Review

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The piRNA pathway in planarian flatworms: new model, new insights

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Abstract: PIWI-interacting RNAs (piRNAs) are small regulatory RNAs that associate with members of the PIWI clade of the Argonaute superfamily of proteins. piRNAs are predominantly found in animal gonads. There they silence transposable elements (TEs), regulate gene expression and participate in DNA methylation, thus orchestrating proper germline development. Furthermore, PIWI proteins are also indispensable for the maintenance and differentiation capabilities of pluripotent stem cells in free-living invertebrate species with regenerative potential. Thus, PIWI proteins and piRNAs seem to constitute an essential molecular feature of somatic pluripotent stem cells and the germline. In keeping with this hypothesis, both PIWI proteins and piRNAs are enriched in neoblasts, the adult stem cells of planarian flatworms, and their presence is a prerequisite for the proper regeneration and perpetual tissue homeostasis of these animals. The piRNA pathway is required to maintain the unique biology of planarians because, in analogy to the animal germline, planarian piRNAs silence TEs and ensure stable genome inheritance. Moreover, planarian piRNAs also contribute to the degradation of numerous protein-coding transcripts, a function that may be critical for neoblast differentiation. This review gives an overview of the planarian piRNA pathway and of its crucial function in neoblast biology.

Keywords: piRNAs; PIWI proteins; planarians; *Schmidtea mediterranea*.

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Introduction

PIWI-interacting RNAs (piRNAs) are short (21–35 nucleotides (nts)) regulatory RNAs that associate with the PIWI clade of the Argonaute family of proteins to form piRNA-guided silencing complexes. Their predominant targets are transposable elements (TEs), which they silence both transcriptionally and post-transcriptionally (Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006; Lau et al., 2006; Vagin et al., 2006). Mutations that disrupt the piRNA pathway result in drastically increased expression of TEs. They also impair germline development, often leading to sterility phenotypes (Lin and Spradling, 1997; Cox et al., 1998; Saito et al., 2006; Watanabe et al., 2006; Brennecke et al., 2007; Houwing et al., 2007; Juliano et al., 2011). Beyond their function in the germline, piRNAs are also found in a broad range of somatic cells (Lewis et al., 2018; Kim, 2019), in lineage-specific stem cells, such as hematopoietic and intestinal cells (Nolde et al., 2013; Ross et al., 2014; Sousa-Victor et al., 2017), and in adult pluripotent stem cells (Juliano et al., 2011; Wolfswinkel, 2014). However, PIWI proteins outside the reproductive system seem dispensable, as their loss evokes no severe phenotype under normal *in vivo* growth conditions (Nolde et al., 2013; Sousa-Victor et al., 2017). In contrast, somatic pluripotent stem cells in metazoans with high regenerative capabilities are detrimentally affected by a loss of function in the piRNA pathway (Juliano et al., 2011; Wolfswinkel, 2014). A knockdown of PIWI proteins in these animals abrogates their regeneration capacity and can even be lethal (Reddien et al., 2005a; Palakodeti et al., 2008). Accordingly, in the cnidarian *Hydra magnipapillata*, the PIWI protein Hywi is essential for the development of the epithelial stem cell lineages that ensure tissue homeostasis (Juliano et al., 2014). Another example is the arrest of cellular expansion and whole-body regeneration triggered by the knockdown of a PIWI protein in colonial ascidian species (Rinkevich et al., 2010, 2013). Finally, *piwi* is also essential for regeneration and embryonic development in acoels, marine worms that represent the

base of Bilateria (Mulder et al., 2009; Srivastava et al., 2014). The piRNA pathway also plays a critical role in the regeneration of vertebrate limbs. While piRNAs and PIWI proteins are predominantly enriched in the germline of vertebrate animals, formation of the regenerating blastema in the Mexican axolotl also requires the expression of two *piwi* genes, PL1 and PL2 (Zhu et al., 2012; Clark and Lau, 2014). Loss of *piwi* expression prevents this specialized tissue from fulfilling its potential to regrow fully functional limbs after amputation.

Intriguingly, parasitic flatworms such as trematodes (flukes) and cestodes (tapeworms) represent an exception to the described connection between adult pluripotent stem cells and piRNA expression. Despite the presence of neoblasts, they lack a canonical piRNA pathway due to the absence of key players such as *piwi*, *vasa* and *tudor* (Tsai et al., 2013; Zheng, 2013; Skinner et al., 2014; Collins, 2017). Instead, numerous other dead-box helicases likely have adopted functions carried out by *vasa* (Skinner et al., 2012; Tsai et al., 2013). Moreover, both flukes and tapeworms have evolved a parasite-specific forth clade of Argonaute proteins, group 4 Argonautes (Wang et al., 2013; Zheng, 2013; Skinner et al., 2014). Group 4 Argonautes are expressed in the germline and in neoblasts. They bind small interfering RNAs (siRNAs) derived from long interspersed nuclear element (LINE) and long terminal repeat (LTR) elements and thereby compensate for the loss of the piRNA machinery (Cai et al., 2012; Wang et al., 2013). Taken together, these data suggest that a thorough understanding of the piRNA pathway and its involvement in stem cell function is required to understand the role of piRNAs and PIWI proteins in regeneration.

To this end, planarian flatworms are an excellent model system in which to uncover the role of the piRNA pathway in somatic pluripotent stem cells. Planarian neoblasts, the adult stem cell population that fuels the regenerative potential of these worms, express three PIWI proteins. Individual knockdown of two PIWI proteins deprives planarians of their ability to regenerate and is lethal, indicating that piRNAs and PIWI proteins are essential for both the regenerative capacity and tissue homeostasis of planarians (Reddien et al., 2005a; Palakodeti et al., 2008; Nakagawa et al., 2012; Zhou et al., 2015; Shibata et al., 2016; Almazan et al., 2018; Kim et al., 2019a). In this review, we summarize our current knowledge of the piRNA pathway in planarian flatworms with an emphasis on its functions in stem cell biology and the mechanisms known to mediate them.

Planarian neoblasts combine features of pluripotent embryonic stem cells and the germline

Planarian flatworms belong to the phylum Platyhelminthes, which includes species with varying degrees of regenerative ability. Regenerative success strongly correlates with the ability to reproduce asexually (Egger et al., 2006; Collins, 2017; Vila-Farré and Rink, 2018). A feature common to both highly regenerative and less regenerative flatworm species is the presence of neoblasts or neoblast-like cells (Figure 1) (Collins, 2017). Neoblasts are the only mitotically active cells in flatworms and they designate a heterogeneous population of pluripotent and multipotent stem cells (Wagner et al., 2011; Wolfswinkel et al., 2014; Fincher et al., 2018; Zeng et al., 2018). Pluripotent (or clonogenic) neoblasts are capable of producing all cell types, including those that give rise to the germline (Wang et al., 2007). Moreover, such pluripotent neoblasts can rescue lethally irradiated animals in single-cell transplantation experiments by restoring their tissue homeostasis and regenerative abilities (Wagner et al., 2011; Zeng et al., 2018). In contrast to other organisms, in which pluripotent stem cells exist only transiently during early embryonic development, planarians thus maintain pluripotent stem cells into adulthood. In addition, the gene expression profile of planarian neoblasts is similar to that of mouse embryonic stem cells (Önal et al., 2012). For example, several genes encoding chromatin regulators [e.g. components of the esBAF, mixed lineage leukemia (MLL), polycomb repressive complex 2 (PRC2) and polymerase-associated factor 1 (PAF1) complexes] show increased expression in neoblasts as do genes that are known targets of the mammalian pluripotency-regulator Sox2, Oct4 and Nanog (Ng and Surani, 2011; Labbé et al., 2012; Önal et al., 2012; Trost et al., 2018).

In addition to features of embryonic stem cells, neoblasts also possess characteristics of the germline (Juliano et al., 2011, 2010; Lehmann, 2012; Solana, 2013; Lai and Aboobaker, 2018). Both the germline and neoblasts possess perinuclear granules, known as chromatoid bodies or the nuage (Coward, 1974; Solana et al., 2009; Voronina et al., 2011; Rouhana et al., 2014; Kashima et al., 2016). Chromatoid bodies are membrane-less cytoplasmic granules with high RNA content. They cluster around the nucleus and are frequently in contact with mitochondria (Coward, 1974). Moreover, neoblasts express the so-called germline multipotency program (GMP) genes, which include *vasa*, *piwi*, *tudor*, *pumilio* and *bruno* (Reddien et al., 2005a; Salvetti et al., 2005; Guo et al., 2006; Palakodeti et al., 2008; Rouhana et al., 2010). GMP genes prevent somatic

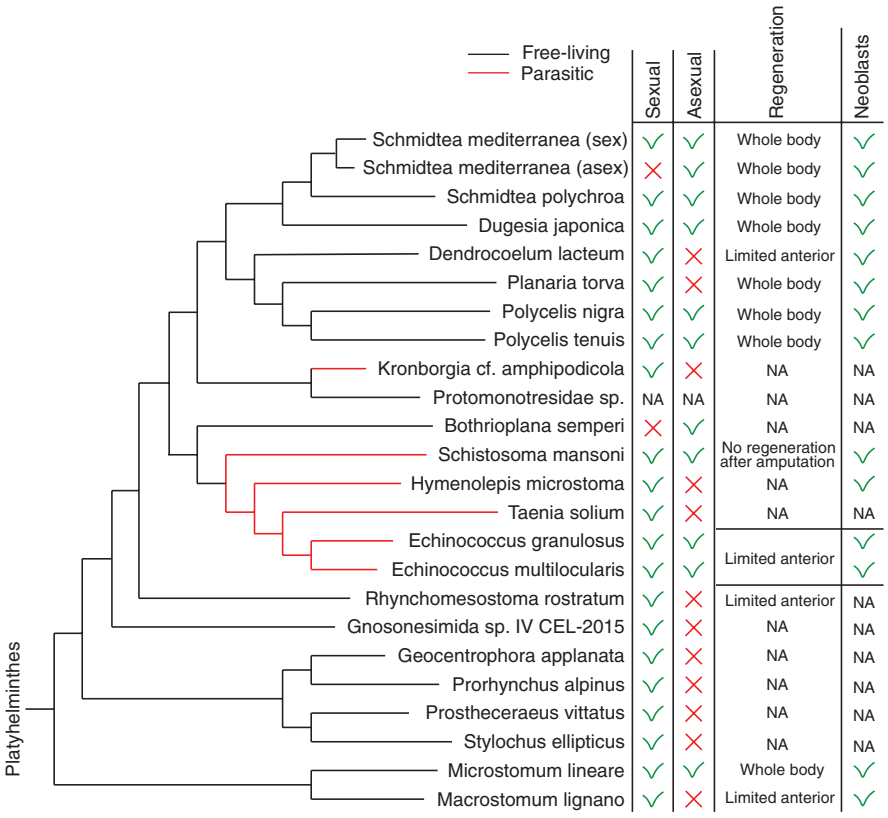


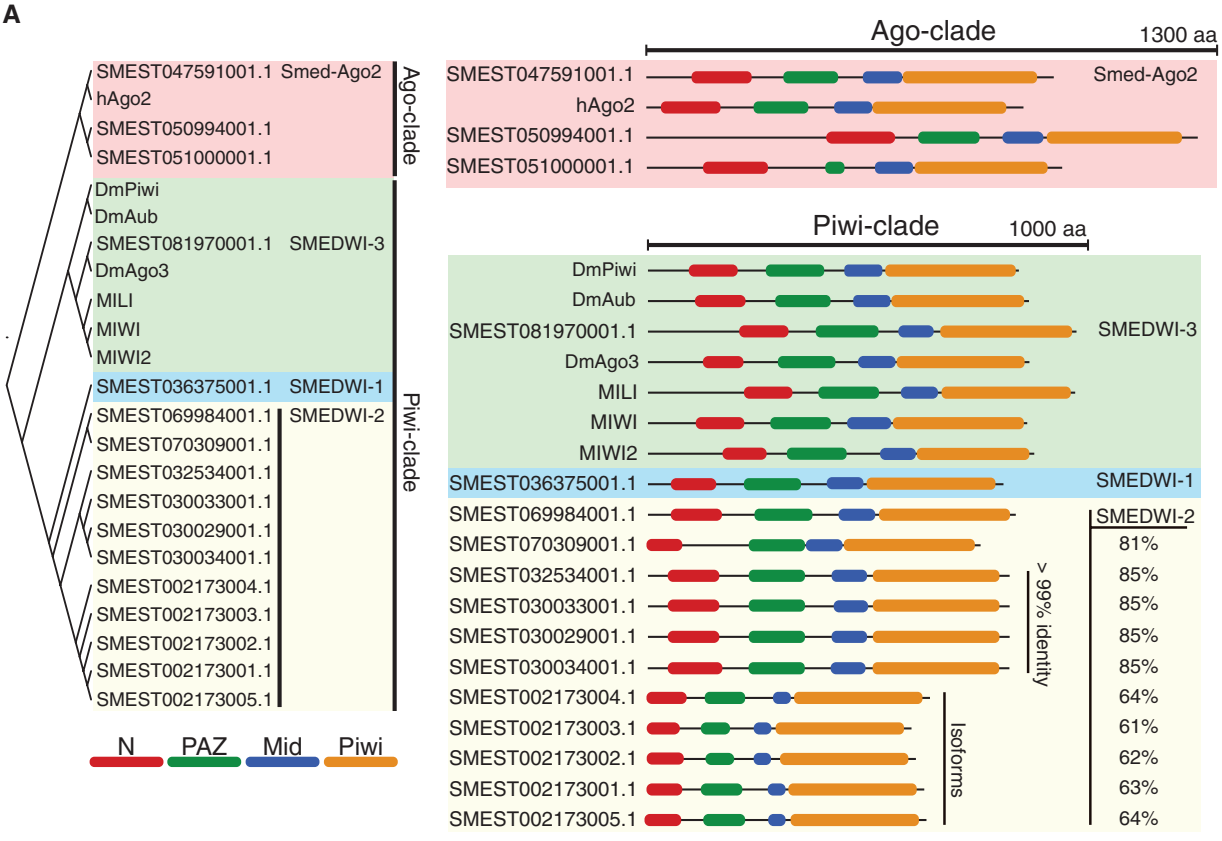
Figure 1: Distribution of regenerative potential amongst Platyhelminthes.
For each listed species of the phylum Platyhelminthes, the reproductive strategy, the ability to regenerate and the possession of neoblasts/neoblast-like cells are indicated. The asexual mode of reproduction includes fission and parthenogenesis. Limited anterior regeneration refers to the inability to restore a head region. The length of bars does not correlate with phylogenetic distance or similarity.

differentiation, regulate cellular proliferation and protect the germline genome (Juliano et al., 2010). Interestingly, some GMP genes such as *vasa*, *piwi* and *tudor* are key members of the piRNA pathway. This highlights that the piRNA pathway is an essential part of the cellular pluripotency program, germline protection and stemness in general. Furthermore, the ability of neoblasts to give rise to the germline provides an unrivalled opportunity to understand the involvement of the piRNA pathway in primordial germ cell segregation and development. In planarians, just like in mammals, the specification of the germline takes place by epigenesis and is thus independent of preexisting maternal information. In contrast, the germline in fruit flies is characterized by the continuous inheritance of a maternally deposited germ plasm (Lehmann, 2012).

The planarian PIWI proteins

A sequence homology search in the genome of the sexual strain of *Schmidtea mediterranea* (version SMESG.1; Grohme et al., 2018) identified 12 potential members of the Argonaute protein superfamily (Figure 2A). Three of the identified Argonautes, including the previously characterized genes for planarian Ago1 (SMEST051000001.1) and Ago2 (SMEST047591001.1) (Li et al., 2011), belong to the Argonaute clade and are closely related to human Argonaute-2 (hAgo2). Of the remaining nine genes, three are clear members of the PIWI clade and have been defined and fairly well characterized in *S. mediterranea* as SMEDWI-1, -2 and -3 (Figure 2A) (Reddien et al., 2005a; Palakodeti et al., 2008). All these three *bona fide* PIWI proteins are likely active endonucleases as they harbor the necessary catalytical DEDH tetrad in their PIWI domains (Figure 2B).

Smedwi-1 (SMEST036375001.1) is expressed in planarian neoblasts (Reddien et al., 2005a; Guo et al., 2006), germ cells and early embryonic cells (Nakagawa et al., 2012; Davies et al., 2017). Its protein product, SMEDWI-1, localizes to the cytoplasm (Guo et al., 2006; Davies et al., 2017). Although both the transcript and the protein are commonly used as a stem cell marker, the expression of *smedwi-1* was found to be heterogeneous amongst



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SMEDWI-1              LDTFHSKTGK-RSVQASVFSISAKFSQYISFVNSSKGKNEFHENLGKNFLTALTTFQNKFNTMPLRLIIYRDGVGDSQLAFTK  649
SMEDWI-2              MDTYHNKSSK-KSIQASVFSINSTFTQYMSFVNSPKGRQEFHETLGKNFNLALEDFKKRYDILPQRILVFRDGVGDNQLQFTK  676
SMEST070309001.1      MDTYHNKSSK-KSIQASVFSINSTFTQYMSFVNSPKGRQEFHETLGKNFGSALGHFKKEYDFFPQRILVYRDGVGDSQLQFTK  596
SMEST032534001.1      LDTYHNKSNK-KSIQASVFSTNESFTQYLSFVNSPKGRQEFHETLGNNFGSALGYFKKQFNIFPQRILVYRDGVGDNQLQFTK  662
SMEST030034001.1      LDTYHNKSSK-KSIQASVFSTNESFTQYLSFVNSPKGRQEFHETLGNNFGSALGYFKKQFNIFPQRILVYRDGVGDSQLQFTK  662
SMEST030033001.1      LDTYHNKSSK-KSIQASVFSTNESFTQYLSFVNSPKGRQEFHETLGNNFGSALGYFKKQFNIFPQRILVYRDGVGDSQLQFTK  662
SMEST030029001.1      LDTYHNKSSK-KSIQASVFSTNESFTQYLSFVNSPKGRQEFHETLGNNFGSALGYFKKQFNIFPQRILVYRDGVGDSQLQFTK  662
SMEST002173001.1      LDTYHNKSSK-KSIQASVFSTNESFTQYLSFVNSPKGRQEFHKTVNNNFGSTLEYFKKQFNISPQRILVYMNGVGDNQLQFTK  470
SMEDWI-3              LDVWHDTKGRSRSVGAVVGSTNALCTRWFSKSHLQEQDKEIMYVLQSCMLSLLKAYFEENNFLPETIFMYRDGVSDGQLGYVQ  823

SMEDWI-1              RLIIYRDGVGDSQLAFTKKFETDAVMKMIEKIYENQT--LPQIIYVVVKKRISVKFFKD-------GANPNPGTVVDEKIVKP  705
SMEDWI-2              RILVFRDGVGDNQLQFTKNFEVDAMKPLIENIYKGNGFPVPQIIYVIVKKRVGTKLFNR-------GNNPNPGTVVDKEIVKP  734
SMEST070309001.1      RILVYRDGVGDSQLQFTKNFEVDAMKQLIEEMYKSNGFPLPQIIYVIVKKRVGTKLFNR-------GNNPNPGTIVDKEIVKP  654
SMEST032534001.1      RILVYRDGVGDNQLQFTKNFEVEAMKLLIENIYKSNGFPAPQIIYVIVKKRIGTKLFNR-------GNNPNPGTIVDKEIVKP  720
SMEST030034001.1      RILVYRDGVGDSQLQFTKNFEVEAMKLLIENIYKSNGFPAPQIIYVIVKKRIGTKLFNR-------GNNPNPGTIVDKEIVKP  720
SMEST030033001.1      RILVYRDGVGDSQLQFTKNFEVEAMKLLIENIYKSNGFPAPQIIYVIVKKRIGTKLFNR-------GNNPNPGTIVDKEIVKP  720
SMEST030029001.1      RILVYRDGVGDSQLQFTKNFEVEAMKLLIENIYKSNGFPAPQIIYVIVKKRIGTKLFNR-------GNNPNPGTIVDKEIVKP  720
SMEST002173001.1      RILVYMNGVGDNQLQFTKNFEVEAMKQLIENIYKSNGLPGPQIIYVIVKKRIGTKLFNR-------GNNPNPRTIVDKEIVKP  528
SMEDWI-3              TIFMYRDGVSDGQLGYVQKTEIEQFFKVFESFSAD---YKPNMVYNVVQKRINTRLYVSDPKNKGQINNPNPGTIVDHTVTRA  885

SMEDWI-1              NFYEFYLVSQKTTKGTASPTNYNVLMDTKFTNKKTNEVSVMSPSVLQQITYSLTHLYFNWMGTIRVPVPTHYAHRL  781
SMEDWI-2              NFYEFFLVSQRTTKGTATPTNYNVLEDTRLLTKKG-TMDPMAPNELQKITYALTHLYFNWMGTIRVPVPVHYAHRL  809
SMEST070309001.1      NFYEFFLVSQRTTKGTATPTNYNVLEDTRLLTKKG-TQDPMPPNELQKITYALTHLYFNWMGTIRVPVPVHYAHRL  629
SMEST032534001.1      NFYEFFLVSQRTTKGTATPTNYNVLEDTRLITKNG-TKEPIPPNELQRITYVLTHLYFNWMGTIRVPVPVHYAHRL  795
SMEST030034001.1      NFYEFFLVSQRTTKGTATPTNYNVLEDTRLITKKG-TKEPIPPNELQRITYILTHLYFNWMGTIRVPVPVHYAHRL  795
SMEST030033001.1      NFYEFFLVSQRTTKGTATPTNYNVLEDTRLITKKG-TKEPIPPNELQRITYILTHLYFNWMGTIRVPVPVHYAHRL  795
SMEST030029001.1      NFYEFFLVSQRTTKGTATPTNYNVLEDTRLITKKG-TKEPIPPNELQRITYILTHLYFNWMGTIRVPVPVHYAHRL  795
SMEST002173001.1      N--GFFLVSQRTTKGTATPTNYNVLEDTRLITKKG-TKEPITPNELQRITYALTHLYFNWMGTIRVPVPVHYAHRL  601
SMEDWI-3              NLYDFFLVSQSVRQGTVTPTHYVVLCDNS----------KYTPHQVQLMAYKTCHIYYNWPGTVRVPAPCMYAHKL  951
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Figure 2: Argonaute superfamily of proteins in *S. mediterranea*.
(A) Phylogenetic relationship of Argonaute superfamily members from *H. sapiens* (hAgo2), *D. melanogaster* (DmPiwi, DmAub, DmAgo3), *M. musculus* (MILI, MIWI, MIWI2) and *S. mediterranea*. Ago clade proteins are highlighted in red. PIWI clade proteins that share similarity to SMEDWI-1, -2 and -3 are highlighted in blue, beige and green, respectively. Classification is based on protein sequences deposited to the UniProt database or based on transcripts identified in the genome (version SMESG.1) of the sexual strain of *Schmidtea mediterranea*. The phylogenetic tree was generated using Clustal Omega (Madeira et al., 2019). Displayed domains (N, PAZ, Mid, Piwi) were identified with InterPro and HHPred (Mitchell et al., 2018; Zimmermann et al., 2018). The similarity of SMEDWI-2 paralogs is indicated. (B) Alignment of the catalytically active residues of all planarian SMEDWI proteins. The catalytic tetrad DEDH is highlighted in yellow.

different sub-populations of neoblasts (Zeng et al., 2018). The highest level of *smedwi-1* is thereby found in epidermal progenitors (ζ neoblasts) and pluripotent tetraspanin-1-positive neoblasts, i.e. cells that are able to produce all planarian stem cell progenitors and, presumably, all planarian cell types (Wolfswinkel et al., 2014; Zeng et al., 2018). Differentiation of pluripotent neoblasts into fate-determined progenitors and terminally differentiated cells is accompanied by a successive reduction of *smedwi-1* levels (Zeng et al., 2018). The differential expression of *smedwi-1* at different stages of neoblast development is reminiscent of the time-regulated expression of murine PIWIs during spermatogenesis. There, nuclear MIWI2 is expressed along with cytoplasmic MILI for a short time in the pre-pachytene stage of mouse spermatogenesis, where both PIWI proteins work together to repress TEs post-transcriptionally and to induce DNA methylation at TEs (Carmell et al., 2007; Aravin et al., 2008). Later, MILI is expressed in conjunction with cytosolic MIWI, which binds pachytene piRNAs that originate from non-repetitive sequences (Aravin et al., 2006; Girard et al., 2006; Gou et al., 2014; Rojas-Ríos and Simonelig, 2018). However, in contrast to murine PIWIs, planarian SMEDWI-2 and -3 are expressed at all stages of neoblast development and differentiation.

The expression of SMEDWI-2 (SMEST069984001.1) and SMEDWI-3 (SMEST081970001.1) is not restricted to planarian neoblasts. The *smedwi-2* transcript, for example, was also detected in the central nervous system and in the epidermis (Solana et al., 2012; Duncan et al., 2015; Kim et al., 2019a). SMEDWI-2 protein shows a predominantly nuclear localization in both neoblasts and differentiated cells (Zeng et al., 2013; Shibata et al., 2016; Kim et al., 2019a; Wang et al., 2019). Similarly, DjPiwiB, the SMEDWI-2 ortholog in *Dugesia japonica*, is present in the nuclei of almost all differentiated cells of the animal (Shibata et al., 2016). The *smedwi-3* transcript is also expressed in non-neoblasts, appearing in the pharynx, nervous system and anterior of the photoreceptors, albeit at low levels (Palakodeti et al., 2008; Kim et al., 2019a). SMEDWI-3 shows cytoplasmic localization in both neoblasts and head cephalic ganglions (Kim et al., 2019a). Furthermore, SMEDWI-3 and its ortholog DjPiwiC in *D. japonica* are enriched in planarian chromatoid bodies, ribonucleoprotein granules that accumulate proteins carrying symmetrical dimethylarginine (sDMA) modifications (Kashima et al., 2016; Kim et al., 2019a). Out of three planarian SMEDWIs, only SMEDWI-3 possesses GRG-motifs that are recognized by the arginine methyltransferase PRMT5 (Rouhana et al., 2012). PRMT5 places an sDMA modification on these GRG motifs, which facilitates the interaction of PIWIs and Tudor domain-containing proteins (Siomi et al., 2010; Rouhana et al., 2012).

Furthermore, six additional PIWI clade genes were identified in the planarian genome, all of which are closely related to *smedwi-2* (Figure 2A). Four of these candidate genes (SMEST323534001.1, SMEST030029001.1, SMEST030033001.1 and SMEST030034001.1) are highly similar to each other (99% identity at the protein level) and to *smedwi-2* (~85% identity). The expression of two paralogs (SMEST070309001.1; SMEST030033001.1 or SMEST030034001.1, both of which are identical at the nucleotide level) was validated previously by real-time quantitative polymerase chain reaction (RT-qPCR) (Palakodeti et al., 2008). However, whether the additional PIWI members encode functional proteins or simply represent pseudogenes is currently unclear. Genome expansion of PIWI-like genes could also be detected in other planarian species through Blast search of the PlanMine database (Rozanski et al., 2018).

Characteristic features of planarian piRNAs and their genomic origin

With few exceptions, the function of PIWI proteins is defined by the sequences of the piRNAs they bind to (Gou et al., 2017; Ozata et al., 2019). Common features of mature piRNAs in all organisms studied to date are the presence of a monophosphate at their 5′-end, the preference for uridine as the first nucleotide (1U bias) or for adenosine at nucleotide position 10 (10A bias), and the modification of their 3′-end by a 2′-O-methyl moiety (Kirino and Mourelatos, 2007; Ohara et al., 2007; Wang et al., 2014; Matsumoto et al., 2016; Stein et al., 2019). In planarian flatworms, PIWI-bound small RNAs exhibit all these canonical characteristics. Planarian piRNAs are 30–35 nt in length (Palakodeti et al., 2008; Friedländer et al., 2009; Zhou et al., 2015; Shibata et al., 2016; Kim et al., 2019a). In *S. mediterranea*, SMEDWI-1- and -2-bound piRNAs exhibit a pronounced 1U bias, while SMEDWI-3-bound piRNAs show a strong 10A bias and a slight conservation of a 5′-terminal uridine (Kim et al., 2019a). At their 3′-ends, planarian piRNAs carry 2′-O-methyl groups (unpublished data), which are likely placed by Hen1 – in analogy to the situation in other organisms (Horwich et al., 2007; Saito et al., 2007). In support, the expression level of a putative planarian ortholog of Hen1 (SMESG000036845.1) (Table 1) is 3 times higher in neoblasts compared to differentiated cells (Labbé et al., 2012; Rozanski et al., 2018).

Table 1: Members of the planarian piRNA pathway.

		S. med proteins	Planmine	Mouse		Drosophila		Function in piRNA pathway	Phenotype	Reference
PIWIs		SMEDWI-1	SMESG000036375.1	MIWI	Id.: 24.8% Sim.: 42.1%	Aubergine	Id.: 25.1% Sim.: 43.7%	Cytoplasmic mRNA/TE surveillance	No robust phenotype	Reddien et al., 2005a,b
		SMEDWI-2	SMESG000069984.1	MIWI2	Id.: 24.6% Sim.: 41.7%	Piwi	Id.: 24.1% Sim.: 41.1%	Transcriptional gene silencing	Lethal	Reddien et al., 2005a,b
		SMEDWI-3	SMESG000081970.1	MILI	Id.: 32.6% Sim.: 49.8%	Ago3	Id.: 34.1% Sim.: 52.3%	Cytoplasmic mRNA/TE surveillance	Lethal	Palakodeti et al., 2008
Secondary piRNA biogenesis	Primary piRNA biogenesis	Smed-DNAJA1	SMESG000012107.1	DNAJA1	Id.: 58.6% Sim.: 76.6%	DnaJ-like-2 IsoformA	Id.: 57.0% Sim.: 70.4%	Binding/stabilization of SMEDWI-1/-2	Lethal	Wang et al., 2019
		Smed-UAP56	SMESG000035241.1	DDX39B	Id.: 75.8% Sim.: 87.4%	UAP56	Id.: 72.9% Sim.: 87.1%	Nuclear export of piRNA precursors	NA	BLAST
		Smed-PLD6	SMESG000034482.1	PLD6	Id.: 28.1% Sim.: 43.5%	Zucchini	Id.: 21.6% Sim.: 36.8%	Primary piRNA biogenesis	NA	BLAST
		Smed-PNLDC1	SMESG000042020.1	PNLDC1	Id.: 23.9% Sim.: 43.3%	–		Trimming of premature piRNAs	NA	BLAST
		Smed-MOV10L1 (a)	SMESG000069862.1	MOV10L1	Id.: 29.5% Sim.: 44.5%	Armitage	Id.: 25.1% Sim.: 42.0%	Loading/unloading of PIWI proteins	NA	Önal et al., 2012
		Smed-Gasz	SMESG000037512.1	ASZ1	Id.: 18.4% Sim.: 35.9%	Gasz	Id.: 18.2% Sim.: 39.2%	Primary piRNA biogenesis	NA	BLAST
		Smed-Mino	SMESG000063488.1	GPAT2	Id.: 19.3% Sim.: 33.4%	Mino	Id.: 19.2% Sim.: 35.2%	Primary piRNA biogenesis	NA	BLAST
		Smed-TDRKH	SMESG000075675.1	TDRKH	Id.: 18.7% Sim.: 33.3%	Papi	Id.: 16.0% Sim.: 29.7%	Pre-piRNA 3′-trimming cofactor	NA	BLAST
		Smed-FKBP2	SMESG000078014.1	FKBP6	Id.: 19.9% Sim.: 33.4%	Shutdown	Id.: 23.8% Sim.: 40.9%	Loading of guide into PIWI protein	NA	Wenemoser et al., 2012
		Smed-Hsp83	SMESG000071533.1	HSP90	Id.: 76.1% Sim.: 88.3%	Hsp83	Id.: 77.6% Sim.: 89.2%	Loading of guide into PIWI protein	NA	BLAST
		Smed-Hen1	SMESG000036845.1	HENMT1	Id.: 21.6% Sim.: 36.2%	Hen1	Id.: 24.8% Sim.: 40.8%	3′-Methylation of piRNAs	NA	BLAST
		Smed-TDRD12	SMESG000065057.1	TDRD12	Id.: 15.5% Sim.: 26.2%	BoYb	Id.: 13.3% Sim.: 23.3%	ping-pong	NA	BLAST
		Smed-vasa-1	SMESG000067941.1	DDX4	Id.: 27.4% Sim.: 36.7%	Vasa	Id.: 23.5% Sim.: 32%	Initiation of ping-pong	Lethal	Rouhana et al., 2010; Wagner et al., 2012

Table 1 (continued)

	S. med proteins	Planmine	Mouse		Drosophila		Function in piRNA pathway	Phenotype	Reference
Chromatoid bodies	Smed-PRMT5	SMESG000023057.1	PRMT5	Id.: 39.1% Sim.: 54.8%	Capsuleen	Id.: 30.4% Sim.: 47.9%	Sym. Dimethylation of CB components	Regeneration defects	Rouhana et al., 2012
	Smedtud-1 (88.3% identity to Spoltud-1)	SMESG000029232.1/ 000029236.1	TDRD6	Id.: 9.4% Sim.: 15.8%	Tudor	Id.: 6.1% Sim.: 11.6%	Scaffolding protein for piRNA players	Lethal	Solana et al., 2012, 2013
	Smed-CBC-1 (94.5% identity to DjCBC-1)	SMESG000073910.1	DDX6	Id.: 53.1% Sim.: 64.7%	Me31b	Id.: 56.5% Sim.: 71.0%	RNA transport and regulation in CB	No robust phenotype	Yoshida-Kashikawa et al., 2007
	Smed-SmB	SMESG000029426.1	SnrpB	Id.: 40.3% Sim.: 50.2%	SmB	Id.: 46.0% Sim.: 56.9%	CB organization and function	Lethal	Fernandéz-Taboada et al., 2010
	Smed-Vret	SMESG000026637.1	TDRD12	Id.: 12.2% Sim.: 22.7%	Vret	Id.: 6.4% Sim.: 12.3%	CB component	NA	BLAST
	Smed-TDRD9	SMESG000016118.1	TDRD9	Id.: 26.8% Sim.: 43.2%	Spindle-E	Id.: 25.8% Sim.: 41.1%	PIWI-interacting	NA	Önal et al., 2012

Putative planarian orthologs of murine and fly piRNA pathway members: PIWI proteins; proteins involved in primary or secondary piRNA biogenesis and other chromatoid body components. The protein name and the annotated genomic ID is given (version SMESG.1). The degree of homology of planarian piRNA pathway members to their murine and fly orthologs is indicated as percentage of amino acid identity (Id.) or similarity (Sim.). Alignments were performed using EMBOSS Needle (Madeira et al., 2019).
piRNA, PIWI-interacting RNA; TE, transposable element.

piRNAs predominantly originate from single-stranded precursors that are transcribed by RNA polymerase II from genomic loci called piRNA clusters (Kawaoka et al., 2013; Thomas et al., 2014). piRNA clusters are divided into three types, based on the mode of transcription and chromatin organization in their vicinity: unidirectional uni-strand clusters, bidirectional uni-strand clusters and dual-strand clusters (Yamanaka et al., 2014; Czech et al., 2018; Ozata et al., 2019). Uni-strand clusters are the most common type amongst animals and potentially also the most ancient ones (Czech et al., 2018; Ozata et al., 2019). Accordingly, more than 90% of all currently annotated piRNA clusters in *S. mediterranea* exhibit a strand bias and therefore belong to the class of uni-strand clusters (Friedländer et al., 2009; Kim et al., 2019a). Uni-strand clusters can harbor remnants of TE sequences and thus produce piRNAs responsible for transposon silencing. This is the case for murine pre-pachytene clusters and for the *flamenco* cluster in somatic follicle cells of *Drosophila*, which suppresses gypsy-like retrotransposons (Aravin et al., 2007; Brennecke et al., 2007; Lau et al., 2009; Gan et al., 2011; Goriaux et al., 2014). Concomitantly, murine pachytene piRNA clusters are relatively depleted of transposon-related sequences as compared to their abundance across the entire genome (Aravin et al., 2006; Li et al., 2013). Despite the highly repetitive nature of the planarian genome [61.7% repetitive sequences (Grohme et al., 2018)], about 70% of all planarian piRNAs originate from non-repetitive, non-TE-related sequences (Friedländer et al., 2009; Shibata et al., 2016; Kim et al., 2019a). Given the complexity of the planarian piRNA pool, this is intriguing and suggests that planarian piRNA clusters resemble murine pachytene clusters. Transcription of murine pachytene clusters is controlled by the transcription factor A-MYB (Li et al., 2013). Whether a similar regulation occurs in planarians is currently unknown. Murine pachytene clusters and planarian piRNA clusters can both be transcribed bidirectionally, thus producing non-overlapping transcripts from the same promoter region (Yamanaka et al., 2014; Kim et al., 2019a). In contrast, to date, dual-strand clusters were only found in arthropods (Klattenhoff et al., 2009; Rangan et al., 2011; Mohn et al., 2014; Andersen et al., 2017).

piRNA biogenesis in neoblasts

Following transcription of their precursors, piRNA biogenesis can be separated into a primary and a secondary

pathway. Primary, or phased, biogenesis entails processing of precursors that were transcribed from piRNA clusters. This mechanism ensures the diversity and complexity of the piRNA population, as encoded in the genome. Processing of piRNA precursors into mature piRNAs is initiated by the endonucleotic cleavage of the piRNA precursor by a member of the PIWI protein family (Han et al., 2015; Homolka et al., 2015; Mohn et al., 2015). The PIWI protein is directed to its cleavage target by a co-bound piRNA. It then cleaves the recognized piRNA precursor opposite nucleotides 10 and 11 of the piRNA guide, as has been established for all Argonaute-type proteins (Elbashir et al., 2001; Leuschner et al., 2006; Brennecke et al., 2007). The initial cleavage event removes the 7-methylguanosine cap that is typical for piRNA precursors in all animals and, in its place, generates a 5′-monophosphate at the newly defined precursor 5′-end (Gainetdinov et al., 2018; Ozata et al., 2019). Now, that newly defined 5′-end is bound by another PIWI family member and is processed in a phased manner with the help of the endonuclease Zucchini-dependent complex (mitoPLD/PLD6 in mice and planarians), which *in vivo* preferentially cleaves piRNA precursors before uridines (Han et al., 2015; Mohn et al., 2015). Planarian piRNAs, in particular those bound by SMEDWI-1 and -2, exhibit a 1U bias (Kim et al., 2019a) and a signature of phased piRNA production (Gainetdinov et al., 2018) (Figure 3). In planarians, phased processing generates relatively long pre-piRNAs (40–45 nt) (Gainetdinov et al., 2018), whose 3′-ends are very likely trimmed to mature piRNAs by exonucleases, in analogy to how 3′-ends are defined in mice by the exonuclease PNLDC1 (Izumi et al., 2016). As we found planarian homologs to all Drosophila and murine proteins involved in phased piRNA production, loading and maturation, e.g. Armitage, Gasz, Minotaur, Vreteno, Shutdown, Hsp83, PNLDC1 and Hen1, we expect primary biogenesis to be conserved and functionally equivalent in planarians (Table 1).

The secondary piRNA biogenesis pathway, also known as the ping-pong cycle, is responsible for silencing of piRNA-targeted transcripts (Brennecke et al., 2007; Gunawardane et al., 2007; Ozata et al., 2019). As the name suggests, such targeted transcripts additionally serve as templates for another, secondary population of piRNAs. In *Drosophila melanogaster*, Aub was shown to initiate the ping-pong cycle and to cleave a targeted transcript. The cleaved target RNA is then loaded onto another PIWI protein, Ago3. It is trimmed to its characteristic length by Nibbler or another unknown exonuclease that uses Papi as a co-factor and thereby generates a so-called responder piRNA (Hayashi et al., 2016). Complementarity of the first 10 nts of an initiator piRNA and the first 10 nts of a responder piRNA creates a so-called ‘ping-pong signature’ that can be used to identify ping-pong relationships between different PIWI proteins. Finally, the responder piRNA starts another ‘ping-pong’ cleavage event by guiding Ago3 to transcripts with antisense orientation to the cleaved TEs (Brennecke et al., 2007; Gunawardane et al., 2007; Ozata et al., 2019). In contrast to fruit flies, planarian ping-pong

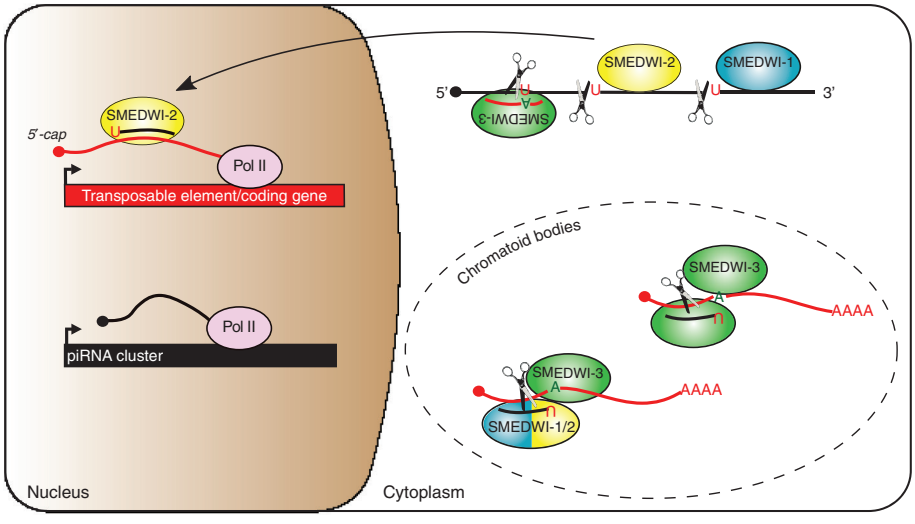

Figure 3: Working model of the piRNA pathway in planarian neoblasts.
In neoblasts, all three planarian PIWI proteins participate in the piRNA pathway. Transcribed piRNA precursors are exported to cytoplasmic processing sites. SMEDWI-2-bound mature piRNAs predominantly relocate to the nucleus and subsequently initiate heterochromatin establishment. Degradation of SMEDWI-1, -2 and -3-targeted transcripts in the ping-pong cycle takes place in chromatoid bodies. piRNA targets comprise transposable elements, yet also protein-coding mRNAs.

pairs can be formed by the use of either of the two PIWI proteins, SMEDWI-1 or -2, each of which can constitute a heterotypic cycle with SMEDWI-3 (Kim et al., 2019a). At the same time, SMEDWI-3 alone is able to initiate the iterative cleavage of targeted transcripts in a so-called homotypic ping-pong cycle (Figure 3). In addition to PIWI proteins, many other proteins make critical contributions to the ping-pong cycle. In particular, Vasa, Qin, Krimper, Shutdown and Tudor-domain-containing proteins (Table 1) are required to achieve coordinated target cleavage and loading of responder piRNAs onto their designated PIWI proteins (Lim and Kai, 2007; Siomi et al., 2010; Zhang et al., 2011; Preall et al., 2012; Xiol et al., 2014).

In all animals, the ping-pong cycle takes place in a specialized membrane-less cytoplasmic compartment called the nuage in Drosophila and the chromatoid body in the mouse germline (Brennecke et al., 2007; Lim and Kai, 2007). These spatially confined piRNA-processing sites increase the local concentration of proteins participating in the pathway and might even protect specific cellular transcripts from unwanted degradation (Ozata et al., 2019). Planarian neoblasts possess chromatoid bodies that are perfect candidates to host secondary piRNA production (Figure 3). Chromatoid bodies in planarians contain homologs of Tudor proteins (Solana et al., 2009), as well as SMEDWI-3 and its ortholog in *D. japonica* (DjPiwiC) (Kashima et al., 2016; Kim et al., 2019a). Some SMEDWI-3 targets, such as *histone* mRNAs and transcripts antisense to the *gypsy* retrotransposon, are accumulated in chromatoid bodies as well (Rouhana et al., 2014; Kashima et al., 2016; Kim et al., 2019a). Furthermore, the RNAi knockdown of SMEDWI-1 and SMEDWI-3 leads to elevated levels of *histone* mRNAs and their delocalization from chromatoid bodies (Rouhana et al., 2014). Taken together, the ping-pong cycle very likely operates in the chromatoid bodies of planarian neoblasts.

The somatic piRNA pathway in planarians

Apart from the primary and secondary piRNA pathway in planarian neoblasts, SMEDWI-2 and -3 are also expressed in neuronal cells of the planarian brain (Palakodeti et al., 2008; Shibata et al., 2016; Kim et al., 2019a). Their presence in the brain suggests that a somatic piRNA pathway exists in planarian neurons, operated by two SMEDWI proteins only (Figure 4A). It is also possible that neuronal cells possess secondary piRNA biogenesis pathways, as SMEDWI-3 is able to perform a homotypic ping-pong cycle, in addition to heterotypic cleavage in cooperation with SMEDWI-2. However, a more thorough experimental investigation of neuron-specific piRNAs in planarians is needed to explore the role of piRNAs in the planarian brain. This seems experimentally feasible, as planarian neuronal cells can be enriched by fluorescence-activated cell sorting (FACS) (Hayashi and Agata, 2018).

Intriguingly, planarians also possess an active piRNA pathway in epidermal cells, which operates using nuclear SMEDWI-2 only (Figure 4B) (Kim et al., 2019a). The use of a single nuclear PIWI protein is reminiscent of the piRNA pathway in ovarian follicular cells in *D. melanogaster* (Li et al., 2009; Malone et al., 2009). Somatic follicular cells surround the Drosophila germline and they only express Piwi, a nuclear PIWI protein. In this case, piRNA biogenesis is only carried out by primary, phased processing of single-stranded precursors in Yb-bodies, cytoplasmic sites of piRNA biogenesis (Qi et al., 2011). However, prior to their transport to Yb-bodies, piRNA precursors accumulate in nuclear structures, termed Dot COM (Dennis et al., 2013). In the planarian epidermis, nuclear foci reminiscent of Dot COM structures were reported (Kim et al., 2019a). Because the planarian epidermis consists of non-dividing terminally differentiated cells, the functional role of the epidermal piRNA pathway likely differs from that in neoblasts (discussed in the following section).

The functional role of the piRNA pathway in planarian flatworms

Silencing of transposable elements

Silencing of TEs is the prime and ancestral function of piRNAs (Ozata et al., 2019). Proper silencing is thereby ensured both at the post-transcriptional and at the epigenetic level by directing heterochromatin formation (Brennecke et al., 2007; Gunawardane et al., 2007; Sienski et al., 2012; Thomas et al., 2013; Batki et al., 2019; Fabry et al., 2019; Murano et al., 2019). In analogy, the piRNA pathway in planarian adult stem cells is responsible for silencing of TEs at both levels. Knockdown of either SMEDWI-2 or SMEDWI-3 leads to the upregulation of TEs and an increase in H3K4me3 levels at the TE loci genome-wide (Zhou et al., 2015; Kashima et al., 2016; Shibata et al., 2016; Kim et al., 2019a). Phenotypically, a SMEDWI-2/-3 knockdown in planarians resembles that of lethally irradiated animals, in which all neoblasts are depleted. Irradiated animals lose their ability to regenerate and to sustain tissue integrity,

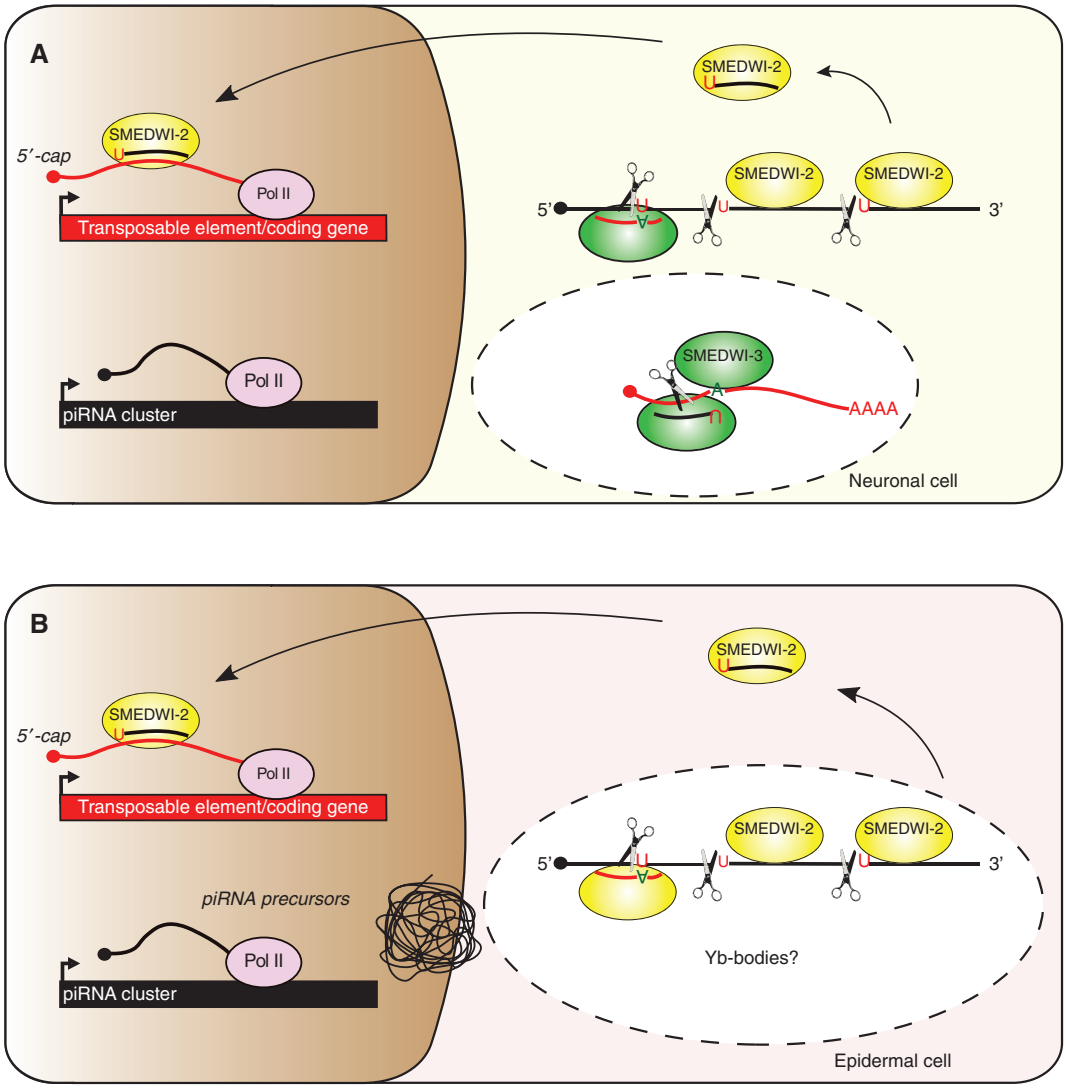

Figure 4: Working models of the piRNA pathway in planarian somatic, non-cycling cells.
(A) The piRNA pathway in neuronal cells is operated by SMEDWI-2 and SMEDWI-3. (B) The piRNA pathway in epidermal cells employs only SMEDWI-2. In epidermal cells, piRNA precursors accumulate in the nucleus close to the nuclear envelope. Phased primary piRNA production may take place in structures similar to Yb bodies found in ovarian somatic cells of *D. melanogaster*.

eventually leading to the death of the animal (Reddien et al., 2005a; Palakodeti et al., 2008). In contrast to irradiated planarians, neoblasts of PIWI knockdown animals are able to proliferate and migrate to wound sites. However, at this stage, their differentiation into lineage-specific progeny is impaired (Reddien et al., 2005a; Wagner et al., 2012; Shibata et al., 2016). Interestingly, in *S. mediterranea*, tissue loss and animal death upon nuclear PIWI knockdown (SMEDWI-2) take place 12 days post feeding (Reddien et al., 2005a; Shibata et al., 2016; Kim et al., 2019a), while irradiation-mediated lysis of animals caused by the neoblast loss occurs only ~30 days after irradiation (Reddien et al., 2005b; Ivankovic et al., 2019). It is therefore possible that the detrimental SMEDWI-2 RNAi phenotype is not confined to neoblasts only. In agreement, the upregulation of TEs in *D. japonica* upon nuclear PIWI knockdown was particularly apparent in *de novo* differentiated somatic cells, but not in neoblasts (Shibata et al., 2016). The elevated expression of TEs caused DNA damage and cell death of the early differentiated progeny. Moreover, in some degenerating cells, the retrotransposon *gypsy* was mobilized in virus-like particles (Shibata et al., 2016). It might hence be possible that these viral particles infect neoblasts and their differentiated progeny, facilitating their dysfunction and death. An analogous situation was found in the Drosophila germline. There, viral *gypsy* particles that originate from follicle and nurse cells invade the oocyte and compromise its genome (Song et al., 1997; Wang et al., 2018).

Epigenetic silencing of TEs by the piRNA machinery could play a role in neoblast differentiation and lineage establishment by co-silencing of developmentally important genes. In *D. melanogaster*, most TEs, whose transcription is regulated by piRNAs, are located in euchromatic regions (Sienski et al., 2012; Iwasaki et al., 2016). Therefore, silencing of transposons inserted near the transcription start site or in an intron of a gene would certainly impact the expression of the affected gene. In *D. melanogaster*, a knockdown of nuclear Piwi or Maelstrom, both of which are required for epigenetic silencing of TEs, results in transcriptional bleeding and upregulation of the expression of coding genes ~15 kb downstream of the TE insertion site (Sienski et al., 2012). This fact underscores the possible distal influence of heterochromatin on gene expression. In planarian flatworms, a number of neoblast-specific genes in *D. japonica*, i.e. *Djmcm2* and *Djhistone h4*, were suggested to be regulated at the epigenetic level by nuclear PIWI and piRNAs (Kashima et al., 2018). Due to the fact that the planarian genome is highly repetitive (Grohme et al., 2018) and over 1000 planarian genes carry either LTR or DNA transposon inserts in their introns (Planmine and RepeatMasker annotations) (Smit et al., 2013; Rozanski et al., 2018), piRNA-mediated heterochromatin establishment across transposons might indeed modulate the expression of planarian coding genes.

piRNAs in post-transcriptional gene regulation

In addition to their function in transposon control, piRNAs also regulate the expression of coding genes (Aravin et al., 2001; Rouget et al., 2010; Gou et al., 2014; Barckmann et al., 2015; Rojas-Ríos and Simonelig, 2018). The piRNA-driven degradation of TEs and of protein-coding mRNAs can thereby be viewed as a reaction of piRNAs to 'non-self' sequences. In the germline of *Caenorhabditis elegans*, piRNAs enable the selective silencing of certain transcripts, while the expression of others is favored (Ashe et al., 2012; Lee et al., 2012; Shirayama et al., 2012; Seth et al., 2013; Wedeles et al., 2013). Much like piRNAs of *C. elegans* and murine pachytene piRNAs, the majority of planarian piRNAs originate from regions that do not resemble TEs (Shibata et al., 2016; Kim et al., 2019a), supporting a regulatory role toward non-TE targets. Indeed, planarian piRNAs and PIWI proteins regulate the accumulation of *histone mRNAs*, *traf* genes, *ank1* and *dapk1*, and others via post-transcriptional piRNA-mediated degradation (Rouhana et al., 2014; Kim et al., 2019a) (Figure 5). To that end, SMEDWI-3 utilizes a homotypic ping-pong cycle to degrade large numbers of protein-coding transcripts, such as *ankyrin*- and *zinc finger*-containing mRNAs, numerous *traf*- and *histone*-encoding transcripts, as well as *actin-like* and *tubulin-like* genes (Kim et al., 2019a). The piRNA-driven modulation of *histone* transcript abundance might be important for neoblast cell cycle control. In human embryonic stem cells, elevated *histone* expression levels induce a G1/S cell cycle transition to ensure stem cell self-renewal (Medina et al., 2012). A similar mechanism might take place in planarian neoblasts, adult pluripotent cells. Interestingly, many of the targeted genes are present in multiple copies in the planarian genome according to a search of the Planmine database (Rozanski et al., 2018). However, to date, it is unclear whether the majority of transcripts degraded by SMEDWI-3 encode functional proteins or represent pseudogenes. Some of the annotated targeted *trafs* most likely are pseudogenes, as they lack intron sequences or encode only a fragment of the parent coding sequence (Kim et al., 2019a). Moreover, the TRAF protein family is expanded in *S. mediterranea*, but not in other Platyhelminthes (Swapna et al., 2018). We therefore speculate that piRNAs in *S. mediterranea* might serve a function reminiscent of dosage compensation by degrading transcripts of the expanded TRAF protein family (Hughes et al., 2007). Furthermore, it is an unsolved question, whether *traf* genes are targeted by piRNAs in other Platyhelminthes species as well that did not undergo the expansion of the TRAF gene family found in *S. mediterranea*.

Apart from SMEDWI-3-mediated degradation of numerous mRNAs in a homotypic ping-pong cycle, SMEDWI-3 binds another class of transcripts without their direct endonucleolytic cleavage and processing into piRNAs (Figure 5). The targeted transcripts are recognized via imperfect piRNA-mRNA base-pairing (Kim et al., 2019a). Studies that reported such mode of target recognition in other organisms revealed two functionally opposed outcomes: targeted mRNAs were either selectively translated or degraded via the CCR4-NOT pathway (Rojas-Ríos and Simonelig, 2018; Dai et al., 2019; Ramat et al., 2020). However, whether cleavage-independent binding of SMEDWI-3 to coding transcripts represents some sort of mRNA surveillance and licensing mechanism remains to be investigated.

piRNAs in germline specification

Of all three planarian PIWI proteins, only the knockdown of SMEDWI-1, a stem cell marker (Zeng et al.,

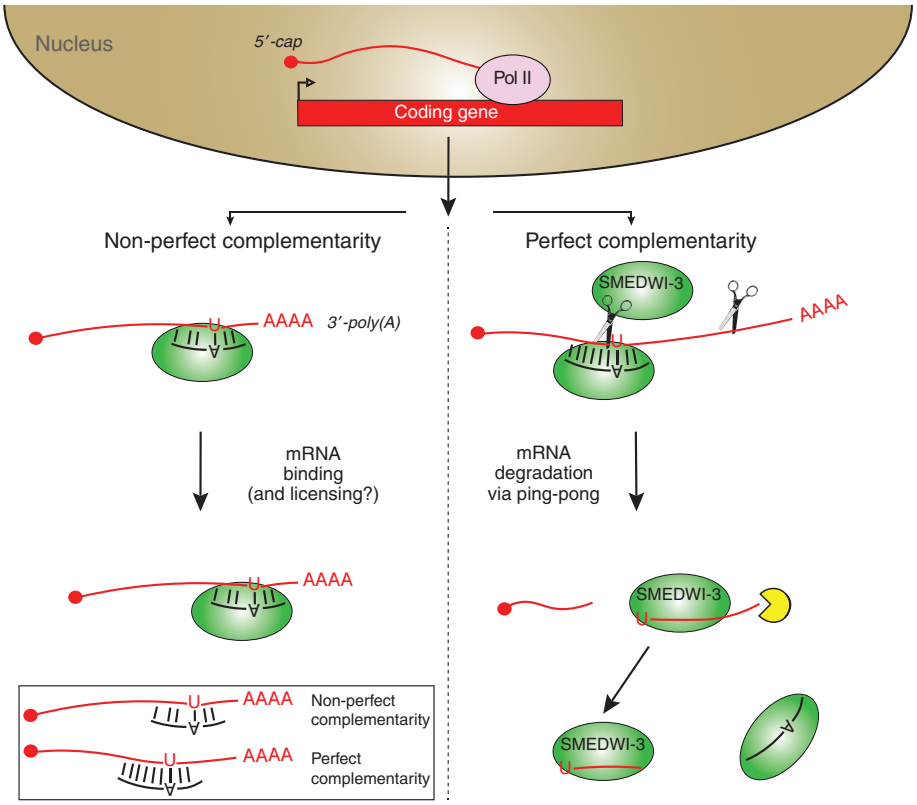

Figure 5: Working model of SMEDWI-3 as gatekeeper for mRNA fate in neoblasts, planarian stem cells.
While SMEDWI-3 degrades a set of mRNAs in a homotypical ping-pong cycle, it binds another set of mRNAs without processing them into piRNAs. Whether an mRNA is degraded or not is determined by the base-paring pattern between antisense piRNAs bound by SMEDWI-3 and target mRNAs.

2018), does not cause any apparent phenotypical defects under normal conditions in the asexual *S. mediterranea* (Reddien et al., 2005a). In contrast, RNAi knockdown of *Drpiwi-1*, a SMEDWI-1 ortholog in the sexualized planarian *Dugesia ryukyuensis*, severely affected germline formation without compromising the regenerative abilities of the animal (Nakagawa et al., 2012). *Drpiwi-1* therefore seems crucial for the smooth transition and differentiation of neoblasts into germline cells without affecting regeneration. As the asexual strain of *S. mediterranea* is derived by a chromosomal translocation event that abolishes the ability of the animals to form gonads (Newmark and Alvarado, 2002), the expression of *smedwi-1* in neoblasts of asexual strains could be a relic of their sexual descent. Nevertheless, the asexual strain of *S. mediterranea* contains a neoblast sub-population that expresses *nanos*, a zinc-finger-encoding transcript that is responsible for germ cell specification and differentiation (Wang et al., 2007). Taken together, it would therefore be of particular interest to analyze the *nanos* expression pattern in neoblasts of the asexual strain of *S. mediterranea* upon *smedwi-1* RNAi knockdown.

piRNAs in the planarian brain

Planarians possess a primitive brain composed of glia and multiple neuronal cell types (Pellettieri, 2019). However, the planarian brain is highly dynamic and undergoes constant cell turnover at a level of 25% of all neuronal cells per week. Upon injury or amputation, the planarian brain can regenerate and functionally reintegrate new tissue in only 7 days (Brown and Pearson, 2017). The presence of SMEDWI-2 and SMEDWI-3 in planarian cephalic ganglia (Palakodeti et al., 2008; Shibata et al., 2016; Kim et al., 2019a) thus raises the fascinating possibility that the piRNA pathway plays a role in planarian brain metabolism and brain regeneration. This speculation is warranted, as piRNAs were shown to play a role in long-term memory formation in *Aplysia*, to be involved in axon regeneration in *C. elegans* and to regulate the expression of LINE elements that are highly expressed in brain tissues (Rajasethupathy et al., 2012; Perrat et al., 2013; Nandi et al., 2016; Kim et al., 2018). In addition, piRNAs and PIWI proteins seem to play a crucial role in the control of tauopathy-mediated neurodegenerative disorders like Alzheimer's disease.

Not only were piRNAs considerably downregulated in Alzheimer's disease affected brains (Qiu et al., 2017; Roy et al., 2017), but the overexpression of neuronal nuclear Piwi significantly reduced tau-induced neurotoxicity and neuronal death in tau-transgenic *Drosophila* (Sun et al., 2018). Because of the unique neuro-regenerative capabilities of planarians, the availability of behavioral tests and similarity to mammalian brain neurochemistry (Hagstrom et al., 2016; Brown and Pearson, 2017), planarians will likely become an exciting model to investigate the role of piRNAs in brain metabolism and plasticity in the future.

piRNAs in the planarian epidermis

The epidermal piRNA pathway in planarians is mechanistically reminiscent of the piRNA pathway in somatic follicular cells in *D. melanogaster*. Both somatic pathways operate by the use of only a nuclear PIWI protein, accumulate piRNA precursors in the nuclear Dot COM structures and their genomes do not contribute to the next generation (see previous text). In *D. melanogaster*, the somatic piRNA pathway restricts the expression of certain tissue-specific TEs (Duc et al., 2019). The retrotransposons *gypsy*, *idefix* and *ZAM*, all of which are expressed in the soma, are able to invade and infect the neighboring germ cells (Chalvet et al., 1999; Li et al., 2009; Malone et al., 2009). However, the Drosophila germline does not produce piRNAs that are able to repress *ZAM*. Therefore, the somatic piRNA pathway indirectly protects the integrity of the germline genome in a tissue-specific manner by utilizing piRNAs generated from *flam* piRNA precursors (Duc et al., 2019). Interestingly, all three planarian genes, *traf-6*, *ank-1* and *dapk-1* found in Dot COM foci (Kim et al., 2019a), harbor domains that are also part of a planarian-specific giant retrotransposon termed Burro (big unknown repeat rivalling *ogre*) (Grohme et al., 2018; Arkhipova and Yushenova, 2019). Burro elements span genomic regions of more than 30 kb, compared to only 5–10 kb for LTRs in vertebrates (Grohme et al., 2018). In addition to traditional LTR domains, which include a viral particle coat (GAG), a protease (Pr), a reverse transcriptase (RT), a ribonuclease H (RH) and an integrase (IN) (Kazazian, 2004), Burro elements also contain AIR1 (arginine methyltransferase-interacting protein with RING Zn-finger), Smc (structural maintenance of chromosomes), MATH_TRAF_C (meprin and TRAF-C homology), BIR (baculoviral inhibition of apoptosis protein repeat domain), BCL2-like (apoptosis regulator protein) and ankyrin repeats (Arkhipova and Yushenova, 2019). Although the family of Burro retrotransposons is expressed in both planarian neoblasts and differentiated cells (Kim et al., 2019b), it remains to be investigated whether selected Burro elements are expressed in a tissue-specific manner.

Another possibility for a functional role of piRNAs in the planarian epidermis is their involvement in the innate immunity of the animals. One example of such connection is the antiviral response in somatic tissues of mosquitos that involves piRNAs and prevents viral replication (Miesen et al., 2015; Varjak et al., 2018; Tassetto et al., 2019). Moreover, a study examining retroviruses recently invading the koala genome revealed that unspliced transcripts of retroviral elements are degraded by a piRNA-mediated mechanism (Yu et al., 2019). Finally, planarians are known to respond to bacterial infections by the secretion of anti-microbial peptides, by the induction of apoptosis at the site of infection, and by the activation of phagocytic cells (Altincicek and Vilcinskas, 2008; Abnave et al., 2014; Peiris et al., 2014; Arnold et al., 2016). It is therefore reasonable to hypothesize that the planarian epidermis, the most exposed cell layer of the animal to its surroundings, uses a piRNA-mediated immune response to fight potentially genome-disrupting invaders.

Conclusion

Planarian regeneration and tissue homeostasis depend on a functional piRNA pathway. The knockdown of either planarian SMEDWI-2 or -3 leads to the impairment of neoblast differentiation, in line with the findings in fruit flies and mice where mutations in piRNA pathway are detrimental for germline development. Over the past decade, it became clear that the role of the piRNA pathway extends beyond silencing of TEs in the germline. It also regulates the stability and expression of coding genes, maintains transcriptome profiles by mRNA surveillance, and ensures antiviral defense. Such flexibility of piRNA function is secured by the highly adaptive and rapidly evolving nature of piRNA clusters. As both the germline and adult pluripotent stem cells of planarians are responsible for the transfer of genomic information, they use the piRNA pathway as the first line of defense in the race against mobile TEs, other repeat expansions and retroviral invasion. Moreover, the complexity of the piRNA pool, especially in planarians, allows these unique small RNAs to efficiently target and eliminate many copies and multiple types of RNA sequences, thus rapidly changing the transcriptome of a cell. In conclusion, the piRNA pathway is an invaluable part of planarian neoblast biology, as changes in a cell's transcriptional profile determine its differentiation capabilities. Thus, a

further elucidation of the piRNA pathway in planarians will provide broad insight into the nature of stem cell pluripotency and animal regeneration.

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