The Roles of Factors Modulating PIWI/piRNA Pathway Silencing of Transposons

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Abstract

The goal of this research project is to ascertain the role of factors in the initiation of PIWI-directed silencing of transposable elements (TEs). There have been genetic studies on the factors involved in the PIWI/piRNA pathway, but the initiation of TE silencing is not fully understood. To study this question, we applied a Piwi-interacting RNA (piRNA)-targeted reporter assay to *Drosophila* OSS cells. We found that piRNA biogenesis factors and PIWI-associated factors have direct roles in the initiation of PIWI silencing and that chromatin-modifying factors may act downstream. Our findings also reveal that RNA Polymerase II associated proteins PAF1 and RTF1 oppose PIWI-directed silencing. When the piRNA targeted transcript region is close to the promoter, PAF1 and RTF1 knockdowns enhance PIWI silencing of the reporters. Our results show that the role of PAF1 in the AGO1/siRNA-directed silencing of fission yeast may be conserved with the role of PAF1 in PIWI/piRNA-directed silencing in *Drosophila*. Our reporter assay was also successfully applied for PIWI knockdown in WRR-1 cells that have a male genotype, confirming that PIWI has a direct role in TE silencing in this cell line. This study indicates that our reporter assay can be used to test other factors in the initiation of PIWI-directed silencing. We hope to apply the assay to OSC-delta-I(3)mbt cell that expresses the secondary piRNA ping-pong pathway, but transfection conditions need to be optimized for the assay to work.

Introduction

Abstract

The current incomplete picture of the PIWI/piRNA mechanism that silences transposable elements warrants more research. The unique and highly conserved PIWI/piRNA pathway needs to be understood to prevent the disastrous effects of uncontrolled transposon propagation. Unregulated transposon activity may lead to genome instability, sterility, and poor organismal health. There is a lack of understanding on many aspects of the natural PIWI/piRNA pathway, so more research needs to be conducted. Specifically the roles of Piwi factors in initiating PIWI-directed TGS and in PTGS and how TGS and PTGS mechanisms interact with each other are not well understood and are the focus of this research project. Using luciferase reporter assays and other techniques on OSS, Δmbt-OSC, and WRR-1 cell line platforms will shed light on these worthwhile and interesting topics.

The unique mechanism of the PIWI/piRNA pathway represses transposable elements

The PIWI/piRNA pathway is particularly interesting to study because of its central role in suppressing transposable elements (TEs). Transposons, mobile DNA sequences, comprise a significant portion of eukaryotic genomes, are conserved among almost all organism genomes, and are important for genome evolution (for review, see Huang, Burns, & Boeke, 2012). However, they can wreak havoc with their unregulated activity, especially in the germ line by affecting reproductive health (Malone et al., 2009). The PIWI/piRNA prevents the insertional mutagenesis, loss of genome integrity, sterility, and decreased progeny fitness associated with

uncontrolled transposon propagation (Iwasaki et al., 2016; Malone et al., 2009). Therefore, understanding the mechanism of this natural line of defense is important.

Previous research on the PIWI/piRNA pathway has made progress on revealing certain mechanistic aspects of the pathway. Piwi proteins, which are members of the evolutionarily conserved Argonaute protein family among eukaryotes, specifically associate with small Piwi-interacting RNAs (piRNAs) that are 23 to 29 nucleotides long. These piRNAs are derived from intergenic regions containing repetitive elements, including transposons, to silence TEs (Brennecke et al., 2007; Gunawardane et al., 2007; Juliano, Wang, & Lin, 2011; Saito et al., 2006; Vagin et al., 2006). The initiation of PIWI silencing begins with PIWI binding to a primary piRNA for transport into the nucleus and the subsequent binding of the piRNA to complementary TE transcripts (Haase et al., 2010; Saito et al., 2010). For PIWI-mediated silencing to occur, a threshold of piRNAs that can bind to nascent transcripts needs to be reached (Post, Clark, Sytnikova, Chirn, & Lau, 2014). PIWI affects TE sequences directly and indirectly affects coding genes adjacent to TEs, because protein-coding transcripts do not have the piRNA bulk targeting requirement for triggering PIWI silencing (Post et al., 2014; Sytnikova, Rahman, Chirn, Clark, & Lau, 2014). The mechanisms for piRNA biogenesis have also been studied.

In *Drosophila*, the two known pathways for piRNA biogenesis differ based on their location and mechanism. The primary piRNA biogenesis pathway occurs in the nucleus of germline nurse cells and of surrounding somatic follicle cells, whereas the secondary piRNA biogenesis pathway only occurs in the cytoplasm of germline nurse cells (Brennecke et al., 2007; Gunawardane et al., 2007; Li et al., 2009). Primary piRNAs are produced from precursors derived from piRNA clusters at granules, perinuclear and cytoplasmic Yb body and nuclear Dot

COM, which then undergo processing at Yb bodies to form mature piRNAs (Dennis et al., 2013; Murota et al., 2014; Saito et al., 2010). Primary piRNAs form complexes with Aub and PIWI (Li et al., 2009; Malone et al., 2009). The Aub/piRNA complex stay in the cytoplasm to exert silencing of TEs by cleavage of their complementary mRNAs transcripts using small RNA-guided endoribonuclease activity (Slicer), while the PIWI/piRNA complex enters the nucleus to exert transcriptional silencing of TEs without Slicer activity (Klenov et al., 2011; Le Thomas et al., 2013; Rozhkov, Hammell, & Hannon, 2013; Saito et al., 2010; Sienski, Dönertas, & Brennecke, 2012; S. H. Wang & Elgin, 2011). Secondary piRNAs arise from Aub/piRNA RNA cleavage of mature TE transcripts, and the piRNAs then attach to AGO3 (Brennecke et al., 2007; Gunawardane et al., 2007). Aub and AGO3 work together in a ping-pong amplification cycle, which is a Slicer-dependent feed-forward loop that amplifies secondary piRNAs that feeds into the PIWI mechanism (Brennecke et al., 2007; Gunawardane et al., 2007). The PIWI/piRNA pathway mechanism is unique compared to previously studied RNA pathways.

Similar to the PIWI/piRNA pathway, the RNA interference (RNAi) pathway and miRNA pathway also function in gene silencing but have different components and mechanisms. The Argonaute protein family consists of AGO1, AGO2, AGO3, Aubergine, and PIWI (P-element-induced wimpy testis) (Williams & Rubin, 2002). The Argonaute (Ago) subfamily members, AGO1 and AGO2, bind to microRNAs (miRNAs) and short interfering RNA (siRNAs), respectively, and are involved in the miRNA pathway and the RNAi pathway, respectively (Feng Jiang et al., 2005; Forstemann et al., 2005; Kim, Han, & Siomi, 2009; Liu et al., 2003; Saito, Ishizuka, Siomi, & Siomi, 2005). Ago proteins are ubiquitously expressed in all cell types and play a role in post-transcriptional gene silencing, by destabilizing mRNA or repressing

translation (Hock & Meister, 2008; Williams & Rubin, 2002). On the other hand, Piwi subfamily members, AGO3, Aubergine (Aub), and PIWI, bind piRNAs and are involved in the PIWI/ piRNA pathway (Williams & Rubin, 2002). Piwi proteins are mainly expressed in germ cells and is the main pathway that silences transposons through transcriptional gene silencing (TGS), post-transcriptional gene silencing (PTGS), and epigenetic gene silencing (Brennecke et al., 2008; Rozhkov et al., 2013). In general, the targets of repression differ between the different types of small RNAs: host genes for miRNAs; host genes, transposable elements (TEs), and viruses for siRNAs; and TEs for piRNAs (Lau et al., 2009). Unlike siRNAs and miRNAs, piRNAs are not produced by endoribonuclease Dicer and do not have 2',3' hydroxyl termini, (Vagin et al., 2006). The PIWI/piRNA pathway is distinct from the other RNA pathways based on the factors involved in the pathway, the location of the Piwi protein expression, the pathway's function, the mechanism of piRNA production, the structure of piRNA, and piRNA targets.

How PIWI/piRNA pathway factors interact to trigger TE silencing

Although a significant number of *Drosophila* PIWI-TGS pathway gene factors have been discovered through genetic and transgenic-RNAi studies, there still is a lack of understanding of the directness of these factors in initiating PIWI-directed silencing and how these factors interact in this mechanism. Genetic studies have identified known factors specific to the Piwi pathway: Panoramix/Silencio (PANX/SILE), Maelstrom (MAEL), Asterix (ARX/GTSF1), Armitage (ARMI), Zucchini (ZUCC), Spindle-E (SPN-E), and Krimper (KRIMP) (Haase et al., 2010; Lim & Kai, 2007; Malone et al., 2009; Saito et al., 2010; Sato et al., 2015; Sienski et al., 2015; Sumiyoshi et al., 2016; Webster et al., 2015; Y. Yu et al., 2015). SILE, ARX, and MAEL are localized in the nucleus and associated to PIWI (Sumiyoshi et al., 2016). SILE binds ARX and

PIWI to initiate heterochromatin formation when PIWI binds a target RNA or a gene (Sienski et al., 2015; Y. Yu et al., 2015). ARMI, a cytoplasmic Yb body component, and ZUCC, a nuclease, are involved in piRNA biogenesis and help form a stable PIWI complex (Haase et al., 2010; Saito et al., 2010). ARMI attaches to PIWI to localize PIWI into Yb bodies for inspection and stabilizes PIWI attachment to piRNA for entry into the nucleus (Saito et al., 2010). ZUCC is a putative nuclease that processes precursor TE transcripts (Haase et al., 2010). SPN-E and KRIMP contain a tudor-domain, are specific to the germline, and are unusually expressed in OSC/OSS cells. KRIMP promotes secondary piRNA biogenesis in the germline and inhibits the binding of AGO3 to primary piRNAs (Lim & Kai, 2007; Malone et al., 2009; Sato et al., 2015; Webster et al., 2015). SPN-E also promotes secondary piRNA biogenesis, and knockdown of this factor in OSC/OSS cells does not change levels of primary piRNAs (Malone et al., 2009; Saito et al., 2010). In addition to these known Piwi-pathway specific factors, the role of chromatin-associated factors linked to the Piwi pathway in TE silencing has not been fully dissected.

Genetic studies have also shown that heterochromatin-associated proteins maintain H3K9me3 marks at piRNA-targeted loci in germ cells to cooperatively compact heterochromatin. These proteins include heterochromatin Protein 1a (HP1A/Su(Var)-205), linker histone H1, and H3K9 histone methyltransferase (HMTase) Eggless (EGG/SetDB1) (Brower-Toland et al., 2007; Iwasaki et al., 2016, 2016; Klattenhoff et al., 2009; Le Thomas et al., 2014; Lu et al., 2013; Mohn, Sienski, Handler, & Brennecke, 2014; Pal-Bhadra et al., 2004; Sienski et al., 2015; S. H. Wang & Elgin, 2011; Zhang, Wang, et al., 2014). PIWI associates with linker histone H1 and regulates H1 binding to target transposon loci to perform transcriptional silencing by modulating chromatin accessibility (Iwasaki et al., 2016). Chromatin accessibility may be the

final effector in the PIWI/piRNA TE silencing pathway that is controlled two mutually interdependent pathways, regulated by H1 and HP1a, which compact chromatin cooperatively mediated recruitment of HP1a, which initiates heterochromatin formation at target loci (Iwasaki et al., 2016). The first pathway is Piwi recruitment of H1, and the second pathway is H3K9me3-mediated recruitment of HP1a, which initiates heterochromatin formation at target loci (Iwasaki et al., 2016). The two previous groups of factors are known to be involved with PIWI-directed silencing, but transcriptional regulators may also influence this pathway.

Genetic studies have also discovered transcriptional regulators that are involved in transposon silencing. The factors in the *Drosophila* PAF1 complex, which are associated with RNA Polymerase II (RNA Pol-II), may modulate PIWI-directed silencing: ATU, CTR9/CG2469, PAF1 (also called antimeros, atms), and RTF1 (Bahrampour & Thor, 2016; Chaturvedi, Inaba, Scoggin, & Buszczak, 2016). ATU is a homolog of Leo1, which is part of the yeast Paf1 complex (Jaehning, 2010). PAF1 and RTF1 help with RNA Pol-II elongation and promote H3 lysine-4 trimethylation (H3K4me3) marks on chromatin to activate transcription (Adelman et al., 2006; Tenney et al., 2006). CTR9 is involved in RNA Polymerase II release of the transcript (Jaehning, 2010).

Comparison of RNA Pol II regulation between yeast and *Drosophila*

The Paf1 complexes in *Schizosaccharomyces pombe* (*S. pombe*), fission yeast, and *Drosophila* have several similarities, but also have critical differences. The Paf1 complex (Paf1C) consists of proteins Paf1, Ctr9, Cdc73/Parafibromin (Hrpt2)/Hyrax, Rtf1, and Leo1 (Jaehning, 2010). The Paf1 complexes in yeast and *Drosophila* both help elongate RNA Pol II and activate genes, but the manner of Pol II association and components of the Paf1 complex

differ between these two species (Adelman et al., 2006). Also, Paf1 and Rtf1 associate differently with Pol II in *Drosophila* and yeast. In yeast, Paf1 association with Pol II and active chromatin depends on Rtf1 being present, but in *Drosophila*, Rtf1 presence is not necessary, whereas Rtf association with Pol II depends on Paf1 presence (Adelman et al., 2006). In addition, methylation patterns differ between the two complexes. Yeast Paf1C marks euchromatin with both H3K4me3 and H3K79me3 (Krogan et al., 2003; Ng, Robert, Young, & Struhl, 2003), while *Drosophila* Paf1 complex factors Paf1, Rtf1, and Ctr9 influence global levels of H3K4me3 (Adelman et al., 2006; Chaturvedi et al., 2016; Tenney et al., 2006).

A paradox for eukaryotic small RNA-induced TGS mechanisms exists in yeast and *Drosophila*, such as the RNAi mechanism and the PIWI-TGS mechanism. In *S. pombe*, the Paf1 complex helps transcription terminate efficiently, allows RNA to be quickly released from the transcription site, and antagonizes targeting of nascent transcripts by the RNAi transcriptional silencing complex (Kowalik et al., 2015). Transcription of a nascent RNA is necessary for RNAi machinery to target a locus and slowly release Pol II, which promotes silencing of the locus by heterochromatin formation (Buhler, Verdel, & Moazed, 2006; Zaratiegui et al., 2011). The activity of Paf1C in the TGS mechanism of fission yeast involving AGO1 and siRNA of must be compromised to decrease the rate of nascent RNA target release to allow AGO1/siRNAs to silence the gene by heterochromatin formation (Chen et al., 2015; Kowalik et al., 2015; Yang et al., 2016; M. Yu et al., 2015). In *Drosophila* follicle cells, RNA Pol-II-associated factors, like cut-off, TFIIs, and ATU, which is a homolog of yeast Paf1 component Leo1, regulate RNA Pol II transcription efficiency and TE silencing (Jaehning, 2010). The transcription and nascent RNA

dynamics in the *Drosophila* PIWI-TGS mechanism are not as well understood as that in the yeast RNAi mechanism and further research is necessary.

Cell culture platform is used for genomic experiments to study the PIWI/piRNA pathway

Several cell lines are useful for performing genomic experiments, such as the OSS and OSC cell lines. Reporter assays are used to measure reporter expression after the knockdown of a certain factor, which help us gain insights into the role of factors in the PIWI/piRNA pathway. The *Drosophila* ovarian somatic sheet (OSS) cell line and the *Drosophila* ovarian somatic cells (OSC) cell line are related lines derived from follicle cells and have been used to extensively study somatic primary piRNA biogenesis in *Drosophila*, which only involves PIWI, but not the other Piwi members Aub and AGO3 (Haase et al., 2010; Lau et al., 2009; Malone et al., 2009; Niki, Yamaguchi, & Mahowald, 2006; Saito et al., 2009). Although OSS cells also express miRNAs and endo-siRNAs, primary piRNAs are produced most abundantly, and no secondary piRNAs are expressed (Lau et al., 2009). The Lau lab and other labs have been independently growing batches of OSS cells that came from the Niki lab, and the Siomi lab renamed their cell line OSC cells (Sytnikova et al., 2014). These OSS and OSC cells have dramatically different TE landscapes and are regulated by the PIWI/piRNA pathway very differently (Sytnikova et al., 2014). These two cell lines present a simpler model to study only PIWI's TE silencing mechanism.

However, the detailed mechanism of the ping-pong cycle involved in secondary piRNA biogenesis in germ cells of *Drosophila* is not well understood. Applying the functional assay to *Drosophila* cell lines that recently appeared in literature and express Piwi pathway genes, such as WRR-1 cells and Δmbt-OSC cells, may lead to new and exciting discoveries relating to this

topic. The WRR-1 cells have a male signature instead the female signature of OSC/OSS cells and only expresses the primary piRNA biogenesis pathway (Fagegaltier et al., 2016). The Δmbt-OSC cell line, an ovarian somatic cell line with the function of lethal (3) malignant brain tumor [l(3)mbt] eliminated by CRISPER, leads to the abnormal expression of germ-specific ping-pong cycle, involving detectable Aub, AGO3, and Vasa (Sumiyoshi et al., 2016). Vasa is a germ-specific DEAD-box RNA helicase that uses ATP hydrolysis to transfer sliced piRNA precursors from Aub/piRNA complexes between ping-pong factors (Nishida et al., 2015; Xiol et al., 2014). The Δmbt-OSC cell line also expresses PIWI that is only involved in primary piRNA silencing mechanism. This Δmbt-OSC cell line may show both transcriptional, such as the primary piRNA silencing, and post-transcriptional transposon silencing, such as the ping-pong cycle, which would elucidate the interaction between these two pathways (Sumiyoshi et al., 2016).

Experimental setup used to investigate PIWI-directed TGS mechanism

The TE-directed or genic piRNAs from piRNA master loci target the reporters that the Lau lab has created to investigate the PIWI-directed TGS mechanism. Genetic studies have revealed several piRNA master loci in somatic and germline cells (Brennecke et al., 2007; Lau et al., 2009). Transposon-rich piRNA master loci are regions in the genome where many piRNAs could uniquely mapped and are mainly located in pericentromeric and subtelomeric heterochromatin (Brennecke et al., 2007). In the Drosophila germline, TEs such as *412*, *mdg1*, *GTWIN*, *idefix*, *copia*, *DM-CR1A*, *DNAREP1/DINE-1*, *roo*, and *297*, create a significant amount of piRNAs that is detectable (Aravin et al., 2003; Brennecke et al., 2007; Lau et al., 2009; Vagin et al., 2006). *Flamenco* (*flam*)/20AB is a piRNA master cluster located on X chromosome that is strongly active and specifically expressed in somatic ovarian follicle cells, like OSS cells. The

flam locus affects PIWI-directed TE silencing (Lau et al., 2009; Post et al., 2014). A less strongly expressed piRNA cluster is 20A, which is active in both somatic and germline cells (Lau et al., 2009). A piRNA cluster mainly active in the germline is 42AB (Lau et al., 2009). Also, the soma-specific, protein-coding, and single exon gene *traffic jam* (tj) locus produces abundant genic piRNAs at its 3'UTR that work with PIWI to regulate TE's (Post et al., 2014; Robine et al., 2009; Saito et al., 2009). *Tj* is a large Maf factor that regulates gonad morphogenesis in *Drosophila* and produces TJ protein that activates PIWI expression and piRNAs that help PIWI silence targets (Saito et al., 2009). *Flam* and other somatic TE master loci are only involved in primary piRNA biogenesis and not the "ping-pong" cycle (Lau et al., 2009), so studying these loci is simpler.

The Lau lab constructed a set of reporter plasmids that are targets for TE-directed piRNAs from the *flam* locus or genic piRNAs from the *tj* locus to investigate how piRNAs interact with the reporter transcript in gene silencing (Post et al., 2014). A 2.4-kb *flam* and a 1.5-kb *tj* 3'UTR element were inserted into the *Renilla* luciferase 3'UTR. These elements were cloned in the "Sense" orientation, in which the luciferase transcript is in the same orientation as endogenous piRNAs and cannot base pair to the piRNAs, or in the "Antisense" orientation, in which the transcript is complementary (Post et al., 2014). The piRNA-targeted reporter assay effectively measures PIWI/piRNA silencing capacity (Post et al., 2014) and can be used to test the effects of other factors on reporter expression. The piRNA-targeted segments in this study were inserted in the 5' intron of our *Renilla* luciferase reporter construct in addition to the 3'UTR. We first knocked down the studied factor by siRNAs or a negative control siGFP siRNA. Then after two days, we transfected these reporter plasmids into OSS cells. After another two

days, the luciferase assay was performed. We did a triple normalization to rule out non-PIWI/ piRNA effects and to simplify interpretations of silencing capacity. First, the *Renilla* values were normalized to the firefly luciferase transfection control. Then the normalized the "Antisense" reporter to the "Sense" reporter. Finally, we normalized these reporter ratios from factor siRNA to the siGFP control. Since the assay only transiently introduces a synthetic piRNA reporter plasmid to the cells (Post et al., 2014), the assay mimics the initial steps of PIWI-directed TGS. We will investigate the roles of particular factors in the initiation of PIWI-directed silencing and how the location of a piRNA-targeted element relative to a transcription promoter affects PIWI-directed silencing capacity.

Conclusion

In order to silence transposons, which could be threatening if unregulated, the unique and highly conserved PIWI/piRNA pathway is constantly active and protecting the organism. This organism's natural way of protecting itself has important implications in fertility and organism health, but there are many aspects of the PIWI/piRNA pathway that are not well studied and require future research. In particular, the roles of Piwi factors, such as Piwi-pathway-specific factors, chromatin-associated proteins, and transcriptional regulators, in triggering the early steps of PIWI-directed TGS and their roles in PTGS are not well understood. The interaction between TGS and PTGS mechanisms are also not well known. These topics are the focus of this research project. OSS, Δmbt-OSC, and WRR-1 cell line platforms will be used to perform genetic experiments, such as luciferase reporter assays, to gain insight into these topics. The interaction between different groups of Piwi factors, quality-control mechanisms used to select transposon

mRNAs for secondary piRNAs, and how transposon desilencing, relate to developmental phenotypes in *piwi* mutants (Juliano et al., 2011) are other interesting areas for future research.

Reporter assays results from knockdown of factors

Introduction

In order to investigate the roles of factors in PIWI-directed silencing, we used siRNAs to knock down known Piwi-pathway-specific, chromatin-associated, and RNA Pol-II associated factors. After, we transfected piRNA targeted plasmid reporters and then performed luciferase assays in OSS cells. Since the piRNA-targeted reporter assay directly introduces a new synthetic piRNA target, the assay mimics the initiating steps of PIWI-directed silencing of a *de novo* TE transposition event (Post et al., 2014). The reporter assays can show how the distance from the piRNA-targeted element to a promoter affects PIWI-directed silencing.

To start the assay, we knock down the factor being studied by siRNA nucleofection. After two days, we transfect plasmids that contain 2.4 kb segment of *Flamenco (Flam)* or a 1.5kb segment of *Traffic jam (Tj)* 3' Untranslated Region (3'UTR) piRNA loci (Figure 1A). These segments, either in the "Sense" orientation (mRNA sequence is the same as endogenous piRNAs) or "Antisense" orientation (mRNA is complementary to piRNAs), were cloned into the 5' intron or 3' UTR of a *Renilla* luciferase reporter (Figure 1A). When a direct factor, such as PIWI, is knocked down and compared to siGFP control, only the "Antisense" reporter is de-repressed, unlike the "Sense" reporter, which shows no change. To solely measure PIWI/piRNA effects and not other effects on silencing, we carried out a triple normalization procedure of normalizing Renilla values to firefly luciferase values, then normalizing the "Antisense" reporter to the "Sense" reporter, and finally normalizing the factor siRNA knockdown to the siGFP control (Figure 1B).

We tested various Piwi-pathway-specific, chromatin-associated, and RNA Pol-II associated factors. The Piwi-pathway factors we tested were PIWI-associated factor PANORAMIX/SILENCIO (PANX/SILE) and primary piRNA biogenesis factors ARMITAGE (ARMI) and ZUCCHINI (ZUCC). We also tested factors that contain the Tudor domain, are germline-specific, and are abnormally expressed in OSS cells: SPINDLE-E (SPN-E), KRIMPER (KRIMP), QIN, and TUDOR (TUD) (Sumiyoshi et al., 2016).

We wanted to study if H3K9me3 is necessary for piRNA reporter silencing, so we tested heterochromatin-associated proteins HP1A and linker histone H1. H3K9me3 marks can accumulate on PIWI-targeted loci on piRNA reporter plasmids turning them into heterochromatin, but the reporter plasmids have fewer marks compared to endogenous loci (Post et al., 2014).

Since several transcriptional regulators were implicated in piRNA-silencing of a *gypsy*-lacZ transgenic reporter in fly ovaries, we tested several transcriptional regulators in this project (Handler et al., 2013). We tested *Drosophila* PAF1, RTF1, CTR9 (also called CG2469, (Bahrampour & Thor, 2016; Chaturvedi et al., 2016)), and HYRAX (HYR, Drosophila homolog of CDC73, (Mosimann, Hausmann, & Basler, 2009)). PAF1 and RTF1 promote RNA Pol-II elongation and histone H3 lysine-4 trimethylation (H3K4me3) (Adelman et al., 2006; Tenney et al., 2006). Finally, we also tested *Drosophila* CSTF64 (Sullivan, Steiniger, & Marzluff, 2009), DRE4 (homolog of Spt16/FACT) (Shimojima et al., 2003), and CDK9/pTEFb (Ni, Schwartz, Werner, Suarez, & Lis, 2004), to investigate how transcription termination, elongation, and modulating paused RNA Pol II impacts PIWI-directed silencing, respectively. In yeast Paf1C mutants, mutations in Ctf1 (also known as CstF64) enhanced siRNA-reporter silencing, which

indicates that the silencing mechanism in yeast Paf1C mutants involves transcription termination (Kowalik et al., 2015).

Piwi-associated and primary piRNA biogenesis factors have a direct role in Piwi silencing

The luciferase assays showed de-repression of the Flam 5' intron and Flam 3' UTR reporters for all the Piwi-pathway-specific factors I tested, except for KRIMP, QIN, AND TUD (Figure 1C). Piwi-associated factor SILE and primary piRNA biogenesis factors ARMI and ZUCC have a direct role in the initiation of PIWI-directed silencing on the Flam reporters. Knockdown of SILE destabilizes PIWI, because SILE associates with a target-engaged PIWI complex, even though SILE does not affect primary piRNA levels (Dönertas, Sienski, & Brennecke, 2013, p. 1; Ohtani et al., 2013; Saito et al., 2010; Sienski et al., 2015; Y. Yu et al., 2015). Knockdown of the primary piRNA biogenesis factors ARMI and ZUCC lead to an absence of piRNAs and destabilizes PIWI (Haase et al., 2010; Han, Wang, Li, Weng, & Zamore, 2015; Klattenhoff et al., 2007; Murota et al., 2014; Olivieri, Sykora, Sachidanandam, Mechtler, & Brennecke, 2010; Pane, Wehr, & Schüpbach, 2007; Saito et al., 2010; Watanabe et al., 2011). Without these factors, PIWI-directed silencing is halted and the reporters are de-repressed.

The results showed that knockdowns for QIN, TUD, and KRIMP did not affect both the Flam 5' intron and Flam 3' UTR reporters. This result is expected because QIN, TUD, and KRIMP promote germline-specific secondary piRNA biogenesis. The OSS cell line that used does not generate secondary piRNAs (Lim & Kai, 2007; Malone et al., 2009; Sato et al., 2015; W. Wang et al., 2015; Webster et al., 2015; Zhang, Koppetsch, et al., 2014). However, de-repression of the Flam reporters with SPN-E knockdown was unexpected, because this factor

also promotes secondary piRNA biogenesis and does not affect primary piRNA levels (Malone et al., 2009; Saito et al., 2010).

There is stronger de-repression in the 5' intron in comparison to the 3' UTR (Figure 1C). These results support that initiating PIWI-directed silencing on the nascent RNA could be adjustable as opposed to an "on-off switch," which is characteristic of the silencing in fission yeast. In fission yeast, genes that regulate transcription can be mutated and result in a complete loss of function, but they are still able to survive. In contrast, *Drosophila* genes that regulate transcription are essential for fly development and cannot be fully mutated (Bahrampour & Thor, 2016; Chaturvedi et al., 2016; Mosimann et al., 2009; Tenney et al., 2006). However, we hypothesize that PIWI silencing capacity is regulated by transcription and can be strengthened when the piRNA-targeting element is near the promoter in the 5' intron as opposed to in the 3' UTR.

PIWI-pathway-specific factor knockdowns displayed de-repression of the Tj-3' UTR reporter in the 5' intron targeted by genic piRNAs (Figure S1C). De-repression of the reporter with SPN-E knockdown confirms that SPN-E directly affects PIWI silencing. The SILE result was interesting because SILE primarily interacts with TE-directed piRNAs and interacts very little with genic piRNAs (Sienski et al., 2015). However, SILE knockdown showed de-repression of the Tj-3'UTR reporter and is required for silencing. These results support the hypothesis proposed by another paper (Sienski et al., 2015) that SILE interacts with PIWI/piRNAs only after they bind a target RNA.

Chromatin-associated factors may act downstream in Piwi silencing

Knockdowns of the chromatin-associated factors HP1a and H1 did not affect the Flam reporters (Figure 1D). Since the reporters are transiently transfected, these results indicate that chromatin-associated proteins are not required for the initiation of PIWI-directed silencing. Even though these reporters do accumulate some H3K9me3 marks during the assay, the plasmids are unlikely to be integrated into the genome due to the short time of assay (Post et al., 2014), and the chromosomal environment may not allow these chromatin factors to act on PIWI-directed silencing on these reporters. These chromatin factors may act downstream of an initial PIWI-directed silencing event. It is possible that linker H1 knockdown in OSS cells allow for the expression of TEs that only partially overlap with PIWI's targets, while HP1a knockdown may overlap slightly more and express TEs more (Iwasaki et al., 2016). This data suggests that the actions of HP1a and H1 on piRNA-targeted loci regulate chromatin downstream.

Several *Drosophila* PAF1 complex factors influence Piwi-directed silencing

Among *Drosophila* PAF1 complex factors, only HYR knockdown caused de-repression of Flam 5' intron and Flam 3' UTR reporters, and PAF1 and RTF1 showed enhanced silencing of the Flam reporters (Figure 1E). De-repression of the Flam reporters with HYR knockdown suggests that HYR has a direct role in initiating PIWI-directed silencing. CTR9 knockdown does not have an effect on silencing of the Flam reporters, suggesting that this factor does not have an affect on PIWI-directed silencing or may act downstream.

Notably, PAF1 and RTF1 knockdowns enhanced PIWI-directed silencing on the Flam 5' intron reporter. This repression of the reporter was unlikely to be due to global transcriptional changes. One reason is that our assays cancel global RNA Pol-II activity by the triple

normalization process. Another reason is that both PAF1 and RTF1 caused small increases in the transfection control firefly luciferase reporter expression normalized to siGFP (Figure S2A). These factors enhance Piwi-directed silencing despite the fact that PAF1 and RTF1 knockdowns cause a reduction in *Flam* and *Tj*-3'UTR piRNAs (Clark et al. manuscript, 2017). Therefore PAF1 and RTF1 both directly antagonize piRNA-reporter silencing.

PAF1 knockdown enhanced silencing only in the Flam 5' intron reporter and not the 3' UTR reporter. This further supports that PIWI silencing depends on the distance of piRNA-targeting element to the promoter and transcription termination signals and is adjustable.

Transcriptional regulators influence Piwi-directed silencing

Knockdowns of CSTF64 and DRE4 enhanced silencing of Flam 5' intron reporter, while knockdown of CDK9 showed de-repression of the Flam 5' reporter (Figure 2D). CSTF64 and DRE4 knockdowns directly enhance PIWI-directed silencing rather than global transcriptional effects, similarly to the PAF1 and RTF1 knockdowns. The fact that transfection control firefly luciferase reporter expression was not affected for CSTF64 and DRE4 knockdowns supports this (Figure S2A). The Flam 5' intron reporter is de-repressed, even though CDK9 knockdown decreased transfection control firefly luciferase reporter expression (Figure S2A). These data support that CDK9 promotes PIWI-directed silencing by promoting paused RNA Pol II to release nascent transcripts and piRNA to associate with PIWI. In contrast, DRE4 and CSTF64 function in general transcript elongation and termination, respectively, and oppose PIWI-directed silencing, which is similar to fission yeast mechanisms that oppose siRNA-directed silencing (Kowalik et al., 2015).

Knockdowns of transcriptional regulators have a stronger effect on the Flam 5' intron as opposed to the 3'UTR, which shows a smaller impact. This again supports that the proximity of the piRNA-targeting element to the promoter affects the strength of PIWI-directed silencing.

Piwi knockdowns have no effect on *Roo*-internal and *Blood*-internal reporters

In order to eliminate the confounding effects from long-terminal-repeat (LTR) sequences that include both transcription termination and promoter elements (Eggermont & Proudfoot, 1993) on reporters that contain TEs, we constructed reporters with just the internal segment of a TE and not the LTR segment. These reporters have the internal segments of two *Drosophila* retrotransposons and lack the LTR segments. Reporter assays of siPIWI knockdowns show no impact on the *roo*-internal and *blood*-internal 5' intron or 3'UTR reporters (Figure S8A). We think that the reporter assays may not be able to detect a PIWI silencing response because the endogenous levels of some TE-directed piRNAs are too low for reporter plasmid silencing.

The PIWI-PAF1 antagonism model applies to other TE reporters

We cloned two other reporters containing TE segments, *mdg1* and *412*, which did have enough piRNAs to see an effect in PIWI-directed silencing in OSS cells. The reporter assays for PIWI knockdown showed consistent de-repression of *Flam*, *mdg1*, and *412* reporters (Figures 4B and S8D). The PAF1 knockdown displayed enhanced silencing on the *Flam* and *mdg1* reporters only when the piRNA segment was put into the 5' intron (Figure 4B). The knockdown of both PIWI and PAF1 together lessened the de-repression effect of only doing a PIWI knockdown on the *Flam* and *mdg1* reporters (Figure 4B). This shows that PAF1 antagonizes PIWI-directed silencing. These results suggest that the balance of PIWI to PAF1 is restored in the cell although

the levels of siPIWI and siPAF1 are reduced overall in the cell (Clark et al. manuscript, 2017). On the other hand, PAF1 knockdown does not have an effect on the *412* 5' intron or 3' UTR (Figure S8D). The combination of PIWI and PAF1 knockdowns does not change the de-repression of the *412* reporters compared to PIWI knockdown (Figure S8D). These data show that the PIWI-PAF1 antagonism model applies to a specific group of endogenous TE sequences in OSS cells.

Efficient knockdown of factors

Quantitative RT-PCR (qPCR) showed that after the corresponding siRNA treatment, mRNA levels greatly decreased for the tested factors (Figure S1A). This indicates that the knockdowns were robust for PAF1, RTF1, SPN-E, KRIMP, CTR9, HYR, QIN, TUD, DRE4, CDK9, and CSTF64. Since ribosomal protein gene RP49 transcripts between siRNA knockdowns did not change (Clark et al. manuscript, 2017), RP49 was used as a control to directly compare fold change measured in Reads Per Million (RPM).

Discussion

From our data, we propose a model for the initiation of PIWI-directed silencing showing the role of various factors (Figure 4C). A group of direct factors, including SPN-E and SILE, help PIWI initiate the destabilization of the nascent or maturing transcript targeted by piRNAs. The proximity of the piRNA-targeted element to the transcript promoter affects the strength of silencing, with stronger silencing near the promoter. The primary piRNA biogenesis factors ARMI and ZUCC allow for the formation of piRNAs that PIWI proteins form a complex with. The loss of the PAF1/RTF1 sub-complex promotes silencing by stopping nascent RNA release by

RNA Pol II. *Drosophila* PAF1 complex factors HYR and CTR9 are associated with RNA Pol II and work together upstream to promote PIWI-directed silencing. Transcriptional regulator CDK9 promotes paused RNA Pol II to release nascent transcripts and PIWI to engage with piRNA. DRE4 and CSTF64 promote general transcript elongation and termination, respectively, and loss of these factors enhances PIWI-directed silencing. Before heterochromatin formation, nascent RNA could be destabilized or RNA Pol II elongation could be stalled by the PIWI/piRNA complex and PIWI-associated factors. Chromatin-associated factors, like HP1a and H1, may have a downstream effect after an initial PIWI-triggered event on a target transcript to compact the chromatin and fully reinforce silencing.

The initiating steps of PIWI-directed silencing involve a variety of Piwi-pathway specific factors and transcriptional regulators. Our piRNA reporter assays can measure 2-8 fold de-repression from the initiation of silencing in comparison to 100-1000 fold de-repression on endogenous TEs due to absolute TE silencing by many mechanisms (Sienski et al., 2015; Y. Yu et al., 2015). We found that transcriptional regulators, such as CSTF64, DRE4, and CDK9, impact piRNA-targeted elements close to the promoter, but PAF1's role in animals in the regulation of transcription elongation, transcription termination, and promoting paused RNA Pol II release still needs to be researched (Chen et al., 2015; Yang et al., 2016; M. Yu et al., 2015). In the future, we hope to apply our reporter assays to Piwi pathway-expressing *Drosophila* cell lines, such as the WRR-1 cell line and the OSC-delta-l(3)mbt cell line.

Future Directions

We wanted to apply the functional assay to newly reported *Drosophila* cell lines that express Piwi pathway genes. The WRR-1 cell line has a male genome, in contrast to the female genome of OSC/OSS cells, and only expresses the primary piRNA biogenesis pathway. This cell line was made by activating oncogenic Ras and negating the Hippo tumor suppressor pathway, leading to a reactivation of the primary piRNA pathway in *Drosophila* somatic cells (Fagegaltier et al., 2016).

Luciferase assay results showed that two trials of PIWI knockdown in WRR-1 cells resulted in de-repression of the Flam 5' intron and Flam 3' UTR reporters (Figures A and B). This suggests that PIWI has a direct role in silencing the piRNA-targeting element in WRR-1 cells. In the first trial, there was not a big difference between PIWI knockdown's effects on the Flam 5' intron reporter compared to the Flam 3' UTR reporter (Figure A). However, in the second trial, there was greater depression of the Flam 5' intron reporter compared to the Flam 3' UTR, which is a similar pattern to knockdown of factors in OSS cells (Figure B). At least one more PIWI knockdown replicate needs to be done to confirm these results. In the future, the same known Piwi-pathway-specific factors, Piwi-pathway-linked chromatin-associated factors, and transcriptional regulators tested on the OSS cells can be used on WRR-1 cells to find out what the role of these factors are in the initiation of Piwi-directed silencing. The role of these factors in the male genome of WRR-1 cells may be different than in the female genome of OSC/ OSS cells.

We also wanted to apply the reporter assays to the OSC-delta-I(3)mbt (OSCΔmbt) cell line, which expresses secondary ping-pong piRNAs. OSCΔmbt cells have the mbt locus deleted

and express the entire secondary piRNA ping-pong pathway, which includes AGO3 and AUB, in addition to the primary piRNA pathway that involves PIWI (Sumiyoshi et al., 2016).

Western blots detected the presence of PIWI, Tubulin, and AGO3 in OSCΔmbt cells (Figure C), which indicates that both primary and secondary piRNA pathways are present in this cell line. The band for AGO3 in the OSCΔmbt cells is not as thick as the bands for PIWI and Tubulin, so a replicate of this result would confirm that AGO3 is actually expressed. Previous trials of western blots were unable to show a band for AUB on the film, so a repeat of a test for AUB is needed to confirm the presence of this protein in the secondary piRNA ping-pong pathway. Also, a better control sample of female fly ovary lysate is needed to make sure that the primary and secondary antibodies are functional in case our sample of OSCΔmbt cells actually does not express these proteins.

In the future, the same factors tested on the OSS cells can be tested on OSCΔmbt cells to figure out what the role of these factors are in the initiation of Piwi-directed silencing. This would elucidate how the primary and secondary piRNA biogenesis pathways interact with each other and which pathway is more important for initiating Piwi-directed silencing.

Figures and Tables

Figure 1A from *Clark et al. manuscript*. The experimental setup for the piRNA-tarted reporter assay.

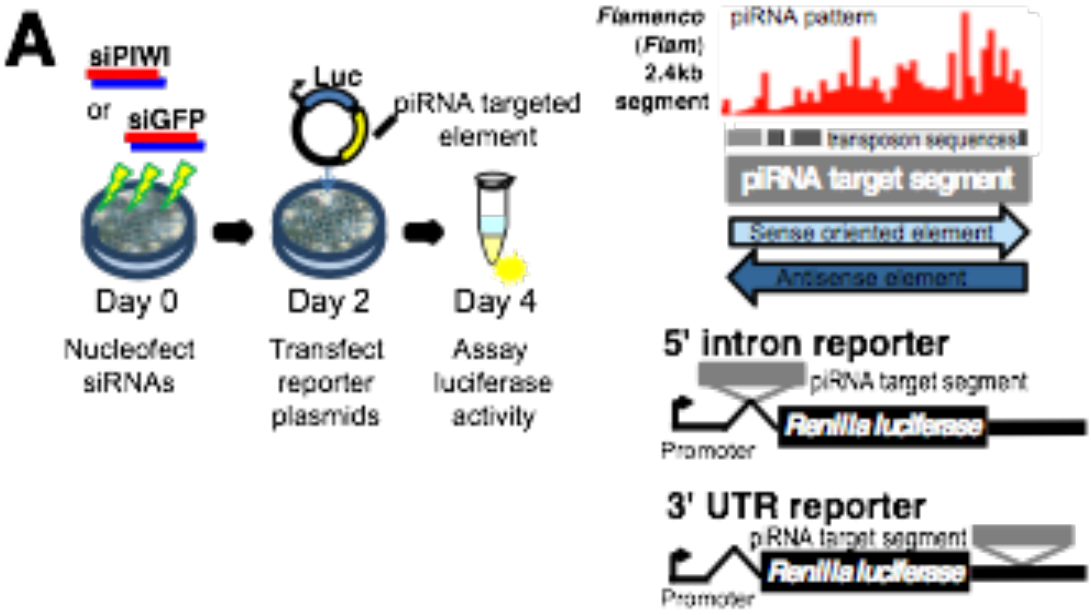
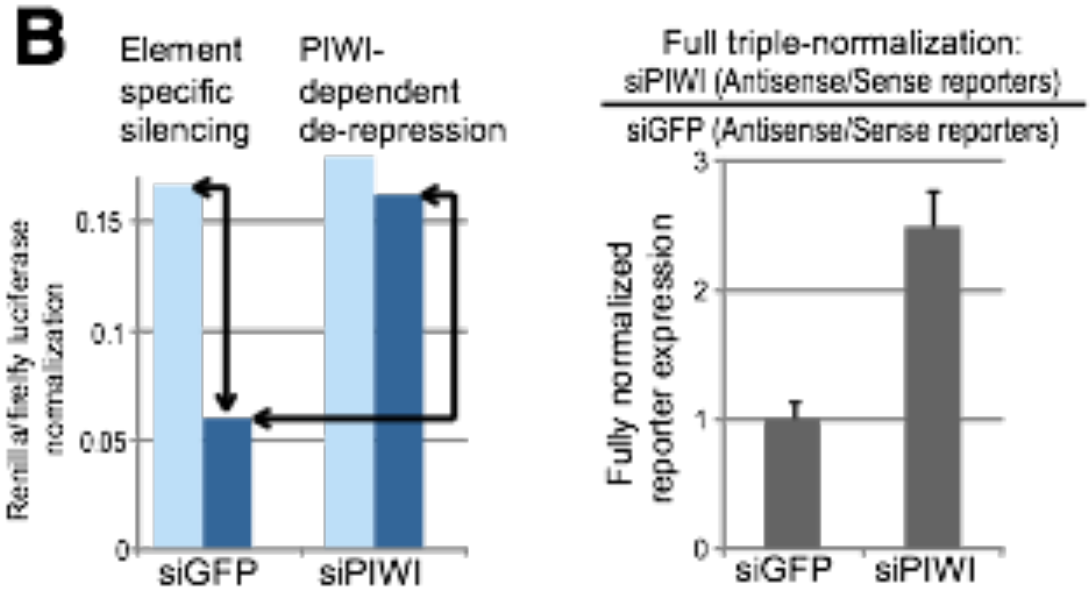


Figure 1B from *Clark et al. manuscript*. The triple normalization procedure to calculate luciferase expression.


B
Element specific silencing
PIWI-dependent de-repression
Renilla/firefly luciferase normalization
0.15
0.1
0.05
0
siGFP
siPIWI
Full triple-normalization:
siPIWI (Antisense/Sense reporters)
siGFP (Antisense/Sense reporters)
Fully normalized reporter expression
3
2
1
0
siGFP
siPIWI

*My contribution is marked with the blue line.

Figure 1C from *Clark et al. manuscript*. Piwi-pathway-specific Factors on Flam Reporters.*

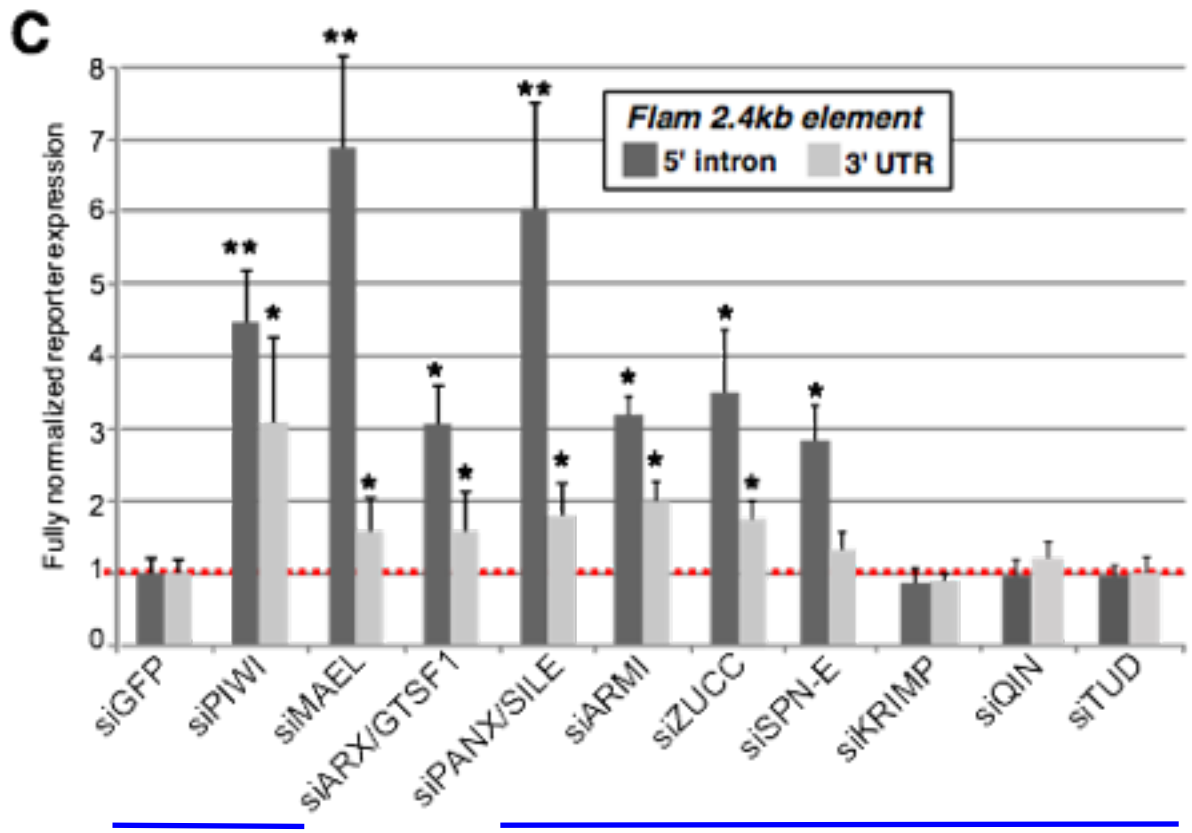


Figure S1C from *Clark et al. manuscript.* Piwi-pathway-specific Factors on TJ Reporter.*

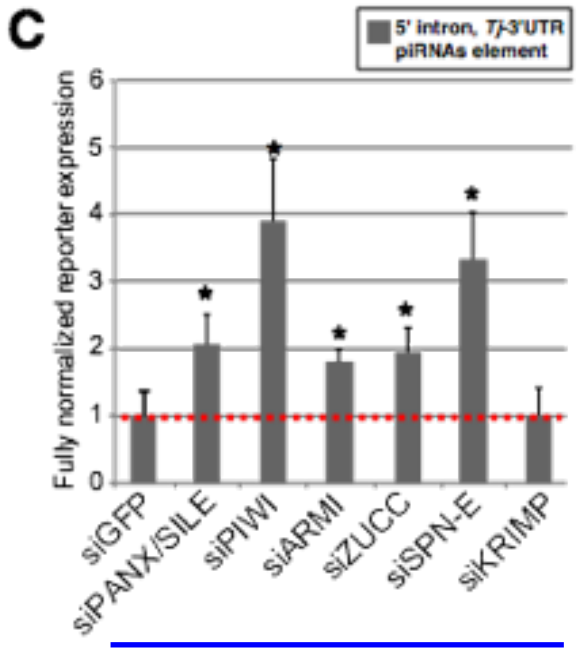


Figure 1D from *Clark et al. manuscript*. Chromatin-associated factors on Flam reporters.*

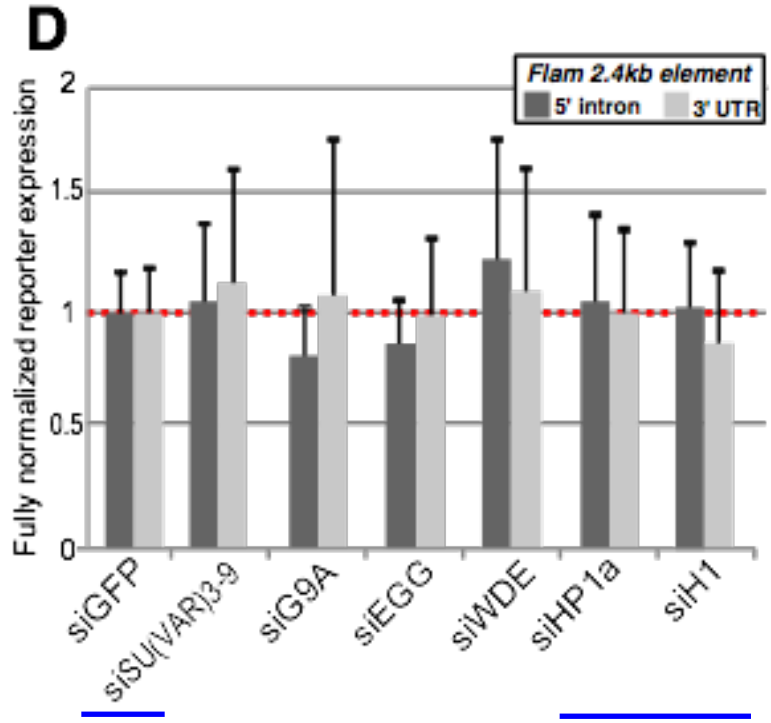


Figure 1E from *Clark et al. manuscript*. *Drosophila* PAF1 complex factors on Flam reporters.*

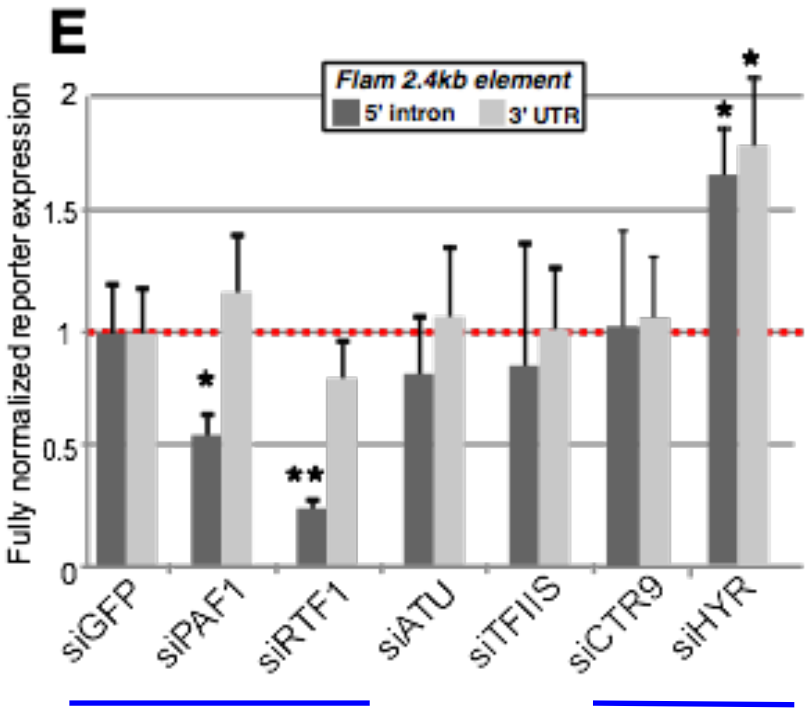


Figure S8A from *Clark et al. manuscript*. PIWI knockdown on *roo-internal* and *blood-internal* reporters.*

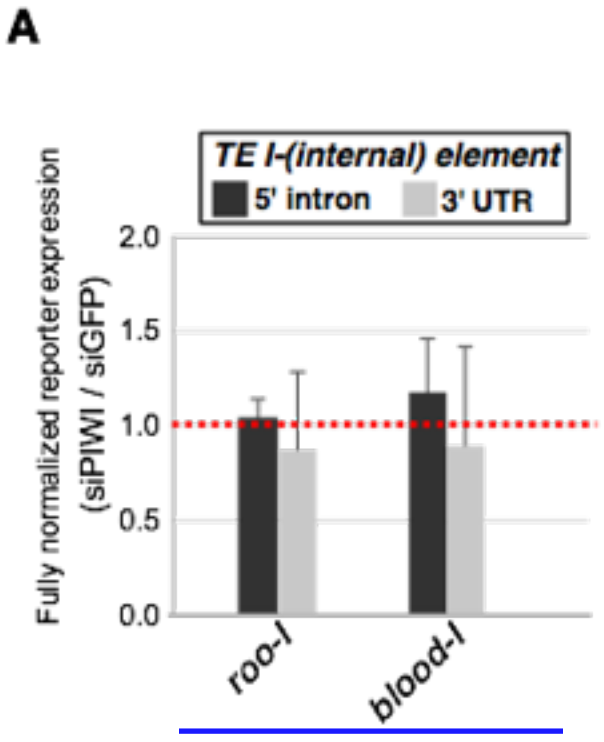
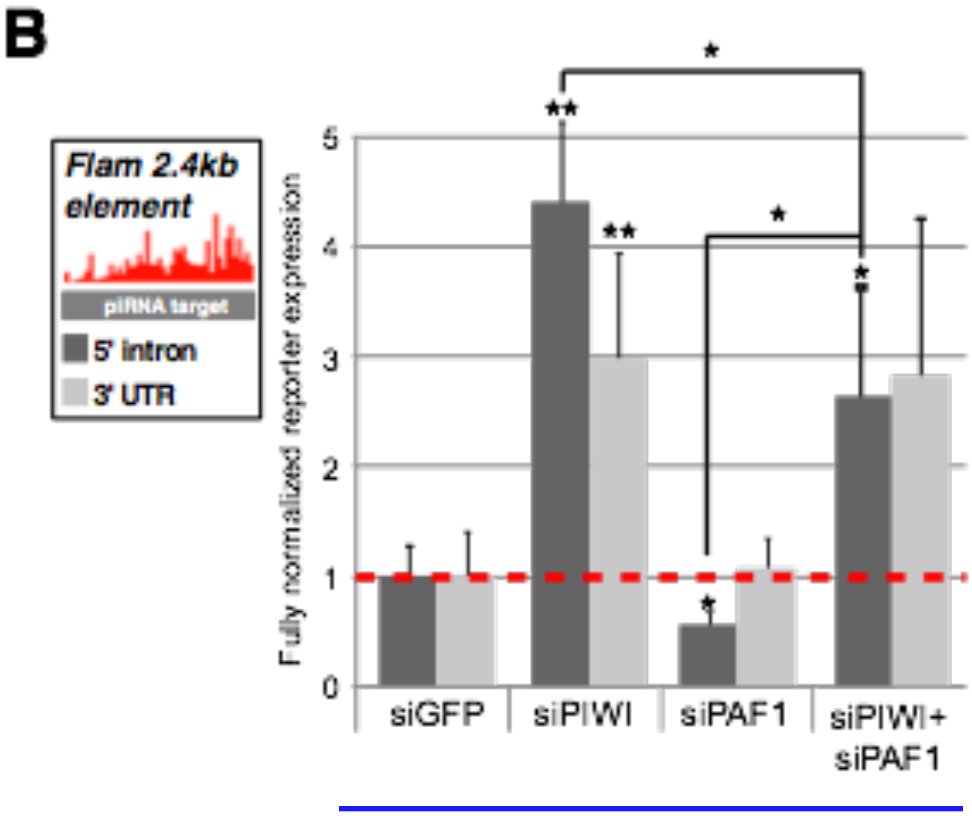


Figure 4B from *Clark et al. manuscript*. PIWI, PAF1, and PIWI+PAF1 knockdowns on *Flam* and *mdg1* reporters.*



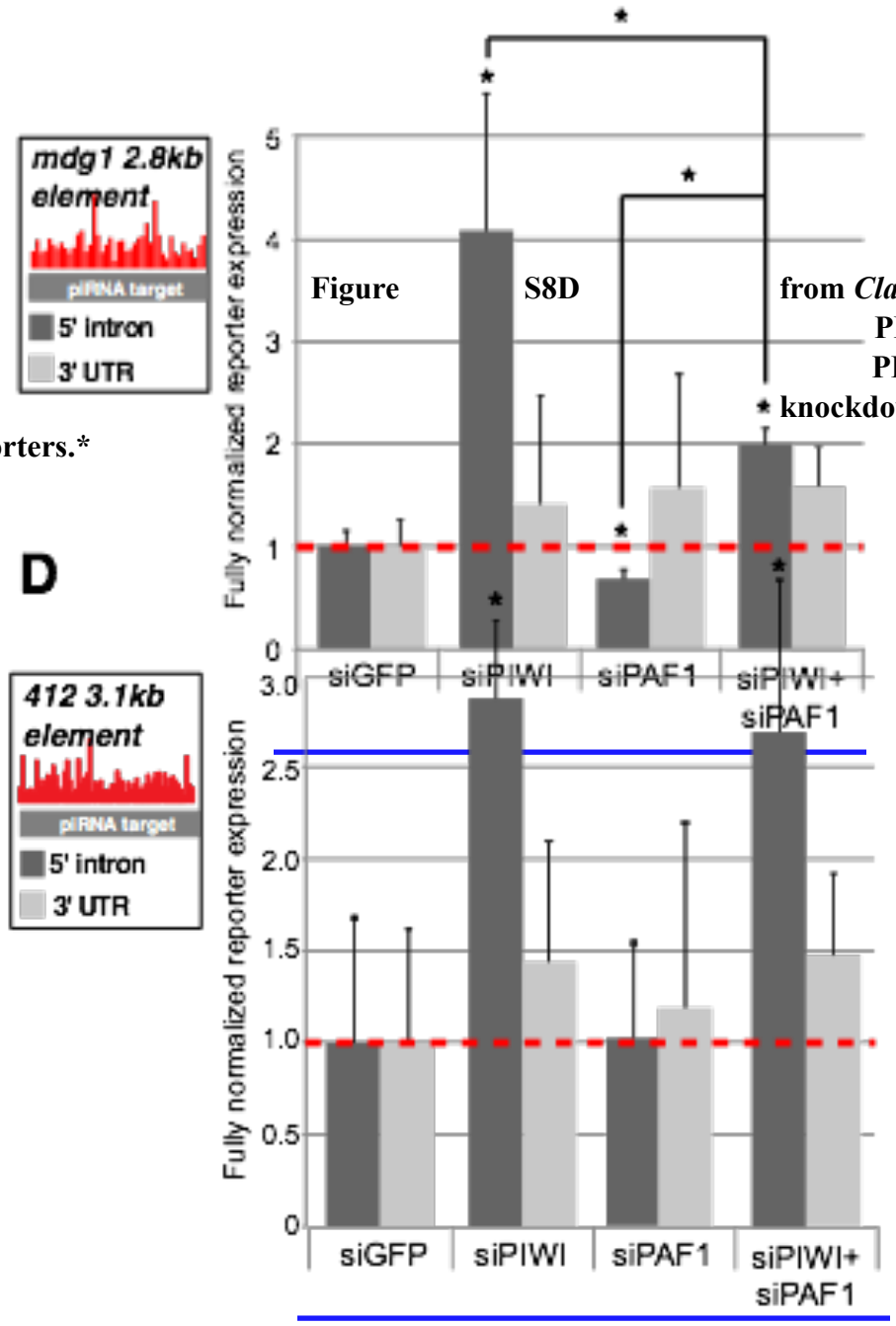


Figure S8D from *Clark et al. manuscript.* PIWI, PAF1, and PIWI+PAF1 knockdowns on *412* reporters.*

Figure S2A from *Clark et al. manuscript*. Global transcriptional regulators on control Firefly luciferase expression.*

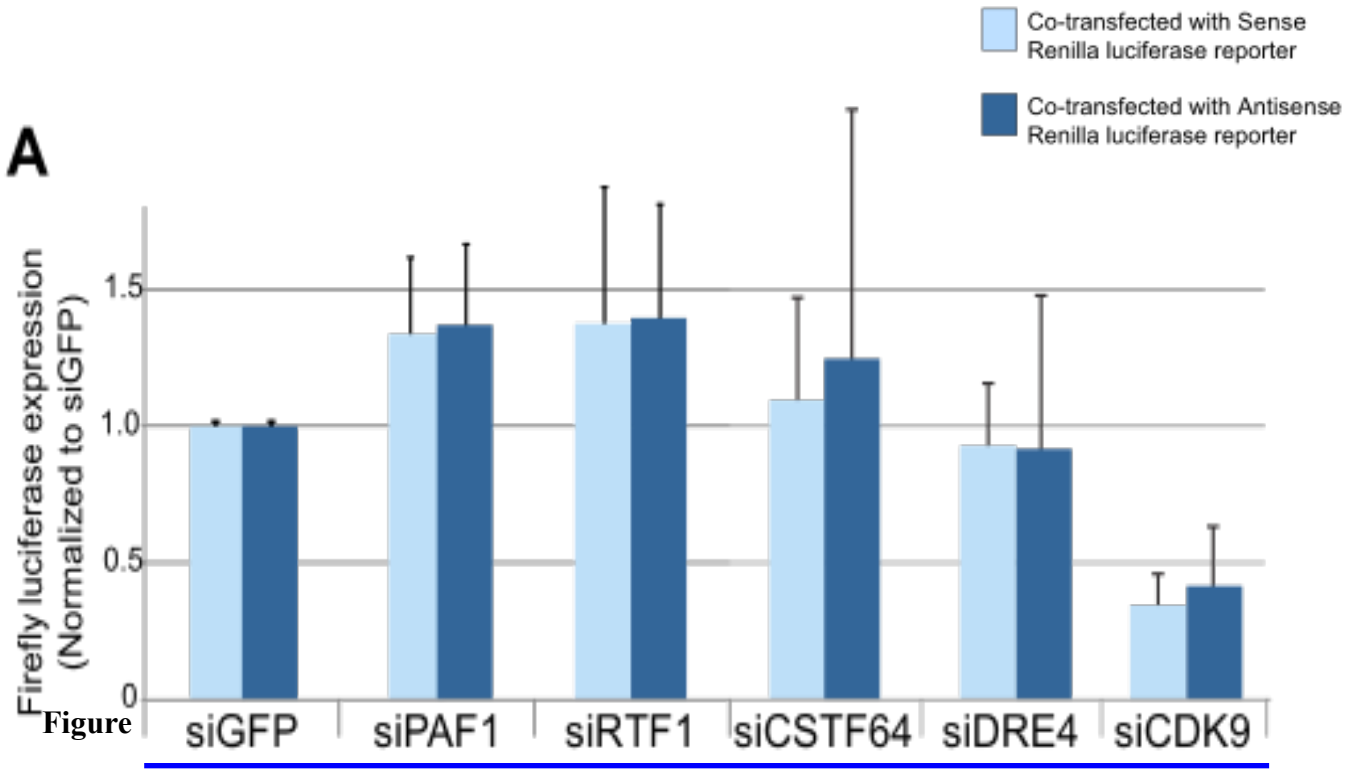


Figure 2D from *Clark et al. manuscript*. Transcriptional regulators on Flam reporters.*

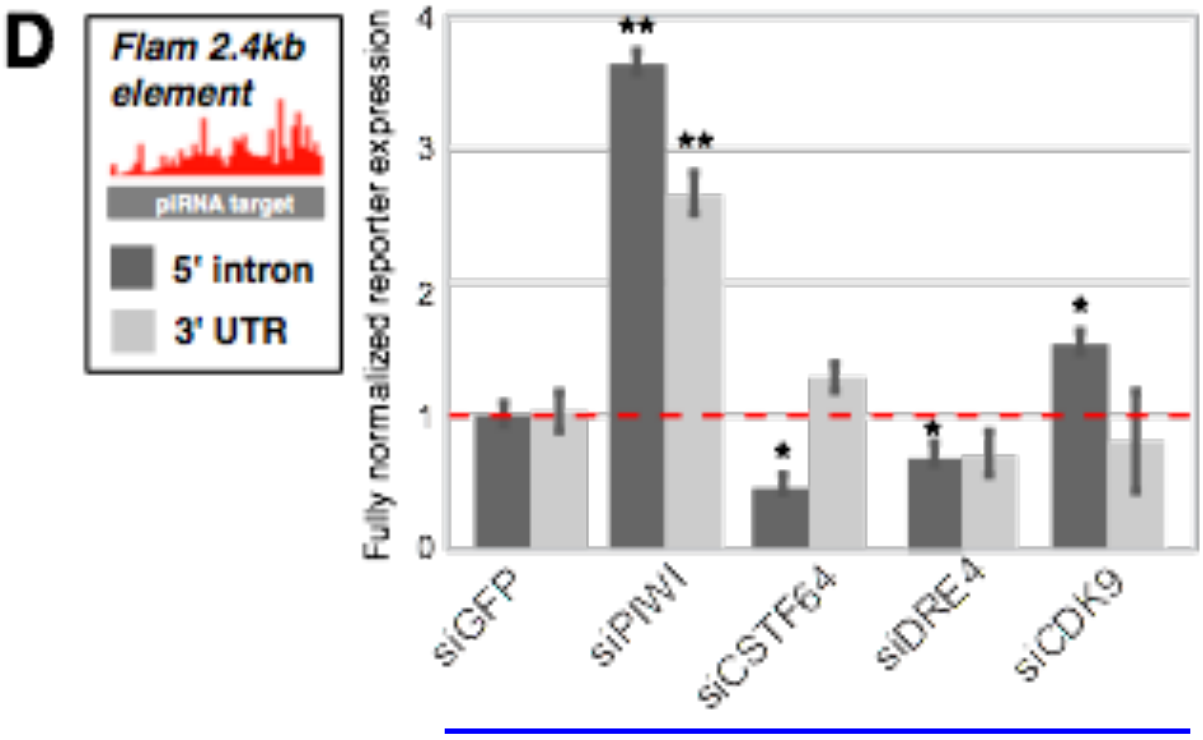


Figure S1A from *Clark et al. manuscript*. Knockdown of the factors were efficient.*

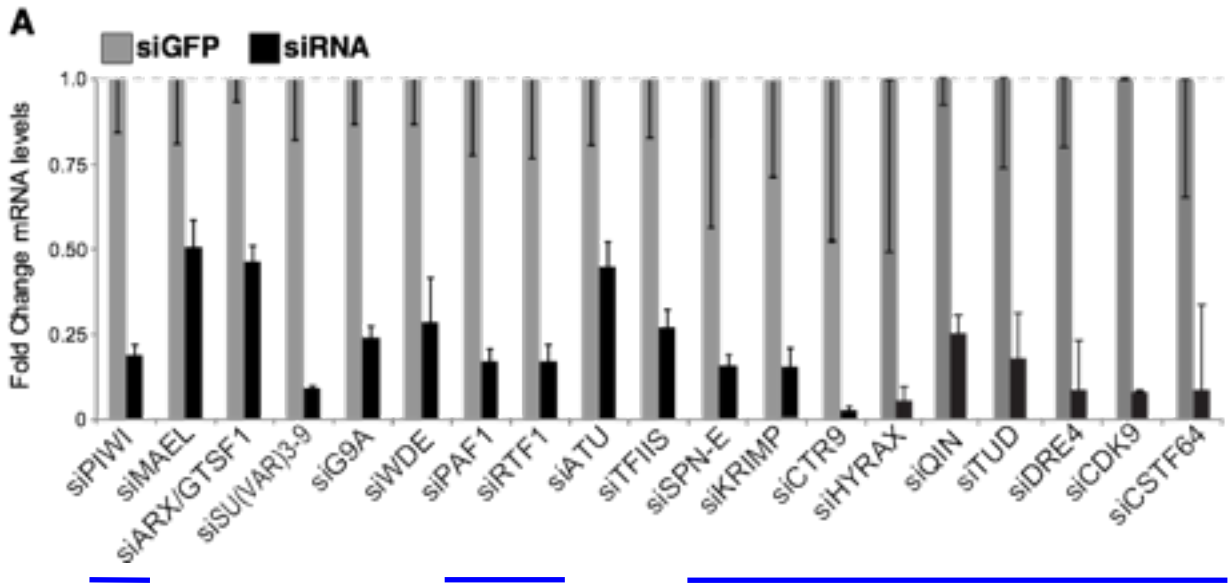


Figure 4C from *Clark et al. manuscript* revised. Model for the initiation of PIWI-directed silencing.

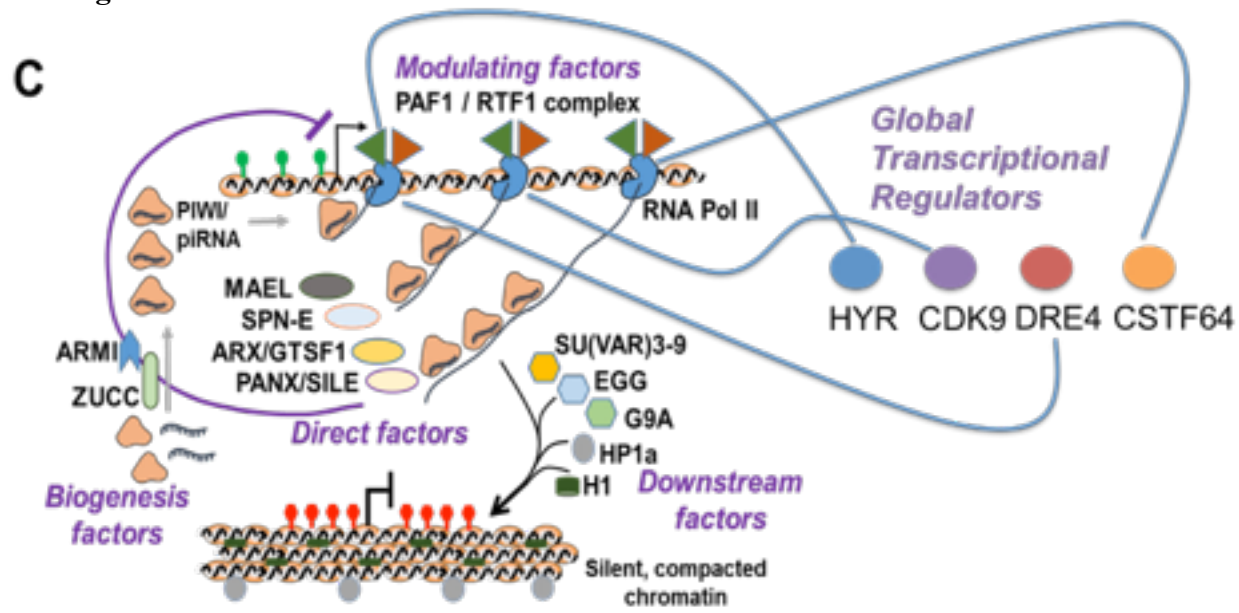


Figure A. Trial 1 of PIWI Knockdown in WRR-1 cells.

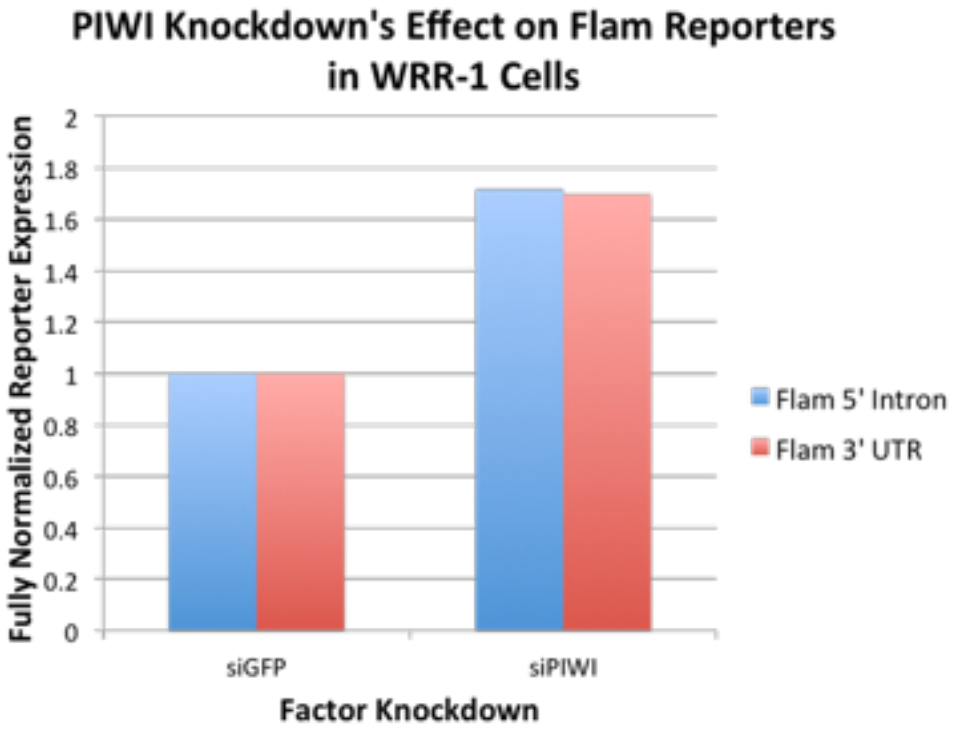


Figure B. Trial 2 of PIWI Knockdown in WRR-1 cells.

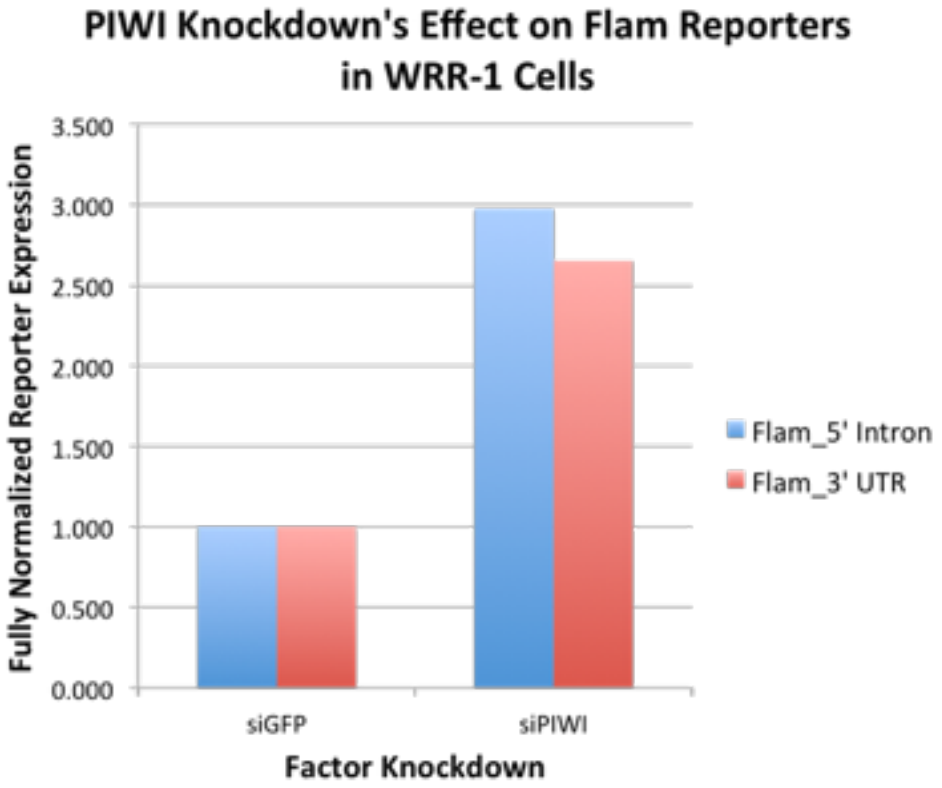


Figure C. Western blots of PIWI, α-Tubulin, and AGO3 in OSCΔmbt cells and female fly abdomen/ovary.

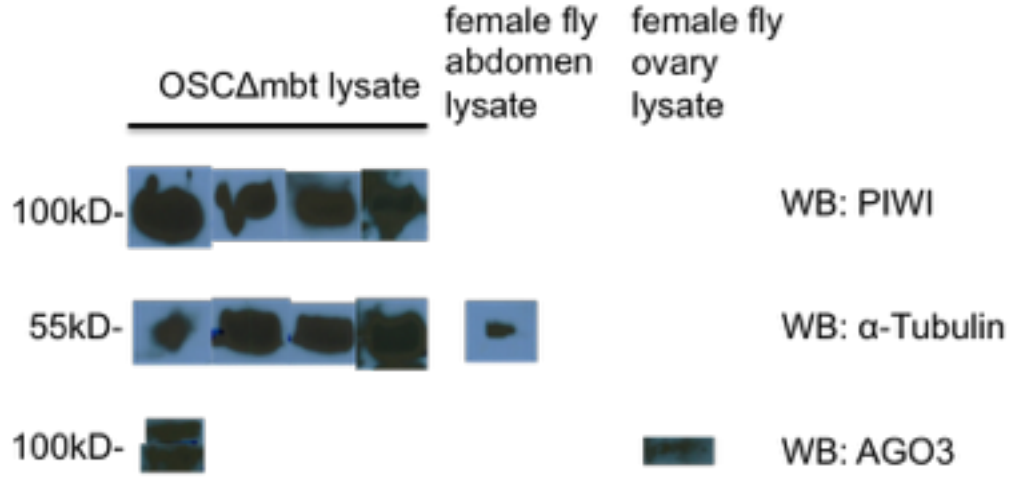


Table S1 adapted from *Clark et al. manuscript*. Primers and siRNAs used in this project.

	Gene name	Forward	Reverse
	RP49	ATGACCATCCGCCCAGCATAC	CTGCATGAGCAGGACCTCCAG

Primers for qPCR	Piwi	TCGTACCCAATGATAACGCCGAAAG	AGTCCGGACAAGGGTAGTTCGATCA
	Paf1	GCATCTACTACAACGAGCTAG	ACGATGCTCCATGCTGTCC
	Rtf1	GGTGCCAAGAAGGACGAAG	GGAGACTGTTTCAGCTGGT
	Krimp	TGTTTTCGTGCACTCGAATC	ATTTAATCAACTGTGATATCG
	SpnE	AGGAAGATGTGGGACACCAG	TGATGGAGCTCTCAGCAATG
	Ctr9	TCAAGGATGAGAAATCTACTC	GGAGATGTAGTCATCTTTACG
	Hyrax/Cdc73	GTCCATCGAAGCGAACATCT	ACTCCTGTTGCGAGAGCTTG
	Tudor	GCAGCACTTGCTCCTACACA	TCGTCATAGGTGCAAAGGAA
	Qin	AGCCCTTTGATCCCAAGTTT	CAATCGTAGGAGGAGCTGGA
	Dre4/Spt16	CAGATGACAAACCACGCAAC	GTCCCGGCTGTTGTACTTGT
	Cdk9/pTEFb	CTACTGCGACGAGAGCAACA	CACCACGTTCTCGTGCTTTA
	CstF64	GATAAGGCGCAGGAACAAAG	GCACTTAAAGCCGTCTCCTG

	siRNA Name	Passenger strand	Guide strand
siRNAs	siGFP	GGCAAGCUGACCCUGAAGUtt	ACUUCAGGGUCAGCUUGCCtt
	siPIWI	GCUCCCAGGCGUGAAGGUGtt	CACCUUCACGCCUGGGAGCtt
	siPANX/SILE	CGGCUACGCUGUACAAGAAtt	UUCUUGUACAGCGUAGCCGtt
	siZUCC	GCAUUGCCGUCAGCACUGUTT	ACAGUGCUGACGGCAAUGCTT
	siARMI	UAAACUUAGCUUGACAGCGTT	CGCUGUCAAGCUAAGUUUATT
	siSPN-E	CUCGCGGCAUGUAAAGAUUTT	AAUCUUUACAUGCCGCGAGTT
	siKRIMP	GGCCAACAAUCCAGUACGUTT	ACGUACUGGAUUGUUGGCCTT
	siHP1A	GAUCUUGGGUGCCUCCGACtt	GUCGGAGGCACCCAAGAUCtt
	siH1	GAAAGAUCCAGAUGCACCCtt	GGGUGCAUCUGGAUCUUUCtt
	siPAF1	GCCACCGCUUCGUGCAGUAtt	UACUGCACGAAGCGGUGGCtt
	siRTF1	GGACGAAGAUGACAUGCAAtt	UUGCAUGUCAUCUUCGUCCtt
	siCTR9/CG2469	GCAUGAAGAAGUUCUACAATT	UUGUAGAACUUCUUCAUGCTT
	siHyrax/Cdc73	GGACAUUAUCAGCAGAGAATT	UUCUCUGCUGAUAAUGUCCTT
	siTudor	CGAUCAGUUUGAAAUGUUAtt	UAACAUUUCAAACUGAUCGtT
	siQin	GGAGGAUUUCUAUGUUCAAtt	UUGAACAUAGAAAUCCUCCtt
	siDre4/Spt16	AGGAAGAUGUGGACGACAAtt	UUGUCGUCCACAUCUUCCUtt
	siCdk9/pTEFb	CGAUGUCCCUGAUGGAGAAtt	UUCUCCAUCAGGGACAUCGtt
	siCstF64	GCCAGAUGCUAAUGCUCAAtt	UUGAGCAUUAGCAUCUGGCtt

Materials and Methods

OSS cells cultivation

M3 media with additional 10% heat inactivated FBS, 1x fly extract, 10 μg/mL insulin, 0.6 mg/mL glutathione according to (Niki, 2009) procedures were used to cultivate the OSS cells (Clark et al., 2017).

Luciferase reporter assays

Each factor being studied was knocked down with electroporation of 500 pmol siRNAs into one-quarter of a T75 flask of OSS cells using Amaxa Kit V and program D013 on Amaxa Nucleofector II device. 48 hours after siRNA electroporation, reporter vectors were transfected into the cells using Fugene HD (1 ug:1 ug, FugeneHD:Luciferase plasmids, equal amount of Firefly and *Renilla* reporter vectors; for 2 cm^2 of cells). 48 hours after reporter transfection, cell lysis was performed and Dual Luciferase Assay Kit (Promega) was used to measure *Renilla* and Firefly luciferases activities. A full triple normalization procedure was performed with *Renilla* (RN) luciferase signal normalized against Firefly (FF) luciferase signal, then RN/FF ratios were normalized siRNA/siGFP, and finally these values were normalized Antisense/Sense (Clark et al., 2017).

Reporter gene cloning

Reporter constructs used in (Post et al., 2014) set the basis for the creation of the *Renilla* and Firefly luciferase reporters. Restriction enzyme sites were introduced into the *Renilla* luciferase coding sequence using site-directed mutagenesis. Table S1 lists the oligonucleotides used for amplifying the *Flam*, Tj 3' UTR, *mdg1*, *roo*, *412*, and *blood* elements. *Renilla* luciferase

reporters with XmaI, KpnI, KasI, and ApaI sites were cloned into the 5' UTR, 5' intron, 3' intron, and 3' UTR of luciferase pFREN vector sites, respectively (Clark et al., 2017).

siRNA design

The Flyrnai.org website was used to find the sense oligo sequence for short hairpins in the desired gene. The UCSC Genome Browser website was used to find the most upstream RNA sequence that is unique. We find a 19-nucleotide RNA sequence that contains a greater number of C-G pairs and add two DNA t's at the end of the sequence to get the guide strand sequence. The passenger strand is the complementary sequence.

RNA extraction and RT-PCR analysis

Extraction of total RNA from OSS cells was performed with TRI-reagent RT (Molecular Research Center, Inc protocol). First strand cDNA synthesis (NEB protocol) was performed with 1 μg of RNA, ProtoScript II reverse transcriptase, and random primer. The standard protocol for regular PCR with dNTPs, Taq polymerase, the designed qPCR primers, and the cDNA was performed to confirmed that the primers were effective and that there was no RNA contamination in the cDNA sample. The standard qPCR protocol (Promega protocol) with the GoTaq qPcR Master Mix, the designed qPCR primers, and cDNA template was performed. The 2ΔΔCt method was used to calculate the relative changes in gene expression. Gene expression was normalized against Rp49 mRNA (Clark et al., 2017).

qPCR primer design

The FlyBase website was used to obtain the gene sequence of the specific factor we want to analyze. The Primer3 website was used to create forward and reverse primer DNA sequences that

span an intron. We picked primers that would show after regular PCR an mRNA length that is at least 150bp shorter than the DNA length that includes the intron, so that we can distinguish between the mRNA and DNA fragments on an agarose gel. We aimed the mRNA PCR length to be within the 100 to 500 bp range. Table S1 lists the oligonucleotides that we designed for qPCR amplification of specific genes of factors we studied.

Western blots and antibodies

The standard protocols for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were used. Primary antibodies to Tubulin, PIWI, AUB, and AGO3 were used 1:1,000 for mouse antibodies from Siomi Lab, rabbit antibodies from Hannon Lab, and mouse anti-α-Tubulin (E7A) purchased from the Developmental Studies Hybridoma Bank. Primary antibodies to AUB and AGO3 from Wang Lab were used 1:5,000. The secondary antibodies, α-mouse IgG, and α-rabbit IgG, were used 1:10,000 or 1:50,000.

Acknowledgements and contributions

I am thankful for Professor Lau's constant guidance as my faculty research sponsor, whom I have worked under for three years. I would not have a senior thesis without him. I appreciate Professor Lau and Josef Clark for writing the Clark et al. manuscript that served as a guide for this thesis. I thank Nachen Yang for helping me perform experiments and for always being willing to help me in the lab. I also thank Josef Clark for helping me learn many lab techniques in Lau lab.

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