djeya* dsRNA-injected tail fragment showing a no-eye phenotype, 5 days after the second transection. (F) A water-injected tail, 5 days after the second transection. Regenerating eyes are indicated by arrows. Scale bar: 250 μm. (G) *DjMCM2*, *DjFGFR1*, *DjMHC-A*, *DjMHC-B*, *DjIFb*, *DjSix-1*, *Djops* and *DjClg3* expression visualized by comparative RT-PCR. RNA from *DjPum* dsRNA-injected planarians and water-injected controls was obtained from intact specimens 4 days after the first injection (1) and from planarian fragments sacrificed 4 days after the first (2) and the second (3) transection. *DjEF2* is used as an internal amplification control.

and passed through the cytofluorimeter. Because neoblasts have a mean diameter of 7 μm, they are the smallest cells in planarians and are the principal cell type expected to be found after the filtering procedure. Indeed, morphological analysis of the enriched fraction, which was obtained from water-injected controls, demonstrated the presence of many small spherical cells (diameter: 7 μm to 13 μm) with scanty cytoplasm (Fig. 7A). Comparative RT-PCR analysis confirmed that this fraction was specifically enriched in *DjMCM2*- and *DjFGFR1*-positive cells (Fig. 7B).

FACS analysis demonstrated that most of the events observed in samples obtained from water-injected controls, showed similar fluorescence intensity values (R2 box, Fig. 8A). Cells in the R2 box shared a similar morphology when analyzed by forward-angle light scatter (FSC) and side-angle light scatter (SSC) (Fig. 8B). When we compared the sorting profile of samples obtained from water-injected controls with that obtained from X-ray-treated planarians, we found that the cell fraction included in the R2 box was nearly absent after irradiation (Fig. 8C,D). Planarian neoblasts are considered a heterogeneous population of undifferentiated cells. Only a fraction of these cells are supposed to proliferate, the rest being determined cells ready to differentiate (Reddien and Sanchez Alvarado, 2004). In our experimental conditions of cell collection and FACS analysis (15 days after X-ray treatment), we were unable to detect any fraction corresponding to determined, not proliferating, neoblasts. A possible explanation is that these cells were already differentiated at that time, and not recovered by the filtering procedure. In order to demonstrate that the cell fraction included in the R2 box was reduced in number in *DjPum* dsRNA-injected planarians, we compared the sorting profile of the neoblast-enriched cell fraction obtained from *DjPum* dsRNA-injected animals (Fig. 8E,F) with that obtained from water-injected controls (Fig. 8A,B). The comparison showed that the number of events in the R2 box was significantly reduced after *DjPum* dsRNA

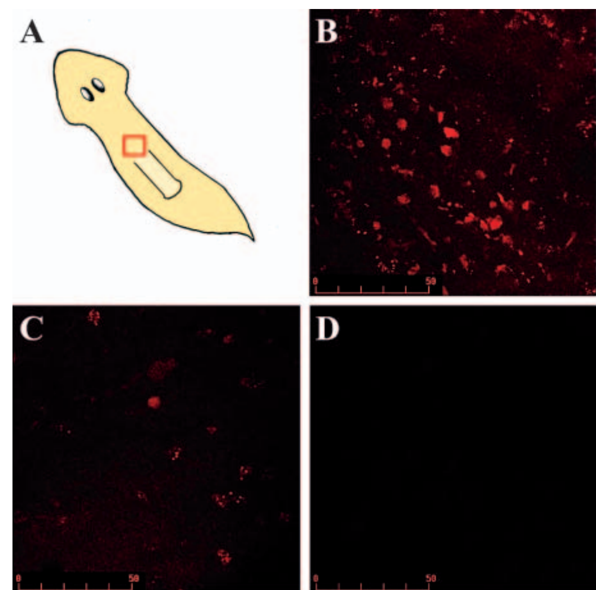


Fig. 6. Distribution of mitoses in *D. japonica*, as detected by anti-phospho-histone H3. Confocal projections of 40 sections taken at 0.9 μm intervals through 36 μm were obtained from whole-mount preparations. The H3P signal is visualized in red. (A) Schematic drawing of a planarian. Red box indicates the body region corresponding to the confocal images depicted in B-D. (B) H3P immunostaining in a water-injected control, 15 days after the first transection. (C) H3P staining in a *DjPum* dsRNA-injected planarian 15 days after the first transection. (D) H3P staining of an intact planarian, 15 days after X-ray irradiation. Scale bars: 50 μm.

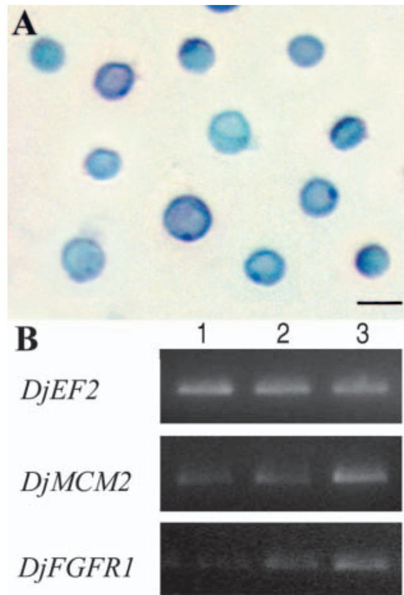


Fig. 7. Characterization of the cell fraction obtained following the serial filtering procedure in *D. japonica*. (A) Bright-field image of cells obtained by filtration through the 8 μm pore size mesh. Scale bar: 10 μm. (B) *DjMCM2* and *DjFGFR1* expression visualized by comparative RT-PCR. RNA was extracted from cell fractions obtained by filtration through nylon meshes of 50 μm (1), 20 μm (2) and 8 μm (3) pore size sequentially. *DjEF2* is used as an internal amplification control.

injection. Indeed, we calculated that *DjPum* RNAi determined a significant reduction, ranging from 55% to 90%, of the proliferating cell fraction, when the arbitrary value of 100% was attributed to water-injected controls (Fig. 8G). The *DjPum* RNAi-induced reduction in number of neoblasts, detected by the FACS analysis, was confirmed by counting the number of neoblast-like cells in dissociated cell preparations. A mean reduction of about 60% in the number of neoblast-like cells was detected in *DjPum* dsRNA-injected organisms, with respect to that found in water-injected controls.

Discussion

DjPum is a planarian PUF-related gene

Neoblast-based planarian regeneration provides us with a unique opportunity to study in vivo the molecular program operating in maintenance of the stem cell state. A key approach to this issue is to determine the role that post-transcriptional gene regulators, such as PUF proteins, play in maintaining stem cell potential to proliferate mitotically or differentiate. We have cloned *DjPum,* a planarian gene that encodes a protein typified by the presence of a RNA-binding domain consisting of eight PUF repeats plus conserved flanking residues. The presence of at least five different subfamilies of PUF proteins has been described by Wickens et al. (Wickens et al., 2002). Our phylogenetic analysis shows that DjPum clusters with high probability into the Pumilio subfamily, including members involved in stem cell maintenance, such as *Drosophila* Pumilio, mammalian PUF proteins and *Dictyostelium* PufA. Such

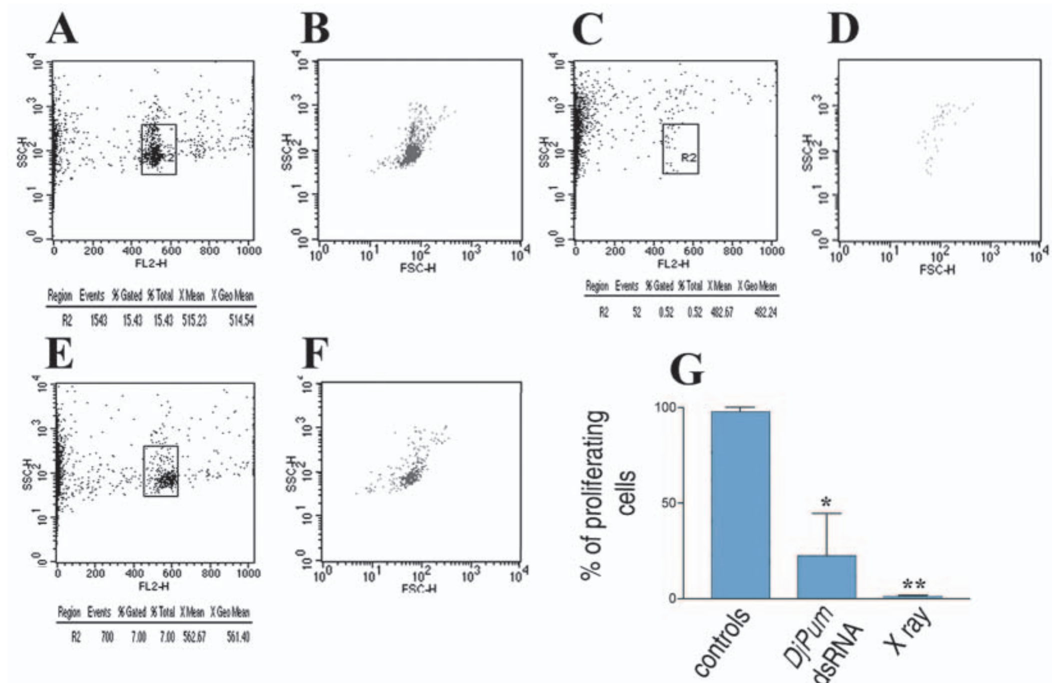


Fig. 8. FACS analysis of neoblasts in *DjPum* dsRNA-injected *D. japonica* demonstrates a consistent reduction in the number of proliferating cells. Graphs from a representative experiment are shown. The cell population individuated as proliferating cells is included in the R2 box. (A) Cells from water-injected planarians, stained with PI and analyzed for the DNA content in the fluorescent channel FL2. (B) Analysis of cells included in the R2 box in A, using FSC and SSC. (C) Cells from X-ray-treated planarians sacrificed 15 days after the treatment were stained with PI and analyzed for the DNA content in the fluorescent channel FL2. (D) Analysis of cells included in the R2 box in C, using FSC and the SSC. (E) Cells from *DjPum* dsRNA-injected planarians were stained with PI and analyzed for the DNA content in the fluorescent channel FL2. (F) FSC and SSC analysis of cells included in the R2 box in E, demonstrates that the cells interpreted as proliferating cells have a similar morphology. The 'nuage' at the left in A, C and E is probably due to residual bacterial contamination. Clusters of cells may generate the high values of fluorescence intensity observed at the right of the R2 box in A, C and E. (G) Graphic representation of proliferating cells (in percentage). GraphPad Prism Version 3.0 computer program was used for data analysis. Each bar shows the mean±s.e.m. of three separate experiments. The results were compared using the unpaired *t*-test; $P<0.05$ was considered statistically significant. *$P<0.05$; **$P<0.01$.

clustering allows us to speculate that DjPum and the other proteins included in the Pumilio subfamily, can recognize related target sequences and play similar regulatory roles. The comparison between DjPum, mammalian and *Drosophila* PUF protein members shows that the eighth motif of the planarian protein contains some additional amino acids. This non-canonical structure could be of functional importance in determining the specificity of protein interactions. Mutational analysis in *Drosophila* indicates that Pumilio interacts with Brat, a member of the NHL family, via the outer face of repeats seven and eight, and the conserved C-terminal region (Goodwin, 2001). Moreover, the eighth repeat of Pumilio plays a key role in recruiting Nos to the Nos response element (NRE). The human relative, which bears an insertion of three amino acids within this motif, also binds the NRE, but is unable to recruit Nos (Sonoda and Wharton, 1999). Because the structural conservation of the eighth repeat appears essential to recruit Nos to target mRNAs, we suggest that DjPum cannot recruit a planarian Nos homologue. DjPum could have other regions, outside the repeats, able to recruit different proteins. It has been recently proposed that PUF proteins can function with alternative partners (Wickens et al., 2002). This suggests that a combinatorial mechanism involving RNA-protein and protein-protein interactions plays a key role to build a variety of PUF-containing complexes, that can regulate different developmental events (Asaoka-Taguchi et al., 1999; Deshpande et al., 1999; Gamberi et al., 2002; Moore et al., 2003; Nakahata et al., 2001; Sonoda and Wharton, 1999).

DjPum is expressed in planarian stem cells

DjPum-positive cells are distributed throughout the planarian parenchyma and are also detected at the level of the cephalic ganglia. In *Drosophila* the *Pumilio/staufen* pathway of translational repression prevents ubiquitous expression of protein products in the neurons (Dubnau et al., 2003). The presence of *DjPum* mRNA in cells of the planarian brain suggests DjPum to be part of a translational repression complex specific for nerve cells, an hypothesis that deserves further investigation. Parenchymal cells that express *DjPum* have all the requirements to be considered neoblasts. Indeed, although X-ray irradiation does not eliminate *DjPum* transcripts from the planarian brain, this treatment dramatically affects the parenchymal expression of this gene. In the parenchyma of intact and regenerating planarians, *DjPum* hybridization signal resembles that of *DjMCM2*. *DjPum* and *DjMCM2* transcripts appear to be preferentially arranged in longitudinal dorsal cords. During regeneration, both these transcripts are preferentially accumulated in the postblastema, a region characterized by intense mitotic activity (Saló and Baguñà, 1989). *DjPum*-expressing cells appear similar to neoblasts in shape and some of the neoblasts expressing *DjPum* were also found positive for *DjMCM2* transcripts. These findings suggest a function for DjPum in proliferating neoblasts.

DjPum is essential for neoblast maintenance

RNAi-mediated gene silencing has been proven to successfully suppress specific gene activity in planarians. *DjPum* RNAi produces a strong reduction of endogenous mRNA level and results in the loss of regenerative capability. A high number of phenotypes lacking a visible blastema were observed after the second transection, being only a small percentage of them detected during the first regeneration. A more effective dsRNA-mediated interference during the second regeneration has already been observed in planarians (Mannini et al., 2004). The detection of few phenotypes lacking a blastema after the first transection is probably due to the presence of the high number of neoblasts in the planarian parenchyma. Head fragments resulted highly sensitive to *DjPum* RNAi, and some of them were unable to form a blastema even after the first transection, probably because a lower number of neoblasts are localized in the head parenchyma (Newmark and Sánchez Alvarado, 2000; Salvetti et al., 2000). *DjPum* dsRNA-injected planarians did not regenerate even after 3 weeks from transection and died after a short time. This may be due to a drastic and irreversible reduction in the number of neoblasts. Consistent with this conclusion is the observation that X-ray irradiation also produces planarian fragments that are unable to regenerate, and die within 3-4 weeks. Ultrastructural investigations demonstrated that *DjPum* RNAi does not interfere with wound closure, because this process occurred normally. However, under the TEM, no accumulation of unspecialized cells was detected beneath the wound epithelium in *DjPum* dsRNA-injected fragments. The *DjPum* RNAi-induced loss of regenerative capability may result from a failure of local neoblasts to migrate and accumulate beneath the wound epithelium. Alternatively, neoblasts might have a reduced proliferative capability and not be activated by local signals to resume proliferation after the cut. As a further possibility, we hypothesize that, after the second transection, the neoblasts in the parenchyma of *DjPum* dsRNA-injected planarians were reduced in number because of *DjPum* dsRNA interference and no new neoblasts had been produced. As part of the evidence that the number of these cells was drastically decreased, we observed that two stem cell markers, associated (*DjMCM2*) or not associated (*DjFGFR1*) with the cell cycle, had a reduced expression in *DjPum* dsRNA-injected specimens. The use of anti-H3P antibodies after *DjPum* RNAi confirmed a dramatic reduction in the number of mitoses. Counting the number of neoblasts after cell dissociation, as well as FACS analysis, demonstrated a substantial reduction in the number of these cells. In conclusion, our data indicate that downregulation of *DjPum* transcripts does not allow regeneration, because planarian stem cells are not maintained. Literature data support the hypothesis that an ancestral function of PUF proteins is that of promoting the mitotic proliferation of stem cells by controlling the translation of mRNAs known to encode for regulators of cell cycle and cell differentiation (Chen and McKearin, 2005; Gerber et al., 2004; Kennedy et al., 1997; Lin and Spradling, 1997). In *Dictyostelium* and yeast, it has been proposed that PUF proteins support mitoses by repressing the expression of cAMP-dependent protein kinase (PKA-c) (Souza et al., 1999). It has been speculated that PUF proteins might also act through a similar mechanism in *Drosophila* and *C. elegans* (Wickens et al., 2002). In order to assess whether *DjPum* RNAi induces cell differentiation, we investigated the expression levels of representative genes of a number of specialized cell types. However, no significant variation was observed in *DjPum* dsRNA-injected planarians with respect to the controls. Interestingly, a significant increase in the expression level of the apoptotic cell marker *DjClg3*, as well as a slight increase in the number of TUNEL-positive cells was observed in *DjPum* dsRNA-injected animals. These results

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indicate that apoptotic cell death occurs as a consequence of *DjPum* RNAi. Knockdown of an essential player for stem cell maintenance, such as *DjPum,* could be sufficient to trigger apoptosis. As a further possibility, *DjPum*-induced arrest of neoblast proliferation, could represent an altered stem cell condition leading to the activation of apoptotic cell death pathways.

We are especially grateful to Kiyokazu Agata and Hwang Jung Shan for providing us with the TUNEL assay protocol. We also thank Claudio Pugliesi for X-ray irradiation, Claudio Ghezzani for technical assistance with TEM and Mrs Tamar Shanks for English revision. This study was supported by a Cofinanziamento Programmi di Ricerca di Interesse Nazionale, MIUR, Italy grant.

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