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RESEARCH ARTICLE

STEM CELLS AND REGENERATION

PIWI homologs mediate Histone H4 mRNA localization to planarian chromatoid bodies

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ABSTRACT

The well-known regenerative abilities of planarian flatworms are attributed to a population of adult stem cells called neoblasts that proliferate and differentiate to produce all cell types. A characteristic feature of neoblasts is the presence of large cytoplasmic ribonucleoprotein granules named chromatoid bodies, the function of which has remained largely elusive. This study shows that histone mRNAs are a common component of chromatoid bodies. Our experiments also demonstrate that accumulation of histone mRNAs, which is typically restricted to the S phase of eukaryotic cells, is extended during the cell cycle of neoblasts. The planarian PIWI homologs SMEDWI-1 and SMEDWI-3 are required for proper localization of *germinal histone H4* (*gH4*) mRNA to chromatoid bodies. The association between histone mRNA and chromatoid body components extends beyond *gH4* mRNA, since transcripts of other core histone genes were also found in these structures. Additionally, piRNAs corresponding to loci of every core histone type have been identified. Altogether, this work provides evidence that links PIWI proteins and chromatoid bodies to histone mRNA regulation in planarian stem cells. The molecular similarities between neoblasts and undifferentiated cells of other organisms raise the possibility that PIWI proteins might also regulate histone mRNAs in stem cells and germ cells of other metazoans.

KEY WORDS: Stem cells, Neoblasts, Chromatoid bodies, RNP granules, PIWI, Histone SLBP

INTRODUCTION

Substantial changes in gene expression programs are required to balance self-renewal and differentiation of stem cells (He et al., 2009; Smith, 2001). Planarian flatworms have become a popular model system for analyzing events in stem cell proliferation and differentiation because of the abundance of pluripotent stem cells present throughout their soma (Elliott and Sánchez Alvarado, 2012; Newmark and Sanchez Alvarado, 2002). Somatic stem cells, known as 'neoblasts', can be observed during the adult life of this organism and give rise to all other somatic cells, which become terminally differentiated and replaced by neoblast progeny upon their death (Newmark and Sanchez Alvarado, 2000; Wagner et al., 2011). Neoblasts proliferate continuously and this process is accelerated in response to nutritional intake or amputation to allow for growth and for regeneration, respectively (Baguñà, 1976; Newmark and Sanchez Alvarado, 2000; Wenemoser and Reddien, 2010). Thus, the anatomical integrity and function of every planarian tissue depends on the proper proliferation and differentiation of neoblasts.

Accumulating evidence shows that factors present in germ cells across metazoans play a prominent role in regulating neoblast gene expression post-transcriptionally (Guo et al., 2006; Juliano et al., 2011, 2010; Palakodeti et al., 2008; Reddien et al., 2005; Rink, 2013; Rouhana et al., 2010; Salvetti et al., 2005; Shibata et al., 2010, 1999). A shared post-transcriptional regulatory network between planarian neoblasts and metazoan germ cells is also suggested by the presence of large cytoplasmic ribonucleoprotein (RNP) granules (Shibata et al., 2010). In planarians, these membrane-less organelles are known as 'chromatoid bodies', and their function has remained elusive for decades (Auladell et al., 1993; Coward, 1974; Hori, 1982; Morita et al., 1969). Planarian chromatoid bodies contain homologs of the RNA helicase DHH1/RCK (Yoshida-Kashikawa et al., 2007), Tudor (Solana et al., 2009), SmB (Fernandez-Taboada et al., 2010) and methylated substrates of the protein arginine methyltransferase 5 (PRMT5) (Rouhana et al., 2012). PRMT5 catalyzes the synthesis of symmetrical dimethylated arginines (sDMA) on SmB, Vasa and PIWI homologs (Blackwell and Ceman, 2012). These sDMA modifications are binding sites for Tudor domain-containing proteins and contribute to the aggregation and function of these granules (Gao and Arkov, 2012; Siomi et al., 2010).

Noncoding RNAs have come to light as major regulators of gene expression (Ghildiyal and Zamore, 2009). PIWI-interacting RNAs (piRNAs) represent an intriguing family of small (24-31 nucleotide long) non-coding RNAs (Siomi et al., 2011). Regulation by piRNA involves base-pairing with target RNA in a PIWI-containing complex that leads to the cleavage of target RNA and production of new piRNAs (Ishizu et al., 2012). Growing evidence for a second mode of action indicates that piRNAs also serve as guides for epigenetic mark deposition and for silencing of specific loci. This double-mode mechanism for gene silencing secures germline survival, where piRNAs repress the expression of transposons that would otherwise cause genomic instability (Siomi et al., 2011). PIWIs and piRNAs also regulate genes with specific roles in stem cell maintenance (Cox et al., 1998; Klenov et al., 2011), spermatogenesis (Aravin et al., 2006; Grivna et al., 2006), somatic cells of ovaries (Malone et al., 2009; Robine et al., 2009; Saito et al., 2009) and synaptic plasticity (Rajasethupathy et al., 2012).

Planarian members of the PIWI protein family are transcriptionally abundant in neoblasts and routinely used as planarian stem cell markers (Guo et al., 2006; Reddien et al., 2005; Rossi et al., 2007; Sanchez Alvarado et al., 2002). Two PIWI homologs in *Schmidtea mediterranea* (*Smedwi-2* and *Smedwi-3*) are required for proper neoblast maintenance and function (Palakodeti et al., 2008; Reddien et al., 2005). In order to gain a better perspective of which genes these PIWIs might regulate, deep sequencing efforts have identified planarian piRNAs (Friedlander et al., 2009; Palakodeti et al., 2008). Surprisingly, only 20-30% of sequenced piRNAs map to transposable

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elements (Resch and Palakodeti, 2012). The targets and function of the remainder of these small RNAs remain to be elucidated.

Here, we present evidence for regulation of histone mRNA by PIWI homologs in planarian stem cells. Histone gene expression in most eukaryotic cells is regulated post-transcriptionally by the conserved stem-loop binding protein (SLBP) and by interacting factors (Marzluff and Duronio, 2002; Marzluff et al., 2008). These ensure that histone mRNAs only accumulate and are translated exclusively during S phase (when DNA is replicated and packaged into chromatin). Although histone mRNA processing by the SLBP pathway appears to be partially conserved in *S. mediterranea*, an additional level of histone mRNA regulation by PIWI homologs is present in planarian stem cells. We find that transcripts representative of each canonical core histone are present in neoblasts throughout the cell cycle and localize to chromatoid bodies. Knockdown of *Smedwi-1* and *Smedwi-3* leads to loss of histone mRNA localization to chromatoid bodies. Increased levels of histone mRNAs and other neoblast markers are also observed after simultaneous knockdown of these PIWI homologs. Altogether, this work uncovers a connection between the piRNA pathway, chromatoid bodies and histone mRNA regulation in planarian stem cells.

RESULTS

Characterization of Histone H4 transcripts and their localization to chromatoid bodies

We previously identified a sequence with perfect homology to human Histone H4 from a *S. mediterranea* cDNA clone collection (Zayas et al., 2005). This sequence was named *germinal histone H4* (*gH4*) and has been used as a marker for planarian neoblasts and germ cells (Collins et al., 2010; Wang et al., 2010, 2007). Unlike many other neoblast-enriched transcripts, *gH4* transcripts localize to chromatoid bodies (Rouhana et al., 2012), as judged by colocalization of fluorescent *in situ* hybridization (FISH) signals with Y12 antibody (Lerner et al., 1981) labeling of chromatoid bodies (Fig. 1A,B; supplementary material Fig. S1). Y12 binds specifically to symmetrical dimethylarginine (sDMA), a post-translational modification that has been detected in RNA-processing factors, such as Sm proteins (Brahms et al., 2000) and PIWI homologs (for full characterization, see Rouhana et al., 2012). It is important to note that *gH4* mRNA is also observed away from chromatoid bodies in the cytoplasm of neoblasts (Fig. 1A). Also of interest is the observation that *gH4* transcripts were not detected in all chromatoid bodies (72-89% of Y12-labeled chromatoid bodies), revealing molecular heterogeneity in chromatoid bodies and their RNA components.

The *S. mediterranea* genome contains numerous Histone H4 loci (supplementary material Table S1). Of these, sequence with the greatest identity to *gH4* corresponds to a locus that contains a *gH4* sequence duplication, flanking transposon remnants and piRNA islands (Rouhana et al., 2012). The transcript that resulted in the *gH4* cDNA clone used in our studies is predicted to originate from a spliced and polyadenylated product from this locus, which contains a long 3′UTR (supplementary material Fig. S2A). Both splicing and polyadenylation are RNA-processing events that are rarely observed in histone mRNAs (Marzluff et al., 2008). Thus, we questioned whether the transcripts detected by our riboprobes were representative of our *gH4* cDNA clone, bona fide histone mRNAs, pseudogene transcripts or some run-on transcript originating from the transposable element present in this locus. To test this, we synthesized riboprobes covering different fragments of this cDNA. FISH analyses showed that only probes corresponding to the open reading frame (ORF) of the *gH4* cDNA sequence, and not to the long 3′UTR, hybridized to transcripts in planarian neoblasts and chromatoid bodies (supplementary material Fig. S2B). Furthermore, northern blot analyses to verify the size and polyadenylation status of these transcripts confirmed that non-polyadenylated material, which migrates as a single band of approximately 350 nucleotides, was detected from irradiation-sensitive cells (supplementary material Fig. S2C-E). Altogether, these results suggest that the original *gH4* cDNA must have come from a rare transcript detected by priming with oligo(dT) during cDNA cloning, and demonstrate that the vast majority of *gH4* RNA detected in planarian neoblasts exhibits the size and polyadenylation status expected of canonical histone mRNAs. These findings imply that chromatoid bodies may be involved in regulation of canonical Histone H4 mRNAs.

Canonical core histone mRNAs are normally restricted to the S phase of the cell cycle, where they are translated and then degraded once DNA replication is complete (Marzluff and Duronio, 2002). Thus, we hypothesized that the presence of planarian histone mRNA would be restricted to the subset of neoblasts undergoing DNA replication. To test this, we analyzed the temporal distribution of Histone H4 mRNAs recognized by *gH4* riboprobes by performing double FISH with commonly used planarian stem cell markers. Surprisingly, we observed an almost complete (>96%) overlap in detection of Histone H4 transcripts with neoblast markers, such as *Smedwi-1* (Fig. 1C) and *Smed-bruno-like* (Fig. 1D), in stem cells of *S. mediterranea*. Given that all dividing cells in adult planarians express *Smedwi-1* (Guo et al., 2006; Reddien et al., 2005; Wagner et al., 2011), and that only a fraction of SMEDWI-1(+) cells are in S phase [~35% are labeled by BrdU 4 hours after BrdU injection (Guo et al., 2006)], we concluded that expression of histone genes is not restricted to S phase and seems to extend throughout the cell cycle of neoblasts. We further validated this notion by labeling S phase neoblasts with BrdU and checking for expression of *gH4* directly. As expected, a large portion of *gH4*-expressing cells (~37%) did not colabel with BrdU following a 2 hour BrdU pulse and 1 hour chase (Fig. 1E). Significantly, no BrdU(+)/*gH4*(−) cells were found during our analyses. These observations indicate that the presence of histone mRNAs is not limited to the S phase of the cell cycle in planarian neoblasts.

SLBP is required for neoblast maintenance and proper histone mRNA processing in planarians

The regulation of canonical histone mRNAs by the SLBP is a conserved feature of cellular proliferation in eukaryotes (Marzluff and Duronio, 2002). Co-transcriptional 3′-end processing of most pre-mRNAs is mediated by recognition of the AAUAAA motif in their 3′ UTR by components of the cleavage and polyadenylation specificity factor complex (Wahle and Keller, 1992; Zarkower and Wickens, 1987). However, 3′-end processing of histone pre-mRNAs is normally mediated by a separate, but conserved, mechanism that involves SLBP recognition of a stem-loop structure just downstream of the stop codon (Marzluff et al., 2008). The characterization of Histone H4 mRNA by *gH4* northern blot analysis displays characteristics of SLBP-processed transcripts. However, the presence of Histone H4 mRNA outside of S phase in neoblasts is contrary to what is expected from mRNAs regulated by SLBP. To address this paradox, we tested the effect of *SLBP* RNA-interference (RNAi) on Histone H4 mRNA. *SLBP* RNAi led to a gradual loss of *gH4* signals in both FISH and northern blot analyses (Fig. 2A-E). Analysis of neoblast distribution in *SLBP(RNAi)* samples by *Smedwi-1* FISH revealed a severe loss of neoblasts (Fig. 2A-D). Furthermore, *Smedwi-1*(+)/*gH4*(−) neoblasts (or vice versa) were not detected in *SLBP* knockdowns (Fig. 2D-D″). These results suggest that *SLBP* RNAi results in rapid loss of neoblasts,

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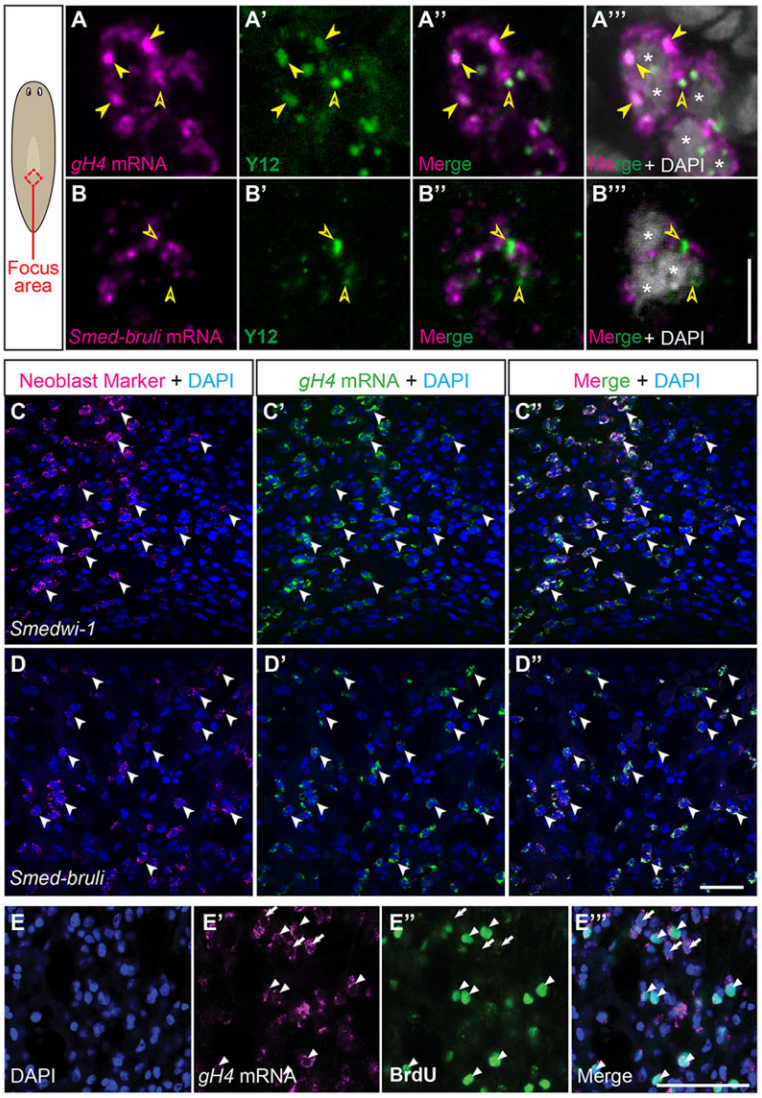

Fig. 1. *germinal histone H4* transcripts are found in the vast majority of neoblasts and localize to chromatoid bodies. (A-B‴) *germinal histone H4* (*gH4*) transcripts specifically localize to chromatoid bodies. Confocal microscopy analyses focused on the neoblast-rich area located posterior to the pharynx (red square). Fluorescent *in situ* hybridization (FISH) analyses of *gH4* mRNA (magenta; A,A″,A‴) and *Smed-bruli* mRNA (magenta; B,B″,B‴) followed by Y12 immunolabeling of chromatoid bodies (green; A′,A‴,B′,B‴) of neoblasts. On average, ~74±5.9% of neoblasts in a single confocal plane (n>100 neoblasts from four biological replicates) contain chromatoid bodies with *gH4* mRNA (filled yellow arrowheads; A″,A‴). *gH4* mRNA is not detected in all chromatoid bodies (empty arrowheads; A″,A‴). *Smed-bruli* mRNA is not detected in chromatoid bodies (empty arrowheads; B″,B‴). Note that *gH4* mRNA is also detected in the cytoplasm outside of chromatoid bodies (A″,A‴). Asterisks (A‴,B‴) indicate cell nuclei labeled with DAPI. (C-D″) *gH4* expression (green; C′,C″,D′,D″) in neoblasts is co-detected by double FISH in a field of cells expressing neoblast markers (magenta) *Smedwi-1* (C,C″) and *Smed-bruli* (D,D″). Nuclei are visualized by DNA stain DAPI (blue). Merged images (C″,D″) reveal *gH4* transcript detection in virtually all neoblasts (>96%, n=308 for C; >98%, n=191 for D), some are pointed out (arrowheads). (E-E‴) Approximately 63% of *gH4*-expressing cells (magenta; E′) were colabeled with BrdU (green; E″) following a 2 hour pulse and 1 hour chase (arrowheads; n>200 cells from six biological replicates). Significantly, ~37% of *gH4*(+) cells were not colabeled by BrdU (arrows), showing *gH4* expression outside of S phase. No BrdU(+)/*gH4*(−) cells were detected. DAPI staining of nuclei (E) and merged images (E‴) are shown. Scale bar in B‴ (applies to A-B‴): 10 μm; in D″ (applies to C-D″): 40 μm; in E‴ (applies to E-E‴): 50 μm.

which is not preceded by a detectable reduction in neoblast histone mRNA levels. Albeit surprising, these results corroborate previous studies in other systems in which compromised SLBP levels led to reduced cellular proliferation without any substantial effects on histone mRNA levels (Sullivan et al., 2009; Zhao et al., 2004).

Loss of SLBP function in *Drosophila* and mice leads to detection of longer polyadenylated histone mRNAs, which result from transcriptional continuation through stem-loop processing signals and eventual processing by canonical cleavage and polyadenylation signals downstream in their 3′UTR (Sullivan et al., 2009; Zhao et al., 2004). Although accumulation of longer histone transcripts after *SLBP* RNAi was not detected by *gH4* northern blot analyses (Fig. 2E), we hypothesized that their detection might be limited due to the rapid loss of neoblasts harboring such transcripts. Thus, in order to enhance detection of aberrant histone mRNA-processing events, we performed a PCR-based analysis selective for polyadenylated transcripts with *gH4* sequence (Fig. 2F). Total RNA was extracted from planarians subjected to control or *SLBP* RNAi. A DNA oligonucleotide was ligated to the 3′-end of the RNA, and a primer of complementary sequence was used to initiate reverse transcription. The complementary primer possessed four thymidines at its 3′-terminus, and thus would only extend reverse transcription of mRNAs ending in minimally four adenosines. Gene-specific primers in the *gH4* ORF were used to prime second-strand synthesis. After PCR, the lengths of Histone H4 transcripts were deduced from the length of DNA fragments. Histone H4 transcripts became progressively longer in planarians subjected to *SLBP* RNAi, with transcriptional termination of transcripts occurring up to 800 nucleotides downstream from that observed in control RNAi samples (Fig. 2G). Taken together, these results demonstrate that SLBP is required for neoblast maintenance and proper Histone H4 mRNA 3′-end processing in planarians.

mRNAs encoding core histones localize to chromatoid bodies

The accumulation of longer polyadenylated transcripts with homology to *gH4* sequence in *SLBP(RNAi)* planarians provided further evidence that transcripts recognized by *gH4* riboprobes in chromatoid bodies are core Histone H4 mRNAs. Since expression of Histone H4 in other systems is synchronized with that of other core histones during the cell cycle, we tested whether other histone mRNAs also localize to chromatoid bodies. We identified and obtained cDNA clones representative of every major histone family (Histone H1, H2A, H2B and H3; supplementary material Table S1). Each of these sequences contains a stem-loop structure 22-31 nucleotides downstream from the stop codon, suggesting that they

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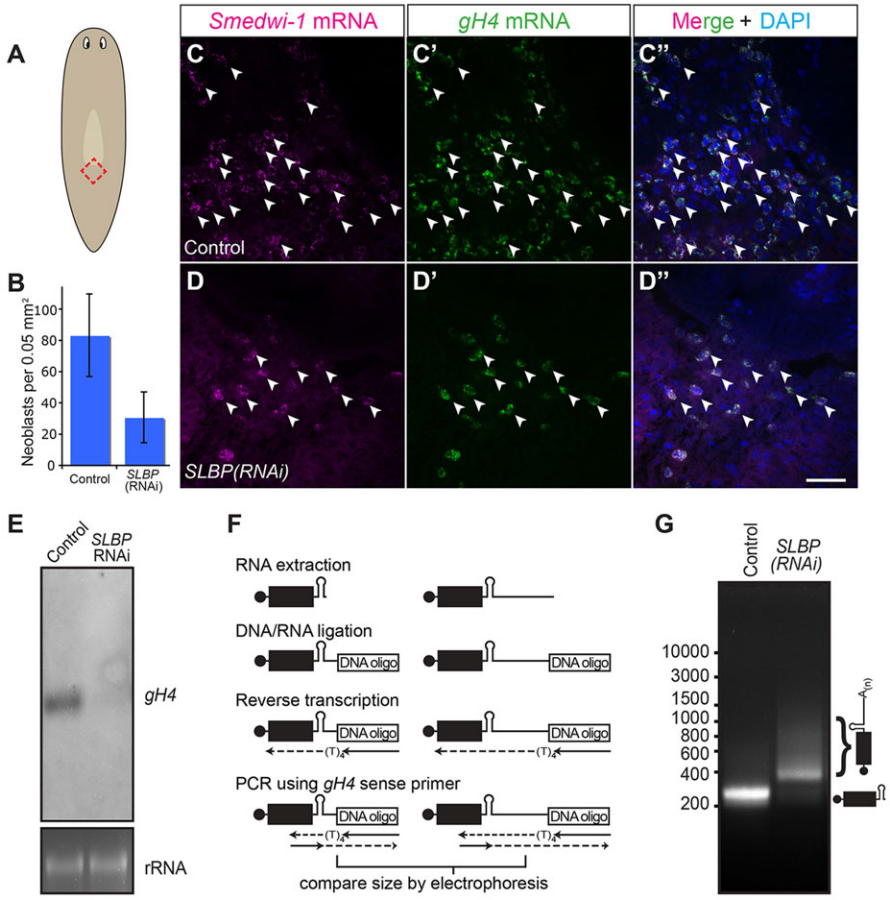

Fig. 2. SLBP is required for neoblast maintenance and *gH4* mRNA 3′-end processing. (A) Illustration indicating the neoblast-rich area (red box) analyzed by FISH and confocal microscopy. (B) The number of neoblasts is significantly reduced in *SLBP(RNAi)* planarians compared with controls between 2 and 3 weeks after the first of two dsRNA feedings (n=4 per group; error bars indicate s.d.; t-test P<0.05). (C-D″) Representative double FISH of cells co-expressing *Smedwi-1* (magenta; C,D) and *gH4* (green; C′,D′) mRNAs reveals a dramatic loss of neoblasts (arrowheads) in *SLBP(RNAi)* (D) compared with control (C) planarians 2 weeks after the first of two dsRNA feedings. Nuclei were observed by DAPI staining (blue, C″,D″). Scale bar: 40 μm. (E) Northern blot analysis reveals the loss of *gH4* signal from total RNA extracts after 3 weeks of *SLBP* RNAi. rRNA levels are shown as loading control. (F) PCR-based 3′-end length analysis modified for selection of polyadenylated RNA (see Materials and Methods for details). (G) Electrophoretic analysis reveals longer 3′-ends in Histone H4 transcripts from animals subjected to 2 weeks of *SLBP* RNAi compared with controls. This assay only requires four terminal adenosines for signal amplification and does not necessarily represent bona fide poly(A) tails. Ladder units: base pairs.

represent canonical histone mRNAs (supplementary material Figs S3, S4). To examine whether these histone mRNAs also localize to chromatoid bodies, we performed FISH combined with immunolabeling of chromatoid bodies using the Y12 antibody as described previously (Fig. 1) (Rouhana et al., 2012). Indeed, we observed enrichment in chromatoid bodies for transcripts corresponding to core Histone H2A and H3 (~74% and 56% of chromatoid bodies, respectively), and to a lesser extent for transcripts corresponding to Histone H1 (~40%) and H2B (~34%) mRNAs (Fig. 3; supplementary material Fig. S3). Localization of these mRNAs to chromatoid bodies overlapped extensively with *gH4* signals (Fig. 3A-D). Histone mRNA localization to chromatoid bodies was also observed in single FISH (supplementary material Fig. S3B-F), demonstrating that localization to chromatoid bodies and partial overlap with *gH4* signals is not a technical artifact of the double FISH procedure. These results suggest that, in addition to mRNA encoding Histone H4, transcripts encoding Histones H1, H2A, H2B and H3 also localize to chromatoid bodies. To our knowledge, these are the only mRNAs thus far shown to localize to chromatoid bodies of planarian stem cells. It is plausible that post-transcriptional regulation of histone mRNAs by chromatoid body components serves as an alternative pathway for their regulation during the cell cycle, which could explain the presence of histone mRNAs in all neoblasts and not only cells transitioning through S phase (Fig. 1C-E).

The localization of histone mRNAs to chromatoid bodies of planarian stem cells raises questions such as: What is the fate of histone transcripts localized to chromatoid bodies? What is the machinery involved in the localization of histone mRNAs to chromatoid bodies? A natural candidate for involvement in histone mRNA localization to chromatoid bodies is SLBP, which is required for transcriptional termination and post-transcriptional regulation of histones via the shared stem-loop structure present in the 3′UTR of histone mRNAs. Analysis of *gH4* FISH signals from remaining neoblasts in planarians subjected to *SLBP* RNAi still showed localization of Histone H4 mRNA to chromatoid bodies (supplementary material Fig. S4A,B). Although it is possible that robust *SLBP* RNAi was not achieved in cells with remaining *gH4* signals, this result suggests that disrupting SLBP function does not directly interfere with localization of histone mRNAs to chromatoid bodies. Additionally, riboprobes corresponding to transcripts of *histone H3* loci that contain single or multiple changes in the stem-loop sequence still recognize transcripts found in chromatoid bodies (supplementary material Fig. S4C-F). Nevertheless, whether planarian SLBP binding specificity allows for such changes in stem-loop sequence has not been established.

SMEDWI-1 and SMEDWI-3 are required for regulation of histone mRNA and localization to chromatoid bodies

Chromatoid bodies have been visualized in this and previous studies (Rouhana et al., 2012) by their immunoreactivity to the Y12 antibody (Lerner et al., 1981). Y12 binds specifically to sDMA, a post-translational modification long known to be present in Sm proteins (Brahms et al., 2000). Although Y12 does recognize planarian material expected to be Sm proteins by western blot analysis, it does not effectively label the nuclei of planarian cells under our current immunofluorescence protocol (Rouhana et al., 2012). Instead, this antibody is completely absorbed by chromatoid bodies, which is partly attributed to the fact that Y12 also recognizes

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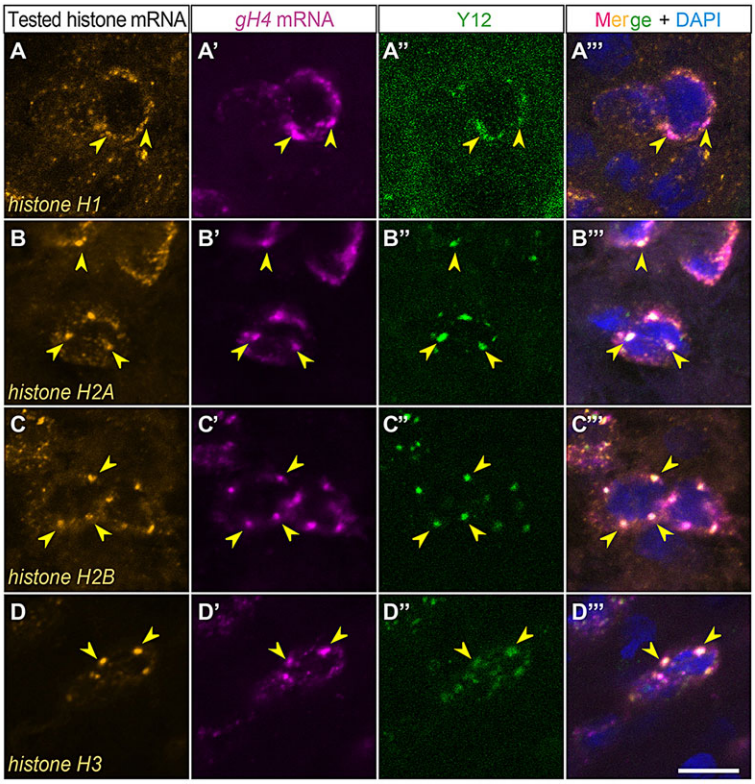

Fig. 3. Core histone mRNAs localize to chromatoid bodies. Double FISH analysis of *histone H1*, *H2A*, *H2B* or *H3* mRNA (gold; A-D) with *gH4* (magenta; A′-D′) followed by Y12 immunofluorescence (green; A″-D″) shows that mRNAs representative of each histone are detected in chromatoid bodies. Merged images and visualization of nuclei by DAPI staining (blue) are shown (A‴-D‴). Arrowheads indicate chromatoid bodies containing the mRNA of tested histones and *gH4*. Scale bar: 10 μm.

sDMA deposited on PIWI homologs (Kirino et al., 2009; Rouhana et al., 2012; Vagin et al., 2009). The physical association between PIWI homologs and Tudor domain-containing proteins is facilitated by sDMA and is crucial for RNP granule integrity and piRNA function (Gao and Arkov, 2012; Siomi et al., 2010).

To examine whether compromising the function of PIWI homologs affects histone mRNA localization to chromatoid bodies, we quantified the number of chromatoid bodies containing Histone H4 mRNA, as visualized by the percentage of Y12 immunofluorescence foci containing *gH4* FISH signals in neoblasts of control and *Smedwi-1*, *Smedwi-2* or *Smedwi-3* knockdown planarians (Fig. 4A-C). We found a decrease of colocalization from 72±5% (n=267) in neoblasts of control animals to 52±8% (n=522) in *Smedwi-1(RNAi)* and 45±6% (n=470) in

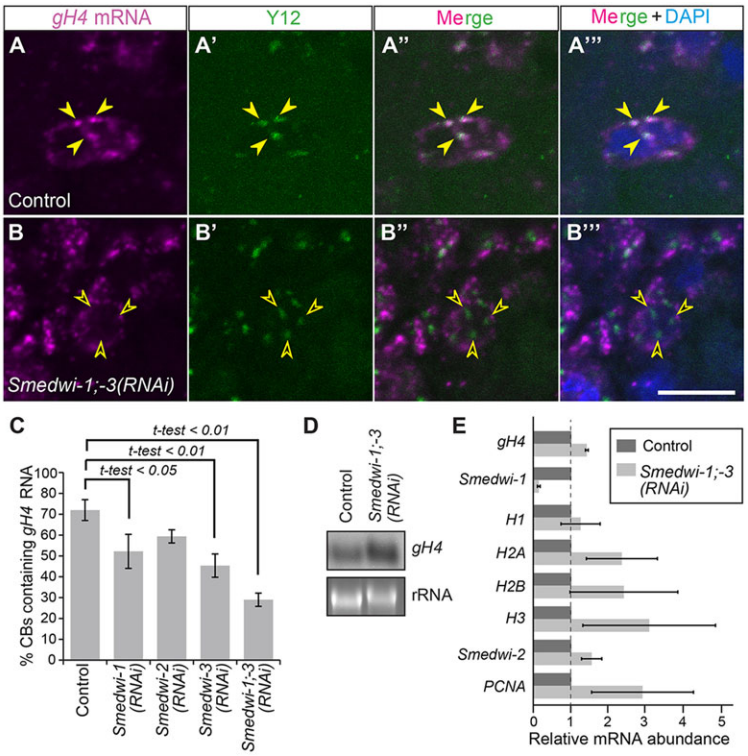

Fig. 4. SMEDWI-1 and SMEDWI-3 are necessary for regulating Histone H4 mRNA localization to chromatoid bodies. (A-B‴) FISH of *gH4* mRNA (magenta; A,B) followed by Y12 immunofluorescence (green; A′,B′) in control (A-A‴) and *Smedwi-1;-3(RNAi)* planarians (B-B‴). Merged images (A″,B″) show reduced *gH4* mRNA localization to chromatoid bodies in *Smedwi-1;-3(RNAi)* samples. DAPI was used to visualize nuclei (blue; A‴,B‴). Filled arrowheads indicate signal colocalization; empty arrowheads represent Y12 signal that does not colocalize with *gH4* mRNA. (C) *Smedwi-1*, *Smedwi-3* and *Smedwi-1;-3* knockdowns lead to a significant reduction in the percentage of chromatoid bodies containing *gH4* mRNA [n≥3 biological replicates; ±s.d.; *t*-test P<0.05 for *Smedwi-1 (RNAi)* and P<0.01 for *Smedwi-3(RNAi)* and *Smedwi-1;-3(RNAi)*]. (D) Northern blot analysis reveals increased *gH4* mRNA levels in total RNA from *Smedwi-1;-3(RNAi)* extracts compared with controls. Ribosomal RNA (rRNA) was used as loading control. (E) Relative abundance of histone and neoblast marker mRNAs in *Smedwi-1; -3(RNAi)* planarians (light gray) relative to controls (dark gray) as measured by RT-qPCR. Transcript levels of genes listed in the *y*-axis were normalized to a *Smed-β-tubulin* internal control. Reduced *Smedwi-1* mRNA levels show effective RNAi in *Smedwi-1;-3(RNAi)* samples. Columns represent averaged values from three biological replicates. Error bars represent s.d. Analyses were performed on planarians 11-12 days after the first of two dsRNA feedings. The second feeding occurred on day 5. Scale bar: 10 μm.

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Smedwi-3(RNAi) (Fig. 4C). Neoblasts of planarians subjected to simultaneous *Smedwi-1* and *Smedwi-3* RNAi [*Smedwi-1;-3(RNAi)*] displayed an even more severe decrease in Y12/*gH4* signal colocalization, which fell to 29±3% (n=486) (Fig. 4A-C). *Smedwi-2(RNAi)* animals showed homeostatic defects on days 11-13 of RNAi treatment, whereas no homeostatic defects were observed in *Smedwi-1(RNAi)*, *Smedwi-3(RNAi)* or *Smedwi-1;-3 (RNAi)* at this time point. The observed homeostatic state of single knockdowns is equivalent to previous observations from studies of homeostatic integrity and neoblast loss dynamics of PIWI homolog knockdowns reported by Palakodeti et al. (2008). Indeed, analysis of neoblast distribution revealed a severe loss of neoblasts in all *Smedwi-2(RNAi)* planarians; however, no significant reduction in *gH4* mRNA localization to chromatoid bodies was observed in lingering neoblasts of *Smedwi-2(RNAi)* at this time point (Fig. 4C). Our attempts at simultaneous RNAi for all three PIWI homologs (*Smedwi-1*, *-2* and *-3*) resulted in variable phenotypes, some of which displayed a severe loss of neoblasts. As RNAi efficiency might be compromised in attempted triple knockdowns we decided to discontinue their analysis. In conclusion, the PIWI homologs SMEDWI-1 and SMEDWI-3 are required for proper localization of Histone H4 transcripts to chromatoid bodies.

The observed reduction in histone mRNA localization in chromatoid bodies after *Smedwi-1* and/or *Smedwi-3* RNAi raised questions about the effect of PIWI function on histone mRNA stability. Thus, we analyzed Histone H4 mRNA levels in planarians subjected to simultaneous *Smedwi-1* and *Smedwi-3* RNAi by northern blot analysis. The levels of Histone H4 mRNA increased in total RNA extracts of *Smedwi-1;-3(RNAi)* planarians compared with those of control animals (Fig. 4D,E), suggesting a correlation between delocalization from chromatoid bodies and increased Histone H4 mRNA levels. A similar trend was observed for the levels of other histone gene mRNAs in *Smedwi-1;-3(RNAi)* planarians compared with controls (*H2A* and *H3*; Fig. 4E). However, when we analyzed the relative levels of mRNA between control and *Smedwi-1;-3(RNAi)* samples for non-histone genes expressed in neoblasts, we also observed an increase in transcript levels (i.e. *Smedwi-2* and *PCNA*; Fig. 4E). These results demonstrate that PIWI homologs are required for regulating the localization of Histone H4 mRNA to chromatoid bodies of planarian stem cells, which correlates with increased levels of this and other neoblast transcripts.

PIWI proteins are conventionally guided by piRNAs that base-pair with respective target mRNAs. Targeted transcripts are cleaved by the 'slicer' activity of PIWI, which in turn generates secondary piRNAs (Siomi et al., 2011). We hypothesized that direct regulation of histone mRNAs by PIWI proteins would require and/or result in piRNAs of histone sequences. In an attempt to elucidate the mechanism by which planarian PIWI proteins regulate histone mRNAs, we searched the *S. mediterranea* genome for previously mapped piRNAs corresponding to histone loci (Friedlander et al., 2009; Palakodeti et al., 2008; Robb et al., 2008). Indeed, we found over 50 piRNA island sequences that correspond to loci representing every core histone family (Fig. 5A; supplementary material Table S1). The identified piRNA sequences matched both intragenic and intergenic sequences, pseudogenes and bona fide histone mRNA coding sequences. Surprisingly, most of the histone piRNA sequences corresponded to the sense orientation of the analyzed histone genes (n=40/50; supplementary material Table S1). These data support the notion that direct contact between PIWI proteins and histone transcripts occurs at some point during the regulation of expression of core histone genes, as piRNAs of histone sequences are likely to be produced by PIWI slicing activity on histone mRNAs.

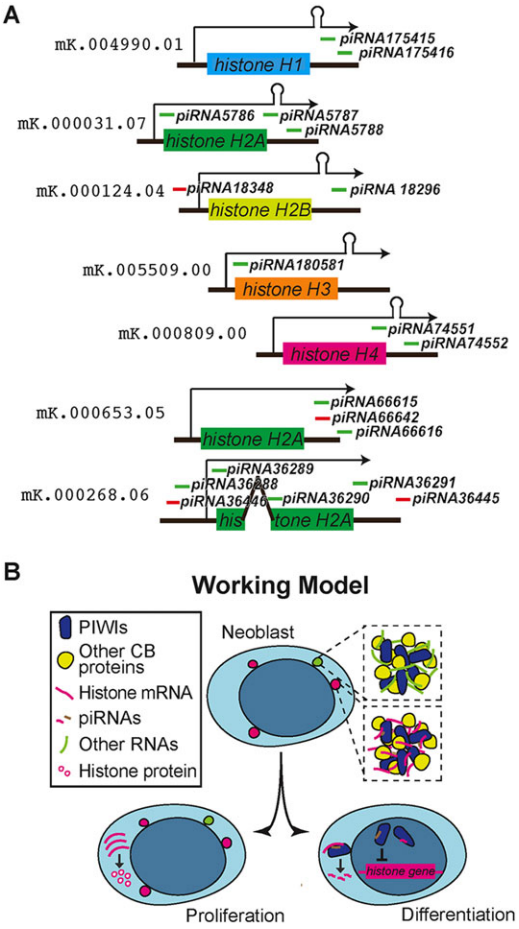

Fig. 5. Regulation of planarian histone mRNAs by the PIWI/piRNA pathway. (A) A subset of histone loci with corresponding piRNA islands. SmedGD locus name is shown (mk.00#####.##). Histone ORF coding sequences are depicted as colored boxes and predicted transcripts as black arrows. Location and name of sense (green) or antisense (red) orientation piRNAs with respect to histone gene mRNAs are depicted as lines. Histone mRNA 3′UTR stem-loop sequences are present in some loci (top), but unrecognizable in others (bottom). (B) Working model. Histone mRNAs localize to chromatoid bodies in a PIWI-dependent manner. Chromatoid bodies lacking histone mRNAs are also present in neoblasts. Histone mRNAs found outside chromatoid bodies are translated to provide histones for DNA replication. Chromatoid bodies and histone mRNAs are lost during differentiation. The loss of histone mRNAs during differentiation and the maintenance of histone gene silencing in differentiated cells could involve 'slicing' of histone mRNAs by PIWI proteins. This mechanism would allow degradation of neoblast histone transcripts no longer required after differentiation, and simultaneous production of secondary piRNAs capable of repressing *de novo* transcription of histone genes by piRNA-mediated gene silencing in differentiated cells.

DISCUSSION

Here, we characterized core histone mRNAs as components of chromatoid bodies in planarian neoblasts. Planarian histone mRNA 3′-end processing is mediated by a conserved mechanism involving SLBP. However, surprising events in histone mRNA biology were observed in planarian stem cells, including a prolonged presence throughout the cell cycle and PIWI-dependent localization to chromatoid bodies. The added complexity of histone mRNA regulation observed in neoblasts might be linked to the unique physiology of planarians, in which all somatic cells (other than neoblasts) are non-proliferative and terminally differentiated. However, histone mRNA post-transcriptional regulation in neoblasts

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could also be reflective of cells in the germline or of early embryos of certain metazoans, in which tight coupling between histone mRNA accumulation and S phase is not observed (Lanzotti et al., 2002; Rouget et al., 2010; Woodland, 1980; Woodland and Adamson, 1977), and where piRNA activity has already been shown to regulate the stability of maternal mRNAs (Rouget et al., 2010).

Histone mRNA regulation by PIWI proteins could also be a common theme in cells in which expression of PIWI proteins is abundant, such as a number of cancer cells (Siddiqi and Matushansky, 2012) and stem cells of basal organisms (Funayama et al., 2010; Juliano et al., 2011). Indeed, recent analysis of the small RNA repertoire in *Hydra* uncovered a population of piRNAs corresponding to sequences along the length of several histone mRNAs (Krishna et al., 2013). Furthermore, Krishna et al. (2013) observed an increase in transcript levels of Late Histone H2A2.2 and Histone H4 mRNAs when corresponding piRNAs were downregulated. Furthermore, work by Juliano et al. (2014) reported that two PIWI homologs are expressed in *Hydra* somatic stem cells and localized to cytoplasmic ribonucleoprotein granules similar to chromatoid bodies. These findings support the idea that an ancestral mechanism of histone mRNA regulation by PIWI proteins is conserved in stem cells of bilaterian and non-bilaterian animals.

SLBP is central to ensuring timely translation and turnover of histone mRNAs within S phase of somatic cells. However, accumulation of histone transcripts and DNA replication are not always coupled. For example, maternal histone mRNA and protein are deposited during oogenesis to provide for early rounds of embryonic division, which require an exponentially increasing number of histones (Marzluff and Duronio, 2002). Although maternal histone mRNAs are regulated differently and protected throughout multiple rounds of proliferation, their 3′-end structure and processing by SLBP are no different from those of somatic cells (Lanzotti et al., 2002; Sullivan et al., 2001). Akin to planarian neoblasts, cells of early embryos rely heavily on post-transcriptional regulation (Wickens et al., 2000), so it is reasonable to assume that similar surrogate pathways of histone mRNA regulation could also be present in stem cells of other metazoans. Supporting this idea, histone mRNA half life is longer in induced pluripotent stem cells than in somatic cells (Neff et al., 2012).

Recently, it has been reported that components of the RNAi pathway in *Caenorhabditis elegans* are required for maintaining sufficient histone mRNA levels and for 3′-end formation downstream of the stem-loop sequence (Avgousti et al., 2012). Indeed, we found piRNAs potentially produced from PIWI slicing immediately downstream of planarian histone mRNA stem-loop sequences (Fig. 5A). However, several lines of evidence indicate that if flatworm PIWIs are indeed involved in regulating the levels of histone mRNA, it would be by a different mechanism from that observed in nematodes. First, the proteins involved in histone 3′-end processing in nematodes belong to the AGO subfamily, whereas planarian histone mRNAs are regulated by PIWI proteins. Second, compromising the levels of the responsible Argonaute in *C. elegans* (CSR-1) by RNAi results in a large reduction in histone mRNA levels, whereas increased levels in histone mRNAs are observed in planarians after *Smedwi-1;-3* RNAi (Fig. 4D,E). Lastly, the endo-siRNAs associated with histone mRNA 3′-end formation in *C. elegans* contain sequences capable of forming base pairs downstream of the stem-loop sequence, whereas planarian histone piRNAs contain sequences found upstream and downstream of the stem-loop, as well as the 5′-end, ORF and intergenic sequences (Fig. 5A). Furthermore, most planarian histone piRNAs correspond to the sense orientation of histone transcripts, although it is possible that unstable antisense piRNAs exist but are barely detectable. How (and if) regulation of histone mRNAs by Argonaute family members occurs in other systems remains to be elucidated.

Since their unveiling by electron microscopy, chromatoid bodies have been postulated to be hubs of RNA regulation. Their presence in neoblasts and their disappearance during differentiation (Coward, 1974) suggest a role for chromatoid bodies in maintaining the stem cell functions of proliferation and/or pluripotency. Chromatoid bodies contain substrates of PRMT5, which include sDMA-SMEDWI-3 (Rouhana et al., 2012), as well as proteins containing the sDMA-binding Tudor domain (Solana et al., 2009). Disruption of PRMT5 expression leads to reduced chromatoid body integrity, increased transposable element transcript levels and, ultimately, loss of neoblasts and regeneration (Rouhana et al., 2012). Thus, the interaction between sDMA and Tudor domain-containing proteins might be the glue that stabilizes chromatoid body structure and function, as is the case in other organisms (Gao and Arkov, 2012). Our observation that histone mRNA levels increase in *Smedwi-1;-3* knockdowns, which correlates with a decrease in histone mRNA localization to chromatoid bodies, could be explained by a model in which these RNP granules serve as sites for priming substrates of PIWI-mediated decay. A different, but plausible, model would be that histone mRNAs are stabilized while in chromatoid bodies, as has been observed for some transcripts in RNA granules in *Drosophila* and *C. elegans* (Bashirullah et al., 1999; Updike and Strome, 2010). Protection of histone mRNAs from degradation by chromatoid bodies could explain the extended presence of these transcripts during the cell cycle of neoblasts (Fig. 1). In this model, the presence of piRNAs corresponding to histone sequences would be the result of degradation of histone mRNAs that have diffused through the cytoplasm or of chromatoid bodies disappearing during differentiation. The increase in histone mRNA levels observed during *Smedwi-1;-3* RNAi could also result from indirect effects on the transcription of histone genes or from subtle increases in neoblast numbers. Nevertheless, the increase in levels of transposable element RNA observed after *PRMT5* RNAi (Rouhana et al., 2012) and the effects on Histone H4 mRNA localization after *Smedwi-1;-3* knockdown both point to PIWI proteins as integral mediators of chromatoid body function. However, chromatoid bodies are heterogeneous, for histone mRNA is only detected on a fraction of these granules and current data do not indicate that all of them contain PIWI proteins (Fig. 1A and Fig. 5B) (Rouhana et al., 2012). Future studies will uncover additional components of chromatoid bodies as well as their connection to proteins, such as the snRNP component SmB (Fernandez-Taboada et al., 2010) and the DEAD box helicase CBC-1/DHH1 (Yoshida-Kashikawa et al., 2007), the functions of which in chromatoid bodies remain to be elucidated.

The prevailing model for piRNA biogenesis, known as the 'ping-pong' cycle, involves the continuous synthesis of sense and antisense piRNAs by reciprocal association of PIWI paralog complexes with guide and target RNAs (Siomi et al., 2011). Given the compartmentalization and physical proximity of PIWI proteins in chromatoid bodies of mammalian male germ cells (Aravin et al., 2009; Shoji et al., 2009), these structures have been postulated to be hubs of piRNA synthesis and function (Siomi et al., 2011). The 3′UTRs of developmentally regulated mRNAs are also substrates for novel piRNA production (Robine et al., 2009; Saito et al., 2009). The regulation of mRNAs that are not related to transposable elements by PIWI proteins could serve two functions. First, the target mRNA is inactivated by endonucleolytic cleavage, causing a reduced expression of the respective gene. Second, secondary piRNAs are synthesized from cleavage of the target mRNAs. Secondary piRNAs could direct downstream gene-

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silencing functions in the nucleus, such as epigenetic marks on DNA and heterochromatin formation (Castel and Martienssen, 2013). We hypothesize that planarian histone gene silencing by piRNAs could occur during neoblast differentiation (Fig. 5B), as histone synthesis is not observed in the differentiated soma, which is non-mitotic. Interestingly, a large fraction of piRNAs that map to histone loci seem to be products of histone mRNA 'slicing' by PIWI homologs (Fig. 5A; supplementary material Table S1). The fact that only 20-30% of piRNAs found in *S. mediterranea* map to transposable elements (Resch and Palakodeti, 2012) suggests that additional groups of genes might be silenced by similar mechanisms during neoblast differentiation. A remaining challenge is to identify such genes and the mechanisms involved in balancing timely synthesis of histones during the cell cycle of proliferating neoblasts.

MATERIALS AND METHODS

Planarian culture and irradiation

A clonal asexual line of *S. mediterranea*, CIW4 (Sanchez Alvarado et al., 2002), was maintained in 0.5 g/l of Instant Ocean salts (Spectrum Brands) in deionized water at 21°C and starved for at least 1 week before each experiment. Planarians of ~8 mm length were used for all experiments. Neoblast depletion was achieved by 40 Gy of gamma irradiation using a Gammacell-220 with a cobalt-60 source (Nordion).

DNA constructs, riboprobes and double-stranded RNA (dsRNA) synthesis

The cDNA constructs utilized for riboprobe and dsRNA synthesis for *germinal Histone H4* (GenBank DN297663.1; Wang et al., 2007), *Smedwi-1* (Guo et al., 2006; Reddien et al., 2005), *Smedwi-2* (Palakodeti et al., 2008; Reddien et al., 2005) and *Smedwi-3* (Palakodeti et al., 2008) have been described previously (Rouhana et al., 2012). Constructs corresponding to Histones H1, H2A, H2B, H3 and SLBP (PL04013A1A11, PL08007A2G03, PL030007A10F10, PL05014A2G10 and PL06013B2C04) from the *S. mediterranea* EST database (newmark13.life.illinois.edu/est) were obtained from cDNA clones collected by Zayas et al. (2005). Characterization of Histone H2B of identical sequence has been reported previously (Solana et al., 2012). PL030012A10A05 was used as a representative for *H3* with an aberrant stem-loop and PL06005A1B05 as a representative for *H3* with an imperfect stem-loop (single nucleotide change). Histone and *SLBP* constructs have been deposited in GenBank (accession numbers: KC916777-KC916784) and plasmids are available through Addgene. Primers used for amplification of templates for different *gH4* cDNA fragment riboprobe synthesis are listed in supplementary material Table S2.

RNAi

DsRNA feeding was performed as described in Rouhana et al. (2013). Planarians were fed liver solution containing dsRNA (0.1 µg/µl). For double or triple gene knockdowns the concentration of total dsRNA was maintained constant (0.1 µg/µl) and gene-specific dsRNA amounts were distributed equally. The bacterial F episome *ccd*B sequence was used as control dsRNA. The frequency and duration of RNAi feedings are specified in the figure legends.

Analysis of chromatoid bodies by whole-mount double FISH and immunofluorescence

Samples were processed for formaldehyde-based whole-mount FISH as described (King and Newmark, 2013), followed by immunofluorescence staining with Y12 antibody (Lerner et al., 1981; Thermo Scientific, MS-450-P; 1:250) as previously described (Rouhana et al., 2012). For double FISH, digoxigenin- and dinitrophenol-labeled probes were included during the hybridization step and developed sequentially after a peroxidase inactivation step, which included incubation in 1% H_2O_2 in PBSTx for 20 min, followed by 4% formaldehyde in PBSTx for 40-60 min. A 1:500 dilution of Alexa-633-labeled goat anti-mouse antibody (Life Technologies, A-21052) was used to visualize Y12 signal after FISH. For analyses, images of single confocal sections from the neoblast-rich region posterior to the pharynx and between gut branches were acquired on a Zeiss LSM710 confocal microscope and analyzed using ZEN 2011 software (Zeiss). Colocalization was scored based on overlapping peaks in intensity between Y12 and FISH signals from single confocal plane images and averaged from three or more biological replicates.

BrdU labeling and analysis

Animals were soaked in 20 mg/ml bromodeoxyuridine (BrdU) and 3% dimethyl sulfoxide diluted in 10× Instant Ocean planarian salts for 2 hours. Several washes in 10× planarian salts were then performed in an hour prior to fixing and processing for FISH and BrdU detection, essentially as described in King and Newmark (2013), with the exception that animals were bleached in 6% H_2O_2 in methanol overnight. BrdU was detected using mouse anti-BrdU (BD Biosciences, cat# 347580; dilution 1:25) and HRP-anti-mouse (Invitrogen, cat# G-21040; at 1:1000) followed by tyramide signal amplification and detection as described above.

Northern blot, RT-qPCR and PCR-based 3′-end polyadenylation analysis

Northern blot analyses using DIG-labeled riboprobes were performed as described (Miller and Newmark, 2012). Polyadenylated and non-polyadenylated fractions for northern blot analysis were obtained using the MicroPoly(A)Purist system (Ambion). PCR-based 3′-end length analyses of polyadenylated RNA were performed as previously described (Rouhana and Wickens, 2007). Briefly, cDNA of polyadenylated RNA was obtained by ligation of the P1 anchor oligo (5′-/5Phos/GGTCACCTTGATCTGAAGC/3AmMO/-3′) to total planarian RNA using T4 RNA ligase (New England BioLabs), reverse transcription primed by P1 antisense (5′-GCTTCA-GATCAAGGTGACCTTTT-3′) and PCR using the *gH4* internal primer (5′-GGAAAATGTTATTAGAGACGCTGTGAC-3′) and the P1 antisense primer. For RT-qPCR, total RNA was extracted from groups of three to five planarians using TRIzol reagent (Invitrogen) and reverse transcribed using random primers. Reverse transcription and PCR were performed using the GoTaq 2-step RT-qPCR system (Promega) as directed by the manufacturer. Primers for *Smed-β-tubulin* and *Smedwi-1* have been described (Miller and Newmark, 2012). Other primers are listed in supplementary material Table S2.

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Competing interests

The authors declare no competing financial interests.

Author contributions

L.R. conceived all experiments and wrote the manuscript. J.A.W. performed analyses of chromatoid bodies in Figs 3 and 4, and supplementary material Figs S1-S4. R.S.K. conducted BrdU/*gH4* analysis of Fig. 1C. P.A.N. assisted with manuscript drafts and participated in the study design and coordination.

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Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.101618/-/DC1

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