piRNA- and siRNA-mediated transcriptional repression in Drosophila, mice, and yeast: new insights and biodiversity

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Abstract

The PIWI-interacting RNA (piRNA) pathway acts as a self-defense mechanism against transposons to maintain germline genome integrity. Failures in the piRNA pathway cause DNA damage in the germline genome, disturbing inheritance of “correct” genetic information by the next generations and leading to infertility. piRNAs execute transposon repression in two ways: degrading their RNA transcripts and compacting the genomic loci via heterochromatinization. The former event is mechanistically similar to siRNA-mediated RNA cleavage that occurs in the cytoplasm and has been investigated in many species including nematodes, fruit flies, and mammals. The latter event seems to be mechanistically parallel to siRNA-centered kinetochore assembly and subsequent chromosome segregation, which has so far been studied particularly in fission yeast. Despite the interspecies conservations, the overall schemes of the nuclear events show clear biodiversity across species. In this review, we summarize the recent progress regarding piRNA-mediated transcriptional silencing in Drosophila and discuss the biodiversity by comparing it with the equivalent piRNA-mediated system in mice and the siRNA-mediated system in fission yeast.

Keywords chromatin segregation; piRNA; siRNA; transcriptional repression

Introduction

PIWI-interacting RNAs (piRNAs) are small noncoding RNAs that are particularly abundant in animal reproductive tissues, where piRNAs protect the germline genome by repressing transposons (Iwasaki et al., 2018; Ozata et al., 2019). Dysfunction of piRNAs results in derepression of transposons, allowing them to move freely across the genome, leading to DNA damage, impaired gonadal development, and infertility (Klattenhoff et al., 2007).

Earlier studies in Drosophila uncovered an association between repetitive DNA sequences and repression of protein-coding genes (Livak, 1984; Palumbo et al., 1994). Later, small RNAs of 25–27 nucleotides (nt) in length derived from the Suppressor of Stellate [Su(Ste)] repeats located on the Y chromosome were found to be involved in repressing the Stellate (Ste) gene located on the X chromosome (Aravin et al., 2001; Stapleton et al., 2001). Deletion of Su(Ste) repeats derepressed the Ste gene. Consequently, Ste protein accumulated and crystallized in sperm cells, causing male infertility.

In 2003, comprehensive sequencing of small RNAs in Drosophila testes and embryos identified many repeat-associated small interfering RNAs (rasiRNAs), including those arising from Su(Ste) repeats and transposons (Aravin et al., 2003). Subsequent investigations using a number of organisms, including Drosophila, mice, and humans, revealed that rasiRNAs are present in their gonads and bind specifically to PIWI members, a subclade of the Argonauta family, but not to ubiquitous AGO (Girard et al., 2006; Grivna et al., 2006; Lau et al., 2006; Saito et al., 2006; Watanabe et al., 2006; Houwing et al., 2007; Nishida et al., 2007; Lau et al., 2009). On the basis of this cross-species observation, PIWI-bound rasiRNAs were collectively termed piRNAs.

PIWI proteins are defined by their greater similarity at the peptide sequence level to other PIWI subclade members than to AGO subclade members (Kim et al., 2009; Iwasaki et al., 2015). This simple definition of PIWI proteins (and their partner piRNAs) is confusing sometimes. For example, plants have no piRNAs. However, this interpretation is based on the fact that plants have no proteins defined as PIWIs. Some AGO members are present in the germline, bind repeat-derived small RNAs, and are required for fertility (Borges & Martienssen, 2015; Araki et al., 2020). In rare animals, such as mosquitoes, planarians, and Aplysia, some PIWI members are present outside of the germlines and are loaded with small RNAs that can map to transposons (Rajasethupathy et al., 2012; Shibata et al., 2016; Halbach et al., 2020; Kim et al., 2020). Tetrahymena expresses PIWI proteins, which bind to small RNAs that are produced in a Dicer-dependent manner (Mochizuki et al., 2002; Mochizuki, 2005). Dicer is otherwise known as a microRNA (miRNA) biogenesis factor (Kim et al., 2009). As piRNA research across species progresses further, such interspecies differences will become even more apparent.

piRNAs repress target genes at the transcriptional or post-transcriptional level. At which level piRNA-mediated silencing
occurs is determined by the subcellular localization of PIWIs binding to piRNAs or the piRNA-induced silencing complex (piRISC). The post-transcriptional event is carried out by cytoplasmic piRISC with a mechanism similar to RNA interference (RNAi), i.e., by cleaving the target mRNAs. PIWI proteins involved in this event are Drosophila Aubergine (Aub) and mouse Mili (Piwi4). These PIWIs have a nuclear import signal (NLS) and inactive slicer activity (Saito et al, 2009; De Fazio et al, 2011; Sienski et al, 2012; Yamaguchi et al, 2020). Thus, they associate with various co-factors, such as histone modifiers, chromatin mark readers, and DNA methyltransferases, to direct epigenetic changes at target loci via heterochromatin formation to repress transcription.

This nuclear silencing provides an additional benefit to the organism because repetitive sequences can disrupt the integrity of the genome through recombination, and heterochromatinization of these loci not only prevents the expression of harmful transposons that impair fertility but also minimizes undesirable recombination events. However, some animals, such as fish and silkworms, only have cytoplasmic PIWIs, despite being recombination positive.

Accumulating evidence shows that the overall framework of nuclear PIWI-mediated transposon silencing is similar to that of chromosomal compaction in fission yeast, which is mediated by Ago1 of the AGO subclade, although the biological purpose and meaning of the two events are distinct. In this review, we summarize mechanistic insights into Piwi-piRISC-mediated transcriptional silencing in Drosophila and compare its features with those of the Mili2-piRISC-mediated mouse system and the Ago1/siRNA-mediated chromatin control in fission yeast.

Piwi-piRISC-mediated transcriptional silencing in Drosophila

This pathway has been well studied using cultured Drosophila ovary-derived somatic cells (OSSs/OSCs), which contain only mitotically active early follicle cells (Niki et al, 2006; Saito et al, 2009). Like follicular somatic cells in the ovary, OSCs express Piwi but not two other PIWI members, Aub and Ago3. Furthermore, Piwi binds piRNAs and represses transposons in OSCs as it does in the ovary. Thus, OSCs are suitable for studying the Piwi-piRNA pathway.

Cytoplasmic production and nuclear localization of Piwi-piRISC

Piwi-piRISC in OSCs is assembled sequentially via two cytoplasmic organelles, Yb bodies and mitochondria (Olivieri et al, 2010; Saito et al, 2010; Hirakata & Siomi, 2016; Yamashiro & Siomi, 2018). Yb bodies are non-membranous perinuclear granules formed through liquid–liquid phase separation (LLPS) (Hirakata et al, 2019). The piRNA precursors are mostly derived from the piRNA cluster flamenco (flam) on the X chromosome (Pelisson et al, 1994; Brenneck et al, 2007). The piRNA precursors contain cis-elements, to which Yb binds specifically as a trans-acting factor, and then multi-merizes, inducing the LLPS-driven assembly of Yb bodies (Ishizu et al, 2015; Hirakata et al, 2019). Unbound Piwi and piRNA biogenesis factors, such as Armitage (Armi) and Vreteno, subsequently
localize to these bodies to initiate Piwi-piRISC production (Handler et al., 2011; Ishizu et al., 2019; Yamashiro et al., 2020).

In the Yb bodies, the piRNA precursors are converted into shorter intermediates by an unknown enzyme(s). This step generates multiple 5'-ends to which Piwi binds, becoming the Piwi-piRISC precursor (pre-piRISC) (Murata et al., 2014). This complex then heads to the mitochondrial surface along with the RNA helicase Armi, where the endonuclease Zucchini (Zuc) processes the Piwi-bound intermediates to mature piRNAs, thereby releasing Piwi-piRISC (Ipsaro et al., 2012; Nishimasu et al., 2012; Han et al., 2015; Mohn et al., 2015). The leftover RNA (i.e., the 3' trailer) is used to produce phased piRNAs (Han et al., 2015; Mohn et al., 2015), which are also bound to nascent Piwi and become Piwi-piRISCs. Two mitochondrial factors, Gasz and Daedalus, serve together as the scaffold for Zuc processing (Munafò et al., 2019; Yamashiro et al., 2020).

Yb is unique to Drosophila OSCs, which indicates that Piwi-piRISCs in ovarian germ cells (OGCs) are produced in an Yb-independent manner (Handler et al., 2013). Piwi-piRISCs in OGCs are produced from RNA fragments cleaved by Ago3-piRISCs (Wang et al., 2015). The piRNAs within Piwi-piRISCs in OGCs are also phased (Han et al., 2015; Mohn et al., 2015; Pandey et al., 2017; Ge et al., 2019). Zuc is involved in the process, but other required factors may not be identical to those in OSCs.

Piwi has a bipartite nuclear localization signal (NLS) at the N-terminal end. The signal is hidden prior to piRISC assembly to ensure unbound Piwi does not enter the nucleus. However, upon piRISC assembly, the Piwi-NLS is exposed to the cytosol and binds to Importinα, which actively transports the piRISC into the nucleus (Yashiro et al., 2018). This “molecular gate” that depends on small RNA binding raises the efficiency of nuclear transposon regulation, because only piRNA-loaded Piwi, not unloaded Piwi, is competent to target transposons.

A Piwi mutant lacking the NLS was fully loaded with piRNAs, but never entered the nucleus. Consequently, transposons were desilenced, proving that Piwi-piRISC must enter the nucleus to exert the silencing effect (Saito et al., 2010; Klenov et al., 2011; Sienski et al., 2012; Yashiro et al., 2018).

**Piwi-piRISC does not have slicer activity**

It was previously thought that slicer activity was not necessary for Piwi, because mutations in the predicted slicer active site did not affect transposon repression (Saito et al., 2010; Sienski et al., 2012; Darricarrère et al., 2013). In fact, Piwi-piRISC in OSCs shows no slicer activity (Yamaguchi et al., 2020), because the canonical Asp–Glu–Asp–His (D–E–D–H) tetrad necessary for exerting slicer activity is substituted by Asp–Val–Asp–Lys in endogenous, wild type Piwi (Sheu-Guttadurra & MacRae, 2017). Changing the peptide back to the D–E–D–H tetrad restored slicer activity (Yamaguchi et al., 2020).

Artificial tethering of Piwi to nascent reporter mRNAs via, for instance, the λN-box8 system did not repress the reporter genes (Sienski et al., 2015; Yu et al., 2015). This strongly supports the notion that Piwi must bind mRNA targets by itself via piRNAs, but not by other means such as the λN-boxB. Furthermore, Piwi must be continuously sustained on the target RNAs to maintain the repressive effect (Sarot et al., 2004; Sienski et al., 2012). The slicer activity given to Piwi by amino acid changes, which were introduced to wild type, naturally non-catalytic Piwi, hardly disturbed its RNA targeting effects, but Piwi was easily displaced from target RNAs upon cleavage (Yamaguchi et al., 2020). The displacement was more obvious when target RNAs were less complementary to Piwi-bound piRNAs. Such mismatches/gaps in base-pairing between Piwi-bound piRNAs and transposon mRNAs are often found within the ovariess. Thus, Piwi lacking the slicer activity might be more effective in transposon repression in the tissue, due to sustained target RNA binding, which is perhaps the reason for waiving its slicer activity in evolution. Without the slicer activity, though, Piwi requires co-factors to accomplish gene silencing. In fact, a number of Piwi co-factors have been identified.

**Piwi-piRISC attenuates target transcription via Maelstrom**

Efficient gene silencing may require transcription attenuation right upon Piwi binding to the target RNA. If RNA synthesis continued at the regular speed, the transcripts would leave the genome before Piwi could trigger the silencing mode of action. A recent study has shown that Maelstrom (Mael) contributes to this process (Onishi et al., 2020) (Fig 1).

Mael was originally identified as a factor regulating anterior–posterior axis formation in early oocytes (Clegg et al., 1997; Sato et al., 2011). More recent studies revealed that Mael is indispensable for transposon silencing and that this Mael function is conserved in mice (Lim & Kai, 2007; Soper et al., 2008; Sienski et al., 2012; Castañeda et al., 2014; Chang et al., 2019). Mael’s points of action in transposon silencing in Drosophila OSCs and OGCs are different: Mael in OSCs is not required for piRNA biogenesis, but it controls the chromatin accessibility of RNA Pol II, with little effect on the abundance of Histone 3 lysine 9 trimethylation (H3K9me3) around the transposon loci, resulting in transcriptional regulation of transposons (Sienski et al., 2012). Mael in OGCs controls piRNA biogenesis by attenuating canonical transcription of transposons in the piRNA clusters (Chang et al., 2019).

Mael in OSCs resides in the nuclear Piwi complex, and this complex further binds to Brahma (Brm), the core unit of the chromatin remodeler SWItch/Sucrose Non-Fermentable (SWI/SNF) complex (Onishi et al., 2020) (Fig 1). SWI/SNF relaxes chromatin structures around promoter regions, allowing RNA Pol II to initiate transcription (Wilson & Roberts, 2011). Piwi-targeted transposons in cultured OSCs are mostly controlled by SWI/SNF, and Piwi in collaboration with Mael reduces the level of SWI/SNF and RNA Pol II around long terminal repeats (LTRs) of target transposons, resulting in transcriptional attenuation (Onishi et al., 2020) (Fig 1). Piwi tethering by artificial piRNAs also repressed Brm-independent genes, but at a slower speed than Brm-dependent genes. Thus, it is likely that Piwi represses gene targets in a Mael-dependent and Mael-independent manner in slightly different ways: If transcription of the target genes is initiated under the control of SWI/SNF, Piwi first attenuates the transcription with help from Mael. If transcription is initiated in a SWI/SNF-independent manner, Piwi attenuates RNA synthesis by other unknown means. In both cases, heterochromatinization eventually takes place at the target loci. A similar Mael-mediated artificial tethering was conducted in germ cells within the ovariess, but it failed to repress the reporter (Sienski et al., 2015). The dependency of Piwi-mediated transcriptional repression on Mael may not be identical in germ and somatic cells.

**The PICTS/PPNP/SSFNX/Pandas complex anchors Piwi-piRISC to target RNAs**

RNAi-based gene screening conducted within the ovaries identified numerous piRNA factors (Czech et al., 2013; Handler et al., 2013;
Muerdter et al., 2013). Of those, some factors were found necessary in both germ (nurse) cells and somatic (follicle) cells, which were presumed to co-function with Piwi, because Piwi is present in both cell types, whereas Aub and Ago3 are germ cell-specific (Handler et al., 2013). Besides Mael, Panoramix/Silencio (Panx) and nuclear RNA export factor 2 (Nxf2) were within this category, namely, necessary in both germ and somatic cells (Czech et al., 2013; Handler et al., 2013; Muerdter et al., 2013).

Artificial tethering of Panx to reporter RNAs aberrantly accumulated H3K9me3 at the gene locus and repressed expression even in the absence of Piwi, which indicates that Panx has an intrinsic ability to induce gene silencing independent of Piwi (Sienski et al., 2015; Yu et al., 2015). Unlike Nxf1, a Nxf2 homolog that functions in mRNA export, Nxf2 does not localize at the nuclear periphery. Structural data showed that the two putative nucleoporin-binding pockets in Nxf2 are restricted, supporting the idea that Nxf2 may have lost the ability to bind nucleoporins and therefore any role in mRNA export (Batki et al., 2019; Zhao et al., 2019).

Nxf1/p15 was originally reported as an Nxf1 co-factor (Suyama et al., 2000). Panx, Nxf2, and Nxt1 assemble a ternary complex, which was termed PICTS, PPNP, SFINX, and Pandas in independent studies (Batki et al., 2019; Fabry et al., 2019; Murano et al., 2019; Zhao et al., 2019). Strictly speaking, the components of the protein complexes may be slightly different from each other, but in this review we call the complex PICTS/PPNP/SFINX/Pandas. The three members of the complex stabilize each other through mutual protein–protein interactions (Batki et al., 2019; Fabry et al., 2019; Murano et al., 2019; Zhao et al., 2019).

Nxf2 binds RNAs through the LRR domain. Nxf2 lacking the LRR domain successfully induced silencing in tethering assays under normal conditions, but failed in the absence of endogenous Nxf2 (Batki et al., 2019; Murano et al., 2019). Thus, it was inferred that the PICTS/PPNP/SFINX/Pandas complex anchors Piwi-piRISC to target RNAs through the LRR domain in Nxf2 (Fig 1). The complex was necessary prior to H3K9me3 accumulation at the Piwi target loci, supporting this notion (Murano et al., 2019).

Cutup/LC8 (Ctp) has recently been found to be the fourth component of the PICTS/PPNP/SFINX/Pandas complex (Eastwood et al., 2021; Schnabl et al., 2021). Ctp is ubiquitous and functions in numerous biological processes by dimerizing its interacting complexes to stabilize them (Jespersen & Barbar, 2020). In the Piwi-piRNA pathway, the PICTS/PPNP/SFINX/Pandas complex should be dimerized to support efficient transcriptional gene silencing and Ctp acts as the dimerization hub (Fig 1). Ctp, however, does not promote Nxf1 dimerization.

**Gtsf1/Arx interconnects Piwi-piRISC with the PICTS/PPNP/SFINX/Pandas complex**

Gametocyte-specific factor/Asterix (Gtsf1/Arx) is a nuclear protein that interacts with Piwi in OSCs (Dönertas et al., 2013; Ohtani et al., 2013;...
Piwi-piRISC is produced and localized to the nucleus in Gtsf1/Arx-deficient OSCs, but the Piwi-dependent transposons were desilenced and the H3K9me3 level at the transposon loci was reduced. Thus, Gtsf1/Arx is not necessary for piRNA biogenesis, but it is necessary for Piwi-mediated silencing. In the nuclear step, Gtsf1/Arx interconnects Piwi-piRISC with the PICTS/PPNP/SFINX/Pandas complex (Onishi et al., 2020) (Fig 1). Gtsf1/Arx has two CHHC-type zinc finger domains. Alteration of conserved residues in these domains desilenced transposons, although the precise function(s) of the domains remains unknown (Dönertas et al., 2013; Ohtani et al., 2013).

Factors co-functioning in Piwi-mediated heterochromatin formation

Drosophila has three H3K9-specific histone methyltransferases, Su(var)3-9, G9a, and Eggless/SetDB1 (Egg). Of these, only Egg functions in Piwi-mediated transcriptional silencing (Sienkiewicz et al., 2015) (Fig 1). Egg is ubiquitinated by Ub2 and this modification is necessary for its methylation activity (Osumi et al., 2019). Windei (Wde), an Egg co-factor, retains Egg on chromatin (Osumi et al., 2019). The H3K4 demethylase, Lsd1, and its co-factor, CoREST, have also been shown to play important roles in Piwi-mediated transcriptional silencing (Yu et al., 2015) (Fig 1).

Mi-2, MEP-1, and Rpd3 assemble in a complex and are recruited together to the Piwi-targeted transposon loci, where they deacetylate H3K9 prior to its methylation by Egg (Mugat et al., 2020) (Fig 1). Mi-2, an ATP-dependent chromatin remodeler, and Rpd3 reside within the Drosophila nucleosome remodeling and deacetylation (dNuRD) complex (Bowen et al., 2004). Mi-2 is also present in the Drosophila MEP-1-containing (dMec) complex, but Rpd3 is not (Kunert et al., 2009). Thus, the complex containing Mi-2, MEP-1, and Rpd3 may be unique to the Piwi-mediated pathway (Mugat et al., 2020).

Su(var)2–10 (Sv210), a SUMO E3 ligase, is necessary for the physical link between Panx and effectors such as Egg, Wde, Mi-2, and MEP-1 (Mugat et al., 2020; Ninova et al., 2020). Sv210 repressed reporter transcription in artificial tethering assays, which required both Wde-Egg and the SUMOylation activity of Sv210 (Ninova et al., 2020). Given that many of the effectors have SUMO-interacting motifs, Sv210 SUMOylates proximal proteins, including itself, which may supply the scaffolding for recruiting Egg, Wde, Mi-2, and MEP-1 (Mugat et al., 2020; Ninova et al., 2020).

Heterochromatin Protein 1a (HP1a) specifically binds to H3K9me3 to maintain the heterochromatin and is necessary for transposon repression (Kienow et al., 2011; Wang & Elgin, 2011; Le Thomas et al., 2014). Histone H1 directly binds to Piwi and controls chromatin accessibility at the Piwi target loci without changing the levels of H3K9me3 and HP1a (Iwasaki et al., 2016) (Fig 1).

Miwi2-piRISC-mediated transcriptional silencing in mice

Drosophila Piwi functions in both ovaries and testes. However, Miwi2 (Piwi4) in mice, the Piwi paralog, is testis-specific (Carmell et al., 2007). The other mouse PIWI members, Miwi (Piwi1) and Mili (Piwi2), are also testis-specific but are cytoplasmic and silence transposons by cleaving the RNA transcripts as Drosophila Aub and Ago3 do (Kuramochi-Miyagawa et al., 2001; Deng & Lin, 2002; Kuramochi-Miyagawa, 2004; Aravin et al., 2006; De Fazio et al., 2011). All mouse PIWI members are necessary for spermatogenesis and fertility, but their expression timing during spermatogenesis differs: Miwi is expressed from the pachytene stage to round spermatid stage (Kuramochi-Miyagawa et al., 2001; Deng & Lin, 2002), Miwi2 is specific to progenitors of spermatogonial stem cells (SSCs) (Carmell et al., 2007), and Mili is expressed in all these cells (Kuramochi-Miyagawa et al., 2001; Kuramochi-Miyagawa, 2004; Aravin et al., 2006). Miwi2- and Mili-bound piRISC-RNA are rich in transposon sequences (Zheng et al., 2010). Conversely, Miwi- and Mili-bound piRISC-RNAs are rich in protein-coding gene sequences (Watanabe et al., 2015; Dai et al., 2019). Promoter deletion of pi6, one of the piRISC-RNA loci on chromosome 6, resulted in reduced male fertility, increased expression of several genes required for sperm function, and reduced production of piRISC-RNAs from other loci (Wu et al., 2020). However, the complete function of piRISC-RNA is still unclear.

Miwi2-dependent de novo methylation in gonocytes

In the mammalian life cycle, major reprogramming occurs twice, first in the fertilized eggs and then in primordial germ cells (PGCs) (Reik, 2001; Sasaki & Matsui, 2008). During these periods, repressive epigenetic marks are erased to reset the epigenetic memory inherited from the parents (Reik, 2001; Sasaki & Matsui, 2008). In germ cells, after this reprogramming event, de novo DNA methylation occurs throughout the genome to ensure proper spermatogenesis (Popp et al., 2010; Seisenberger et al., 2012; Kobayashi et al., 2013; Molaro et al., 2014; Kubo et al., 2015). The male germ cells, in which de novo DNA methylation takes place, are called gonocytes, and they serve as progenitors of SSCs.

Gonocytes express Mili and Miwi2 (Kuramochi-Miyagawa, 2004; Aravin et al., 2006; Carmell et al., 2007). The Mili-piRISC cleaves target RNAs in the cytoplasm, from which Miwi2-bound piRNAs are produced (De Fazio et al., 2011). This resembles the situation in Drosophila, where Piwi- and Aub-bound piRNAs are produced from RNAs cleaved by Ago3-piRISC (Wang et al., 2015). The Miwi2-piRISC then enters the nucleus to repress transposon transcription via DNA methylation (Carmell et al., 2007; Kuramochi-Miyagawa et al., 2008). Evolutionarily young long interspersed nuclear element-1 (LINE1) and intracisternal A-particle (IAP) are the main targets of Miwi2 (Pezic et al., 2014; Kojima-Kita et al., 2016). Importantly, the region that gains DNA methylation is the target gene promoter (Pastor et al., 2014; Barau et al., 2016). This suggests that Miwi2 triggers de novo methylation specifically on the promoter region of certain types of transposons.

Mice express four Dnmt3 members, Dnmt3A, Dnmt3B, Dnmt3L, and Dnmt3C, and all these enzymes are required for spermatogenesis (Chedin et al., 2002; Bourc’his & Bestor, 2004; Kameda et al., 2004; Suetake et al., 2004; Barau et al., 2016; Veland et al., 2019; Gao et al., 2020). Of those, Dnmt3C is gonocyte-specific and rodent-specific. Its deletion causes DNA demethylation of transposon promoter regions specifically, as seen in mili mutant cells (Barau et al., 2016). Dnmt3C-mediated de novo DNA methylation is specific for the Miwi2 pathway (Barau et al., 2016) (Fig 2).

Factors co-functioning in Miwi2-dependent de novo DNA methylation

Miwi2 interacts with multiple co-factors in gonocytes (Fig 2). One representative is TDRD9 (Shoji et al., 2009; Wenda et al., 2017).
TDRD9 is a DExH-box helicase/ATPase containing a Tudor domain and is expressed in the testis of both embryos and adults. In gonocytes, TDRD9 is detected in cytoplasmic non-membranous granules and in the nucleus (Shoji et al., 2009; Wenda et al., 2017). 
tdrd9 point mutation mice show defects in DNA methylation of LINE1 elements, leading to developmental arrest at the pachytene stage and infertility (Shoji et al., 2009; Wenda et al., 2017). However, these mutant mice produced piRNAs normally. This highlights the importance of TDRD9 in Miwi2-mediated transcriptional silencing (Shoji et al., 2009). However, its functional contribution remains undetermined. The fly homolog of TDRD9 is Spindle-E, which is essential in piRNA biogenesis, although its functional contribution also remains unknown (Lim et al., 2009).

SPOCD1 and TEX15 are necessary for de novo DNA methylation of transposons (Scho¨pp et al., 2020; Yang et al., 2020; Zoch et al., 2020) (Fig 2). SPOCD1 encodes a protein with a TFIIS-M and a SPOC domain. Tex15 harbors DUF3715 and two TEX15 domains and is expressed exclusively in both adult and fetal testis. Tex15 deficiency leads to early meiotic arrest and complete infertility. Moreover, the DNA methylation level over young transposons decreases in the mutant, showing striking similarity with piRNA pathway mutants. Both SPOCD1 and TEX15 proteins are nuclear proteins whose expressions are restricted to testes, and interact with Miwi2 (Schöpp et al., 2020; Zoch et al., 2020). SPOCD1 recruits de novo DNA methyltransferases, Dmnt3A and Dmnt3L, to the sites where Miwi2 functions (Zoch et al., 2020). The SPOCD1 interacting factors include components of the NuRD and BAF complexes (Zoch et al., 2020). The BAF complex is one of the SWI/SNF complexes in mice (Wilson & Roberts, 2011).

Morc1 belongs to the Morc family of GHKL ATPases and its depletion causes male infertility (Watson et al., 1998; Inoue, 1999). Morc1 is abundant in blastocysts and gonocytes, but it is not present in differentiated cells (Hruz et al., 2008). In morc1 mutant mice, male germ cells undergo transposon hypomethylation and derepression (Pastor et al., 2014). However, the abundance of piRNAs is maintained (Pastor et al., 2014). The differentially methylated regions (DMRs) in the mutant mice were concentrated around the transposon promoters (Pastor et al., 2014). This phenocopies mili and dnmt3c, but not dnmt3l, mutant mice (Barau et al., 2016). Morc1 may play an important role in Miwi2-dependent transposon silencing in gonocytes (Fig 2).

There is an intimate interplay between de novo DNA methylation and specific sets of histone modifications. In the early developmental stage of spermatogenesis and oogenesis, methylation of H3K9 at the young transposon loci by SetDB1 prior to de novo DNA methylation is important for subsequent proper de novo DNA methylation.
Drosophila between role in the Miwi2 system, is rodent-specific (Barau noncanonical Dnmt2 (Kunert weakly during fly embryonic development, but it is mediated by canonical DNMTs (Kunert (Fig 3). This is basically attributable to the fact that mediated RNA silencing is the involvement of DNA methylation Silencing step noted that the methylation of H3K36, followed by the demethylation at H3K4, is a common molecular cascade that leads to de novo DNA methylation in both oocytes and gonocytes.

Comparison of Piwi- and Miwi2-mediated transcriptional silencing

piRISC biogenesis Piwi- and Miwi2-piRISC bind nascent transposon mRNAs through RNA–RNA base-paring while transcription is still undergoing. Piwi- and Miwi2-bound piRNAs are mostly “antisense” to transposon mRNAs; thus, the two PIWI proteins target the mRNAs. Piwi- and Miwi2-bound piRNAs are produced mainly from piRNA cluster transcripts cleaved by Ago3- and Mili-piRISC, respectively (De Fazio et al, 2011; Wang et al, 2015) (Fig 3). It should be noted that Drosophila OSCs do not express Ago3; hence, Piwi-piRISC in the cells is produced in an Ago3-piRISC-independent manner (Saito et al, 2009) (Fig 3). Other aspects of the production of the Piwi- and Miwi2-piRISC have been summarized elsewhere (Ozata et al, 2019).

Silencing step The major difference between Piwi- and Miwi2-mediated RNA silencing is the involvement of DNA methylation (Fig 3). This is basically attributable to the fact that Drosophila lacks canonical DNMTs (Kunert et al, 2003). DNA methylation may occur weakly during fly embryonic development, but it is mediated by noncanonical Dnmt2 (Kunert et al, 2003). Dnmt3C, which plays a role in the Miwi2 system, is rodent-specific (Barau et al, 2016).

Although this DNA methylation issue is a major difference between Drosophila and mice, the piRNA pathway in these two organisms eventually forms heterochromatin at the target locus (Fig 3). However, it has been noted that only a few factors are commonly used in the two systems. For example, Panx, which is important for the Piwi-mediated system, is an orphan gene that only exists in Drosophila (Sienski et al, 2015; Yu et al, 2015). These differences may be due to convergent evolution of the piRNA systems in different species to counteract transposons. Gtsf1/Arx is one of the factors that appear in both the mouse (as Gtsf1) and fly systems as an interacting factor of Miwi2 and Piwi, respectively (Donertas et al, 2013; Ohtani et al, 2013; Yoshimura et al, 2018). However, it shows distinct functions in these species. Mouse Gtsf1 is cytoplasmic and contributes to the production of Miwi2-bound piRNAs, while Drosophila Gtsf1/Arx localizes to the nucleus and cofunctions with Piwi-piRISC to repress transposons (Donertas et al, 2013; Ohtani et al, 2013; Yoshimura et al, 2018).

Histone modifiers, such as H3K4 demethylases and H3K9 methyltransferases, as well as the NuRD complex with the H3K9 deacetylation activity are likely to be used in both the mouse and Drosophila systems (Liu et al, 2014; Pezic et al, 2014; Sienski et al, 2015; Yu et al, 2015; Nagamori et al, 2018) (Figs 1 and 2). Furthermore, Piwi and Miwi2 interact with components of the BAF complex (Zoch et al, 2020) (Figs 1 and 2). Considering the Brm function in the Piwi-mediated system (shown above), Miwi2 may also regulate the Brm homolog to accomplish the task of transcriptional silencing.

Ago1/siRNA-mediated heterochromatin formation in Schizosaccharomyces pombe

In addition to the interspecies divergence of the molecular mechanism by which the nuclear Piwi-piRNA pathway represses transposons, there is another diversity in regard to how the small RNA-mediated silencing pathway in the nucleus affects the chromatin state. In fission yeast, Ago1/siRNA-mediated constitutive heterochromatin formation occurs at three genomic regions, the pericentromeric and sub-telomeric regions, and the mating-type locus, which we refer to as cen, tel, and mat, respectively. Each of the loci contains unique repetitive elements known as dg/dh, cenH, and cenH-like, respectively. The repetitive elements give rise to siRNAs
that nucleate chromatin compaction with help from multiple cofactors (Reinhart & Bartel, 2002; Volpe et al., 2002; Verdel et al., 2004). The chromatin compaction is spread to flanking areas toward both sides to fully cover the loci (Motamedi et al., 2004; Noma et al., 2004). This siRNA-dependent constitutive heterochromatin formation has a broad effect on chromosome homeostasis, such as suppression of recombination, faithful segregation of chromosomes during cell division, and switching of mating-type information (Egel, 1984; Klar & Bonaduce, 1991; Thon & Klar, 1993; Allshire et al., 1995; Provost et al., 2002; Hall et al., 2003; Jia et al., 2004; Tuzon et al., 2004).

**Key RNAi components involved in heterochromatin assembly in fission yeast**

The repetitive elements are located within the constitutive heterochromatin regions, but they must be transcribed to a minor extent to produce siRNAs (Kato et al., 2005). Upon transcription, the nascent, single-stranded (ss) RNAs are converted to double-stranded (ds) RNAs by Rdpl, the core resident of the RNA-directed RNA polymerase complex (RDRC) (Motamedi et al., 2004; Sugiyama et al., 2005) (Fig 4). The RDRC contains two other components, the putative helicase, Hrr1, and noncanonical poly(A) polymerase family member Cid12 (Motamedi et al., 2004). The dsRNAs are subsequently processed into 21-nt-long siRNA duplexes by Dcr1 (Fig 4), the fission yeast ortholog of human Dicer, and fly Dicer2 (Provost et al., 2002; Lee et al., 2004; Colmenares et al., 2007). The siRNA duplexes are loaded onto Ago1 in a stoichiometric manner, where the passenger strand of the siRNA duplex is then cleaved by the slicer activity of Ago1 and ejected into the nucleoplasm for degradation (Buker et al., 2007). The guide strand remains with Ago1, which assembles the RNA-induced initiation of transcriptional gene silencing (RTSi) complex with chromodomain protein Chp1 and Gly-Trp (GW)-repeat protein Tas3, which serves as a platform for gene silencing by RTSi (Verdel et al., 2004; Probst et al., 2011) (Fig 4).

A mutation introduced into Rpb2, a subunit of RNA Pol II, reduced the levels of siRNAs derived from the repetitive elements, causing misregulation of the siRNA-mediated heterochromatin formation. Nonetheless, the cell growth of this rpb2 mutant was comparable to that of the wild-type strain (Kato et al., 2005). This indicates that the transcription state of repetitive elements is somewhat distinct from the transcription state of other euchromatic genes. For example, the dg/dh repeat within the cen locus is transcribed in both directions (Volpe et al., 2002). Furthermore, some splicing mutants disrupted the siRNA production from centromeric regions, although canonical splicing remained active. This also supports the notion that some features of RNA metabolism trigger RNAi-dependent heterochromatin formation (Bayne et al., 2008).

**The RTSi complex deposits repressive histone marks in the silencing loci**

The RTSi complex targets nascent RNAs and recruits the Clr4 methyltransferase complex (CLRC) to the loci (Horn et al., 2005; Jia et al., 2005; Zhang et al., 2008) (Fig 4). Clr4 is an H3K9 methyltransferase that shows similarities, both structurally and functionally, to mammalian SuV39h and Drosophila Su(var)3–9 (Rea et al., 2000; Nakayama et al., 2001; Schotta et al., 2002). The CLRC methylates H3K9 to H3K9me3, to which Chp1 within the RTSi complex binds through the chromodomain, which stabilizes the RTSi association to the heterochromatin (Noma et al., 2004; Cam et al., 2005; Petrie et al., 2005; Schalch et al., 2009). In this vicinity, Stc1 interconnects the CLRC to the RTSi complex. The artificial tethering of Stc1 to arbitrary genomic regions recruits CLRC and induces heterochromatin formation in a RTSi-independent manner, even in euchromatin regions, highlighting the role of Stc1 in CLRC recruitment to chromatin (Bayne et al., 2010) (Fig 4). It has been noted that Stc1 is structurally similar to Gtsf1/Arx; thus, its function may also be similar to that of Gtsf1/Arx (Bayne et al., 2010; Onishi et al., 2020) (Fig 5).

**Chromosomal compaction spreading throughout the heterochromatin regions**

siRNAs are derived mainly from repetitive elements embedded within the constitutive heterochromatin regions (Cam et al., 2005; Bühler et al., 2008). However, chromosomal compaction is not restricted to these elements. Rather, it spreads beyond these elements throughout the heterochromatin regions (Cam et al., 2005). Indeed, reporter genes artificially inserted very close to the cen locus, outside of dg/dh, were repressed depending on RTSi, RDRC, CLRC, and Dcr1 (Volpe et al., 2002; Motamedi et al., 2004; Verdel et al., 2004; Jia et al., 2005). The key factor in this heterochromatin spreading is Swi6, an HP1 homolog in fission yeast (Hall et al., 2002; Noma et al., 2004). Swi6 binds H3K9me2 and H3K9me3 through its chromodomoid and self-oligomerizes to build a platform for recruiting other chromatin proteins, such as histone deacetylases and chromatin remodelers (Nakayama et al., 2001; Fischer et al., 2009; Canzio et al., 2011) (Fig 4). In addition to Swi6, Tas3, one of the RTSi complex components, is involved in cis-spreading of heterochromatin silencing (Li et al., 2009). Its C-terminal domain self-associates and forms a polymer structure in solution. In vivo, this domain is essential for RTSi spreading over centromeric heterochromatin regions (Li et al., 2009).

Sir2, Clr3, and Clr6 are three major histone deacetylases in fission yeast, which play essential roles in suppressing genes located within heterochromatin regions (Grewal & Klar, 1997; Bjerling et al., 2002; Shankaranarayana et al., 2003; Hansen et al., 2005) (Fig 4). Sir2 is an NAD+-dependent deacetylase and is required for the heterochromatin gene silencing at cen, tel, and mat. In sir2 mutant cells, the acetylation level of H3K9 increases in these heterochromatin regions. In contrast, the methylation level of H3K9 as well as the abundance of Swi6 decreases. Clr3 assembles the Snf2/Hdac-containing repressor complex (SHREC) with the SNF2 remodeling factor Mit1 (Sugiyama et al., 2007). Clr3 maintains nucleosome occupancy at specific regions within the heterochromatin (Garcia et al., 2010; Yamane et al., 2011). Additionally, in clr3 mutant cells, the nucleosome turnover rate increases all over the heterochromatin regions (Aygün et al., 2013). These nucleosome states are in favor of transcription, supporting direct involvement of Clr3 in transcriptional gene silencing over these regions. Histone chaperone Asf1/HIRA also plays a role in maintaining nucleosome occupancy over heterochromatin regions together with Clr6 (Yamane et al., 2011). The clr3 and asf1 double mutation causes a severer reduction in nucleosome occupancy within the heterochromatin regions, suggesting a redundant role of Clr3 and Clr6 in ensuring the chromatin state that suppresses transcription. Swi6 is involved in recruiting Clr3, Clr6, and Asf1 to heterochromatin regions (Motamedi et al., 2008). Overall, the Swi6-centered protein network ensures the repressed chromatin state within the heterochromatin regions.
Accessory proteins that modulate heterochromatin formation

Recent studies have revealed the link between RNA homeostasis and siRNA-dependent constitutive heterochromatin formation. One example involves the Mlo3 function in mRNA export in fission yeast (Thakurta et al., 2005). This protein is an RRM-containing protein homologous to Aly/REF. In mlo3 mutant cells, the level of centromeric transcripts is increased, while the level of siRNAs derived from the transcripts is severely reduced (Zhang et al., 2008).

Remarkably, Clr4 methyltransferase methylates Mlo3. However, alteration of Lys9 of histone H3 to arginine did not affect the level of siRNAs, highlighting the specific importance of Clr4-mediated Mlo3 methylation in siRNA production. The levels of H3K9me3 and Swi6 over centromeric heterochromatin regions are maintained in mlo3 mutant cells, while siRNA production is attenuated. Thus, siRNA production can be uncoupled from the maintenance of heterochromatin marks as observed in the mat locus (Hall et al., 2002).

The Trf4/Air2/Mtr4p polyadenylation (TRAMP) complex, together with the nuclear exosome, plays an important role in processing ribosomal RNA, small nuclear RNA, and small nucleolar RNA (LaCava et al., 2005; Vanácková et al., 2005; Wyers et al., 2005). One subunit of TRAMP, Cid14, is a poly(A)-specific nucleotidyl transferase (Bühler et al., 2007). In cid14 mutant cells, siRNAs from constitutive heterochromatin regions are mostly lost, while the precursors accumulate (Bühler et al., 2007). Similarly to the mlo3 mutant cells, the levels of H3K9me3 and Swi6 in cid14 mutant cells are hardly affected, further supporting the notion that siRNA production is uncoupled from the maintenance of heterochromatin marks (Bühler et al., 2007).

Conversely, Rrp6, a subunit of the nuclear exosome, has a distinct role in the formation of constitutive heterochromatin. In ago1 mutant cells, the repetitive elements are transcriptionally upregulated, while the levels of H3K9me3 and Swi6 are decreased. Interestingly, the ago1/rrp6 double mutant shows an even severer phenotype than the ago1 mutant; consequently, the constitutive heterochromatin turns into the euchromatic state (Reyes-Turcu et al., 2011). This finding indicates that Ago1 and Rrp6

Figure 4. RITS-mediated heterochromatin formation model.

The RITS complex targets nascent RNA of dg/dh repeats. Chp1 in the RITS complex binds to H3K9me3 via its chromodomain and stabilizes RITS on the chromatin. RITS recruits RdRC and reverse transcribes dg/dh repeat sequences to produces double-stranded siRNA precursors. The precursors are processed by Dicer into secondary siRNAs. RITS also recruits CLRc and Stc1 interconnects the two complexes. Clr4 of CLRc interacts with H3K9me3 using its chromodomain and methylates neighboring H3K9. Chp2 and Swi6 recognize H3K9me3 and recruit SHREC and Clr6 to repress transcription and form heterochromatin.
### siRNA pathway in fission yeast

**ARGONAUTE**
- Ago1
  - N
  - PAZ
  - MID
  - PHB

**HUB**
- Zn-f (LIM-type)

**SAFFOLD**
- Chp1
  - CD (Chromo domain)
- Tas3
  - Argonaute hock

### Histone: writers

**Histone: writers**
- Ctr4
  - CD
  - Pre-NT
  - SET
  - SET
  - SET
- Cul4
  - Cullin N-terminal
  - Cullin protein neddylation domain
- Raf1
  - WD40 repeats
- Raf2
  - DNA (cytosine-5)-methyltransferase 1, replication foci domain
- Rik1
  - CPSF A subunit, N-terminal
  - CPSF A subunit, C-terminal

### Histone: readers

**Histone: readers**
- Swi6
  - CD
  - CSD
  - (Chromo shadow domain)
- Chp2
  - CD
  - CSD

### Histone: erasers

**Histone: erasers**
- Mit1
  - Znf (PHD-type)
  - Chromo-like
  - ATPase
  - Hel-C
  - Znf (C2H2-type)
- Histone deacetylase
  - Bjerling et al., 2002
- Ctr6
  - Histone deacetylase
  - Sugiyma et al., 2007
- Ctr3
  - Histone deacetylase
  - Arb2
- Sir2
  - Sir2 family
  - N-terminal

### Others

**Others**
- Cid12
  - DNA2/NAM7 helicase, helicase domain
  - DNA2/NAM7 helicase, C-terminal
- Hrr1
  - DNA-dependent RNA polymerase, eukaryotic-type
- Rdp1
  - DNA-dependent RNA polymerase, eukaryotic-type

### piRNA pathway in Drosophila

**piRNA pathway in Drosophila**
- Piwi
  - N
  - PAZ
  - MID
  - PHB

**HUB**
- Zn-f (U11-48K-like CHHC)

**SAFFOLD**
- Gtsf1
  - TNF (PHD-type)

### Histone: writers

**Histone: writers**
- Egg
  - Tudor
  - MBD
  - Pre-SET
  - Post-SET
- Wde
  - FN3

### Histone: erasers

**Histone: erasers**
- HP1a
  - CD
  - CSD

### Others

**Others**
- Lsd1
  - SWIRM
  - Amine oxidase
- Co-REST
  - ELM2
  - SANT1
- Sv210
  - SAP
  - PINT
  - Znf (SP-RING-type)
- Mael
  - HMG
  - MAEL
- H1
  - H15

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Figure 5.
independently trigger heterochromatin formation to suppress transcription. The redundancy in maintaining heterochromatin indicates the existence of an RNAi-independent heterochromatin formation pathway that includes Rrp6. This redundancy creates the additive effect on heterochromatin formation of the RNAi machinery and a protein that is involved in nuclear RNA processing. This phenotype was not observed in the cid14/ago1 double mutant (Reyes-Turcu et al., 2011), because the cid14 mutation suppresses the defect in heterochromatin formation caused by the Ago1 deletion. These data support the idea that TRAMP and the nuclear exosome play distinct roles in heterochromatin formation.

Transcription-independent, DNA motif-dependent heterochromatin assembly

Two members of the ATF/CREB family, Atf1 and Pcr1, induce heterochromatin formation at the mat locus in an siRNA-independent manner (Jia et al., 2005). The ATF/CREB family of transcription factors are leucine zipper proteins that bind to the cAMP response element (CRE). At the mat locus, in contrast to the cen locus, depletion of RNAi components hardly affects the heterochromatin status as well as the gene silencing status, although these components localize to the mat locus. Interestingly, depletion of the ATF/CREB-binding motif (CRE) in combination with the loss of RNAi machinery causes severe defects in heterochromatin formation. This shows that at the mat locus, the DNA elements (CRE) trigger an siRNA-independent pathway to ensure the heterochromatin status by recruiting Clr3 through Atf1/Pcr1. Similarly, at the tel locus, the DNA-binding protein, Taz1, induces siRNA-independent heterochromatin formation (Kanoh et al., 2005). Such DNA motif has not been found at the cen locus, although heterochromatin can be formed in an siRNA-independent but Rrp6-dependent manner.

Comparison of the Piwi-piRISC and RITS pathways

Chromodomain dependency In fission yeast, siRNAs are produced from repetitive elements within constitutive heterochromatin, which are rich in H3K9me3. Nonetheless, the elements are weakly transcribed to generate siRNAs only during S phase (Chen et al., 2008; Kloc et al., 2008). The siRNA production takes place within the vicinity and the siRNAs target the parental loci to maintain the heterochromatin state. Thus, it is reasonable that many key factors, such as Chp1, Chp2, Swi6, Mit1, and Clr4, possess the chromodomain H3K9me3-reading domain (Fig 5).

In contrast, the chromodomain is not common among Piwi co-factors, and only HP1 members and Mi-2 have this domain (Fig 5). The piRNA clusters used in OSCs are rich in H3K9me3 and are bound to the HP1 homolog, Rhino (Rhi), through the chromodomain. Rhi then recruits other factors to activate transcription internally within the clusters (Mohn et al., 2014; Zhang et al., 2014; Andersen et al., 2017). Rhi is unique to OSCs and absent in OSCs (Sumiyoshi et al., 2016). The main piRNA source in OSCs is flam, which is H3K9me3-free (Sienski et al., 2012). Transposons targeted by piRNAs are normally located within euchromatin, which is fundamentally H3K9me3-free before the action of Piwi (Sienski et al., 2012). Thus, Piwi co-factors do not have to rely on the chromodomain to function in this pathway. Clr4 in fission yeast contains a chromodomain together with a Pre-SET and SET domain (Fig 5). The Drosophila counterpart, Egg/SetDB1, has a TUDOR domain instead of the chromodomain (Fig 5). The TUDOR domain binds the active histone mark, H3K14ac, but not H3K9me3 (Jurkowska et al., 2017).

Determining the genomic sites where Piwi localizes is experimentally a difficult task. On the basis of this, the “target-engaged piRISC” model was proposed (Huang et al., 2013; Lin et al., 2015; Marinov et al., 2015; Ilyin et al., 2017). According to this model, nuclear Piwi-piRISCs scan nascent transcripts until they find landing sites, which should be highly complementary to the piRNAs. Once piRISCs bind the target RNAs, they transform to “target-engaged piRISCs” and start recruiting other co-factors (Sienski et al., 2015). This model is based on the structural change found in AGO-RISC before and after its binding to the target RNA (Wang et al., 2008). In fission yeast, the genomic sites to which Ago1, and even its co-factors, bind were easily determined experimentally. These findings prompted us to think that in Drosophila, the sequence specificity that is inherently buried in the nascent transcript triggers heterochromatin formation more than the chromatin status on such genomic regions does. Conversely, RNAi in fission yeast is forced to locate at heterochromatin regions and its activity is regulated by the chromatin status with the help of RNA recognition by the RITS complex. Thus, the difference in the number of chromodomain-containing proteins between the two pathways may reflect the mode of action that triggers heterochromatin formation.

Trans-silencing In most organisms, including Drosophila, siRNAs arising from dsRNAs expressed either exogenously or endogenously efficiently recognize the corresponding RNA sequences and silence them effectively. This indicates that siRNAs can target RNA transcripts in trans no matter where they originated from. Similarly, Drosophila piRNAs are produced from piRNA clusters (strictly speaking, this has only been demonstrated for the flam locus) and silence transposons located in euchromatin (Yamanaka et al., 2014; Sato & Siomi, 2018). In contrast to this fly system, siRNAs in fission yeast, which are not currently known to affect sites in trans, have a different role in heterochromatin formation.
yeast do not induce heterochromatin assembly in trans, i.e., they avoid “trans-silencing”. Namely, siRNAs in fission yeast are restricted to act on the parental elements from which they were produced (Fig 6). However, upon mutating Paf1, an RNA pol II interactor, the effect of trans-silencing was increased, and even genes located outside of the heterochromatin regions were repressed if their RNA transcripts were complementary to the siRNAs (Kowalki et al., 2015). In the paf1 mutant cells, nascent transcripts are not efficiently released from the genes and the lingering RNAs become the target of the silencing machinery, leading to heterochromatin formation. This increase in the range of RNAi-targeting was also observed upon mutating Mst2, a histone acetyltransferase (Flury et al., 2017). The mutation may also retain nascent transcripts aberrantly long on the gene as seen in paf1 mutant cells. Consistent with this notion, the defect in nuclear RNA processing at the transcription site caused by Rrp6 depletion led to efficient targeting of RNAi and subsequent heterochromatin formation on actively transcribed gene regions (Yamanaka et al., 2013).

In fission yeast, the length of introns within protein-coding genes is relatively small, and the average number of introns is smaller than that in humans (Mourier & Jefares, 2003). Moreover, many genes in fission yeast do not even have introns. Conversely, pre-mRNAs in Drosophila are normally much longer than those in fission yeast and thus are theoretically retained on the genome for a longer period of time. This may contribute to the increased rate of “trans-targeting” in organisms other than fission yeast. Moreover, as discussed above, at least in Drosophila, the transcription rate of the Piwi targets is reduced by Mael, or other alternative means, before heterochromatin formation occurs at the target loci (Sienski et al., 2012; Chang et al., 2019; Onishi et al., 2020). Whether or not mice have a similar system remains undetermined.

**piRNAs versus siRNAs**
Fission yeast are PIWI-free and piRNA-free, but Drosophila produce siRNAs and express AGO members in the germline. Endogenous siRNAs in Drosophila are the products of long dsRNAs, and the majority have transposon sequences. In cultured Schneider 2 (S2) cells, a Drosophila cell line of embryo origin, the loss of Dicer2 and Ago2 caused derepression of transposons, which indicates that RNAi contributes to transposon silencing at least in these non-gonadal somatic cells (Kawamura et al., 2008). Such endogenous RNAi may contribute to transposon silencing in the ovaries in addition to the piRNA pathway, but to a much lower, perhaps negligible, extent, because the PIWI mutants, but not the ago2 mutant flies, show infertility (Cox et al., 1998; Kim et al., 2007).

**Perspective**
piRNA-mediated transcriptional transposon silencing in Drosophila and mice is essential for maintaining the genomic integrity of reproductive tissues. A more detailed understanding of the molecular mechanisms of piRNA-mediated transcriptional silencing has been gained in Drosophila (Iwasaki et al., 2015; Czech et al., 2018; Ozata et al., 2019). Recently, it has been reported that Piwi-piRISC efficiently represses transposon transcription by co-activating H3K9me3-independent and H3K9me3-dependent transcriptional repressive mechanisms (Murano et al., 2019; Onishi et al., 2020). Additionally, it has been suggested that H3K9 deacetylation and H3K4 demethylation are important for Piwi-mediated silencing (Yu et al., 2015; Mugat et al., 2020). The next challenge is to clarify the order of these inhibitory mechanisms and the detailed molecular functions of the co-factors.

In recent years, the detailed mechanism of Miwi2-mediated properties has become better understood. So far, phenotypic analysis has been the main method for analyzing Miwi2-mediated transcriptional repression systems. For more detailed mechanistic understanding, biochemical analysis of gonocytes is necessary. Recently, cell sorting technology and biochemical analysis using a small number of cells or a single cell have been developed, and it is expected that these technologies will be used to elucidate the molecular mechanism of Miwi2-mediated transcriptional silencing. Moreover, further progress will be made by comparing the Miwi2-mediated transcriptional silencing mechanism with that in Drosophila.

The model of Ago1-mediated heterochromatin formation in fission yeast has been used as a reference for a piRNA-mediated transcriptional repression model. However, as research on piRNA-mediated transcriptional silencing has progressed, differences between the two mechanisms have been revealed. First, piRNAs mainly target transposons integrated in euchromatic loci and convert them into a transcriptionally inert state, whereas siRNAs in fission yeast target repetitive elements transcribed from constitutive heterochromatin regions. It is interesting to note that the piRNA system seems to be specialized in the repression of active transcription (Aravin et al., 2008; Nagamori et al., 2018; Onishi et al., 2020). This unique feature of the piRNA system may be the result of an arms race between piRNAs and transposons. Therefore, clarifying the origin of piRNA-specific factors will shed light on the evolution of the piRNA system.

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**Conflict of interest**
The authors declare that they have no conflict of interest.
References

Allshire RC, Nimmo ER, Ekwall K, Javerzat JP, Cranston G (1995) Mutations derepressing silent centromeric domains in fission yeast disrupt chromosome segregation. Genes Dev 9: 218 – 233

Andersen PR, Tirian L, Yunjak M, Brennecke J (2017) A heterochromatin-dependent transcription machinery drives piRNA expression. Nature 549: 54 – 59

Araki S, Le NT, Koizumi K, Villar-Briones A, Nonomura K-I, Endo M, Inoue H, Saze H, Komiya R (2020) miR2118-dependent U-rich phased RNA production in rice anther wall development. Nat Commun 11: 3115

Aravin AA, Naumova NM, Tulin AV, Vagin VV, Rozovsky YM, Czovkova VA (2001) Double-stranded RNA-mediated silencing of genomic tandem repeats and transposable elements in the D. melanogaster germline. Curr Biol 11: 1017 – 1027

Aravin AA, Lagos-Quintana M, Yalcin A, Zavolan M, Marks D, Snyder B, Hannon GJ (2007) The DNA methyltransferase-like protein DNMT3L stimulates de novo DNA methylation in mice. Mol Cell 31: 785 – 799

Ayyin O, Mehta S, Grewal SIS (2013) HDAC-mediated suppression of histone turnover promotes epigenetic stability of heterochromatin. Nat Struct Mol Biol 20: 547 – 554

Barau J, Teissandier A, Zamudio N, Roy S, Nalesso V, Hannon GJ (2014) Mutations in the TRAMP-mediated RNA surveillance pathway prevent entry of RNAs into the Schizosaccharomyces pombe siRNA pathway. Nat Struct Mol Biol 15: 1015 – 1023

Baker SM, Iida T, Bühler M, Villén J, Cygi SP, Nakayama J-I, Moazed D (2007) Two different Argonaute complexes are required for siRNA generation and heterochromatin assembly in fission yeast. Nat Struct Mol Biol 14: 200 – 207

Cam HP, Sugiyama T, Chen ES, Chen X, FitzGerald PC, Grewal SIS (2005) Comprehensive analysis of heterochromatin- and RNAi-mediated epigenetic control of the fission yeast genome. Nat Genet 37: 809 – 819

Canzio D, Chang EY, Shankar S, Kuchenbecker KM, Simon MD, Madhani HD, Ryo Onishi et al (2020) Chromodomain-mediated oligomerization of HSP1 suggests a nucleosome-bridging mechanism for heterochromatin assembly. Mol Cell 41: 67 – 81

Carmell MA, Girard A, van de Kant HJG, Bouërch D, Bestor TH, de Rooij DG, Hannon GJ (2007) MIWIZ is essential for spermatogenesis and repression of transposons in the mouse male germline. Dev Cell 12: 503 – 514

Castañeda J, Genzor P, van der Heijden GW, Sarkeshik A, Yates JR, Inglia NT, Bortvin A (2014) Reduced pachytene piRNAs and translation underlie spermiogenic arrest in Maelstrom mutant mice. EMBO J 33: 1999 – 2019

Chang TH, Mattei E, Gaintedino V, Colpan C, Weng Z, Zamore PD (2019) Maelstrom represses canonical polymerase II transcription within bi-directional piRNA clusters in Drosophila melanogaster. Mol Cell 73: 291 – 303.e6

Chedin F, Lieber MR, Hsieh C-L (2002) The DNA methyltransferase-like protein DNMT3L stimulates de novo methylation by Dnmt3a. Proc Natl Acad Sci USA 99: 16916 – 16921

Chen ES, Zhang K, Nicolas E, Cam HP, Zofall M, Grewal SIS (2008) Cell cycle control of centromeric repeat transcription and heterochromatin assembly. Nature 451: 734 – 737

Clegg N, Frost DM, Larkin MK, Subrahmanyam L, Bryant Z, Ruohola-Baker H (1997) Maelstrom is required for an early step in the establishment of Drosophila oocyte polarity: posterior localization of grk mRNA. Development 124: 4661 – 4671

Colmenares SU, Buker SM, Bühler M, Diakì M, Moazed D (2007) Coupling of double-stranded RNA synthesis and siRNA generation in fission yeast RNAs. Mol Cell 27: 449 – 461

Cox DN, Chao A, Baker J, Chang L, Qiao D, Lin H (1998) A novel class of evolutionarily conserved genes defined by piwi are essential for stem cell self-renewal. Genes Dev 12: 3715 – 3727

Czech B, Munafò M, Ciabrelli F, Eastwood EL, Fabry MH, Kneuss E, Hannon GJ (2018) piRNA-guided genome defense: from biogenesis to silencing. Annu Rev Genet 52: 131 – 157

Czech B, Preall JB, McGinn J, Hannon GJ (2013) A transcriptome-wide RNAI screen in the Drosophila ovary reveals factors of the germline piRNA pathway. Mol Cell 50: 749 – 761

Dai P, Wang X, Gou L-T, Li Z-T, Wen ZE, Chen Z-G, Hua M-M, Zhong AI, Wang L, Su H et al (2019) A translation-activating function of MIWI/piRNA during mouse Spermiogenesis. Cell 179: 1566 – 1581.e16

Darricarrère N, Liu N, Watanabe T, Lin H (2013) Function of Piwi, a nuclear Piwi/Argonaute protein, is independent of its slicer activity. Proc Natl Acad Sci USA 110: 1297 – 1302

De Fazio S, Bartonicek N, Di Giacomo M, Abreu-Goodger C, Sankar A, Funaya C, Antony C, Moreira PN, Enright AJ, O’Carroll D (2011) The endonuclease activity of Mili fuels piRNA amplification that silences LINE1 elements. Nature 480: 259 – 263
Deng W, Lin H (2002) miwi, a murine homolog of piwi, encodes a cytoplasmic protein essential for spermatogenesis. *Deu Cell* 2: 819–830

Döntertas D, Sienksi G, Bremenece J (2013) *Drosophila* GSTf1 is an essential component of the Piwi-mediated transcriptional silencing complex. *Genes Deu* 27: 1693–1705

Eastwood EL, Jara KA, Bornelov S, Munafó M, Frantzis V, Kneuss E, Barbier Ej, Czech B, Hannon GJ (2021) Dimethylation of the PICTS complex via LCB/Cut-up drives co-transcriptional transposon silencing in *Drosophila*. *Elife* 10: e65557

Egel R (1984) Two tightly linked silent cassettes in the mating-type region of *Schizosaccharomyces pombe*. *Curr Genet* 8: 199–203

Fabry MH, Ciabrelli F, Munafò M, Eastwood EL, Kneuss E, Falcioni I, Falconio FA, Hannon GJ, Czech B (2019) piRNA-guided co-transcriptional silencing coops export factors. *Elife* 8: e47999

Fischer T, Cui B, Dhakshnamoorthy J, Zhou M, Rubin C, Zafall M, Veenstra TD, Grewal SIS (2009) Diverse roles of HP1 proteins in heterochromatin assembly and functions in fission yeast. *Proc Natl Acad Sci USA* 106: 8998–9003

Flury V, Georgescu PR, Iesmiantavicius V, Shimada Y, Kuzdere T, Braun S, Gao L, Emperle M, Guo Y, Grimm SA, Ren W, Adam S, Uryu H, Zhang Z-M, Halbach R, Miesen P, Joosten J, Tass
d

Han BW, Wang W, Li C, Weng Z, Zamore PD (2015) Noncoding RNA. piRNA-guided transposon cleavage initiates Zucchini-dependent, phased piRNA production. *Science* 348: 817–821

Handler D, Oliviari D, Novatchkova M, Gruber FS, Meixner K, Mechtler K, Stark A, Sachidanandaram R, Brennecke J (2011) A systematic analysis of *Drosophila* TUDOR-domain-containing proteins identifies Vreteno and the Tdrd12 family as essential primary piRNA pathway factors. *EMBO J* 30: 3977–3993

Handler D, Meixner K, Pizka M, Lauss K, Schmied C, Gruber FS, Brennecke J (2013) The genetic makeup of the *Drosophila* piRNA pathway. *Mol Cell* 50: 762–777

Hansen KR, Berns G, Mata J, Wolpe TA, Martienssen RA, Bähler J, Thon G (2005) Global effects on gene expression in fission yeast by silencing and RNA interference machineries. *Mol Cell Biol* 25: 590–601

Hirakata S, Siomi MC (2016) piRNA biogenesis in the germline: from transcription of piRNA genomic sources to piRNA maturation. *Biochim Biophys Acta* 1859: 82–92

Hirakata S, Ishizu H, Fujiy A, Tomoe Y, Siomi MC (2015) Requirements for multivalent Yb body assembly in transposon silencing in *Drosophila*. *EMBO Rep* 20: e47708

Horn PJ, Bastie J-N, Peterson CL (2005) A Rik1-associated, cullin-dependent E3 ubiquitin ligase is essential for heterochromatin formation. *Genes Deu* 19: 1705–1714

Houwing S, Kamingma LM, Berezikov E, Cronembold D, Girard A, van den Elst H, Filippov DV, Blaser H, Raz E, Moens CB et al (2007) A role for Piwi and piRNAs in germ cell maintenance and transposon silencing in Zebrafish. *Cell* 129: 69–82

Hruz T, Laule O, Szabo G, Wessendorf F, Bleuler S, Oertle L, Widmayer P, Grussien W, Zimmermann P (2008) Genevestigator V3: a reference expression database for the meta-analysis of transcriptomes. *Adu Bioinform* 2008: 1–5

Huang YA, Yin H, Sweeney S, Raha D, Snyder M, Lin H (2013) A major epigenetic programming mechanism guided by piRNAs. *Deu Cell* 24: 502–516

Ilin AA, Ryazansky SS, Doronin SA, Olenkina OM, Mikhailova EA, Yaksheev EY, Abramov YA, Belyakin SN, Ivankin AV, Pindyurin AV et al (2017) Piwi interacts with chromatin at nuclear pores and promiscuously binds nuclear transcripts in *Drosophila* ovarian somatic cells. *Nucleic Acids Res* 45: 7666–7680

Inoue N (1999) New gene family defined by MORC, a nuclear protein required for mouse spermatogenesis. *Hum Mol Genet* 8: 1201–1207

Ipsaro J, Haase AD, Knott SR, Joshua-Tor L, Hannon GJ (2012) The structural biochemistry of Zucchini implicates it as a nuclease in piRNA biogenesis. *Nature* 491: 279–283

Ishizu H, Iwasaki YW, Hirakata S, Ozaki H, Iwasaki W, Siomi H, Siomi MC (2015) Somatic primary piRNA biogenesis driven by cis-acting RNA elements and trans-acting Yb. *Cell Rep* 12: 429–440

Ishizu H, Kinoshita S, Hirakata S, Komatsu S, Siomi MC (2019) Distinct and collaborative functions of Yb and Armitage in transposon-targeting piRNA biogenesis. *Cell Rep* 27: 1822–1835.e8

Iwasaki YW, Siomi MC, Siomi H (2015) Piwi-Interacting RNA: its biogenesis and functions. *Annu Rev Biochem* 84: 405–433

Iwasaki YW, Murano K, Ishizu H, Shibuya A, Iyoda Y, Siomi MC, Siomi H, Saito K (2016) Piwi modulates chromatin accessibility by regulating multiple factors including histone H1 to repress transposons. *Mol Cell* 63: 408–419

Jesperse N, Barbier E (2020) Emerging features of linear motif-binding hub proteins. *Trends Biochem Sci* 45: 375–384

Jia S, Noma K, Grewal SIS (2004) RNA1-dependent heterochromatin nucleation by the stress-activated ATR/CREB family proteins. *Science* 304: 1971–1976

Jia S, Kobayashi R, Grewal SIS (2005) Ubiquitin ligase component Cul4 associates with Ctr4 histone methyltransferase to assemble heterochromatin. *Nat Cell Biol* 7: 1007–1013
Kawamura Y, Saito K, Kin T, Ono Y, Asai K, Sunohara T, Okada TN, Siomi MC, Kowalik KM, Shimada Y, Flury V, Stadler MB, Batki J, Kaneda M, Okano M, Hata K, Sado T, Tsujimoto N, Li E, Sasaki H (2002) Drosophila endogenous small RNAs bind to Argonaute 2 in somatic cells. Nature 430: 467 – 469

Kawamata Y, Saito K, Ono Y, Asai K, Sunohara T, Okada TN, Siomi MC, Siomi H (2008) Drosophila endogenous small RNAs bind to Argonaute 2 in somatic cells. Nature 453: 793 – 797

Kim K, Lee YS, Carthew RW (2007) Conversion of pre-RISC to holo-RISC by Ago2 during assembly of RNAi complexes. RNA 13: 22 – 29

Kim VN, Han J, Siomi MC (2009) Biogenesis of small RNAs in animals. Nat Rev Mol Cell Biol 10: 126 – 139

Kim IV, RiedeIbauch S, Kuhn C-D (2020) The piRNA pathway in planarian flatworms: new model, new insights. Biol Chem 401: 1123 – 1141

Klar AJ, Bonaduce MJ (1991) siwi, a gene required for mating-type switching, prohibits meiotic recombination in the mat2-mat3 ‘cold spot’ of fission yeast. Genetics 129: 1033 – 1042

Klattenhoff C, Bratu DP, McGinnis-Schultz N, Koppetsch BS, Cook HA, Theurkauf WE (2002) Drosophila rasiRNA pathways disrupt embryonic axis specification through activation of an ATR/Chk2 DNA damage response. Dev Cell 12: 45 – 55

Klenov MS, Sokolova OA, Yakushev EF, Stolyarenko AD, Mikhailova EA, Lavrov SA, Gvozdev VA (2011) Separation of stem cell maintenance and transposon silencing functions of Piwi protein. Proc Natl Acad Sci USA 108: 18760 – 18765

Kloc A, Zaratiegui M, Nora E, Martinezs R (2008) RNA interference guides histone modification during the S phase of chromosomal replication. Curr Biol 18: 490 – 495

Kobayashi H, Sakurai T, Miura F, Imai M, Mochiduki K, Yanagisawa E, Sakashita A, Wakai T, Suzuki Y, Ito T et al (2013) High-resolution DNA methylation analysis of primordial germ cells identifies gender-specific reprogramming in mice. Genome Res 23: 616 – 627

Kojima-Kita K, Kuramochi-Miyagawa S, Nagamori I, Ogunuki N, Ogura A, Hasuwa H, Akazawa T, Inoue N, Nakano T (2016) MIWI2 as an effector of DNA methylation and gene silencing in embryonic male germ cells. Cell Rep 16: 2819 – 2828

Kowalik KM, Shimada Y, Flury V, Stadler MB, Batski J, Bühler M (2015) The Pa2 complex represses small-RNA-mediated epigenetic gene silencing. Nature 520: 248 – 252

Kubo N, Tosh H, Shirane K, Shirakawa T, Kobayashi H, Sato T, Sone H, Sato Y, Tomizawa S-I, Tsurusaki Y et al (2015) DNA methylation and gene expression dynamics during spermatogonial stem cell differentiation in the early postnatal mouse testis. BMC Genom 16: 624

Kunert N, Marhold J, Stanke J, Stach D, Lyko F (2003) A Dnmt2-like protein mediates DNA methylation in Drosophila. Development 130: 5083 – 5090

Kunert N, Wagner E, Murawska M, Klinker H, Kremmer E, Brehm A (2009) dMec: a novel Mi-2 chromatin remodelling complex involved in transcriptional repression. EMBO J 28: 533 – 544

Kuramochi-Miyagawa S, Kimura T, Yomogida K, Kuroiwa A, Tadokoro Y, Fujita Y, Sato M, Matsuda Y, Nakano T (2001) Two mouse piwi-related genes: miwi and mil1. Mech Dev 108: 121 – 133
Molmar A, Falcicatore I, Hodges E, Aravin AA, Mannan K, Rafii S, McCombie WR, Smith AD, Hannon GJ (2014) Two waves of de novo methylation during mouse germ cell development. Genes Dev 28: 1544–1549

Motamedi MR, Verdel A, Colmenares SU, Gerber SA, Cygi SP, Moazed D (2004) Two RNAi complexes, RITS and RDRC, physically interact and localize to noncoding centromeric RNAs. Cell 119: 789–802

Motamedi MR, Hong E-JE, Li X, Gerber S, Denison C, Cygi S, Moazed D (2008) HP1 proteins form distinct complexes and mediate heterochromatic gene silencing by nonoverlapping mechanisms. Mol Cell 32: 778–790

Mourier T, Jefrakes DC (2003) Eukaryotic intron loss. Science 300: 1393

Muerder F, Guzzardo PM, Gillis J, Luo Y, Yu Y, Chen C, Fekete R, Hannon GJ (2013) A genome-wide RNAi screen draws a genetic framework for transposon control and primary piRNA biogenesis in Drosophila. Mol Cell 50: 736–748

Mugat B, Nicot S, Varela-Chavez C, Jourdan C, Sato K, Basyuk E, Juge F, Siomi H, Ishizu H, Saito K, Fukuhara S, Kamatani MK, Bonnefond L, Motamedi MR, Verdel A, Colmenares SU, Gerber SA, Gygi SP, Moazed D, Nishimori I, Kobayashi H, Nishimura T, Yamagishi R, Katahira J, Kuramochi-K, Okano H, Siomi H, Siomi MC (@ 2013) The Rpd3 histone deacetylase is involved in piRNA-guided heterochromatin formation. Nat Commun 11: 2818

Munafò M, Manelli V, Falconio FA, Sawle A, Kneuss E, Eastwood EL, Seah JWE, Czech B, Hannon GJ (2019) Daedalus and Gasz recruit Armitage to mitochondria, bringing piRNA precursors to the biogenesis machinery. Genes De3 33: 844–856

Murano K, Iwasaki YW, Ishizu H, Mashiko A, Shibuya A, Kondo S, Adachi S, Suzuki S, Saito K, Natsume T et al (2019) Nuclear RNA export factor variant initiates piRNA-guided co-transcriptional silencing. EMBO J 38: e102870

Murota Y, Ishizu H, Nakagawa S, Iwasaki YW, Shibata S, Kamatani MK, Saito K, Okano H, Saito K, Siomi H, Siomi MC (2014) Yb integrates piRNA intermediates and processing factors into perinuclear bodies to enhance piRISC assembly. Cell Rep 8: 103–113

Nagamoni I, Kobayashi H, Nishimura T, Yamagishi R, Katahira J, Kuramochi-Miyagawa S, Nono T, Nakano T (2018) Relationship between PIWIL4-Mediated H3K4me2 demethylation and piRNA-dependent DNA methylation. Cell Rep 25: 350–356

Nakayama J, Rice JC, Strahl BD, Allis CD, Grewal SI (2001) Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. Science 292: 110–113

Niki Y, Yamaguchi T, Mahowald AP (2006) Establishment of stable cell lines of Drosophila germ-line stem cells. Proc Natl Acad Sci USA 103: 16325–16330

Ninova M, Chen Y-CA, Godneeva B, Rogers AK, Luo Y, Fejes Tóth K, Aravin AA (2020) Su(var)2–10 and the SUMO pathway link piRNA-guided target recognition to chromatin silencing. Mol Cell 77: 556–570.e6

Nishida KM, Saito K, Mori T, Kawamura Y, Nagami-Okada T, Inagaki S, Siomi H, Siomi MC (2007) Gene silencing mechanisms mediated by Aubergine piRNA complexes in Drosophila male gonad. RNA 13: 1911–1922

Nishimasu H, Ishizu H, Saito K, Fukushima S, Kamatani MK, Bonnefond L, Matsumoto N, Nishizawa T, Nakamura K, Aoki J et al (2012) Structure and function of Zucchini endoribonuclease in piRNA biogenesis. Nature 491: 284–287

Noma K, Sugiyama T, Cap H, Verdel A, Zofall M, Jia S, Moazed D, Grewal SIS (2004) RITS acts in cis to promote RNA interference-mediated transcriptional and post-transcriptional silencing. Nat Genet 36: 1174–1180

Ohtani H, Iwasaki YW, Shibuya A, Siomi H, Siomi MC, Saito K (2013) DmGTSF1 is necessary for Piwi-piRISC-mediated transposon silencing in the Drosophila ovary. Genes Dev 27: 1656–1661

Olivieri D, Sykora MM, Sachidanandam R, Mechtler K, Brennecke J (2010) An in vivo assay identifies major genetic and cellular requirements for primary piRNA biogenesis in Drosophila. EMBO J 29: 3301–3317

Onishi R, Saito K, Murano K, Negishi L, Siomi H, Siomi MC (2020) Piwi suppresses transcription of Brahma-dependent transposons via Maelstrom in ovarian somatic cells. Sci Adv 6

Osumi K, Saito K, Murano K, Siomi H, Siomi MC (2019) Essential roles of Windei and nuclear monoubiquitination of Eggless/SETDB1 in transposon silencing. EMBO Rep 20: e48296

Ozata DM, Gainetdinov I, Zoch A, O’Carroll D, Zamore PD (2019) Piwi-interacting RNAs: small RNAs with big functions. Nat Rev Genet 20: 89–108

Palumbo C, Bonaccorsi S, Robbins LG, Pimpinelli S (1994) Genetic analysis of Stellate elements of Drosophila melanogaster. Genetics 138: 1181–1197

Pandey RR, Homolka D, Chen K-M, Sachidanandam R, Fauvarque M-O, Pillai RS (2017) Recruitment of Armitage and Yb to a transcript triggers its phased processing into primary piRNAs in Drosophila ovaries. PLoS Genet 13: e1006956

Pastor WA, Stroud H, Nee K, Liu W, Pezic D, Manakov S, Lee SA, Moiissard G, Zamudio N, Bourchís D et al (2014) MORC1 represses transposable elements in the mouse male germline. Nat Commun 5: 5795

Pelission A, Song SU, Prud’homme N, Smith PA, Bucheton A, Corces V (1994) Gypsy transposition correlates with the production of a retroviral envelope-like protein under the tissue-specific control of the Drosophila flamenco gene. EMBO J 13: 4401–4411

Petrie VJ, Wittschick JD, Givens CD, Kosinski AM, Partridge JF (2005) RNA interference (RNAi)-dependent and RNAi-independent association of the Chp1 chromodomain protein with distinct heterochromatic loci in fission yeast. Mol Cell Biol 25: 2331–2346

Pezic D, Manakov SA, Sachidanandam R, Aravin AA (2014) piRNA pathway targets active LINE1 elements to establish the repressive H3K9me3 mark in germ cells. Genes Dev 28: 1410–1428

Popp C, Dean W, Feng S, Cokus SJ, Andrews S, Pellegri M, Jacobsen SE, Reik W (2010) Genome-wide erasure of DNA methylation in mouse primordial germ cells is affected by AID deficiency. Nature 463: 1101–1105

Provost P, Silverstein RA, Dishart D, Walfidsson J, Djupedal I, Kniola B, Wright A, Samuelsen B, Radmark O, Ekwall K (2002) Dicer is required for chromosome segregation and gene silencing in fission yeast cells. Proc Natl Acad Sci USA 99: 16648–16653

Rajaseethupathy P, Antonov I, Sheridan R, Frey S, Sander C, Tuschi T, Kandel ER (2012) A role for neuronal piRNAs in the epigenetic control of memory-related synaptic plasticity. Cell 149: 693–707

Rea S, Eisenhaber F, O’Carroll D, Strahl BD, Sun Z-W, Schmid M, Opravil S, Mechtler K, Ponting CP, Allis CD et al (2000) Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature 406: 593–599

Reik W (2001) Epigenetic reprogramming in mammalian development. Science 293: 1089–1093

Reinhart BJ, Bartel DP (2002) Small RNAs correspond to centromere heterochromatic repeats. Science 297: 1831

Reyes-Turcu FE, Zhang K, Zofall M, Chen E, Grewal SIS (2011) Defects in RNA quality control factors reveal RNAi-independent nucleation of heterochromatin. Nat Struct Mol Biol 18: 1132–1138

Saito K, Nishida KM, Mori T, Kawamura Y, Miyoshi K, Nagami T, Siomi H, Siomi MC (2006) Specific association of Piwi with rasiRNAs derived from retrotransposon and heterochromatic regions in the Drosophila genome. Genes De3 20: 2214–2222
Saito K, Inagaki S, Mituyama T, Kawamura Y, Ono Y, Sakota E, Kotani H, Asai K, Siomi H, Siomi MC (2009) A regulatory circuit for piwi by the large Maf gene traffic jam in Drosophila. Nature 461: 1296–1299

Saito K, Ishizu H, Komai M, Kotani H, Kawamura Y, Nishida KM, Siomi H, Siomi MC (2010) Roles for the Yb body components armitage and Yb in primary piRNA biogenesis in Drosophila. Genes Dev 24: 2493–2498

Sarot E, Payen-Groschén G, Bucheton A, Péllisson A (2004) Evidence for a piwi-dependent RNA silencing of the gypsy endogenous retrovirus by the Drosophila melanogaster flamenco gene. Genetics 166: 1313–1321

Sasaki H, Matsui Y (2008) Epigenetic events in mammalian germ-cell development: reprogramming and beyond. Nat Rev Genet 9: 129–140

Sato K, Nishida KM, Shibuya A, Siomi MC, Siomi H (2011) Maelstrom coordinates microtubule organization during Drosophila oogenesis through interaction with components of the MTCG. Genes Dev 25: 2361–2373

Sato K, Siomi MC (2018) Two distinct transcriptional controls triggered by nuclear Piwi-piRISCs in the Drosophila piRNA pathway. Curr Opin Struct Biol 53: 69–76

Schalch T, Job G, Noffsinger VJ, Shanker S, Kuscu C, Joshua-Tor L, Partridge JF (2008) Mouse maelstrom, a component of nuage, is essential for spermatogenesis and transposon repression in meiosis. Dev Cell 15: 285–297

Stapleton W, Das S, McKee BD (2001) A role of the Drosophila homelife gene in repression of Stellate in male meiosis. Chromosoma 110: 228–240

Stewart KR, Veselovska L, Kim J, Huang J, Saadeh H, Tomizawa S, Smallwood SA, Chen T, Kelsey G (2015) Dynamic changes in histone modifications precede de novo DNA methylation in oocytes. Genes Dev 29: 2449–2462

Suetake I, Shinozaki F, Miyagawa J, Takeshima H, Tajima S (2004) DNMT3L stimulates the DNA methylation activity of Dnmt3a and Dnmt3b through a direct interaction. J Biol Chem 279: 27816–27823

Sugiyama T, Cam H, Verdel A, Moazed D, Grewal SIS (2005) RNA-dependent RNA polymerase is an essential component of a self-enforcing loop coupling heterochromatin assembly to siRNA production. Proc Natl Acad Sci USA 102: 152–157

Sugiyama T, Cam HP, Sugiyama R, Noma K, Zofall M, Kobayashi R, Grewal SIS (2007) SHREC, an effector complex for heterochromatic transcriptional silencing. Cell 128: 491–504

Sumiyoshi T, Sato K, Yamamoto H, Iwasaki Y, Siomi H, Siomi MC (2016) Loss of (3)mbt leads to acquisition of the ping-pong cycle in Drosophila ovarian somatic cells. Genes Dev 30: 1617–1622

Suyama M, Doersk T, Braun IC, Sattler M, Izaurralde E, Bork P (2000) Prediction of structural domains of TAP reveals details of its interaction with p15 and nuclearopins. EMBO Rep 1: 53–58

Thakurta AG, Copal G, Yoon JH, Kozak L, Dhar R (2005) Homolog of BRCA2-interacting Dss1p and Uap56p link Mlo3p and Rae1p for mRNA export in fission yeast. EMBO J 24: 2512–2523

Thon G, Klar AJ (1993) Directionality of fission yeast mating-type interconversion is controlled by the location of the donor loci. Genetics 134: 1045–1054

Tuzon CT, Borgstrom B, Weilguny D, Egel R, Cooper JP, Nielsen O (2004) The fission yeast heterochromatin protein Rik1 is required for telomere clustering during meiosis. J Cell Biol 165: 759–765

Vagin VV (2006) A distinct small RNA pathway silences selfish genetic elements in the germline. Science 313: 320–324

Vanáčová S, Wolf J, Martin G, Blank D, Dettwiler S, Friedlein A, Langen H, Keith G, Keller W (2005) A new yeast poly(A) polymerase complex involved in RNA quality control. PLoS Biol 3: e189

Veland N, Lu Y, Hardikar S, Gaddis S, Zeng Y, Liu B, Estacio MR, Takata Y, Lin K, Tomida MW et al (2019) DNMT3L facilitates DNA methylation partly by maintaining DNMT3A stability in mouse embryonic stem cells. Nucleic Acids Res 47: 152–167

Verdel A, Jia S, Gerber S, Sugiyama T, Gygi S, Grewal SIS, Moazed D (2004) RNAi-mediated targeting of heterochromatin by the RITS complex. Science 303: 672–676

Volpe TA, Kidner C, Hall IM, Teng G, Grewal SIS, Martienssen RA (2002) Methylation and heterochromatin assembly – deposed H3 histone – is required to establish centromeric heterochromatin. Genes Dev 16: 1067–1078

Volpe TA, Kidner C, Hall IM, Teng G, Grewal SIS, Martienssen RA (2002) Homolog of BRCA1 facilitates DNA methylation partly by maintaining DNMT3A stability in mouse embryonic stem cells. Nucleic Acids Res 47: 152–167

Wang Y, Juranek S, Li H, Sheng G, Tuschi T, Patel D (2008) Structure of an argonaute silencing complex with a seed-containing guide DNA and target RNA duplex. Nature 456: 921–926

Wang SH, Elgin SCR (2011) Drosophila Piwi functions downstream of piRNA production mediating a chromatin-based transposon silencing mechanism in female germ line. Proc Natl Acad Sci USA 108: 21164–21169
Wang W, Han BW, Tipping C, Ge DT, Zhang Z, Weng Z, Zamore PD (2015) Slicing and binding by Ago1 or Aub trigger piwi-bound piRNA production by distinct mechanisms. *Mol Cell* 59: 819 – 830

Watanabe T, Takeda A, Tsukiyama T, Mise K, Okuno T, Sasaki H, Minami N, Imai H (2006) Identification and characterization of two novel classes of small RNAs in the mouse germline: retrotransposon-derived siRNAs in oocytes and germline small RNAs in testes. *Genes Dev* 20: 1732 – 1743

Watanabe T, Cheng E, Zhong M, Lin H (2015) Retrotransposons and pseudogenes regulate mRNAs and IncRNAs via the piRNA pathway in the germine. *Genome Res* 25: 368 – 380

Watson ML, Zinn AR, Inoue N, Hess KD, Cobb J, Handel MA, Halaban R, Duchene CC, Albright CM, Moreadith RW (2012) Distinct roles of RNA helicases MVH and TDRD1 in piRNA precursor splicing. *Cell* 148: 13461 – 13466

Wenda JM, Homolka D, Yang Z, Spinelli P, Sachidanandam R, Pandey RR, Pillai RS (2017) Distinct roles of RNA helicases MVH and TDRD1 in piRNA precursor splicing. *Cell* 148: 13461 – 13466

Wilson BC, Roberts CWM (2011) SWI/SNF nucleosome remodelers and cancer. *Nat Rev Cancer* 11: 481 – 492

Wu P-H, Fu Y, Cecchini K, Ozata DM, Anf A, Yu T, Colpan C, Gainetdinov I, Weng Z, Zamore PD (2020) The evolutionarily conserved piRNA-producing locus pi6 is required for male mouse fertility. *Nat Genet* 52: 728 – 739

Wyers F, Rougemolle M, Badis G, Rousselle J-C, Dufour M-E, Boulay J, Régnault B, Devaux F, Namane A, Séraphin B et al (2005) Cryptic pol II transcripts are degraded by a nuclear quality control pathway involving a new poly(A) polymerase. *Cell* 121: 725 – 737

Xu Q, Xiang Y, Wang Q, Wang L, Brind’Amour J, Bogutz AB, Zhang Y, Zhang B, Yu G, Xia W et al (2019) SETD2 regulates the maternal epigenome, genomic imprinting and embryonic development. *Nat Genet* 51: 844 – 856

Yamaguchi S, Oe A, Nishida KM, Yamashita K, Kajiy A, Hirano S, Matsumoto N, Dohmae N, Ishitani R, Saito K et al (2020) Crystal structure of *Drosophila* Piwi. *Nat Commun* 11: 858

Yamanaka S, Mehta S, Reyes-Turcu FE, Zhuang F, Fuchs RT, Rong Y, Robb GB, Grewal SIS (2013) RNAI triggered by specialized machinery silences developmental genes and retrotransposons. *Nature* 493: 557 – 560

Yamanaka S, Siomi MC, Siomi H (2014) piRNA clusters and open chromatin structure. *Mol DNA S* 2: 22

Yamashiro H, Siomi MC (2018) PIWI-Interacting RNA in *Drosophila* biogenesis, transposon regulation, and beyond. *Chem Rev* 118: 4404 – 4421

Yamashiro H, Negishi M, Kinoshita T, Ishizu H, Ohtani H, Siomi MC (2020) Armitage determines Piwi-piRISC processing from precursor formation and quality control to inter-organelle translocation. *EMBO Rep* 21: e48769

Yang F, Lan Y, Pandey RR, Homolka D, Berger SL, Pillai RS, Bartolomei MS, Wang PJ (2020) TEX15 associates with MiLi and silences transposable elements in male germ cells. *Genes Dev* 34: 745 – 750

Yashiro R, Murota Y, Nishida KM, Yamashiro H, Fujii K, Ogai A, Yamanaka S, Negishi L, Siomi H, Siomi MC (2018) Piwi nuclear localization and its regulatory mechanism in *Drosophila* ovarian somatic cells. *Cell Rep* 23: 3647 – 3657

Yoshimura T, Watanabe T, Kuramochi-Miyagawa S, Takamoto N, Shiromoto Y, Kudo A, Kanai-Azuma M, Tashiro F, Miyazaki S, Katanaya A et al (2018) Mouse GTSF1 is an essential factor for secondary piRNA biogenesis. *EMBO Rep* 19: e42054

Yu Y, Gu J, Jin Y, Luo Y, Preall JB, Ma J, Czech B, Hannon GJ (2015) Panoramix enforces piRNA-dependent cotranscriptional silencing. *Science* 350: 339 – 342

Zhang K, Mosch K, Fischle W, Grewal SIS (2008) Roles of the CIR4 methyltransferase complex in nucleation, spreading and maintenance of heterochromatin. *Nat Struct Mol Biol* 15: 381 – 388

Zhang Z, Wang J, Schultz N, Zhang F, Parhad SS, Tsu S, Vreven T, Zamore PD, Weng Z, Theurkauf WE (2014) The HP1 homolog rhino anchors a nuclear complex that suppresses piRNA precursor splicing. *Cell* 157: 1333 – 1363

Zhao K, Cheng S, Miao NA, Xu P, Lu X, Zhang Y, Wang M, Ouyang X, Yuan X, Liu W et al (2019) A Pandas complex adapted for piRNA-guided transcriptional silencing and heterochromatin formation. *Nat Cell Biol* 21: 1261 – 1272

Zheng K, Xiol J, Reuter M, Eckardt S, Leu NA, McLaughlin KJ, Stark A, Sachidanandam R, Pillai RS, Wang PJ (2010) Mouse MOV10L1 associates with Piwi proteins and is an essential component of the Piwi-interacting RNA (piRNA) pathway. *Proc Natl Acad Sci USA* 107: 11841 – 11846

Zoch A, Auchynnikava T, Berrens RV, Kabayama Y, Schöpp T, Heep M, Vasiliauskaitė L, Pérez-Rico YA, Cook AG, Shukumatava A et al (2020) SPOCD1 is an essential executor of piRNA-directed *de novo* DNA methylation. *Nature* 584: 635 – 639

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