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

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**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

**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 (Baguña, 1998; Brönnsted, 1969; Greignigni, 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 (Oiri 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 kanamycin 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 IQKKFEEF and INNYVIQ 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′-TAATTACTGATTCCCTCGAAATTCA-3′. The 3′ region was amplified with the sequence-specific sense primer 5′-GTACCCAGAAGACCCGCCCTCGG-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 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 Apal and PstI 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 1010-1011 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′-TGGGAACACCTGAGCCAACATCTCTAC-3′ and reverse 5′-CTGGAGAACACATCTCTAC-3′. The primers utilized to investigate the expression level of stem cell markers were: DjMCM2, forward 5′-CAGGGCAATCTCCAAGACTG-3′; reverse 5′-GTTAGCGCATTTAGTACATAG-3′; DjFGR1, forward 5′-TGAGCTACTGATACCTTCTG-3′; reverse 5′-TAGATTTGAACCTGAGTGAG-3′; Djeya, forward 5′-CTGGGAAACACATCTCTAC-3′ and reverse 5′-CTGGGAAACACATCTCTAC-3′; Djops, forward 5′-CAAGAAGACCTGCAAAGG-3′; reverse 5′-CAAGAAGACCTGCAAAGG-3′; DjClg3, forward 5′-CTTCCCGGACGACCCGAC-3′; reverse 5′-CTTCCCGGACGACCCGAC-3′.

The primers utilized to investigate the expression level of differentiated cell markers were: DjMH2-B, forward 5′-CAAGTACATGAAACCCGCC-3′; reverse 5′-ATTGCCGGAACACCGAAG-3′; DjMHC-A, forward 5′-CAAGAAGACCTGCAAAGG-3′; reverse 5′-ATTGCCGGAACACCGAAG-3′; DjIFb, forward 5′-CTGGTATCCCTCAATTTGATCT-3′; reverse 5′-CTGGTATCCCTCAATTTGATCT-3′; Djops, forward 5′-ATTATCAAACTGTAAGG-3′; reverse 5′-ATATAAAGGGATTGTACATAG-3′.

The primers utilized to investigate the expression level of the apoptotic cell marker DjClg3 were: forward, 5′-GGGAATCTACCAAGGATGAC-3′; reverse, 5′-ATATAAAGGGATTGTACATAG-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′-TGAATCTACCAAGGATGAC-3′ and reverse 5′-ATATAAAGGGATTGTACATAG-3′.

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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 Ca²⁺/Mg²⁺-free solution (CMF: NaH₂PO₄·H₂O 2.56 mM; KCl 10.21 mM; NaCl 14.28 mM; NaHCO₃ 9.42) containing 30 µg/µl 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 cytometer (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 Wikens 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* expression at the level of the brain (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 hybridized signal oriented 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).

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), C26234; Ce6 (PUF-6), T21080; Ce7 (PUF-8), T25238; Ce8 (PUF-8), T15717; Ce9 (PUF-9), T26218; Ce10 (PUF-10), AAF60691; Ce11 (PUF-11), AAK68592; Drosophila melanogaster Pumilio (Pm), AAD39751; Homo sapiens, Hs1, AAG31807; Hs2, AAG31806; Mus musculus, Mm1, AAG42319; Mm2, AAG31805; Oryza sativa Os, AAK73144; Populus tremula/H11003 Populus tremuloides Pt, AAF71823; Pisum sativum/Pv, AAK73144; Prunus domestica/Pd, AAG31830; Schizosaccharomyces pombe Sp1, CAB60694; Sp2, Q09829; Sp3, Q92359; Sp4, Q10238; Sp5, CAA20674; Sp6, CAA18887; Sp7, CAB54807; Neurospora crassa (NCBI), T49434; Dictyostelium discoidum (Dd), AAD39751; Homo sapiens (Hs1), AAG31807; Hs2, AAG31806; Mus musculus (Mm1), AAG42319; Mm2, AAG31805; Oryza sativa Os, AAK73144; Populus tremula × Populus tremuloides Pf, AAF71823.
In situ hybridization on dissociated cells showed the presence of \textit{DjPum} transcripts in some neoblast-like cells (Fig. 2I). Double fluorescent in situ hybridization provided direct evidence that \textit{DjMCM2} and \textit{DjPum} transcripts are co-expressed in some neoblast-like cells (Fig. 2L-N). Although these results suggest that \textit{DjMCM2}-positive cells also express \textit{DjPum} transcripts, the faint \textit{DjPum} expression makes difficult to unambiguously demonstrate the presence of these transcripts in all \textit{DjMCM2}-positive cells.

\textbf{DjPum is involved in the formation of the regenerative blastema}

We analyzed the effect of RNAi-mediated gene silencing of \textit{DjPum} during planarian regeneration. After transection, we observed that about 10\% (6/57) of \textit{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 \textit{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, \textit{DjPum} 440 and \textit{DjPum} 550, which target different regions of \textit{DjPum}. Both water or \textit{β-Gal} dsRNA-injected fragments always regenerated a well-formed blastema (Fig. 3B-D). \textit{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 \textit{DjPum} RNAi was further supported by the observation that, in our experimental conditions, the RNAi-mediated inactivation of a planarian homologue of \textit{eyes absent} (\textit{Djeya}) never inhibited blastema formation also after the second transection. The specimens injected with \textit{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 \textit{DjPum} expression in \textit{DjPum} dsRNA-injected animals by comparative RT-PCR. We observed that, although \textit{DjPum} RNAi drastically decreased endogenous \textit{DjPum} RNA, no detectable reduction in the expression level of endogenous \textit{DjPum} mRNA was found in planarians injected with \textit{Djeya} dsRNA or with water (Fig. 3G). TEM analysis of some \textit{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).

\textbf{Loss of neoblasts in \textit{DjPum} dsRNA-injected planarians}

Both the similar expression pattern of \textit{DjPum} and \textit{DjMCM2} and the selective destruction of \textit{DjPum} and \textit{DjMCM2}-positive cells by X-ray irradiation prompted us to determine whether proliferating, \textit{DjMCM2}-positive neoblasts were reduced in number after \textit{DjPum} dsRNA injection. To address this issue, we performed in situ hybridization with \textit{DjMCM2} on \textit{DjPum} dsRNA-injected animals. Compared with water and \textit{Djeya} dsRNA-injected controls, \textit{DjPum} dsRNA-injected planarians showed a dramatic reduction of \textit{DjMCM2} hybridization signal (Fig. 5A-G). This reduction was observed both in \textit{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 \textit{DjMCM2} in planarians

\begin{figure}[h]
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\caption{Analysis of \textit{DjPum} expression in intact and regenerating \textit{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) \textit{DjPum} expression. (C) \textit{DjPum} expression, 10 days after X-ray irradiation. (D) \textit{DjPum} expression. (E) Expression of \textit{DjMCM2} is shown for comparison with the \textit{DjPum} expression depicted in D. (F) \textit{DjPum} expression. (G) Expression of \textit{DjMCM2} is shown for comparison with the \textit{DjPum} expression depicted in F. (H) Schematic drawing of a regenerating lateral fragment showing the distribution of \textit{DjPum}- and \textit{DjMCM2}-positive cells (blue lines). Scale bar: 400 µm. (I) Visualization of two neoblast-like cells expressing \textit{DjPum} mRNA after in situ hybridization on dissociated cells. (L-N) Double fluorescent in situ hybridization on dissociated cells. (L) Expression of \textit{DjMCM2} mRNA in two neoblast-like cells. (M) \textit{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.}
\end{figure}
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.

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. 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.

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.
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. 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 planarians 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.
DjPum and stem cell maintenance in planarians

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

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References


Development

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