Cell Reports

Piwi Nuclear Localization and Its Regulatory Mechanism in *Drosophila* **Ovarian Somatic Cells**

Graphical Abstract



Highlights

- Importinα plays a pivotal role in Piwi nuclear localization in OSCs
- Piwi has a classical bipartite nuclear localization signal at the N terminus
- piRNA loading triggers conformational change in Piwi
- Piwi autoregulates its nuclear localization by exposing NLS to environment

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In Brief

Piwi represses transposons transcriptionally to maintain genome integrity. Yashiro et al. show that Importin α plays a pivotal role in Piwi nuclear localization and that Piwi has a bipartite nuclear localization signal at the N terminus. Piwi autoregulates its nuclear localization by exposing the nuclear localization signal to Imp α upon piRNA loading.





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Piwi Nuclear Localization and Its Regulatory Mechanism in *Drosophila* Ovarian Somatic Cells

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SUMMARY

In Drosophila ovarian somatic cells (OSCs), Piwi represses transposons transcriptionally to maintain genome integrity. Piwi nuclear localization requires the N terminus and PIWI-interacting RNA (piRNA) loading of Piwi. However, the underlying mechanism remains unknown. Here, we show that Importina (Impa) plays a pivotal role in Piwi nuclear localization and that Piwi has a bipartite nuclear localization signal (NLS). Imp α 2 and Imp α 3 are highly expressed in OSCs, whereas Impa1 is the least expressed. Loss of Impa2 or Impa3 forces Piwi to be cytoplasmic, which is rectified by overexpression of any Impa members. Extension of Piwi-NLS with an additional Piwi-NLS leads Piwi to be imported to the nucleus in a piRNA-independent manner, whereas replacement of Piwi-NLS with SV40-NLS fails. Limited proteolysis analysis suggests that piRNA loading onto Piwi triggers conformational change, exposing the N terminus to the environment. These results suggest that Piwi autoregulates its nuclear localization by exposing the NLS to $Imp\alpha$ upon piRNA loading.

INTRODUCTION

PIWI proteins are a subclade of the Argonaute family of proteins enriched in the gonads, where PIWI binds PIWI-interacting RNAs (piRNAs) to assemble piRNA-induced silencing complexes (piRISCs) to repress transposons to maintain genome integrity (Aravin et al., 2007; Ghildiyal and Zamore, 2009; Iwasaki et al., 2015; Juliano et al., 2011; Malone and Hannon, 2009; Yamashiro and Siomi, 2018). Loss-of-function mutations of PIWI proteins and piRNA factors essential in the pathway cause defects in the genome surveillance system, leading to transposon derepression, failures in gametogenesis, and infertility (Khurana and Theurkauf, 2010).

Drosophila expresses three genes encoding members of the PIWI protein family, Piwi, Aubergine (Aub), and Ago3 in both ovaries and testes (Cox et al., 1998; Li et al., 2009; Nagao et al., 2011; Schmidt et al., 1999). In the ovaries, Aub and Ago3 are expressed specifically in germ cells, where they silence transposons post-transcriptionally by cleaving their transcripts using their endonuclease activity known as Slicer (Brennecke et al., 2007; Gunawardane et al., 2007; Li et al., 2009; Nishida et al., 2007). Aub- and Ago3-bound piRNAs are mainly antisense and sense to transposon-coding sequences, respectively, and Aub and Ago3 reciprocally cleave transposon mRNAs and antisense transcripts, respectively. The resultant RNA fragments are further processed to mature piRNAs; thereby Aub/Ago3-Slicer-dependent transposon silencing in germ cells is considered to be coupled with piRNA biogenesis.

Piwi is expressed in both germ cells and ovarian somatic cells (OSCs), and in both cell types, Piwi is localized to the nucleus and silences transposons transcriptionally by inducing heterochromatinization at the target loci with co-factors such as Panoramix/Silencio, Asterix/GTSF1, Maelstrom (Mael), and linker histone H1 (Czech et al., 2013; Dönertas et al., 2013; Handler et al., 2013; Iwasaki et al., 2016; Kalmykova et al., 2005; Le Thomas et al., 2013; Muerdter et al., 2013; Ohtani et al., 2013; Saito et al., 2006, 2010; Sienski et al., 2012, 2015; Yu et al., 2015). Piwi nuclear localization requires both the N-terminal region of Piwi and piRNA loading onto Piwi, which may be particularly obvious in OSCs (Klenov et al., 2011; Saito et al., 2009, 2010). A proportion of Piwi in germ (nurse) cells may be translocated to the nucleus even under conditions where the piRNA binding activity was impeded by mutations (Le Thomas et al., 2013). This layered, "gatekeeping" mechanism ensures PiwipiRISC-mediated nuclear transposon silencing, because piRNA-free Piwi in the nucleus would be useless with no ability to find its targets (Ishizu et al., 2011). However, how piRNA loading-dependent Piwi nuclear localization is regulated mechanistically is still unclear. The nuclear import machinery required for Piwi nuclear import also remains unclear.

The N-terminal region of Piwi, which has been shown to be necessary for its nuclear import, is rich in basic residues, a characteristic typical of classical nuclear localization signals (NLSs) whose nuclear import is mediated by an Importin α/β (Imp α/β) heterodimer (Görlich, 1998; Stewart, 2007). The NLS of SV40 large T antigen (SV40-NLS) (PKKKRKV) (Kalderon et al., 1984) and that of *Xenopus laevis* nucleoplasmin (NP-NLS) (KRPAATKKAGQAKKKK) (Robbins et al., 1991) are the

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prototypic monopartite and bipartite NLSs, respectively (Goldfarb et al., 2004; Stewart, 2007). Monopartite NLSs consist of a single stretch of basic residues, while bipartite NLSs contain two stretches of basic residues with a linker region between them. In the Imp α/β pathway, the adaptor molecule Imp α protein first binds cargos in the cytoplasm through their NLSs. Then, Imp β associates with the heterodimer through the Imp β binding (IBB) domain of Imp α to assemble a NLS-cargo/Imp α /Imp β trimeric complex, which facilitates the nucleocytoplasmic translocation of the complex across the nuclear pores located on the nuclear envelope. Upon transport, RanGTP in the nucleus binds and disassembles the complex, releasing the cargos to the nucleoplasm, where they exert their nuclear functions (Görlich, 1998; Stewart, 2007).

Members of the Impa family of proteins can be divided into three phylogenetic clades, $\alpha 1$, $\alpha 2$, and $\alpha 3$ (Mason et al., 2009; Pumroy and Cingolani, 2015). Drosophila contains a single representative of each clade, Impa1, Impa2, and Impa3 (Küssel and Frasch, 1995; Goldfarb et al., 2004; Török et al., 1995; Mason et al., 2002, 2003). All Impa proteins contain one IBB domain at the N-terminal end, and the rest of the sequence contains 10 Armadillo (ARM) repeats, which were originally identified in the protein encoded by the Drosophila segment polarity gene armadillo (Riggleman et al., 1989). While the IBB domain binds Impß during nuclear import through the nuclear pore, the ARM repeats assemble a scaffold structure that can accommodate monopartite or bipartite NLSs (Conti et al., 1998; Fontes et al., 2003). When the ARM repeats are not bound with cargos, the IBB domain binds the ARM repeats intramolecularly, autoinhibiting nuclear import of cargo-free Impa (Kobe, 1999; Stewart, 2007). Drosophila possibly contains a fourth Impa member, Impa4, which, however, lacks the IBB domain and is testis specific (Phadnis et al., 2012). A single $Imp\beta$ gene known as ketel was found in the Drosophila genome (Lippai et al., 2000). Drosophila Imp α 1 null flies developed to adulthood but showed severe defects in gametogenesis and infertility (Ratan et al., 2008). Similarly, homozygous $Imp\alpha 2$ mutants developed normally to adulthood, but mutant females, and not males, were completely sterile (Mason et al., 2002). Impa3 mutants showed embryonic lethality (Mason et al., 2003).

On the basis of all these earlier findings, we speculated that Impa may be responsible for Piwi nuclear localization. To test this, we first examined the levels of Impa proteins in cultured OSCs. To this end, we produced monoclonal antibodies for each Impa member and performed western blotting, which revealed that Impa2 and Impa3 were fairly highly expressed in OSCs, whereas Impa1 was the least expressed. Loss of Impa2 or Impa3 by RNAi treatment disturbed nuclear localization of Piwi. However, nuclear localization of SV40-NLS-cargo was not affected, suggesting that Piwi-NLS may have lower nuclear localization efficiency than SV40-NLS. We defined the N-terminal 36 residues of Piwi as a bipartite NLS. Interestingly, bipartite NP-NLS-cargo behaved similarly to Piwi in OSCs lacking Impa2 or Impα3. Impα proteins may prioritize monopartite NLSs over bipartite NLSs, at least in OSCs. Replacement of Piwi-NLS with SV40-NLS failed to obviate the requirement of piRNA loading in Piwi nuclear localization. However, Piwi was imported to the nucleus piRNA independently upon duplication of the Piwi-NLS. Substitution of the second NLS with a peptide that lacks the NLS activity little changed the outcome. Limited proteolysis assays showed that about 90 residues in the N-terminal end of Piwi were inaccessible when Piwi was free from piRNAs, but piRNA loading made the region accessible to the environment, most likely through a conformational change in Piwi. piRNA loading increased the ability of Piwi to interact with Impa2 and Impa3. Thus, Piwi autoregulates its nuclear localization by exposing the NLS to Impa upon piRNA loading. Addition of Piwi-NLS caused the otherwise cytoplasmic Aub to be imported to the nucleus in a piRNA-dependent manner, as was Piwi. Piwi-NLS may have a unique property to be "hidden" intramolecularly prior to piRNA loading, possibly by domains conserved in PIWI members.

RESULTS

Our previous RNA-sequencing data (Sumiyoshi et al., 2016) suggested that $Imp\alpha 2$ was relatively highly expressed in OSCs, accounting for 70.7% of the total $Imp\alpha$ expression, while the expression level of $Imp\alpha 1$ was much lower (4.0%) (Figure S1A). The expression level of $Imp\alpha 3$ was between those of $Imp\alpha 2$ and $Imp\alpha 1$ (25.3%) (Figure S1A). To estimate the levels of the Imp α proteins by western blotting, we first produced monoclonal antibodies against each of the proteins (Figures S1B and S1C). Comparison of the signals of the endogenous Imp α proteins in OSCs with those of recombinant Imp α 1, Imp $\alpha 2$, and Imp $\alpha 3$ proteins in OSCs was about 5:77.5:17.5 (Figures S1A and S1D). The corresponding monoclonal antibodies detected each Imp α protein in OSCs in immunofluorescence analyses (Figure S1E).

To examine whether Impa was responsible for Piwi nuclear localization, we expressed myc-Piwi in OSCs in which each Impa had been individually depleted by RNAi. The efficiency and specificity of the RNAi treatment were determined by western blotting (Figure S2A). Immunofluorescence showed that myc-Piwi still accumulated in the nucleus in Impa1-depleted OSCs, as it did in normal OSCs (Figure 1A). We considered these results to be reasonable because $Imp\alpha 1$ was the least abundant Impa in the OSCs (Figure S1A), and Impa2 and Impa3 remained highly abundant in OSCs even after RNAi treatment (Figure S2A). However, myc-Piwi was no longer restricted in the nucleus and was detected in the cytoplasm in Impa2- and Impa3-depleted OSCs (Figures 1B and 1C). piRNA loading of myc-Piwi was barely affected by lack of Impα2 and Impα3 (Figure S2B; data not shown). These results support our original idea that Impa plays a role in Piwi nuclear localization.

Imp α 2 accounted for about 78% of total Imp α proteins in the OSCs (Figure S1A). Therefore, after depletion of Imp α 2, the amounts of Imp α 1 and Imp α 3 proteins in the OSCs may not be enough to import all cargos to the nucleus. Indeed, depletion of Imp α 2 strongly affected the cellular localization of Piwi (Figure 1B). However, after depletion of Imp α 3, the amount of Imp α 2 remained high in OSCs (Figure S2A). Therefore, the finding that Piwi nuclear localization was affected by loss of Imp α 3 (Figure 1C) was somewhat confusing. The simplest explanation for this result is that Piwi nuclear localization requires both Imp α 2 and Imp α 3 simultaneously. To test this hypothesis, we



Figure 1. Requirement of Imp α in Piwi Nuclear Localization in OSCs (A) Nuclear localization of myc-Piwi (red) was not affected by loss of Imp α 1 (green) in OSCs. EGFP siRNA was used as a control. DAPI signals (blue) show the nuclei. Scale bars, 5 μ m.

(B) Nuclear localization of myc-Piwi (red) was affected by loss of Imp α 2 (green) in OSCs. EGFP siRNA was used as a control. DAPI signals (blue) show the nuclei. Scale bars, 5 μ m.

(C) Loss of Imp α 3 (green) also affected nuclear localization of myc-Piwi (red) in OSCs. EGFP siRNA was used as a control. DAPI signals (blue) show the nuclei. Scale bars, 5 μ m.

See also Figures S1 and S2 and Table S1.

performed rescue experiments. First, Imp α 2- and Imp α 3-lacking OSCs were prepared; then each *Imp* α gene was expressed by cotransfection with FLAG-Piwi in the cells. If both Imp α 2 and Imp α 3 were simultaneously required for Piwi nuclear localization, ectopic expression of *Imp* α 3 in Imp α 2-lacking OSCs would not restore Piwi nuclear localization, while ectopic expression of *Imp* α 3 should rescue the defective phenotype. Examination revealed that not only *Imp* α 2 expression, but also *Imp* α 1 and *Imp* α 3 expression (all *Imp* α genes were RNAi-resistant) rectified Piwi nuclear localization (Figure 2A). Similarly, ectopic expression of all individual *Imp* α 3 were simultaneously required for Piwi nuclear localization of Imp α 3 (Figure 2B). Therefore, the idea that Imp α 2 and Imp α 3 were simultaneously required for Piwi nuclear localization was excluded.

An alternative explanation may be that the binding activity of Piwi-NLS to Impa2 was relatively low, and therefore Impa2 prioritized cargos other than Piwi in nuclear import when the total amount of Impa proteins was reduced, albeit only by about 18%, as a consequence of, for instance, Impa3 depletion. To check this, we examined whether the subcellular localization of SV40-NLS-cargo was affected by lack of Impa3 as well as Impa2. To this end, Mael fused with a myc-tag, and a monopartite SV40-NLS at the N- and C-terminal ends of Mael, respectively, was employed. Mael is one of the piRNA factors required in germ cells, but is dispensable for the somatic piRNA pathway in OSCs (Matsumoto et al., 2015; Sienski et al., 2012). We found that depletion of neither Impa2 nor Impa3 affected cellular localization of myc-Mael-SV40-NLS (cargo-SV40-NLS in Figures 3A and 3B), suggesting that efficiency of nuclear import of Piwi-NLS was somewhat lower than that of monopartite SV40-NLS.

Previously, we showed that a Piwi mutant Piwi- ΔN that lacked 72 amino acid residues from the N-terminal end was mislocalized to the cytoplasm, although Piwi-AN formed piRISCs with piRNAs as well as wild-type (WT) Piwi (Saito et al., 2009). The 72 residues were very basic, and arginine and lysine residues together accounted for 23.6% (17 out of 72 residues), which is a typical feature of classical NLSs bound with Impa proteins (Figure 4A). We fused the peptide consisting of the 72 residues to EGFP and found that this fusion protein was localized exclusively to the nucleus (Figure 4B). However, the peptide was too long to be defined as a monopartite or bipartite NLS. Thus, we deleted 36 N-terminal residues from Piwi and examined the cellular localization. This mutant Piwi-AN36 failed to accumulate in the nucleus (Figure 4C). We then fused the 36 residues to EGFP and found that this fusion protein, Piwi-N36-EGFP, strongly accumulated in the nucleus similar to Piwi-N72-EGFP (Figure 4C). In contrast, Piwi-N20-EGFP, which contained only 20 residues from the N-terminal end of Piwi, was found to be throughout the cells, the same as EGFP alone (Figures 4B and 4C), although the 20 residues included a basic stretch 7-RGRRR-11. Based on these results, we defined the 1-36 residues at the N-terminal end of Piwi as the Piwi-NLS.

Next, we produced a series of Piwi-NLS mutants in the context of full-length Piwi. We first mutated four residues 9-RRRP-12 of Piwi to four alanine residues. This mutant Piwi-NLS-M1 appeared mostly in the cytoplasm, but was slightly detected also in the nucleus (Saito et al., 2009) (Figure 4D). Piwi-NLS-M2, where Arg22 was mutated to alanine, was nearly exclusively



Figure 2. All Three Impα Members Restore Piwi Nuclear Localization Affected by Loss of Impα2 and Impα3 in OSCs

(A) Expression of individual Imp α members (green), but not EGFP, in Imp α 2-depleted OSCs restored nuclear localization of Piwi (red). DAPI signals (blue) show the nuclei. Scale bars, 5 µm. (B) Expression of individual Imp α proteins (green) in Imp α 3-depleted OSCs restored nuclear localization of Piwi (red). DAPI signals (blue) show the nuclei. Scale bars, 5 µm. See also Table S1.

(sDMA) (Figure S3A). sDMA is one of the post-translational modifications that are observed in the majority of PIWI proteins across species (Yamashiro and Siomi, 2018). Arginines within Piwi-NLS may also be sDMA-modified, but only weakly (Figure S3A). Piwi was undetected with Y12 and SYM11, the antibodies specifically reacting to sDMAs (Figure S3B and data not shown). These results suggest that sDMA modification of Piwi hardly impacts on Piwi nuclear localization in OSCs.

We then took NP-NLS, a representative of bipartite NLSs, and fused it to EGFP to produce NP-NLS-EGFP. In contrast to SV40-NLS-containing cargo (Figures 3A and 3B), NP-NLS-EGFP was localized exclusively to the nucleus of normal OSCs, as expected, but was detected in the cytoplasm under conditions where Impa2 or Impa3 had been depleted (NP-NLS-cargo in Figures 4E and 4F), similar to Piwi (Figures 1B and 1C). These results suggest that the sensitivity that Piwi showed to a subtle reduction in the total amount of Impa proteins in OSCs was not unique to Piwi, and that monopartite NLSs likely have higher efficiency in nuclear transport than bipartite NLSs, at least in OSCs.

Piwi nuclear localization requires not only its NLS but also piRNA loading, sug-

localized to the nucleus (Figure 4D), and Piwi-NLS-M3, where Arg34 was mutated to alanine, was found in both the nucleus and the cytoplasm nearly evenly. However, Piwi-NLS-M4 and Piwi-NLS-M5, where Lys31 and Lys31/Arg34 were mutated to alanine residues, respectively, were not localized to the nucleus (Figure 4D). Piwi-NLS-M1 and Piwi-NLS-M5 only weakly bound to Imp α 3 (Figure S2C). In summary, two small basic islands around 9-RRP-12 and 31-KVFR-34 within the 36-residue Piwi-NLS sequence were necessary for Piwi nuclear localization. Thus, we defined Piwi-NLS as a bipartite NLS.

Mass spectrometric analysis of Piwi in OSCs showed that Arg82 might be deeply modified to be a symmetric dimethyl arginine

gesting that Piwi-NLS is not accessible for Imp α in the cytosol until Piwi is loaded with piRNA. One hypothesis was that an unknown protein (temporally termed as protein X) specifically occupied the NLS of piRNA-free Piwi, blocking Imp α binding to the NLS. Then, piRNA loading onto Piwi triggered displacement of the protein X from Piwi-NLS, allowing Piwi-piRISC to be successfully localized to the nucleus. To test this model, we exchanged Piwi-NLS with SV40-NLS to determine whether the Piwi mutant SV40-NLS-Piwi- Δ N required piRNA loading or not to be localized to the nucleus. Because SV40-NLS-containing cargo was localized to the nucleus with no specific treatments (Figures 3A and 3B), we assumed that SV40-NLS-Piwi- Δ N would



Figure 3. Effects of $\text{Imp}\alpha$ Depletion on SV40-NLS Localization in OSCs

(A and B) Depletion of Imp α 2 (green in A) and Imp α 3 (green in B) only marginally affected cellular localization of cargo-SV40-NLS (myc-Mael-SV40-NLS) (red) in OSCs. DAPI signals (blue) show the nuclei. Scale bars, 5 μ m. See also Table S1.

also be translocated to the nucleus without piRNA loading. However, against our expectation, the mutant failed to be localized to the nucleus when piRNA biogenesis had been compromised by Yb depletion (Figure 5A). Thus, the "protein X" hypothesis was unlikely.

An alternative model is that Piwi can hide its own NLS intramolecularly from $Imp\alpha$ until piRNA is loaded onto Piwi. To examine this possibility, we fused another Piwi-NLS composed of 36 residues to the N-terminal end of WT Piwi (Figure 5B). This process extended the NLS by duplication of the sequence in the context of full-length Piwi by fusing Piwi-NLS to the N terminus of WT Piwi. If this additional Piwi-NLS projected into the cellular environment beyond the surface of piRNA-free Piwi, we considered Imp α would bind to it and translocate the mutant Piwi-NLS-Piwi to the nucleus, regardless of piRNA loading. Indeed, Piwi-NLS-Piwi was localized to the nucleus even after Yb depletion. Two independent Piwi-NLS-Piwi mutants in which the second NLS was mutated to impede the activity as NLS behaved similarly to Piwi-NLS-Piwi (Figures S4A and S4B), suggesting the internal NLS in Piwi-NLS-Piwi is dispensable for rendering the piRNA-independent, nuclear localization activity to the protein.

Human Ago2 (hAgo2) that was not bound with guide RNA was fragmented to domains in limited proteolysis assays, but guide RNA loading made hAgo2 resistant to the proteolysis treatment (Elkayam et al., 2012). We tested whether Piwi behaved similarly in the assays. To this end, we expressed Piwi in cultured Drosophila Schneider 2 (S2) cells, which are non-gonadal somatic cells, so they do not operate the piRNA pathway. Indeed, Piwi expressed in S2 cells was devoid of piRNAs or any other RNAs, and was cytoplasmic (Figures S5A and S5B). We then immunopurified piRNA-free Piwi from S2 cells using anti-Piwi monoclonal antibody 3G11. In parallel, Piwi-piRISC was immunopurified from OSCs using 3G11. The epitope of 3G11 was within the 20 residues at the N-terminal end that corresponded to the first half of the Piwi-NLS (Figure S5C). In accordance with this fact, we found that immunopurification of piRNA-free Piwi from S2 cells was much more difficult than immunopurification of Piwi-piRISC from OSCs, so to obtain comparable amounts of Piwi, we prolonged the duration of immunoprecipitation from S2 cells. Also, the cell number of S2 cells used was about 10 times higher compared with that of OSCs. Both Piwi fractions were subjected to limited proteolysis assays. First, we used thermolysin because it was employed in the original hAgo2 study (Elkayam et al., 2012). However, even a low final thermolysin concentration of 0.08 ng/µL nearly completely degraded even Piwi-piRISC (data not shown). We then used chymotrypsin, which we found successfully released a large portion of Piwi-piRISC to the supernatant (Figure 5C), as was the case for Siwi-piRISC in our previous study (Siwi is one of two PIWI members in silkworm; Matsumoto et al., 2016). The large portion of Piwi in the supernatant was detected by another anti-Piwi monoclonal antibody 4D2 (Saito et al., 2009) by western blotting. The epitope of 4D2 was determined to be located between Trp72 and Lys89 (Figure S5D), suggesting that the proteolysis occurred upstream of the 4D2 epitope. In contrast, for piRNA-free Piwi, multiple 4D2-positive bands appeared exclusively in the bead fraction, implying that proteolysis occurred at multiple sites downstream of the 4D2 epitope. These results strongly suggest that the N-terminal region of approximately 90 residues of Piwi (including Piwi-NLS and 3G11/4D2 epitopes) was hardly accessible when Piwi was devoid of piRNAs, but piRNA loading induced a major conformational change in Piwi, as in hAgo2, exposing the N-terminal region to the environment.

Similar results were obtained when Piwi (with no tag) exogenously expressed in OSCs before and after depletion of Gasz, an essential piRNA biogenesis factor (Handler et al., 2013), was employed (Figure 5D). In this particular experiment, we knocked down Gasz, but not Yb, because piRNA-free Piwi was somewhat more stable than that in Yb-depleted cells (data not shown). Thus, our earlier observation that Piwi-piRISC (from OSCs) and piRNA-free Piwi (from S2 cells) showed distinct proteolytic patterns (Figure 5C) was not because of use of two different cell lines in the assays.

We then examined whether Piwi binding with $Imp\alpha$ is affected by piRNA loading status of Piwi. To this end, we expressed FLAG-Piwi in Gasz-depleted OSCs, immunoprecipitated the



Figure 4. Piwi Contains a Bipartite NLS at the N-Terminal End

(A) Domain structure and amino acid sequence of the N-terminal 72 residues of Piwi (N72) are shown. L0 domain: 38 residues (Y76–R113); N domain: 73 residues (I114–I186); L1 domain: S187–T263 (77 residues); PAZ domain: I264–T371 (108 residues); L2 domain: G372–T470 (99 residues); MID domain: P471–I601 (131 residues); PIWI domain: E602–L843 (242 residues). Three residues, 73-GDQ-75, do not belong to any of the domains indicated. The domain structure was defined by sequence alignment with the peptide sequence of Siwi (Matsumoto et al., 2016). Lysine and arginine residues are shown in red.

(B) myc-Piwi-N72-EGFP (green), but not myc-EGFP, was exclusively localized to the nucleus. EGFP has a small molecular weight (about 25 kDa), which allows EGFP to diffuse passively in and out of the nucleus in a receptor-independent fashion. DAPI signals (blue) show the nuclei. Scale bars, 5 μm.

(C) Deletion of 36 residues from the N-terminal end of Piwi caused mislocalization of Piwi (green: myc-Piwi- Δ N36) in the cytoplasm. Piwi-N36-EGFP (green), but not Piwi-N20-EGFP, was nearly exclusively localized to the nucleus. DAPI signals (blue) show the nuclei. Scale bars, 5 μ m.

(D) myc-Piwi and myc-Piwi-NLS-M2 were nearly exclusively localized to the nucleus. Piwi-NLS-M3 can be detected in both the nucleus and the cytoplasm evenly in OSCs. Piwi-NLS-M1, -M4, and -M5 strongly accumulated in the cytoplasm. DAPI signals (blue) show the nuclei. Scale bars, 5 μ m.

(E and F) Depletion of Imp $\alpha 2$ (in E) and Imp $\alpha 3$ (in F) affects cellular localization of NP-NLS-cargo (green: myc-NP-NLS-EGFP) in OSCs. DAPI signals (blue) show the nuclei. Scale bars, 5 μm . See also Figures S2 and S3 and Table S1.

biogenesis, as was found for Piwi (Figure 6A). Similar results were obtained when Aub was fused to SV40-NLS

Imp α 3 complex, and probed it with anti-FLAG antibody. As expected, depletion of Gasz severely reduced the levels of Piwi-Imp α 3 interaction (Figure 5E). The level of Piwi-Imp α 2 interaction was similarly reduced (Figure S5E). These results strongly support our original idea that Piwi-piRNA loading is necessary for Piwi being tightly associated with Imp α *in vivo*. It was experimentally confirmed that Piwi exogenously expressed in Gasz-depleted OSCs was loaded with piRNAs to a much lesser extent than Piwi expressed in normal OSCs (Figures S5F and S5G).

Aub exogenously expressed in OSCs by transfection was loaded with piRNAs that are normally loaded onto endogenous Piwi in the cells (Sato et al., 2015). However, Aub-piRISC remained to be in the cytoplasm, as was the case in germ cells, because it did not possess an NLS. We then fused Piwi-NLS to Aub and found that the Piwi-NLS-Aub protein was imported to the nucleus in OSCs (Figure 6A). However, Aub was forced to be in the cytoplasm when Yb was depleted to block piRNA (Figure 6A). Both Piwi-NLS-EGFP and SV40-NLS-EGFP were insensitive to Yb depletion and localized to the nucleus in Yb-depleted OSCs (Figure 6B). Addition of another Piwi-NLS to Piwi-NLS-Aub caused the protein to be nuclear localized in a manner independent of piRNAs (Figure S5H). These results suggest that piRNA loading-driven conformational change, which controls the accessibility of the PIWI N terminus, is a common feature of PIWI proteins.

DISCUSSION

In this study, we revealed that Imp α played a vital role in Piwi nuclear localization in OSCs, and that Piwi autoregulated its cellular localization by controlling the accessibility of its own N-terminal bipartite NLS to Imp α , by changing the structure in a piRNA loading-dependent manner. With this "layered" regulation, Piwi was strictly localized to the cytoplasm before piRNA loading.



Figure 5. Addition of Piwi-NLS to Full-Length Piwi Achieves piRNA-Independent Piwi Nuclear Localization

(A) SV40-NLS-Piwi- ΔN (green) requires piRNA loading for Piwi to be localized to the nucleus in OSCs. Yb was detected in the control using anti-Yb antibody (red dots). DAPI signals (blue) show the nuclei. Scale bars, 5 μ m.

(B) Piwi-NLS-Piwi (red) is imported to the nucleus piRNA independently in OSCs. Yb was detected in the control using anti-Yb antibody (green dots). Scale bars, 5 μ m. The proposed models are shown (bottom).

(C) Limited proteolysis of Piwi immunoisolated from S2 cells (i.e., piRNA-free Piwi) and OSCs (i.e., piRNA-bound Piwi). The anti-Piwi antibody 3G11 that reacts with the N-terminal end (1-20 residues) of Piwi (Figure S5C) was used for immunoprecipitation. Western blotting using 4D2 whose epitope was between Try72-Lys89 of Piwi (Figure S5D) shows that piRNA-bound Piwi was digested between the 3G11 and 4D2 epitopes, so the majority of the core region of Piwi positive for 4D2 was detected in the supernatant after chymotrypsin treatment. piRNA-free Piwi was digested downstream of the 4D2 epitope, so the N-terminal regions containing both 3G11 and 4D2 epitopes mostly remained in the bead fraction (Piwi peptides), suggesting that the N-terminal region containing approximately 90 residues of Piwi was difficult to access when Piwi was free from piRNA. The bands indicated with asterisks correspond to full-length Piwi.

(D) Limited proteolysis of Piwi exogenously expressed (with no tag) in and immunoisolated from OSCs using anti-Piwi antibody before (Piwi-piRISC) and after (piRNA-free Piwi) Gasz depletion. It should be noted that the latter material also contains endogenous Piwi-piRISC (~50%) that was formed before Gasz depletion.

(E) Depletion of Gasz severely reduced the activity of Piwi to interact with Imp₂3 in OSCs. See also Figures S4 and S5 and Table S1.

However, once piRNA was loaded onto Piwi, Piwi-NLS was exposed to the cellular environment, where the adaptor protein Imp α bound to it and triggered the nuclear import of Piwi-piRISC together with Imp β . Unfortunately, depletion of Imp β in OSCs by RNAi caused severe damage to the cells; thus, the necessity of Imp β in the processing remains unclear.

The limited proteolysis analysis provided compelling evidence that Piwi drastically changed the conformation in a piRNAdependent manner, similar to what was reported for hAgo2. However, direct evidence is still missing. For this, crystal structural analysis of Piwi in the presence and absence of piRNAs is desirable. However, this is currently difficult because it is extremely challenging to purify a large amount of recombinant PIWI proteins without piRNAs owing to their high instability in cellular environments. Nonetheless, we recently solved the crystal structure of Siwi-piRISC (Matsumoto et al., 2016). To make the crystals, we collected endogenous Siwi-piRISC by immunoisolating the complex from cultured BmN4 cells using anti-Siwi antibody. Because the vast majority of Siwi in BmN4 cells are loaded with piRNAs and the interaction between them is so tight, removing piRNAs from the piRISCs was the issue to be solved. Using a powerful electron-cryo-microscopy may be another way to go; however, the molecular mass of PIWI proteins is around 90–100 kDa, which may be too small for this type of analysis. We are currently exploring gentle but efficient ways of removing piRNAs from PIWI-piRISCs.

Our rescue experiments revealed that all three Imp α proteins could individually rescue mislocalization of Piwi to the cytoplasm caused by loss of Imp α 2 and Imp α 3 in OSCs. This clearly excludes a paralog-specific role of Imp α in Piwi nuclear localization. However, previous genetic studies showed that sterility caused by *Imp\alpha2* mutations was not rescued by ectopic expression of *Imp\alpha1* or *Imp\alpha3*, although the levels of *Imp\alpha* mRNAs (and Imp α proteins) ectopically expressed in the mutant ovaries were



Figure 6. SV40-NLS-Aub and Piwi-NLS-Aub Were Imported to the Nucleus in a piRNA-Dependent Manner, Similar to Piwi, in OSCs (A) Both Piwi-NLS-Aub and SV40-NLS-Aub (green) were imported to the nucleus in OSCs. Their import was blocked when piRNA biogenesis was impaired by Yb RNAi, so Piwi-NLS-Aub and SV40-NLS-Aub were localized in the cytoplasm. Yb was detected in the control using anti-Yb antibody (red dots). DAPI signals (blue) show the nuclei. Scale bars, 5 μm.

(B) Nuclear localization of Piwi-NLS-EGFP and SV40-NLS-EGFP (green) was unaffected by Yb depletion in OSCs. Scale bars, 5 μ m. See also Figure S5 and Table S1.

heterochromatinization of the loci. Aub exhibits a strong Slicer activity, so nuclear Aub-piRISC may immediately cleave the target RNAs upon association. If this were the case, Aub-piRISC, even after being successfully localized to the nucleus in a similar way as PiwipiRISC, would not induce transcriptional

comparable with endogenous ones (Mason et al., 2002). Embryonic lethality of Imp α 3 mutants was also not rescued by overexpression of *Imp\alpha1* or *Imp\alpha2* (Mason et al., 2003). These results suggest that some cargo proteins, crucial for gonadal development and fertility in *Drosophila*, were forced to remain cytoplasmic under the circumstance where other Imp α isoforms were expressed in the mutant ovaries. Identification of cargos for which nuclear import is tightly controlled by a particular Imp α protein is awaited.

Piwi bears a bona fide bipartite NLS. Why a bipartite, not monopartite, NLS evolved in Piwi is still unknown. Our results revealed that bipartite NLSs, from both Piwi and NP, showed less efficiency in nuclear import than monopartite NLSs, at least in OSCs. Therefore, it might be expected that having a monopartite NLS would simply be more effective and secure for exerting Piwi function in the nucleus. However, interestingly, our results also showed that Piwi containing its own NLS was clearly more sensitive to loss of piRNA than Piwi bearing SV40-NLS instead of its own NLS. This result suggests that Piwi autoregulation may be more controllable with bipartite NLSs than with monopartite NLSs, and this is most likely the reason why Piwi evolutionally acquired a bipartite NLS rather than a monopartite NLS. Notably, the N-terminal sequence of MIWI2, one of three PIWI proteins expressed in mouse testis whose nuclear localization and subsequent piRNA-mediated transposon silencing in the nucleus also requires piRNA loading (Carmell et al., 2007; Siomi et al., 2011), is similar to the N-terminal sequence of Piwi, including the NLS. It is likely that MIWI2 also evolutionally acquired a bipartite NLS rather than a monopartite NLS (Figure S5I).

Piwi-NLS-Aub was able to be localized in the OSCs in a piRNA-dependent manner, similar to endogenous Piwi protein. To accomplish transposon transcriptional silencing, Piwi-piRISC has to target and remain bound with nascent transcripts that are attached to the target loci upon transcription to efficiently induce

silencing, as opposed to Piwi-piRISC, which can. However, Aub-piRISC would efficiently cleave transposon mRNAs Slicer dependently even in the nucleus, which should lead to post-transcriptional silencing of transposons. We are currently examining the effect of nuclear Aub-piRISC in transposon silencing.

A recent Arabidopsis study showed that heterochromatic small interfering RNAs (siRNAs) (hc-siRNAs) drive AGO4, a ubiguitously expressed Argonaute member, into the nucleus to facilitate target gene silencing via DNA methylation (Ye et al., 2012). In the study, however, not only AGO4 WT but also AGO4 mutant incapable of unwinding hc-siRNA duplexes to single-strand remained to be cytoplasmic, suggesting that the siRNA duplex loading onto AGO4 was insufficient for driving nuclear localization of AGO4. Rather, it further required AGO4 endonucleasedependent, passenger strand cleavage and displacement, i.e., activation of AGO4-RISC. The Ye et al. (2012) report also revealed that AGO4 contains a monopartite NLS at Asp176-Pro183 residues (DRKRLRRP), and the relocation of NLS to the very N-terminal end of AGO4 drove AGO4 nuclear localization in an hc-siRNA-independent manner. Furthermore, dissociation of a protein chaperone Hsc70/HSP90 from AGO4 was also necessary for AGO4-RISC nuclear localization. Therefore, Drosophila Piwi and Arabidopsis AGO4 show rather distinct features regarding their nuclear localization, although both Argonaute proteins are key components of nuclear transcriptional silencing in the respective species.

EXPERIMENTAL PROCEDURES

Cell Culture, RNAi, and Transfection with Plasmids

OSCs and S2 cells were cultured as described previously (Miyoshi et al., 2005; Saito et al., 2009). To perform RNAi, we transfected OSCs with 200 pmol siRNAs using Nucleofector 2b (Lonza). The siRNA sequences used in this study are listed in Table S1. OSC transfection with plasmids was carried out using Xfect Transfection Reagent (Clontech) as described previously (Murota et al., 2014). S2 cells were transfected using Lipofectamine Transfection Reagents (Invitrogen).

Production of Anti-Impa Monoclonal Antibodies

Recombinant proteins of full-length Imp α 1, Imp α 2, and Imp α 3 fused to glutathione S-transferase (GST) (GST-Imp α 1, GST-Imp α 2, and GST-Imp α 3) were expressed in and purified from *E. coli* and then injected into mice for immunization. Production and selection of hybridomas that produced anti-Imp α monoclonal antibodies were performed as described previously (Nishida et al., 2015). To examine the specificity of anti-Imp α monoclonal antibodies raised in this study, we performed western blotting as described previously (Miyoshi et al., 2005) using OSC lysates before and after RNAi treatment for each Imp α member. β -Tubulin (β Tub) was detected as a loading control using anti- β Tub mouse monoclonal antibody (1:2,500 dilution; obtained from the Developmental Studies Hybridoma Bank).

Plasmid Construction

We used plasmids pAcM-Piwi and pAcM-EGFP that were constructed and used previously (Saito et al., 2009) to express myc-Piwi and myc-EGFP in OSCs. To yield pAcF-Piwi for expressing FLAG-Piwi in OSCs and S2 cells, we exchanged the region encoding a myc peptide in pAcM-Piwi with a DNA fragment encoding a FLAG peptide using NEBuilder HiFi DNA Assembly Master Mix (NEB). The PCR primers used were F-Piwi-I-F/F-Piwi-I-R and F-Piwi-V-F/F-Piwi-V-R (Table S1). Construction of all other plasmids is described in the Supplemental Information.

Estimation of Impa Protein Levels in OSCs

Recombinant GST-Imp α proteins isolated from *E. coli* and BSA (NEB) were run on protein gels. Protein concentrations of full-length GST-Imp α in each solution were estimated by staining them with Coomassie Brilliant Blue (CBB) and comparing them with BSA stained with CBB. Western blotting was then performed using anti-Imp α monoclonal antibodies (this study) on GST-Imp α and OSC whole lysates. Signal intensity was calculated using ImageJ (NIH).

Immunofluorescence

Immunofluorescence was performed as described previously (Saito et al., 2010). The primary antibodies used in this study were anti-Imp α monoclonal antibodies (this study), anti-myc rabbit polyclonal antibodies (1:500 dilution; Sigma), anti-FLAG mouse monoclonal antibody (1:1,000 dilution; Sigma), anti-myc mouse monoclonal antibody (1:1,000 dilution; Sigma), and anti-Yb monoclonal antibody (1:500 dilution) (Murota et al., 2014). The secondary antibodies used were Alexa 488-conjugated anti-mouse immunoglobulin M (IgM; Abcam), Alexa 488-conjugated anti-mouse IgG (Molecular Probes), Alexa 488-conjugated anti-rabbit IgG (Molecular Probes), Alexa 546-conjugated anti-mouse IgG (Molecular Probes), and Alexa 546-conjugated anti-rabbit IgG (Molecular Probes). Transfection of OSCs prior to immunofluorescence with plasmids was performed as described previously (Saito et al., 2009). Impa and Yb were depleted by RNAi by introducing Impa-siRNAs and Yb-siRNA into OSCs prior to overexpression of myc-Piwi (Figure 1), myc-Mael-SV40-NLS (Figure 3), myc-NP-NLS-EGFP (Figures 4E and 4F), myc-SV40-NLS-Piwi-ΔN (Figure 5A), myc-Piwi, (Figure 5B), myc-Piwi-NLS-Piwi (Figure 5B), myc-Piwi-NLS-Aub (Figure 6A), myc-SV40-NLS-Aub (Figure 6A), myc-Piwi-NLS-EGFP (Figure 6B), and myc-SV40-NLS-EGFP (Figure 6B). EGFP-siRNA or luciferase (luc)-siRNA was used as a negative control. Between 12 and 15 cells were analyzed per Impa member, where individual Impa proteins were efficiently depleted, and representative images were shown in corresponding figures (Figures 1A-1C, 3A, 3B, 4E, and 4F). Between 10 and 16 cells were analyzed before and after Yb depletion, and representative images were shown in corresponding figures (Figures 5A, 5B, 6A, 6B, S4A, S4B, and S5H). Approximately 10 cells were analyzed, and representative images were shown in corresponding figures (Figures 4B-4D).

Rescue Assays

To knock down $Imp\alpha 1$, $Imp\alpha 2$, and $Imp\alpha 3$ in OSCs, we introduced $Imp\alpha 1$ -siRNAs, $Imp\alpha 2$ -siRNAs, and $Imp\alpha 3$ -siRNAs separately into OSCs. Two days later, the cells were transfected again with the $Imp\alpha$ -siRNAs to raise

the RNAi effect. Plasmids to overexpress FLAG-Piwi, together with myc-Imp α 1, myc-Imp α 2, or myc-Imp α 3, were simultaneously introduced into the cells. Myc-EGFP was expressed as a negative control. One day later, immunofluorescence was performed as described above (see Immunofluorescence). Between 15 and 18 cells were analyzed per Imp α member, and representative images were shown in corresponding figures (Figures 2A and 2B).

Limited Proteolysis Assays

Piwi with no tags was expressed in S2 cells by transfection and then immunoprecipitated using anti-Piwi antibody 3G11, to obtain piRNA-free Piwi. Piwi-piRISC was immunoprecipitated from OSCs using 3G11. After extensive washing, chymotrypsin was added to both immunoprecipitated samples to give a final concentration of 2.0 ng/µL. After incubation at 37°C for 30 min, the bead fractions were separated from the supernatants, and both fractions were subjected to western blotting using 4D2. Western blotting was performed as described previously (Miyoshi et al., 2005). To perform RNAi, we transfected OSCs with 200 pmol EGFP-siRNA or Gasz-siRNA using Nucleofector 96-well shuttle (Lonza). To obtain piRNA-free Piwi and Piwi-piRISC, Piwi with no tag was expressed in OSCs treated with EGFP-siRNA or Gasz-siRNA using Nucleofector 2b in Nucleofection Solution (5 mM KCl, 15 mM MgCl₂, 50 mM D-mannitol) added to 180 mM Church phosphoric acid buffer (pH 7.2) instead of 60 mM Na₂PO₄ and 60 mM NaH₂PO₄ (Nye et al., 2014) and then immunoprecipitated using anti-Piwi antibody 3G11. After extensive washing, chymotrypsin was added to both immunoprecipitated samples to give a final concentration of 2.0 ng/µL. After incubation at 37°C for 30 min, the beads fractions were separated from the supernatants, and both fractions were subjected to western blotting using 4D2.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.05.051.

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AUTHOR CONTRIBUTIONS

R.Y., H.S., and M.C.S. conceived the project and designed the experiments. R.Y., Y.M., K.M.N., H.Y., and S.Y. performed the experiments with K.F.'s and A.O.'s help. L.N. performed mass spectrometric analysis. R.Y., Y.M., K.M.N., H.S., and M.C.S. analyzed the data and wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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