Development 139, 1083-1094 (2012) doi:10.1242/dev.076182

PRMT5 and the role of symmetrical dimethylarginine in chromatoid bodies of planarian stem cells

Labib Rouhana, Ana P. Vieira, Rachel H. Roberts-Galbraith and Phillip A. Newmark*

SUMMARY

Planarian flatworms contain a population of adult stem cells (neoblasts) that proliferate and generate cells of all tissues during growth, regeneration and tissue homeostasis. A characteristic feature of neoblasts is the presence of chromatoid bodies, large cytoplasmic ribonucleoprotein (RNP) granules morphologically similar to structures present in the germline of many organisms. This study aims to reveal the function, and identify additional components, of planarian chromatoid bodies. We uncover the presence of symmetrical dimethylarginine (sDMA) on chromatoid body components and identify the ortholog of protein arginine methyltransferase PRMT5 as the enzyme responsible for sDMA modification in these proteins. RNA interference-mediated depletion of planarian PRMT5 results in defects in homeostasis and regeneration, reduced animal size, reduced number of neoblasts, fewer chromatoid bodies and increased levels of transposon and repetitive-element transcripts. Our results suggest that PIWI family member SMEDWI-3 is one sDMA-containing chromatoid body protein for which methylation depends on PRMT5. Additionally, we discover an RNA localized to chromatoid bodies, *germinal histone H4*. Our results reveal new components of chromatoid bodies and their function in planarian stem cells, and also support emerging studies indicative of sDMA function in stabilization of RNP granules and the Piwi-interacting RNA pathway.

KEY WORDS: Planarian, Stem cells, Chromatoid bodies, PIWI, PRMT5

INTRODUCTION

The replenishment, survival and proper differentiation of stem cells is necessary for development, continuous production of gametes and the repair of lost or damaged tissues. It is therefore important to understand the mechanisms responsible for regulating the maintenance, proliferation and proper differentiation of stem cells. The capabilities of stem cells are vividly displayed during post-embryonic development of planarian flatworms. A population of planarian adult stem cells, known as neoblasts (Dubois, 1948; Dubois and Wolff, 1947), are the only dividing cells in the planarian soma: they are the sole source of additional neoblasts and differentiated cells during regeneration, growth and homeostasis (Baguñà, 1976; Newmark and Sanchez Alvarado, 2000; Newmark and Sanchez Alvarado, 2002). Single cells within this population (clonogenic neoblasts; cNeoblasts) are sufficient to rescue planarians depleted of stem cells, further demonstrating the self-renewal and differentiation capacities of this cell type (Wagner et al., 2011). Thus, planarians provide a valuable opportunity for understanding the mechanisms involved in maintenance and function of stem cells in vivo.

Many studies have identified genes required for neoblast maintenance and function in planarians (Bonuccelli et al., 2010; Conte et al., 2009; Fernandez-Taboada et al., 2010; Guo et al., 2006; Oviedo and Levin, 2007; Palakodeti et al., 2008; Pearson and Sanchez Alvarado, 2010; Reddien et al., 2005a; Reddien et al., 2005b; Rouhana et al., 2010; Salvetti et al., 2005; Scimone et al., 2010; Solana et al., 2009; Tasaki et al., 2011a; Tasaki et al., 2011b). The vast majority of genes required specifically for neoblast function encode conserved factors involved in post-transcriptional regulation of gene expression (reviewed by Shibata et al., 2010). For example, homologs of Pumilio (Salvetti et al., 2005), Bruno (Guo et al., 2006) and Argonaute-2 (Rouhana et al., 2010) are required for maintenance of neoblasts and, consequently, regeneration. Conversely, RNA helicases (Vasa, Me31b and UPF1) and exoribonuclease 1 (XRN1) are required for regeneration but not for neoblast maintenance, suggesting a role in differentiation (Rouhana et al., 2010). Members of the PIWI subfamily of proteins, which promote genomic stability by repressing transposition in metazoan germ cells (reviewed by Siomi et al., 2008; Siomi et al., 2011), are required for neoblast function in planarians (Reddien et al., 2005b; Palakodeti et al., 2008).

Large cytoplasmic ribonucleoprotein (RNP) granules, named chromatoid bodies (CBs), were described in neoblasts decades ago (Coward, 1974; Hay and Coward, 1975; Hori, 1982; Morita et al., 1969). CBs contain conserved post-transcriptional regulators, such as the DEAD box RNA helicase CBC-1 (CB component-1/Me31b/Dhh1/DDX6) (Yoshida-Kashikawa et al., 2007), the small nuclear ribonucleoproteins (snRNP) core protein SmB (Fernandez-Taboada et al., 2010) and a Tudor homolog (Spoltud-1; Solana et al., 2009). All of these factors are required for regeneration in planarians (Fernandez-Taboada et al., 2010; Rouhana et al., 2010; Solana et al., 2009).

Tudor domain-containing proteins (TDRDs) control the assembly of polar granules, RNP organelles required for germ cell formation in *Drosophila* (Boswell and Mahowald, 1985; Mahowald, 1962; Thomson and Lasko, 2004), and CBs in mice (Yabuta et al., 2011). It has been proposed that TDRDs serve as docking platforms for the assembly of RNP granules involved in germ cell formation (Arkov et al., 2006). Physical interaction of TDRDs with snRNP proteins SmB and SmD3 is enabled by symmetrical dimethylarginine (sDMA) modification of RG motifs of Sm proteins (Anne et al., 2007; Brahms et al., 2001; Cote and Richard, 2005; Friesen et al., 2001; Sprangers et al., 2003). This

Howard Hughes Medical Institute, Department of Cell and Developmental Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA.

*Author for correspondence (pnewmark@life.illinois.edu)

Accepted 9 January 2012

post-translational modification is catalyzed by the Type II protein arginine methyl transferase PRMT5 in mammals (Meister et al., 2001) and its ortholog Capsuléen in flies (Anne et al., 2007).

sDMAs form the major epitope for recognition of Sm proteins by the widely used Y12 monoclonal antibody (Brahms et al., 2000; Lerner et al., 1981), which also recognizes sDMAs on mouse and fly PIWI (Kirino et al., 2009) and Vasa (Kirino et al., 2010a) proteins. Indeed, PIWI complexes containing PRMT5 and TDRDs have been isolated from mouse germline cells (Reuter et al., 2009; Vagin et al., 2009b; Vasileva et al., 2009; Wang et al., 2009). sDMA modifications on PIWI and Vasa are also mediated by PRMT5, are required for the physical interaction between PIWI and TDRDs, and drive localization of PIWI to cytoplasmic foci (Kirino et al., 2009; Liu, H. et al., 2010; Liu, K. et al., 2010; Nishida et al., 2009; Vagin et al., 2009b).

The composition of cytoplasmic RNP granules in somatic stem cells has been investigated less extensively than that of their germline-restricted counterparts. Here, we examine sDMA modifications in CB components of planarian neoblasts, identify the enzyme responsible for this modification and reveal novel CB components.

MATERIALS AND METHODS

Planarian culture

The clonal asexual strain CIW4 (Sanchez Alvarado et al., 2002) and a hermaphroditic strain (Zayas et al., 2005) of *Schmidtea mediterranea* were used and maintained as described by Wang et al. (Wang et al., 2007).

Irradiation

Asexual planarians 3-5 mm in length were exposed to 40 Gy of gamma irradiation using a Gammacell-220 Excel with a cobalt-60 source (Nordion, Ottawa, ON, Canada) in 2 ml of planarian salts (Cebria and Newmark, 2005) and processed at the indicated time points.

Whole-mount and fluorescent in situ hybridization (ISH)

ISH was performed as described (Pearson et al., 2009), with modifications as per Wang et al. (Wang et al., 2010) for large hermaphrodites.

Whole-mount immunofluorescence

Animals for anti-PCNA (1:500; Orii et al., 2005) and anti-phospho-histone H3 (Ser10) D2C8 (1:1000; Cell Signaling Technology, Danver, MA, USA) analyses were killed in 2% HCl and fixed for two hours in 4% formaldehyde, 5% methanol in PBS. Y12 (NeoMarkers, Fremont, CA, USA) was used at 1:250. Goat anti-mouse Alexa-488 (1:1000) and goat anti-rabbit Alexa-568 (1:500) (Molecular Probes, Eugene, OR, USA) were used for detection of primary antibodies. Immunofluorescence was performed and visualized as described by Forsthoefel et al. (Forsthoefel et al., 2011) following overnight bleaching or fluorescent ISH.

RNA interference (RNAi)

Double-stranded RNA (dsRNA) feedings were performed as described by Rouhana et al. (Rouhana et al., 2010) with modifications (Collins et al., 2010). Briefly, dsRNA diluted to 0.1 μg/μl in 2:1 minced liver:ultra-pure water was presented to planarians twice a week (250 ng per animal). Non-planarian dsRNA encoded in pJC53.2 (Collins et al., 2010) served as negative control.

Electron microscopy (EM)

Polysciences (Warrington, PA, USA) provided chemicals unless otherwise stated. We processed and analyzed three animals per condition for EM and immuno-EM. Animals were fixed in iced 2% formaldehyde, 2.5% glutaraldehyde in EMBuffer (70 mM sodium cacodylate, 1 mM $CaCl_2$, pH 7.4), excised, fixed for four additional hours, washed twice in EMBuffer, post-fixed (1% OsO_4, 90 minutes, 4°C in dark), washed twice (EMBuffer), dehydrated (20%+2% uranyl acetate, 40%, 60%, 80%, 100% ethanol), gradually placed in acetone, and infiltrated (Mollenhauer, 1964). Sections (80 nm) were stained as described by Vennable and Coggeshall (Vennable and Coggeshall, 1965) and were imaged using a Phillips CM-200.

For immuno-EM, fixed samples were rinsed twice (EMBuffer), quenched with 0.1 M glycine, and dehydrated, infiltrated and cured using Lowicryl-K4M according to the manufacturer's instructions. Sections collected on FORMVAR-coated grids were treated with IEMBlock (TBST, IgG-free 0.5% BSA, 0.45% fish gelatin), incubated for 1 hour in IEMBlock with or without Y12 (1:1), washed in TBST, incubated for 1 hour with 10 nm immunogold bead-conjugated anti-mouse antibodies (Molecular Probes) in IEMBlock (1:2), TBST-washed, rinsed in water, stained with 4% uranyl acetate, and imaged described as above.

Immunoblotting

Groups of four 3-4 mm asexual or two 1-1.2 cm sexual planarians (rinsed) were snap-frozen, resuspended in 50 or 100 μl SDS loading dye, respectively, homogenized with Kontes pestles, snap-frozen once again, boiled for three minutes, vortexed and centrifuged for 10 minutes (20,000 ***g***), all at 4°C. The equivalent of half to three-fourths of an asexual planarian, or one-fifth of a sexual planarian was used for immunoblotting. Membranes blocked in 1% BSA in PBS, were incubated with Y12 (1:250, NeoMarkers, Fremont, CA, USA) or anti-actin A2066 (1:1000, Sigma, St Louis, MO, USA) in 0.05% Tween-20 PBS overnight at 4°C.

Immunoprecipitation and labeling of planarian RNAs

All steps were performed in medium salt buffer [MSB; 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 0.05% NP-40, Roche EDTA-free protease inhibitors] at 4°C. Extracts were prepared from eighteen 5-7 mm asexual planarians snap-frozen, homogenized in 200 μl MSB, snap-frozen once again, supplemented with 200 μl MSB, further disassociated by pipetting, snap-frozen, thawed, centrifuged for ten minutes (8000 ***g***), re-cleared for five minutes (20,000 ***g***) and split between 4 μl of control or Y12 mAb [courtesy of Drs J. Steitz (Yale University) and G. Dreyfuss (University of Pennsylvania)] (Lerner et al., 1981; Pettersson et al., 1984) bound to 30 μl of protein G-Dynabeads slurry (Invitrogen, Carlsbad, CA, USA). FE-J1 antibodies (Federson et al., 1984) (Developmental Studies Hybridoma Bank, University of Iowa, IA, USA) served as a negative control. Immunoprecipitation and RNA analysis were performed as per Nishida et al. (Nishida et al., 2009) and Kirino et al. (Kirino et al., 2011) with minor modifications. [γ-^{32}P] ATP-labeled RNA was RQ1 DNase-treated (Promega, Madison, WI, USA) or RNaseT1-treated (Ambion, Austin, TX, USA), and resolved by 12% denaturing polyacrylamide gel electrophoresis.

RNAseq and RT-qPCR

RNA from two-month-old control and *PRMT5* RNAi-treated planarians was isolated using TRIzol (Invitrogen), DNase-treated, purified with RNA Clean and Concentrator kit (Zymo, Irvine, CA, USA) and submitted to the W. M. Keck Center for Comparative and Functional Genomics for Illumina sequencing. Reads were mapped to a reference transcriptome (https://www.ideals.illinois.edu/handle/2142/28689) using CLC Genomics Workbench and compared as per Marioni et al. (Marioni et al., 2008). Sequence Read Archive (SRA) data were deposited at NCBI under project accession PRJNA79031.

Transcript quantification in control and *PRMT5(RNAi)* animals was performed as described by Miller and Newmark (Miller and Newmark, 2012) using GoTaq qPCR reagent (Promega), with the following primers: 5′-CCCACCGAGATATTCCAAAA-3′, 5′-CCCCATCCTCTTTGACTTTG-3′ for *Smed-PRMT5*; 5′-GGGCTAATCCAAATCCTGGT-3′, 5′-TGCTGCAATACACTCGGAGA-3′ for *Smedwi-1* (Eisenhofer et al., 2008); and 5′-TGGCTGCTTGTGATCCAAGA-3′, 5′-AAATTGCCGCAACAGTCAAATA-3′ for β-*tubulin*.

Sequences

cDNA clones for *gH4* and *nanos* (Wang et al., 2007), *Smedwi-1* (Reddien et al., 2005b), *Smedwi-2* (Palakodeti et al., 2008; Reddien et al., 2005b) and *Smedwi-3* (Palakodeti et al., 2008), were obtained from an expressed sequence tag (EST) library (Zayas et al., 2005). PRMT homologs were amplified from planarian cDNA and cloned into pJC53.2 using the following: 5′-ATGTTGATGTTAGCTTTGCCCAAGTCTCCAGTCGATCAG-3′, 5′-GGACATGGAATAAGAGCGGCCATTCGGGTTATGAATCGACG-3′ for *Smed-PRMT5* (GenBankID:JQ035529); 5′-ATGG-

AGCACGCATCAATCAATGGAGATGCTATTGAAGCATC-3′, 5′-CCAGCTCAAATTATCGCATTCGATAACTGAATGTTTCCTCC-3′ for *Smed005105* (GenBankID:JQ035531); and 5′-CAATGGACCACAATAGCAATGCAATGGATACAAATGAC-3′, 5′-TCTCCCTTAAACTTCAGATCAATACTGACATCAATTGCTCTAGC-3′ for *Smed001246* (GenBankID:JQ035530).

RESULTS

Planarian neoblasts are enriched with sDMA-containing protein localized to chromatoid bodies

Previous studies of germline development in the sexual strain of *S. mediterranea* revealed the presence of Y12-immunoreactive material in the cytoplasm of spermatogonia (Fig. 1A) (Wang et al., 2010). Y12-immunoreactive material is also detected in neoblasts of asexual planarians (supplementary material Fig. S1). Y12 specifically recognizes sDMA (Brahms et al., 2000), suggesting that neoblasts express machinery necessary for this modification. Recent reports have shown that interactions between proteins containing sDMA and TDRDs are important for RNP aggregation in mouse and fly germlines (Anne, 2010; Gonsalvez et al., 2010; Kirino et al., 2010b; Vagin et al., 2009b). Given that neoblasts are enriched with expression of *tudor* homologs (Rouhana et al., 2010; Yoshida-Kashikawa et al., 2007) and that one homolog is a neoblast CB component (Solana et al., 2009), we analyzed sDMA-containing proteins in neoblasts.

Neoblasts are the only proliferating somatic cells in planarians and expression of proliferating cell nuclear antigen (PCNA) has been used as a marker for this cell type (Orii et al., 2005). We performed simultaneous immunofluorescence staining with Y12 and anti-PCNA antibodies. Indeed, PCNA-expressing cells contained Y12-immunoreactive material in their cytoplasm (Fig. 1B), confirming the presence of sDMAs in neoblast proteins. PCNA was absent from some Y12-labeled cells (~31%; n=94), as is expected given the cyclic expression of this gene and the fact that not all neoblasts are in S-phase at any given time (Newmark and Sanchez Alvarado, 2000). The presence of sDMA-containing protein in the cytoplasm of neoblasts was confirmed further by performing simultaneous Y12 immunofluorescence and in situ hybridization (ISH) for *germinal histone H4* (*gH4*) RNA, a known neoblast and germ-cell marker (Wang et al., 2007). Indeed, Y12-labeled cytoplasmic aggregates and *gH4* transcripts were detected in the same cells (Fig. 1C; 98.6%; n=139), and often expressed in the same cytoplasmic foci (Fig. 1C). Together, these data show that Y12-immunoreactive material is present in neoblasts and preferentially localized to cytoplasmic RNP foci.

The distribution of Y12-labeled cells corresponds to that described for undifferentiated neoblasts (supplementary material Fig. S1) (Eisenhoffer et al., 2008; Newmark and Sanchez Alvarado, 2000; Orii et al., 2005; Shibata et al., 1999). Neoblasts reside in the mesenchyme of intact planarians posterior to the photoreceptors and accumulate in the pre-existing tissue near the wound site in regenerating animals; neoblast progeny reside inside the emerging stump (the blastema) of regenerating planarians. Whole-mount immunofluorescence staining of intact and regenerating asexual planarians using Y12 and antibodies against the mitotic marker anti-phospho-histone H3 (Ser10) (PH3), showed that the distribution of Y12-immunoreactive material reflected that of undifferentiated neoblasts (supplementary material Fig. S1). Y12 staining was most intense in the region immediately posterior to the blastema, as originally observed by EM analyses of neoblasts (Morita et al., 1969). Nevertheless, a relatively weak and much less

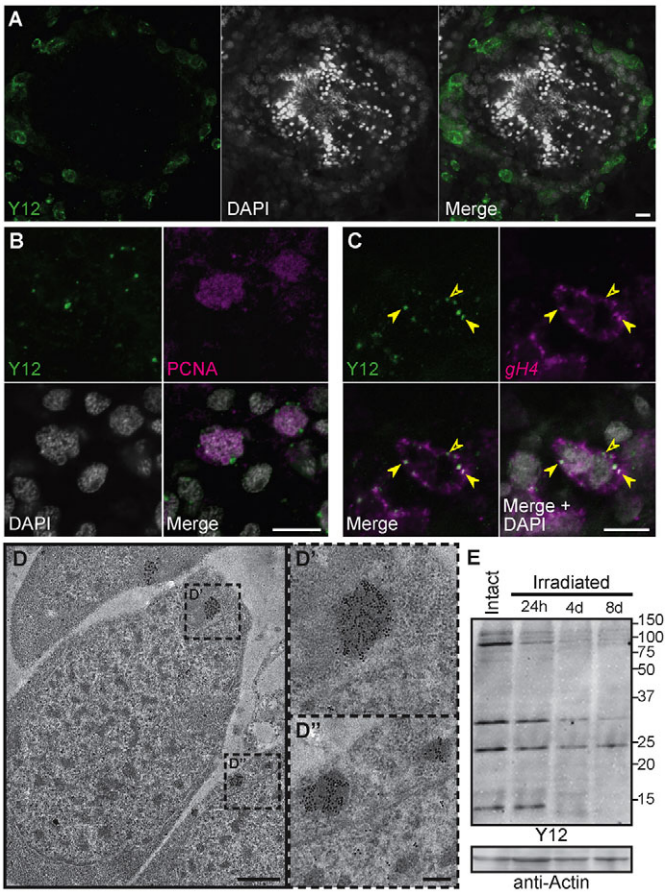

Fig. 1. Chromatoid body components of neoblasts show enrichment of sDMA modification. (**A**) sDMA-specific antibody Y12 (green) and DAPI (gray) staining of planarian testis lobe. Y12 labels spermatogonia in the outer layer of testes lobes, but not more differentiated cells (spermatocytes, spermatids, sperm) located towards the lumen. (**B**) Y12 (green), anti-PCNA (magenta) and DAPI staining of neoblasts located between posterior intestinal branches of asexual planarians. Y12 recognizes cytoplasmic aggregates in neoblasts, verified by the presence of PCNA, a marker of proliferative neoblasts. (**C**) Y12, *gH4* RNA fluorescence ISH (magenta) and DAPI staining of neoblasts in asexual planarians. Y12 recognizes cytoplasmic RNP aggregates in planarian neoblasts; some aggregates contain *gH4* RNA (filled arrowheads) whereas others do not (empty arrowheads). (**D-D″**) Immuno-EM reveals Y12 epitopes as components of CBs. Material recognized by Y12 is visualized by10 nm immunogold-conjugated anti-mouse secondary antibodies. D′ and D″ show magnified views of the boxed areas in D. (**E**) Immunoblot analysis using Y12 reveals that proteins containing sDMA decrease in asexual animal extracts following gamma irradiation (post-irradiation times indicated). Anti-actin serves as loading control. Scale bars: in A-C, 10 μm; in D, 1 μm; in D′ and D″, 200 nm.

aggregated signal was also observed in the cytoplasm of cells in the blastema during regeneration (supplementary material Fig. S1), suggesting the persistence of some methylated material in neoblast progeny.

Among planarian cell types, CBs are an unambiguous characteristic of neoblasts and germ cells (Coward, 1974; Hori, 1982; Morita et al., 1969). We hypothesized that epitopes recognized by Y12 are CB components based on their perinuclear localization in neoblasts and colocalization with RNA (Fig. 1B,C). To test this idea, we performed immuno-EM on regenerating

Table 1. Sequence analysis of putative sDMA-containing proteins in *S. mediterranea*

Protein	Motif sequence	Protein length (number of amino acids)	Molecular weight (kDa)
Homologs of validated targets			
SmB	**GRG, GGRG**	177	18.7
SmD1	**ARGRGRG, GRGRG, GRG**	122	13.6
SmD3	**GRG, GRGRG, GRG**	122	13.5
Histone H2A	**GRG**	127	13.8
Histone H3	–	134	15.1
Histone H4	**GRG**	103	11.4
Smedwi-1	RGG	808	92.9
Smedwi-2	RGG	833	95.8
Smedwi-3	**GRGR, GRGRG, GRGRG**	974	110.1
SmedVAS-1	**ARG, GRA**	626*	71.1*
SmedVAS-2	**GRGR**	541	60.6
LSM-4	**GRG**G, RGG, RGG, **ARGGRGGRG**	135	14.6
Predicted targets			
Y-box protein, CSDA	**GRA, GRGG, GRGRGRG, GRGR, GRGRGRGRGRG**	470	54.0
Y-box protein homolog	**GRG, GRG, GRGRGRGRGRG, GRGR, GRGR, GRGG**	566	64.6
Y-box protein (SmedMSY4)	**GRA, GRG, GRGG, GRGGR, GGRA**	378	42.4
Ribosomal protein S10	**GRG**	150	16.9
String of pearls/40S Ribosomal protein S2	**GRGRRGRGRGRGRGRGRG**	247	247.3
Fetal Alzheimer antigen/enhancer of bithorax	**GRGRGRGRGRGRGR**	1062	122.6
Similarity with FAM98A	**GRG, GGRGRGRGRGG**	455	51.9

Planarian orthologs of validated PRMT5 targets show conservation of symmetrical (sDMA, bold font; GRG, ARG or GRA) (Brahms et al., 2001) and asymmetrical (aDMA; RGG) (Gary and Clarke, 1998) dimethylarginine motifs. Primary sequence search for sDMA motifs identified additional predicted targets of methylation machinery.
*Initiator methionine remains to be determined.

planarians three days post-amputation, because neoblasts accumulate at the base of the blastema. We found strong Y12 immunoreactivity in neoblasts, particularly in CBs (Fig. 1D), which did not appear in control sections prepared without primary antibody. We counted the number of colloidal gold-particles present in areas containing CBs (~564.3 counts/μm^2; n=8 cells), compared with nuclear (~36.4 counts/μm^2) or cytoplasmic neighboring areas (~8.4 counts/μm^2). This 15-fold higher Y12 signal in CBs than elsewhere in cells reveals that CBs are indeed enriched with sDMA-containing protein.

Immunoblot analysis of planarian protein extracts revealed Y12-reactive proteins of approximately 13, 24, 28, 85 and 110 kDa, and a minor polypeptide migrating at ~15 kDa (Fig. 1E). The estimated sizes of some of these polypeptides correspond to planarian homologs of sDMA-containing proteins [such as SmB, SmD and PIWI (Table 1)], recognition of which by Y12 is evolutionarily conserved between flies and mammals (Anne et al., 2007; Gonsalvez et al., 2006; Kirino et al., 2009; Nishida et al., 2009; Vagin et al., 2009b). The conservation of canonical sDMA methylation motifs in planarian Sm and PIWI homologs was confirmed by sequence analysis (Table 1). Exposure to gamma irradiation, which leads to neoblast loss within 24 hours and ablation of differentiating neoblasts within four days (Eisenhoffer et al., 2008), caused a noticeable reduction of ~85 and ~110 kDa Y12 immunoreactive material within 24 hours of treatment, and complete loss of ~13, ~85 and ~110 kDa immunoreactive material within four days of treatment (Fig. 1E). The detection of two other major polypeptides by Y12 (~24 kDa and 28 kDa) was reduced, although not completely eliminated, in irradiated animals (Fig. 1E). The location of residual protein recognized by Y12 in differentiated cells is not clear; we cannot detect Y12 signal by immunofluorescence after irradiation (supplementary material Fig. S2A). From these observations, we conclude that Y12 labels material enriched in CBs of planarian neoblasts, suggesting that they contain sDMA-modified ribonucleoproteins.

PRMT5 is required for methylation of proteins reactive to Y12 in planarians

To examine whether material recognized by Y12 in neoblasts is indeed sDMA, we identified homologs of Type I (asymmetrical) and Type II (symmetrical) protein arginine methyltransferases in the *S. mediterranea* genome (supplementary material Table S1) (Robb et al., 2008). Among these genes was *Smed-PRMT5* (*S. mediterranea protein arginine methyltransferase 5*) mammalian and fly orthologs of which methylate PIWI (Kirino et al., 2009; Nishida et al., 2009), Vasa (Kirino et al., 2010a) and Sm proteins (Anne et al., 2007; Gonsalvez et al., 2006; Meister et al., 2001). Methylation by PRMT5 is required for recognition of these proteins by Y12 (Anne et al., 2007; Kirino et al., 2009; Kirino et al., 2010a). Thus, we suspected that PRMT5 could be responsible for the methylation of at least some of the sDMA-containing proteins present in planarians. To test this, we inhibited the expression of *PRMT5* by RNA interference (RNAi) and tested for levels of sDMA recognized by Y12. In *PRMT5(RNAi)* asexual planarians, we found a dramatic reduction of Y12-immunoreactive material in neoblasts (supplementary material Fig. S2B) and in protein extracts (Fig. 2A). Similarly, sDMA modifications were prominent in spermatogonia of sexual control animals (Fig. 2B), but were undetectable in *PRMT5(RNAi)* animals (Fig. 2C). Disrupting expression of homologs of PRMT1, the predominant mammalian Type I arginine methyltransferase (supplementary material Table S1) (Tang et al., 2000), did not alter sDMA detection by Y12 (Fig. 2D,E), validating the specificity of effects seen after *PRMT5* RNAi. From these results, we conclude that material recognized by Y12 in planarian cells and CBs is indeed sDMA-containing protein and that the enzyme responsible for this modification in *S. mediterranea* is PRMT5.

Characterization of *Smed-PRMT5* expression

To identify the cell types in which *PRMT5* is expressed, we performed ISH on sexual and asexual planarians. *PRMT5* transcript was detected in the brain, neoblasts and testes of sexual planarians,

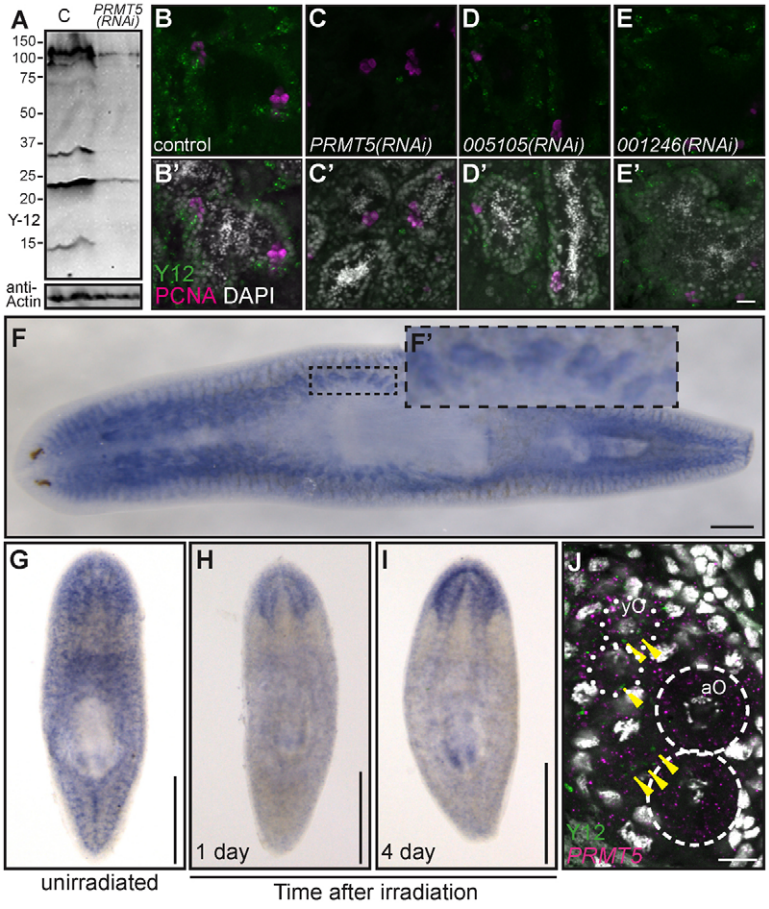

Fig. 2. *PRMT5* mediates sDMA modification of proteins in neoblasts and germline cells of *S. mediterranea*. (**A**) Y12 epitopes are dramatically reduced in extracts of asexual *PRMT5(RNAi)* but not control (C) animals after three weeks of RNAi. Anti-actin serves as loading control. (**B-E'**) Y12 (green) and phospho-histone H3 (magenta) staining showing the loss of sDMA in testes lobes of *PRMT5(RNAi)* (C), but not in control (B) nor knockdowns of PRMT1 homologs *Smed-005105* (D) or *Smed-001246* (E) after three weeks of RNAi treatment. Images were taken at the same magnification and exposure. DAPI staining is shown in gray in B'-E'). Scale bar: 20 μm. (**F**) Detection of *PRMT5* transcript in the outer layer of testes lobes in a sexually mature animal detected by ISH. F' shows a magnified view of the boxed area. (**G-I**) *PRMT5* is expressed in neoblasts of asexual planarians. Detection of *PRMT5* transcript in mesenchymal cells and cephalic ganglia of asexual animals (G); mesenchymal labeling is dramatically reduced one (H) and four (I) days after gamma irradiation. (**J**) *PRMT5* expression and sDMA-containing granules are present during oogenesis. *PRMT5* transcripts were detected in oocytes by fluorescence ISH (magenta). sDMA-containing granules (arrowheads) were observed in the cytoplasm of young oocytes (yO; dotted circles; mid-sized ovarian cells localized more anteriorly) by Y12 immunofluorescence (green). Advanced oocytes (aO; dashed circles; larger ovarian cells with meiotic chromosomes) are indicated. Scale bars: in F-I, 0.5 mm; in J, 10 μm.

predominantly in the outer layer spermatogonia (Fig. 2F). In asexual animals, *PRMT5* expression was detected in mesenchymal cells and the cephalic ganglia (Fig. 2G-I). As expected for genes expressed in neoblasts, we observed reduction of mesenchymal *PRMT5* transcripts in animals fixed 24- and 96-hours post-irradiation (Fig. 2H,I). *PRMT5* expression was also detected in the ovaries of sexually mature planarians (Fig. 2J), leading us to examine sDMA-containing proteins in ovaries. We found that Y12 recognized ooplasmic material present in large granules (Fig. 2J). However, these granules seemed to be lost during progression through oogenesis and were not detected in more mature oocytes (Fig. 2J). Expression of *PRMT5* in neoblasts and germ cells is consistent with a role for PRMT5 in generating sDMA epitopes in these tissues.

PRMT5 promotes proper neoblast function during growth, homeostasis and regeneration

The detection of PRMT5 activity in planarian neoblasts and the enrichment of its product (sDMA) in CBs suggest an involvement of PRMT5 in neoblast function. To test the role of PRMT5 in planarian neoblasts, we inhibited *PRMT5* expression by RNAi. As shown above, *PRMT5* RNAi specifically reduces Y12-detectable sDMA modifications (Fig. 2A-C). Animals were subjected to a long-term regimen of biweekly *PRMT5* and control double-stranded RNA (dsRNA) treatments (see Materials and methods). After two- to three-months of RNAi, asexual *PRMT5(RNAi)* animals were ~35% smaller than control animals subjected to the same diet and amounts of dsRNA [average 5.86±0.65 mm (*n*=19) vs 3.77±0.56 mm (*n*=15)] (Fig. 3A). At this point, anterior blisters and head defects were observed in >25% of asexual *PRMT5(RNAi)* (*n*=21/76), some of which exhibited complete loss of the head region (*n*=6/76; Fig. 3B; supplementary material Fig. S3A). Head loss was also observed in *PRMT5(RNAi)* sexual animals (*n*=9/11; supplementary material Fig. S3B).

To analyze whether the defects observed after *PRMT5* RNAi could be due to changes in neoblasts, we analyzed neoblast distribution by *gH4* ISH. A substantial decrease in the number of *gH4*-expressing cells was found in severely affected *PRMT5(RNAi)* animals, which was not observed in controls (Fig. 3C-E). However, this reduction in neoblast number was not observed in all individuals nor distributed evenly throughout the entire body of single individuals subjected to *PRMT5* RNAi (supplementary material Fig. S3C), complicating the quantification of neoblasts by whole-mount *gH4* ISH (Fig. 3C-E). Thus, we analyzed the expression of another well-characterized neoblast marker, *Smedwi-1* (Reddien et al., 2005b), in *PRMT5(RNAi)* and control animals. Total RNA from groups of *PRMT5(RNAi)* and control animals was extracted and subjected to reverse transcription-coupled quantitative PCR analysis (qRT-PCR; Fig. 3F). *PRMT5* RNAi resulted in a modest yet reproducible reduction of *Smedwi-1* RNA levels compared with controls (30-45% reduction; Fig. 3F), confirming an overall reduction of neoblasts after *PRMT5* RNAi. The range of observed phenotypes (Fig. 3B-E; supplementary material Fig. S3) is unlikely to be due to insufficient knockdown, as *PRMT5* transcript levels (Fig. 3F) and detection of sDMA by Y12 (Fig. 2A) are both dramatically reduced by this RNAi regimen. The compromised ability of *PRMT5(RNAi)* animals to sustain

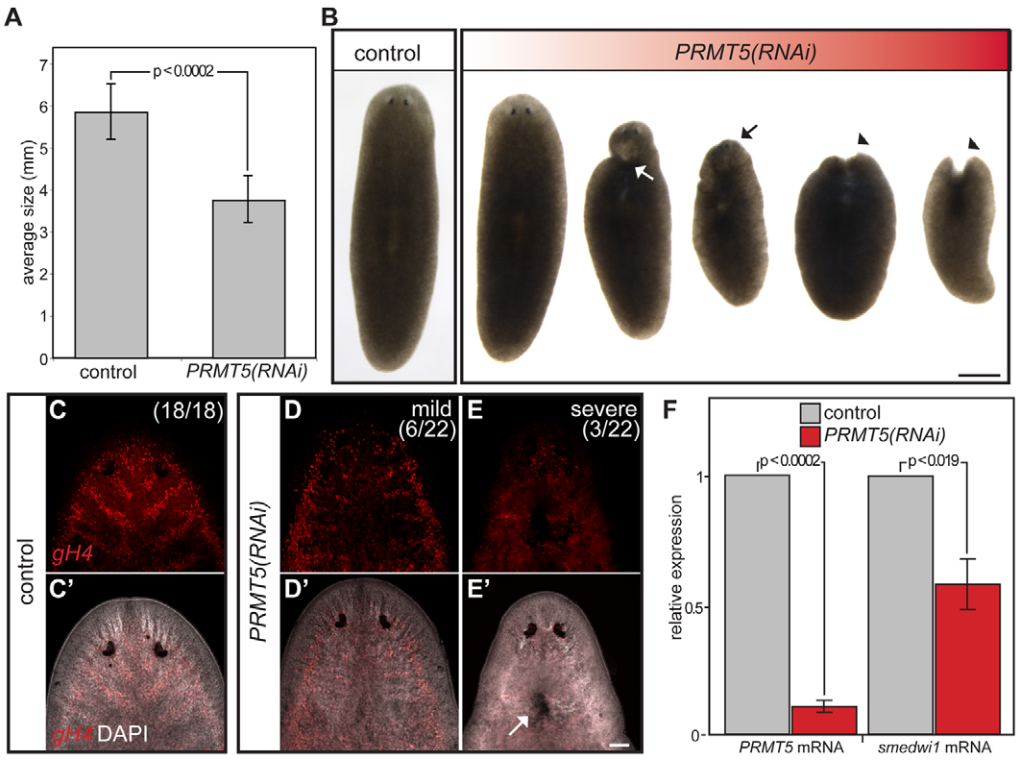

Fig. 3. Knockdown of *PRMT5* results in growth and homeostasis defects. (**A**) *PRMT5(RNAi)* animals are smaller than control animals. Graph showing average size of control and *PRMT5(RNAi)* animals. (**B**) *PRMT5* RNAi leads to blister formation and head loss. Images of asexual control and different *PRMT5(RNAi)* animals. Dorsal blisters (white arrow), head regression (black arrow) and head loss (arrowheads) are indicated. Scale bar: 0.5 mm. (**C-E′**) A reduction in neoblast number is seen in severe *PRMT5(RNAi)* animals. Neoblast abundance visualized by *germinal histone H4* fluorescence ISH (red) revealed similar neoblast distribution in control (C) and *PRMT5(RNAi)* animals with mild defects (D), but noticeable loss of neoblasts in severely affected samples (E). Nuclear staining by DAPI is shown in gray in C′-E′. Images were taken at same magnification and exposure. Dorsal blister (white arrow) is indicated. Scale bar: 0.1 mm. (**F**) Overall number of neoblasts is reduced in *PRMT5(RNAi)* planarians. Relative transcript levels of the neoblast marker *Smedwi-1*, and *PRMT5*, measured by qRT-PCR from biological triplicates of control (gray) and *PRMT5(RNAi)* (red) animals. *Smedwi-1* and *PRMT5* transcript levels were normalized to endogenous β-*tubulin* mRNA. *P*-values from two-tailed unpaired *t*-test are shown for A and F. Error bars represent s.d.

homeostatic size and growth, as well as the development of blisters, head loss and reduction of neoblasts, raised the possibility that PRMT5 could be required for proper neoblast function.

The highest demand for neoblasts is during regeneration, when amputation-induced signals drive an increase in proliferation; differentiation of newly born neoblasts will help replace missing body parts (Baguñà, 1976; Best et al., 1968; Saló and Baguñà, 1984; Wenemoser and Reddien, 2010). The homeostatic defects observed as a result of *PRMT5* RNAi suggest that regeneration might also be compromised in these animals. Thus, after one month of RNAi, we challenged fragments of *PRMT5(RNAi)* animals to regenerate their heads and tails after amputation anterior and posterior to the pharynx. Under these conditions, the effect upon neoblasts should be less severe than those observed in two-month knockdowns (Fig. 3D-F). Indeed, 11% and 10% of *PRMT5(RNAi)* trunk and tail fragments (n=36 and n=29, respectively) failed to regenerate normal heads, as judged by the presence of photoreceptors on the seventh day after amputation (Fig. 4A-D). Additionally, 24% of tail fragments showed abnormal head regeneration with only a single photoreceptor, and 21% of *PRMT5(RNAi)* head fragments (n=34) failed to regenerate a tail (Fig. 4A). Conversely, all control fragments (n=38 and n=32, respectively) displayed successful head regeneration and only one of 38 head fragments failed to regenerate a tail (Fig. 4A,B). These results demonstrate that PRMT5 promotes proper regeneration.

Ultrastructural analysis of trunk fragments three days after amputation revealed additional differences between head-regenerating regions of control and *PRMT5(RNAi)* samples. The organized architecture of the blastema and postblastema region observed in controls (Fig. 5A) seemed distorted in *PRMT5(RNAi)* samples (Fig. 5B). Qualitative analyses revealed that neoblasts of similar shape and size covered the area close to the site of amputation in control animals (Fig. 5A). However, irregularities in shape, size and distribution of neoblasts were observed in *PRMT5(RNAi)* fragments (Fig. 5B). Differentiated structures, such as ciliated ducts of the protonephridial system (not shown) and secretory cell structures (Fig. 5A, arrows), were observed more often in sections of control animals by this time point, than in *PRMT5(RNAi)* sections (Fig. 5B). Similarly, germ cell clusters, which arise from neoblasts in adult planarians (Wang et al., 2007; reviewed by Newmark et al., 2008), were reduced in regenerating *PRMT5(RNAi)* head fragments relative to comparatively sized control fragments (P<0.05, two-tailed unpaired t-test; supplementary material Fig. S4). Furthermore, a 52% reduction in the number of CBs per neoblast was observed in sections of *PRMT5(RNAi)* animals (P<0.05, two-tailed unpaired t-test, n=82

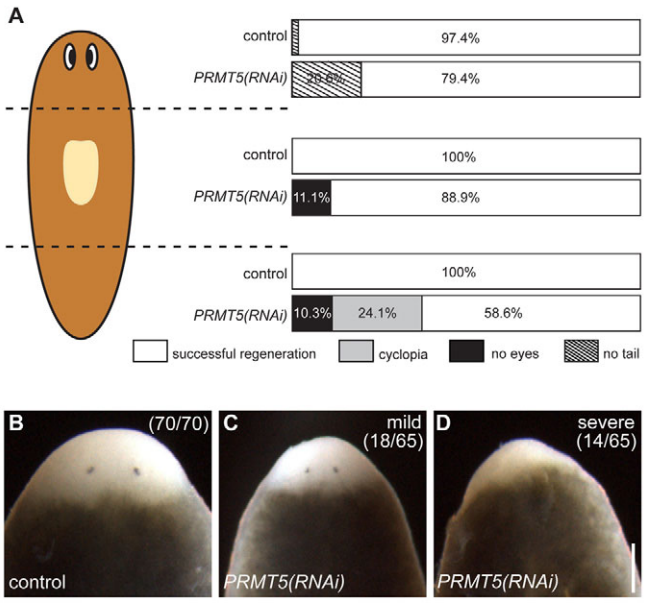

Fig. 4. *PRMT5* RNAi decreases regeneration capacity. (**A**) Regeneration of asexual animals subjected to control and *PRMT5* RNAi for over one month. Animals displaying blister or head-regression prior to the time of amputation were not used for this analysis. Percentage of animals showing normal regeneration within seven days (unshaded); failed posterior regeneration (transverse shading); failed anterior regeneration (black shading) and partial anterior regeneration (gray shading; cyclopia). (**B-D**) *PRMT5(RNAi)* animal amputated trunk fragments show variable head regeneration defects ranging from reduced blastema size (mild, C) to eye regeneration failure (severe, D). Scale bar: 0.2 mm.

controls and *n*=60 *PRMT5(RNAi)* neoblasts; Fig. 5C). These results suggest that PRMT5 plays a role in promoting CB stability or nucleation.

Analysis of planarian sDMA-containing proteins reveals a connection between chromatoid bodies and PIWI proteins

Examples of Sm and PIWI protein family methylation by PRMT5 have been observed in flies and mice, and primary sequence analysis suggested that the same could be true of planarian homologs (Table 1). To verify that planarian PIWI proteins are PRMT5 substrates, we analyzed the expression of polypeptides recognized by Y12 after knockdown of three PIWI homologs. Detection of the 110 kDa polypeptide by Y12 was lost after *Smedwi-3* RNAi (Fig. 6A), and was often reduced (but not eliminated entirely) in extracts of *Smedwi-1(RNAi)* animals (Fig. 6A). The designation of this polypeptide as sDMA-SMEDWI-3 is corroborated by the predicted molecular weight of SMEDWI-3 and the presence of multiple PRMT5 substrate motifs in the primary sequence of SMEDWI-3 (but not SMEDWI-1 or SMEDWI-2; Table 1). However, indirect effects of *Smedwi-3* RNAi on some other sDMA-modified protein are still possible.

Recognition of sDMA-SMEDWI-3 by Y12 suggests that CBs might be associated with piRNAs. Indeed, RNAs within the size range of piRNA (26-32 nts) (Palakodetti et al., 2008; Friedlander et al., 2009) were enriched in material from planarian extracts specifically co-immunoprecipitated with Y12, but not with control antibodies (Fig. 6B, single asterisk). RNA species of higher molecular weight were also detected in Y12 immunoprecipitates, as expected from this antibody's likely affinity for methylated Sm proteins (Fig. 6B, double asterisks). These results strongly suggest that sDMA-SMEDWI-3 is one of the CB factors recognized by Y12 in planarian neoblasts, and that methylated planarian PIWI proteins are capable of direct or indirect association with PIWI-interacting RNAs (piRNAs).

The identification of sDMA-SMEDWI-3 raised the possibility that PRMT5 might influence repression of transposon activity by SMEDWI-3. Indeed, Illumina-based sequencing analyses revealed that the most over-represented transcripts in *PRMT5(RNAi)*, compared with control planarians, corresponded to transposon and repetitive-element sequences (supplementary material Tables S2 and S3). This result suggests that RNAs localized to CBs might be substrates or components of the piRNA pathway, rather than representatives of the translatable pool of neoblast mRNAs. To test whether localization of RNAs to CBs was a general phenomenon, we analyzed the subcellular localization of a number of neoblast-enriched transcripts by ISH followed by Y12 immunofluorescence. Surprisingly, only *gH4* RNA seemed to localize to CBs (Fig. 6C). Transcripts that encode proteins known to be required for neoblast function, such as SMEDWI-2 and SMEDWI-3 (Palakodeti et al., 2008; Reddien et al., 2005b), were not detected in CBs (Fig. 6C). Similarly, transcripts of *Smed-nanos*, function of which is required for germ cell maintenance, did not colocalize with Y12 in presumptive germ cells of asexual animals (Fig. 6C). These results

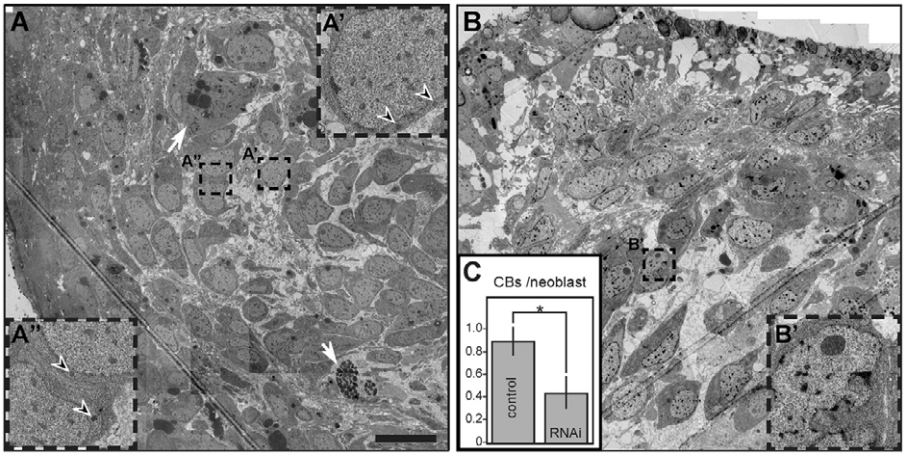

Fig. 5. Neoblasts in *PRMT5(RNAi)* animals have reduced numbers of chromatoid bodies and develop blastemal defects during regeneration. (**A**,**B**) Ultrastructural analysis of representative sections from animals subjected to one month of control (A) or *PRMT5* (B) RNAi, three-days post-amputation. Note the absence of some differentiated cells in B (such as secretory cells; arrows in A). A′, A″ and B′ show magnified views of representative neoblasts, with some CBs indicated (arrowheads). Scale bars: 20 μm. (**C**) A reduced number of CBs was observed in neoblasts of *PRMT5(RNAi)* animals. Neoblasts were defined as ovoid cells with large nucleocytoplasmic ratio that are devoid of cytoplasmic organelles, except CBs or mitochondria. **P*<0.05; two tailed unpaired *t*-test. Error bars represent s.d.

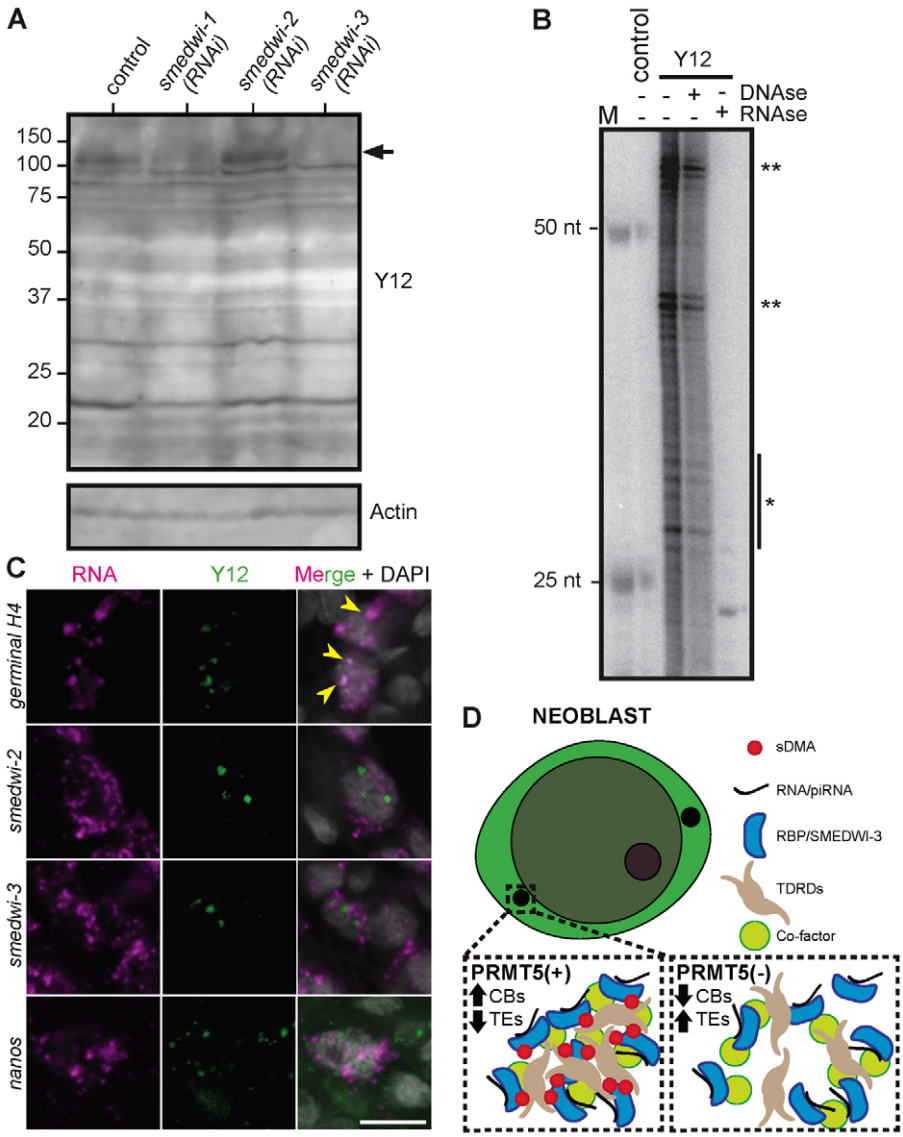

Fig. 6. Components of the piRNA machinery localize to chromatoid bodies. (**A**) sDMA modification is detected on SMEDWI-3. Immunoblot analysis using Y12 reveals absence of a protein with predicted size for SMEDWI-3 (~110 kDa, arrow) in *Smedwi-3(RNAi)*, but not *Smedwi-1(RNAi)*, *Smedwi-2(RNAi)* or control extracts. (**B**) RNAs corresponding to the size of piRNAs are associated with sDMA-containing protein. Visualization of [γ-^{32}P] ATP-labeled 27-32 nt RNAs (single asterisk) and longer RNAs (double asterisks) co-immunoprecipitated from planarian extracts using Y12, but not control antibodies. RNAse and DNAse treatment validated signal to be RNA. M, DNA markers. (**C**) *gH4* transcripts specifically localize to CBs. Fluorescence ISH analysis (magenta) of *gH4*, *Smedwi-2*, *Smedwi-3* and *nanos*, followed by Y12 immunofluorescence (green), shows that only *gH4* transcripts localize to CBs (arrowheads). Scale bar: 10 μm. (**D**) Current model. PRMT5-catalyzed sDMA reinforces interactions between TDRDs and RNA-binding proteins, such as SMEDWI-3, leading to their accumulation in CBs. The proximity of factors allows for proper piRNA processing and repression of transposable elements (TEs) in neoblasts, thus maintaining genomic stability.

suggest that mRNAs actively translated in neoblasts and presumptive germ cells (in the case of *nanos*) are not retained in CBs.

What dictates the localization of *gH4* transcripts to CBs remains unclear. Processing or translational regulation of *gH4* mRNA could occur in CBs. Alternatively, a subset of *gH4* RNA could be a substrate for piRNA machinery localized to CBs. In support of this latter idea, the 13.7 kbp genomic region containing the *gH4* locus harbors several piRNA islands, a recent inverted duplication of *gH4* with >97% nucleotide sequence identity, and a Ty3/gypsy-type transposon (supplementary material Fig. S5A-B). Transcription from regions neighboring *gH4* sequences could result in imperfect but detectable substrates of the piRNA machinery. Transposon-derived transcripts were not localized to CBs and were barely detected in neoblasts (supplementary material Fig. S6A). However, transcripts of one transposon (*PL06017B1E01*) were readily detected in a number of differentiated cell types (supplementary material Fig. S6B), raising the possibility that transposons are not transcribed and/or are effectively degraded in neoblasts. The identification of *gH4* RNA as a CB component warrants future experiments addressing the nature, metabolism and regulation of this RNA to understand better the events that occur in CBs.

DISCUSSION

In this article, we demonstrate a role for the conserved methyltransferase PRMT5 in promoting stem cell function. We show that PRMT5 methylates CB components in planarian neoblasts. PRMT5 depletion leads to loss of protein sDMA methylation in neoblasts, reduced CB number and defects in regeneration, growth and homeostasis. Neoblast loss was observed in severely affected individuals after *PRMT5* RNAi. However, complete loss of neoblasts does not seem to be required for manifestation of PRMT5 deficiency, as homeostatic defects in both size and blistering were seen in animals without a complete loss of neoblasts. Expression of PIWI homologs is characteristic of planarian neoblasts (Palakodeti et al., 2008; Reddien et al., 2005b; Rossi et al., 2006) and, similar to *PRMT5(RNAi)*, defects in regeneration and homeostasis of PIWI knockdowns are not based upon complete loss of neoblasts (Reddien et al., 2005b), although decreased neoblast numbers are observed in *Smedwi-2*, *Smedwi-3* and *Smedwi-2/-3* RNAi-treated animals (Palakodeti et al., 2008). One possible explanation for this observation is that neoblasts in PIWI knockdown animals have decreased piRNA function and increased transposable element activity. Although neoblasts might be lost owing to disruptive activity of transposable elements in

genes required for neoblast maintenance, transposition could also lead to mutation of genes involved in differentiation, repression of neoblast proliferation, or some other neoblast function. The increase in transposon and repetitive element transcripts in *PRMT5(RNAi)* and the detection of sDMA on SMEDWI-3, links PRMT5 and CBs to the piRNA pathway. Nevertheless, PRMT5 might also influence piRNA-independent processes in neoblasts.

Planarian chromatoid body structure

Several ribonucleoprotein granules have been studied intensively in the last decade, and the mechanism by which they maintain structural stability continues to be the focus of much research. Interactions within prion-like domains of some RNP granule components might be important for formation of P-bodies and neuronal RNP granules (Decker et al., 2007; Mazzoni et al., 2007; Reijns et al., 2008; Salazar et al., 2010). Mechanisms involved in RNP granule assembly in the nematode germline include hydrophobic interactions between the phenylalanine-glycine repeat domains of VASA-related proteins GLH-1/2/4 (Updike et al., 2011), and self-interaction domains of P-granule abnormality family member (PGL) proteins (Hanazawa et al., 2011). A conserved interaction between TDRDs and sDMA on RNA-binding proteins appears to be responsible for the formation of germ granules in mice and flies (reviewed by Arkov and Ramos, 2010; Siomi et al., 2010). Our data suggest that planarian CBs are stabilized by this same mechanism and that PRMT5 function in RNP-granule formation is also conserved. The identification of *SpolTud-1*, a planarian gene encoding a protein that contains three Tudor domains and localizes to CBs, supports this idea (Solana et al., 2009).

Like *PRMT5(RNAi)*, *Spoltud-1(RNAi)* animals display regeneration defects and a reduction in neoblast number; however, these phenotypes are only observed several weeks after protein depletion (three weeks for protein depletion versus seven for phenotypic manifestation) (Solana et al., 2009). A similar long-term delay between loss of gene product and phenotypic manifestation occurs after *PRMT5* RNAi. Loss of methylation indicative of PRMT5 depletion is observed within three weeks of dsRNA treatment (Fig. 2A); however, most homeostatic defects were not observed until two months after the start of RNAi treatment (Fig. 3). The phenotypic similarities between *PRMT5* and *Spoltud-1* knockdowns suggest that both factors could be working together to promote CB function and neoblast stability. How might these factors act together? One model proposed from work in mice and flies posits that interactions between PIWI proteins create an amplification loop of piRNA production (reviewed by Siomi et al., 2011). PIWI protein aggregates are stabilized by bridging interactions between the Tudor domains and sDMAs of different PIWI proteins (reviewed by Vagin et al., 2009a). Given that many TDRDs contain two or more Tudor domains, their interaction with multiple sDMA-containing proteins would lead to macromolecular aggregation and compartmentalization of piRNA processing (Fig. 6D) (Aravin et al., 2009; reviewed by Arkov and Ramos, 2010; Chuma and Pillai, 2009).

Planarian chromatoid body function and the role of methylation

The effect of methylation on protein-protein interactions in RNPs came from studies of Sm proteins and the survival of motor neuron protein, a TDRD assembly factor for snRNPs and other ribomacromolecules (Brahms et al., 2001; Friesen et al., 2001). Tudor domains mediate binding to sDMA-modified RG repeats of SmB/B′, SmD1, SmD3 (Brahms et al., 2001; Cote and Richard, 2005; Selenko et al., 2001; Sprangers et al., 2003) and PIWI proteins (Kirino et al., 2010b; Liu, H. et al., 2010; Nishida et al., 2009; Vagin et al., 2009b). Functional analyses have shown that the PRMT5/PIWI/TUDOR network plays a role in the piRNA processing pathway, retrotransposon silencing and, consequently, genomic stability (Nishida et al., 2009; Reuter et al., 2009; Shoji et al., 2009; reviewed by Siomi et al., 2011). By contrast, the PRMT5/Sm/TUDOR network present in fly oocytes is involved in localization of *oskar* mRNA and specification of germ cell fate (Anne et al., 2007; Anne, 2010; Boswell and Mahowald, 1985; Gonsalvez et al., 2006; Gonsalvez et al., 2010). Our biochemical and sequence analyses suggest that both SMEDWI-3 and Sm protein family members are methylated in planarians. Recent work has shown that both SmB and PIWI are required for neoblast function (Fernandez-Taboada et al., 2010; Palakodeti et al., 2008; Reddien et al., 2005a). Furthermore, SmB was shown to localize to planarian CBs (Fernandez-Taboada et al., 2010) and our results suggest that the same is true for sDMA-SMEDWI-3. Thus, planarian CBs might be sites of piRNA processing, as well as other RNA-processing events. However, further characterization of *gH4* RNA and identification of additional CB RNA components are necessary to fully elucidate the post-transcriptional processes that take place in CBs and their influence on planarian stem cell stability and function.

Riboregulation of ancestral stem cells: germline versus soma

Given their transcriptionally quiescent state, germ cells are hubs of post-transcriptional regulation. The establishment, proliferation, differentiation and function of the germline are centered around RNA-binding proteins and RNA-modifying enzymes that regulate the timing, location and translational activity of specific transcripts (reviewed by Seydoux and Strome, 1999; Seydoux and Braun, 2006; Thompson et al., 2007; Voronina et al., 2011). *nanos* function is required for maintenance of the germline in asexual and sexual strains of *S. mediterranea* (Wang et al., 2007). To our knowledge, *gH4* RNA is the first transcript reported to be abundant in CBs of asexual planarian neoblasts. We failed to see accumulation of *nanos* transcripts in CBs of asexual *S. mediterranea* presumptive germ cells (Fig. 6C). *nanos* transcripts accumulate in spermatogonial CBs of *Dugesia japonica*, but their localization to CBs in sexually immature animals is less clear (Sato et al., 2006). Interestingly, *gH4* colocalization with Y12 was observed only infrequently in testes lobes (supplementary material Fig. S7). Thus, it is likely that regulation of piRNA and mRNA by CBs depends on sexual specification.

Recent studies in non-traditional model organisms have shown that a number of RNA-binding proteins often considered to be restricted to germline functions, such as Vasa, Nanos and PIWI, operate in multipotent progenitors and stem cells of many metazoans (reviewed by Juliano and Wessel, 2010; Juliano et al., 2010). These studies led to the suggestion of a ‘germline multipotency program’ present in multipotent stem cells that generate somatic cell types. Indeed, PIWI homologs are expressed in stem cells of sponge (Funayama, 2010; Funayama et al., 2010), acoels (De Mulder et al., 2009), sea urchin (Juliano et al., 2006), jellyfish (Seipel et al., 2004), annelids (Rebscher et al., 2007) and ctenophores (Alie et al., 2011). Methylation of RNP components by PRMT5 may also be considered a constituent of such a ‘germline multipotency program’.

Acknowledgements
We thank John Brubacher, Jim Collins, Ryan King and James Sikes for helpful comments on the manuscript; Alvaro Hernandez for guidance with next generation sequencing; Yuying Wang, Frank Echtenkamp, Brian Freeman and members of the Siomi and Agata laboratories for helpful discussions; Stoyan Toshkov for help with gamma-irradiation; and Drs Joan Steitz, Gideon Dreyfuss, Hidefumi Orii and Kenji Watanabe for antibodies.

Funding
This work was supported by an award from the National Institutes of Health (NIH) [R01 HD043403 to P.A.N.]; an award from the National Science Foundation (NSF) [IOS-0744689 to P.A.N.]; a Jane Coffin Childs Fellowship to R.R.G.; and an NSF Minority Postdoctoral Fellowship [0804021 to L.R.]. P.A.N. is a Howard Hughes Medical Institute Investigator. Deposited in PMC for release after 6 months.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.076182/-/DC1

References

Alie, A., Leclere, L., Jager, M., Dayraud, C., Chang, P., Le Guyader, H., Queinnec, E. and Manuel, M. (2011). Somatic stem cells express Piwi and Vasa genes in an adult ctenophore: ancient association of 'germline genes' with stemness. *Dev. Biol.* **350**, 183-197.
Anne, J. (2010). Arginine methylation of SmB is required for *Drosophila* germ cell development. *Development* **137**, 2819-2828.
Anne, J., Ollo, R., Ephrussi, A. and Mechler, B. M. (2007). Arginine methyltransferase Capsuleen is essential for methylation of spliceosomal Sm proteins and germ cell formation in Drosophila. *Development* **134**, 137-146.
Aravin, A. A., van der Heijden, G. W., Castaneda, J., Vagin, V. V., Hannon, G. J. and Bortvin, A. (2009). Cytoplasmic compartmentalization of the fetal piRNA pathway in mice. *PLoS Genet.* **5**, e1000764.
Arkov, A. L. and Ramos, A. (2010). Building RNA-protein granules: insight from the germline. *Trends Cell Biol.* **20**, 482-490.
Arkov, A. L., Wang, J. Y., Ramos, A. and Lehmann, R. (2006). The role of Tudor domains in germline development and polar granule architecture. *Development* **133**, 4053-4062.
Baguñà, J. (1976). Mitosis in the intact and regenerating planarian *Dugesia mediterranea* n.sp. II. Mitotic studies during regeneration, and a possible mechanism of blastema formation. *J. Exp. Zool.* **195**, 65-80.
Best, J. B., Hand, S. and Rosenvold, R. (1968). Mitosis in normal and regenerating planarians. *J. Exp. Zool.* **168**, 157-167.
Bonuccelli, L., Rossi, L., Lena, A., Scarcelli, V., Rainaldi, G., Evangelista, M., Iacopetti, P., Gremigni, V. and Salvetti, A. (2010). An RbAp48-like gene regulates adult stem cells in planarians. *J. Cell Sci.* **123**, 690-698.
Boswell, R. E. and Mahowald, A. P. (1985). tudor, a gene required for assembly of the germ plasm in *Drosophila melanogaster*. *Cell* **43**, 97-104.
Brahms, H., Raymackers, J., Union, A., de Keyser, F., Meheus, L. and Luhrmann, R. (2000). The C-terminal RG dipeptide repeats of the spliceosomal Sm proteins D1 and D3 contain symmetrical dimethylarginines, which form a major B-cell epitope for anti-Sm autoantibodies. *J. Biol. Chem.* **275**, 17122-17129.
Brahms, H., Meheus, L., de Brabandere, V., Fischer, U. and Luhrmann, R. (2001). Symmetrical dimethylation of arginine residues in spliceosomal Sm protein B/B' and the Sm-like protein LSm4, and their interaction with the SMN protein. *RNA* **7**, 1531-1542.
Cebria, F. and Newmark, P. A. (2005). Planarian homologs of netrin and netrin receptor are required for proper regeneration of the central nervous system and the maintenance of nervous system architecture. *Development* **132**, 3691-3703.
Chuma, S. and Pillai, R. S. (2009). Retrotransposon silencing by piRNAs: ping-pong players mark their sub-cellular boundaries. *PLoS Genet.* **5**, e1000770.
Collins, J. J., 3rd, Hou, X., Romanova, E. V., Lambrus, B. G., Miller, C. M., Saberi, A., Sweedler, J. V. and Newmark, P. A. (2010). Genome-wide analyses reveal a role for peptide hormones in planarian germline development. *PLoS Biol.* **8**, e1000509.
Conte, M., Deri, P., Isolani, M. E., Mannini, L. and Batistoni, R. (2009). A mortalin-like gene is crucial for planarian stem cell viability. *Dev. Biol.* **334**, 109-118.
Cote, J. and Richard, S. (2005). Tudor domains bind symmetrical dimethylated arginines. *J. Biol. Chem.* **280**, 28476-28483.
Coward, S. J. (1974). Chromatoid bodies in somatic cells of the planarian: observations on their behavior during mitosis. *Anat. Rec.* **180**, 533-545.
De Mulder, K., Pfister, D., Kuales, G., Egger, B., Salvenmoser, W., Willems, M., Steger, J., Fauster, K., Micura, R., Borgonie, G. et al. (2009). Stem cells are differentially regulated during development, regeneration and homeostasis in flatworms. *Dev. Biol.* **334**, 198-212.
Decker, C. J., Teixeira, D. and Parker, R. (2007). Edc3p and a glutamine/asparagine-rich domain of Lsm4p function in processing body assembly in *Saccharomyces cerevisiae*. *J. Cell Biol.* **179**, 437-449.
Dubois, F. (1948). Sur les conditions de la migration des cellules de régénération chez les planaires d'eau douce. *Soc. Biol. Strasbourg* **142**, 533-535.
Dubois, F. and Wolff, E. (1947). Sur une méthode d'irradiation localisée permettant de mettre en évidence la migration des cellules de régénération chez les planaires. *Soc. Biol. Strasbourg* **141**, 903-909.
Eisenhoffer, G. T., Kang, H. and Sanchez Alvarado, A. (2008). Molecular analysis of stem cells and their descendants during cell turnover and regeneration in the planarian *Schmidtea mediterranea*. *Cell Stem Cell* **3**, 327-339.
Fenderson, B. A., O'Brien, D. A., Millette, C. F. and Eddy, E. M. (1984). Stage-specific expression of three cell surface carbohydrate antigens during murine spermatogenesis detected with monoclonal antibodies. *Dev. Biol.* **103**, 117-128.
Fernandez-Taboada, E., Moritz, S., Zeuschner, D., Stehling, M., Scholer, H. R., Salo, E. and Gentile, L. (2010). Smed-SmB, a member of the LSm protein superfamily, is essential for chromatoid body organization and planarian stem cell proliferation. *Development* **137**, 1055-1065.
Forsthoefel, D. J., Park, A. E. and Newmark, P. A. (2011). Stem cell-based growth, regeneration, and remodeling of the planarian intestine. *Dev. Biol.* **356**, 445-459.
Friedlander, M. R., Adamidi, C., Han, T., Lebedeva, S., Isenbarger, T. A., Hirst, M., Marra, M., Nusbaum, C., Lee, W. L., Jenkin, J. C. et al. (2009). High-resolution profiling and discovery of planarian small RNAs. *Proc. Natl. Acad. Sci. USA* **106**, 11546-11551.
Friesen, W. J., Paushkin, S., Wyce, A., Massenet, S., Pesiridis, G. S., Van Duyne, G., Rappsilber, J., Mann, M. and Dreyfuss, G. (2001). The methylosome, a 20S complex containing JBP1 and pICln, produces dimethylarginine-modified Sm proteins. *Mol. Cell. Biol.* **21**, 8289-8300.
Funayama, N. (2010). The stem cell system in demosponges: insights into the origin of somatic stem cells. *Dev. Growth Differ.* **52**, 1-14.
Funayama, N., Nakatsukasa, M., Mohri, K., Masuda, Y. and Agata, K. (2010). Piwi expression in archeocytes and choanocytes in demosponges: insights into the stem cell system in demosponges. *Evol. Dev.* **12**, 275-287.
Gary, J. D. and Clarke, S. (1998). RNA and protein interactions modulated by protein arginine methylation. *Prog. Nucleic Acid Res. Mol. Biol.* **61**, 65-131.
Gonsalvez, G. B., Rajendra, T. K., Tian, L. and Matera, A. G. (2006). The Sm-protein methyltransferase, dart5, is essential for germ-cell specification and maintenance. *Curr. Biol.* **16**, 1077-1089.
Gonsalvez, G. B., Rajendra, T. K., Wen, Y., Praveen, K. and Matera, A. G. (2010). Sm proteins specify germ cell fate by facilitating oskar mRNA localization. *Development* **137**, 2341-2351.
Guo, T., Peters, A. H. and Newmark, P. A. (2006). A bruno-like gene is required for stem cell maintenance in planarians. *Dev. Cell* **11**, 159-169.
Hanazawa, M., Yonetani, M. and Sugimoto, A. (2011). PGL proteins self associate and bind RNPs to mediate germ granule assembly in C. elegans. *J. Cell Biol.* **192**, 929-937.
Hay, E. D. and Coward, S. J. (1975). Fine structure studies on the planarian, Dugesia. I. Nature of the 'neoblast' and other cell types in noninjured worms. *J. Ultrastruct. Res.* **50**, 1-21.
Hori, I. (1982). An ultrastructural study of the chromatoid body in planarian regenerative cells. *J. Electron Microsc.* **31**, 63-72.
Juliano, C. and Wessel, G. (2010). Developmental biology. Versatile germline genes. *Science* **329**, 640-641.
Juliano, C. E., Voronina, E., Stack, C., Aldrich, M., Cameron, A. R. and Wessel, G. M. (2006). Germ line determinants are not localized early in sea urchin development, but do accumulate in the small micromere lineage. *Dev. Biol.* **300**, 406-415.
Juliano, C. E., Swartz, S. Z. and Wessel, G. M. (2010). A conserved germline multipotency program. *Development* **137**, 4113-4126.
Kirino, Y., Kim, N., de Planell-Saguer, M., Khandros, E., Chiorean, S., Klein, P. S., Rigoutsos, I., Jongens, T. A. and Mourelatos, Z. (2009). Arginine methylation of Piwi proteins catalysed by dPRMT5 is required for Ago3 and Aub stability. *Nat. Cell Biol.* **11**, 652-658.
Kirino, Y., Vourekas, A., Kim, N., de Lima Alves, F., Rappsilber, J., Klein, P. S., Jongens, T. A. and Mourelatos, Z. (2010a). Arginine methylation of vasa protein is conserved across phyla. *J. Biol. Chem.* **285**, 8148-8154.
Kirino, Y., Vourekas, A., Sayed, N., de Lima Alves, F., Thomson, T., Lasko, P., Rappsilber, J., Jongens, T. A. and Mourelatos, Z. (2010b). Arginine methylation of Aubergine mediates Tudor binding and germ plasm localization. *RNA* **16**, 70-78.
Kirino, Y., Vourekas, A., Khandros, E. and Mourelatos, Z. (2011). Immunoprecipitation of piRNPs and directional, next generation sequencing of piRNAs. In *Argonaute Proteins: Methods and Protocols* (ed. T. C. Hobman and T. F. Duchaine), pp. 281-293. New York: Humana Press.
Lerner, E. A., Lerner, M. R., Janeway, C. A., Jr and Steitz, J. A. (1981). Monoclonal antibodies to nucleic acid-containing cellular constituents: probes

for molecular biology and autoimmune disease. *Proc. Natl. Acad. Sci. USA* **78**, 2737-2741.
Liu, H., Wang, J. Y., Huang, Y., Li, Z., Gong, W., Lehmann, R. and Xu, R. M. (2010). Structural basis for methylarginine-dependent recognition of Aubergine by Tudor. *Genes Dev.* **24**, 1876-1881.
Liu, K., Chen, C., Guo, Y., Lam, R., Bian, C., Xu, C., Zhao, D. Y., Jin, J., MacKenzie, F., Pawson, T. et al. (2010). Structural basis for recognition of arginine methylated Piwi proteins by the extended Tudor domain. *Proc. Natl. Acad. Sci. USA* **107**, 18398-18403.
Mahowald, A. P. (1962). Fine structure of pole cells and polar granules in *Drosophila melanogaster*. *J. Exp. Zool.* **151**, 201-215.
Marioni, J. C., Mason, C. E., Mane, S. M., Stephens, M. and Gilad, Y. (2008). RNAseq: An assessment of technical reproducibility and comparison with gene expression arrays. *Genome Res.* **18**, 1509-1517.
Mazzoni, C., D'Addario, I. and Falcone, C. (2007). The C-terminus of the yeast Lsm4p is required for the association to P-bodies. *FEBS Lett.* **581**, 4836-4840.
Meister, G., Eggert, C., Buhler, D., Brahms, H., Kambach, C. and Fischer, U. (2001). Methylation of Sm proteins by a complex containing PRMT5 and the putative U snRNP assembly factor pICln. *Curr. Biol.* **11**, 1990-1994.
Miller, C. A. and Newmark, P. A. (2012). An insulin-like peptide regulates size and adult stem cells in planarians. *Int. J. Dev. Biol.* doi: 10.1387/ijdb.113443cm.
Mollenhauer, H. H. (1964). Plastic embedding mixtures for use in electron microscopy. *Stain Technology* **39**, 111-114.
Morita, M., Best, J. and Noel, J. (1969). Electron microscopic studies of planarian regeneration. I. Fine structure of neoblasts in *Dugesia dorotocephala*. *J. Ultrastruct. Res.* **27**, 7-23.
Newmark, P. A. and Sanchez Alvarado, A. (2000). Bromodeoxyuridine specifically labels the regenerative stem cells of planarians. *Dev. Biol.* **220**, 142-153.
Newmark, P. A. and Sanchez Alvarado, A. (2002). Not your father's planarian: a classic model enters the era of functional genomics. *Nat. Rev. Genet.* **3**, 210-219.
Newmark, P. A., Wang, Y. and Chong, T. (2008). Germ cell specification and regeneration in planarians. *Cold Spring Harb. Symp. Quant. Biol.* **73**, 573-581.
Nishida, K. M., Okada, T. N., Kawamura, T., Mituyama, T., Kawamura, Y., Inagaki, S., Huang, H., Chen, D., Kodama, T., Siomi, H. et al. (2009). Functional involvement of Tudor and dPRMT5 in the piRNA processing pathway in *Drosophila* germlines. *EMBO J.* **28**, 3820-3831.
Orii, H., Sakurai, T. and Watanabe, K. (2005). Distribution of the stem cells (neoblasts) in the planarian *Dugesia japonica*. *Dev. Genes Evol.* **215**, 143-157.
Oviedo, N. J. and Levin, M. (2007). smedinx-11 is a planarian stem cell gap junction gene required for regeneration and homeostasis. *Development* **134**, 3121-3131.
Palakodeti, D., Smielewska, M., Lu, Y. C., Yeo, G. W. and Graveley, B. R. (2008). The PIWI proteins SMEDWI-2 and SMEDWI-3 are required for stem cell function and piRNA expression in planarians. *RNA* **14**, 1174-1186.
Pearson, B. J. and Sanchez Alvarado, A. (2010). A planarian p53 homolog regulates proliferation and self-renewal in adult stem cell lineages. *Development* **137**, 213-221.
Pearson, B. J., Eisenhoffer, G. T., Gurley, K. A., Rink, J. C., Miller, D. E. and Sanchez Alvarado, A. (2009). Formaldehyde-based whole-mount in situ hybridization method for planarians. *Dev. Dyn.* **238**, 443-450.
Pettersson, I., Hinterberger, M., Mimori, T., Gottlieb, E. and Steitz, J. A. (1984). The structure of mammalian small nuclear ribonucleoproteins. Identification of multiple protein components reactive with anti-(U1)ribonucleoprotein and anti-Sm autoantibodies. *J. Biol. Chem.* **259**, 5907-5914.
Rebscher, N., Zelada-Gonzalez, F., Banisch, T. U., Raible, F. and Arendt, D. (2007). Vasa unveils a common origin of germ cells and of somatic stem cells from the posterior growth zone in the polychaete *Platynereis dumerilii*. *Dev. Biol.* **306**, 599-611.
Reddien, P. W., Bermange, A. L., Murfitt, K. J., Jennings, J. R. and Sanchez Alvarado, A. (2005a). Identification of genes needed for regeneration, stem cell function, and tissue homeostasis by systematic gene perturbation in planaria. *Dev. Cell* **8**, 635-649.
Reddien, P. W., Oviedo, N. J., Jennings, J. R., Jenkin, J. C. and Sanchez Alvarado, A. (2005b). SMEDWI-2 is a PIWI-like protein that regulates planarian stem cells. *Science* **310**, 1327-1330.
Reijns, M. A., Alexander, R. D., Spiller, M. P. and Beggs, J. D. (2008). A role for Q/N-rich aggregation-prone regions in P-body localization. *J. Cell Sci.* **121**, 2463-2472.
Reuter, M., Chuma, S., Tanaka, T., Franz, T., Stark, A. and Pillai, R. S. (2009). Loss of the Mili-interacting Tudor domain-containing protein-1 activates transposons and alters the Mili-associated small RNA profile. *Nat. Struct. Mol. Biol.* **16**, 639-646.
Robb, S. M., Ross, E. and Sanchez Alvarado, A. (2008). SmedGD: the Schmidtea mediterranea genome database. *Nucleic Acids Res.* **36**, D599-D606.
Rossi, L., Salvetti, A., Lena, A., Batistoni, R., Deri, P., Pugliesi, C., Loreti, E. and Gremigni, V. (2006). DjPiwi-1, a member of the PAZ-Piwi gene family, defines a subpopulation of planarian stem cells. *Dev. Genes Evol.* **216**, 335-346.
Rouhana, L., Shibata, N., Nishimura, O. and Agata, K. (2010). Different requirements for conserved post-transcriptional regulators in planarian regeneration and stem cell maintenance. *Dev. Biol.* **341**, 429-443.
Salazar, A. M., Silverman, E. J., Menon, K. P. and Zinn, K. (2010). Regulation of synaptic Pumilio function by an aggregation-prone domain. *J. Neurosci.* **30**, 515-522.
Saló, E. and Baguñà, J. (1984). Regeneration and pattern formation in planarians. I. The pattern of mitosis in anterior and posterior regeneration in *Dugesia (G) tigrina*, and a new proposal for blastema formation. *J. Embryol. Exp. Morphol.* **83**, 63-80.
Salvetti, A., Rossi, L., Lena, A., Batistoni, R., Deri, P., Rainaldi, G., Locci, M. T., Evangelista, M. and Gremigni, V. (2005). DjPum, a homologue of *Drosophila* Pumilio, is essential to planarian stem cell maintenance. *Development* **132**, 1863-1874.
Sanchez Alvarado, A., Newmark, P. A., Robb, S. M. and Juste, R. (2002). The *Schmidtea mediterranea* database as a molecular resource for studying platyhelminthes, stem cells and regeneration. *Development* **129**, 5659-5665.
Sato, K., Shibata, N., Orii, H., Amikura, R., Sakurai, T., Agata, K., Kobayashi, S. and Watanabe, K. (2006). Identification and origin of the germline stem cells as revealed by the expression of nanos-related gene in planarians. *Dev. Growth Differ.* **48**, 615-628.
Scimone, M. L., Meisel, J. and Reddien, P. W. (2010). The Mi-2-like Smed-CHD4 gene is required for stem cell differentiation in the planarian *Schmidtea mediterranea*. *Development* **137**, 1231-1241.
Seipel, K., Yanze, N. and Schmid, V. (2004). The germ line and somatic stem cell gene Cniwi in the jellyfish *Podocoryne carnea*. *Int. J. Dev. Biol.* **48**, 1-7.
Selenko, P., Sprangers, R., Stier, G., Buhler, D., Fischer, U. and Sattler, M. (2001). SMN tudor domain structure and its interaction with the Sm proteins. *Nat. Struct. Biol.* **8**, 27-31.
Seydoux, G. and Strome, S. (1999). Launching the germline in *Caenorhabditis elegans*: regulation of gene expression in early germ cells. *Development* **126**, 3275-3283.
Seydoux, G. and Braun, R. E. (2006). Pathway to totipotency: lessons from germ cells. *Cell* **127**, 891-904.
Shibata, N., Umesono, Y., Orii, H., Sakurai, T., Watanabe, K. and Agata, K. (1999). Expression of vasa(vas)-related genes in germline cells and totipotent somatic stem cells of planarians. *Dev. Biol.* **206**, 73-87.
Shibata, N., Rouhana, L. and Agata, K. (2010). Cellular and molecular dissection of pluripotent adult somatic stem cells in planarians. *Dev. Growth Differ.* **52**, 27-41.
Shoji, M., Tanaka, T., Hosokawa, M., Reuter, M., Stark, A., Kato, Y., Kondoh, G., Okawa, K., Chujo, T., Suzuki, T. et al. (2009). The TDRD9-MIWI2 complex is essential for piRNA-mediated retrotransposon silencing in the mouse male germline. *Dev. Cell* **17**, 775-787.
Siomi, M. C., Saito, K. and Siomi, H. (2008). How selfish retrotransposons are silenced in Drosophila germline and somatic cells. *FEBS Lett.* **582**, 2473-2478.
Siomi, M. C., Mannen, T. and Siomi, H. (2010). How does the royal family of Tudor rule the PIWI-interacting RNA pathway? *Genes Dev.* **24**, 636-646.
Siomi, M. C., Sato, K., Pezic, D. and Aravin, A. A. (2011). PIWI-interacting small RNAs: the vanguard of genome defence. *Nat. Rev. Mol. Cell Biol.* **12**, 246-258.
Solana, J., Lasko, P. and Romero, R. (2009). Spoltud-1 is a chromatoid body component required for planarian long-term stem cell self-renewal. *Dev. Biol.* **328**, 410-421.
Sprangers, R., Groves, M. R., Sinning, I. and Sattler, M. (2003). High-resolution X-ray and NMR structures of the SMN Tudor domain: conformational variation in the binding site for symmetrically dimethylated arginine residues. *J. Mol. Biol.* **327**, 507-520.
Tang, J., Frankel, A., Cook, R. J., Kim, S., Paik, W. K., Williams, K. R., Clarke, S. and Herschman, H. R. (2000). PRMT1 is the predominant type I protein arginine methyltransferase in mammalian cells. *J. Biol. Chem.* **275**, 7723-7730.
Tasaki, J., Shibata, N., Nishimura, O., Itomi, K., Tabata, Y., Son, F., Suzuki, N., Araki, R., Abe, M., Agata, K. et al. (2011a). ERK signaling controls blastema cell differentiation during planarian regeneration. *Development* **138**, 2417-2427.
Tasaki, J., Shibata, N., Sakurai, T., Agata, K. and Umesono, Y. (2011b). Role of c-Jun N-terminal kinase activation in blastema formation during planarian regeneration. *Dev. Growth Differ.* **53**, 389-400.
Thompson, B., Wickens, M. and Kimble, J. (2007). Translational control in development. In *Cold Spring Harbor Monograph Series*, vol. 48 (ed. M. B. Mathews, N. Sonenberg and J. W. B. Hershey), pp. 507-544. Cold Spring Harbor, NY: CSH Laboratory Press.
Thomson, T. and Lasko, P. (2004). *Drosophila* tudor is essential for polar granule assembly and pole cell specification, but not for posterior patterning. *Genesis* **40**, 164-170.
Updike, D. L., Hachey, S. J., Kreher, J. and Strome, S. (2011). P granules extend the nuclear pore complex environment in the C. elegans germ line. *J. Cell Biol.* **192**, 939-948.

Vagin, V. V., Hannon, G. J. and Aravin, A. A. (2009a). Arginine methylation as a molecular signature of the Piwi small RNA pathway. *Cell Cycle* **8**, 4003-4004.
Vagin, V. V., Wohlschlegel, J., Qu, J., Jonsson, Z., Huang, X., Chuma, S., Girard, A., Sachidanandam, R., Hannon, G. J. and Aravin, A. A. (2009b). Proteomic analysis of murine Piwi proteins reveals a role for arginine methylation in specifying interaction with Tudor family members. *Genes Dev.* **23**, 1749-1762.
Vasileva, A., Tiedau, D., Firooznia, A., Muller-Reichert, T. and Jessberger, R. (2009). Tdrd6 is required for spermiogenesis, chromatoid body architecture, and regulation of miRNA expression. *Curr. Biol.* **19**, 630-639.
Vennable, J. H. and Coggeshall, R. (1965). A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.* **25**, 407-408.
Voronina, E., Seydoux, G., Sassone-Corsi, P. and Nagamori, I. (2011). RNA granules in germ cells. *Cold Spring Harb. Perspect. Biol*. **3**, a002774.
Wagner, D. E., Wang, I. E. and Reddien, P. W. (2011). Clonogenic neoblasts are pluripotent adult stem cells that underlie planarian regeneration. *Science* **332**, 811-816.
Wang, J., Saxe, J. P., Tanaka, T., Chuma, S. and Lin, H. (2009). Mili interacts with tudor domain-containing protein 1 in regulating spermatogenesis. *Curr. Biol.* **19**, 640-644.
Wang, Y., Zayas, R. M., Guo, T. and Newmark, P. A. (2007). nanos function is essential for development and regeneration of planarian germ cells. *Proc. Natl. Acad. Sci. USA* **104**, 5901-5906.
Wang, Y., Stary, J. M., Wilhelm, J. E. and Newmark, P. A. (2010). A functional genomic screen in planarians identifies novel regulators of germ cell development. *Genes Dev.* **24**, 2081-2092.
Wenemoser, D. and Reddien, P. W. (2010). Planarian regeneration involves distinct stem cell responses to wounds and tissue absence. *Dev. Biol.* **344**, 979-991.
Yabuta, Y., Ohta, H., Abe, T., Kurimoto, K., Chuma, S. and Saitou, M. (2011). TDRD5 is required for retrotransposon silencing, chromatoid body assembly, and spermiogenesis in mice. *J. Cell Biol.* **192**, 781-795.
Yoshida-Kashikawa, M., Shibata, N., Takechi, K. and Agata, K. (2007). DjCBC-1, a conserved DEAD box RNA helicase of the RCK/p54/Me31B family, is a component of RNA-protein complexes in planarian stem cells and neurons. *Dev. Dyn.* **236**, 3436-3450.
Zayas, R. M., Hernandez, A., Habermann, B., Wang, Y., Stary, J. M. and Newmark, P. A. (2005). The planarian *Schmidtea mediterranea* as a model for epigenetic germ cell specification: analysis of ESTs from the hermaphroditic strain. *Proc. Natl. Acad. Sci. USA* **102**, 18491-18496.