ADMA-histones play a crucial role in the initial recognition of dual-strand piRNA cluster regions by Rhino

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Abstract

In *Drosophila* germ cells, piRNAs arise from dual-strand piRNA clusters, which are marked by a repressive histone mark H3K9me3, but are transcribed from internal sites in a manner dependent on the HP1 homolog Rhino. Rhino binds to H3K9me3 on these clusters, yet the mechanism controlling its binding to clusters remains unclear. Here, we used cultured ovarian somatic cells (OSCs), which lack endogenous Rhino and its stabilizer Kipferl, whose absence renders the dual-strand clusters inert, and found that exogenous Rhino tends to bind to the ends of dual-strand clusters with asymmetric dimethylarginine histones (ADMA-histones). Depletion of the arginine methyltransferases responsible for ADMA modifications, such as DART4, affected the genomic localization of Rhino in both OSCs and ovaries. We also identified primitive, cluster-like genomic regions, termed DART4 clusters, where Rhino propagates in an DART4-dependent but unstable manner. Our study proposes that ADMA-histones play a crucial role in the initial genome loading of Rhino and in establishing the sites of its propagation, which are subsequently stabilized to support piRNA production.

Introduction

PIWI-interacting RNAs (piRNAs) are germline-enriched small non-coding RNAs that repress transposons in collaboration with PIWI proteins to maintain fertility[1,2,3]. In *Drosophila* germ cells, piRNAs are primarily derived from dual-strand piRNA clusters, intergenic elements rich in transposon fragments[4,5]. These clusters are occupied with the repressive histone mark H3K9me3 and lack typical promoters, but are transcribed in both directions from the internal sites[6,7]. This unconventional transcription requires special factors, including heterochromatin protein 1 (HP1) homolog HP1d/Rhino (Rhino), and provides piRNA precursors with a variety of sequences, which are then processed to mature piRNAs in the cytoplasm[5,6,7,8,9,10,11,12,13,14]. The mechanism dedicated to this piRNA processing is known as the ping-pong cycle, where Aubergine and Argonaute 3 cleave the precursors in a manner dependent on the sequences of piRNAs already bound to them[1,2,3]. This pathway also consumes transposon RNA transcripts as the sources of mature piRNAs, resulting in transposon repression at the post-transcriptional level[2,3].

Like other HP1 family members, Rhino has a chromodomain through which it binds to H3K9me3 scattered on dual-strand piRNA clusters[6,8,9,12,13,14,15,16]. Deadlock and Cutoff then join Rhino, assembling the Rhino–Deadlock–Cutoff (RDC) complex, which further recruits the transcription factor IIA-L (TFIIA-L) paralogue Moonshiner, the transcription factor IIA-S (TFIIA-S), and the animal TATA-box-binding protein (TBP) paralog TRF2, initiating transcription of the clusters[6,7,10]. Upon transcription, Cutoff binds to piRNA precursors and protects them from splicing, contributing to the production of an abundance of piRNAs[17,18,19,20].

A recent study identified the zinc-finger protein Kipferl as a factor required for

Rhino to distinguish between H3K9me3 located on transposons and dual-strand piRNA clusters[12]. Kipferl stabilizes Rhino on guanine-rich sequence motifs present in dual-strand clusters in an H3K9me3-independent manner[12]. Maternally inherited piRNAs also play a role in the localization of Rhino to dual-strand clusters during early embryogenesis via Piwi-dependent H3K9me3 epigenetic marks [9,21].

Rhino has a propensity to spread across the genome. For example, depletion of the histone demethylase Kdm3 in the female germline causes Rhino to spread to adjacent chromatin[13]. Deletion of the region proximal to the transcription start site of the *phospholipase D* gene, which is in the region flanking piRNA cluster 42AB, also resulted in Rhino spreading toward the deleted genomic region[10]. In addition, Rhino tethering experiments with artificial constructs showed that Rhino spreads as the constructs are transcribed[7]. In each case, production of piRNAs from regions after Rhino spreading was detected. These findings suggest that piRNA clusters are defined by Rhino spreading over the genome and imply that the process of piRNA cluster formation is composed of three steps: Rhino spreading, Kipferl-dependent stabilization of Rhino–genome association, and epigenetic inheritance of the Rhino positional memory by maternally inherited piRNAs. Since the genomic localization of both Rhino and Kipferl is interdependent, it is hypothesized that the initial loading of Rhino on the genome precedes its spread or stabilization, and that this process is independent of Kipferl. To investigate this, it is necessary to observe the behavior of Rhino in a cell environment where piRNAs are functional, but where both Kipferl and Rhino are not expressed. However, the mechanism of such Rhino behavior remains unclear.

In this study, we describe the important role of asymmetric dimethylarginine histones (ADMA-histones) in the initial loading of Rhino onto the genome and their

stabilization. We first established cultured ovarian somatic cells (OSCs)[22] expressing FLAG-Rhino by induction with doxycycline (Dox). Unlike ovarian germ cells, OSCs lack Rhino and other factors required for the activation of dual-strand piRNA clusters, such as Deadlock and Cutoff[23]; therefore, dual-strand clusters are not in an active state for piRNA biogenesis[24]. We found that Rhino in OSCs assembles nuclear foci similar to Rhino foci in the ovary. We then performed chromatin immunoprecipitation sequencing (ChIP-seq) and showed that Rhino tends to bind to the ends of dual-strand clusters with ADMA-histones, such as H3R17me2a. Depletion of the arginine methyltransferases responsible for ADMA modifications, such as *Drosophila* arginine methyltransferase 4 (DART4), affected the genomic localization of Rhino in OSCs and in the ovary. We also identified genomic regions where Rhino binds at the ends and propagates in the areas in a DART4-dependent manner, but not stably anchored. Kipferl is present at the regions but is not sufficient to stabilize Rhino-genomic binding after Rhino propagates. piRNAs were mapped to these regions, albeit at low levels. The levels of these piRNAs decreased also in a DART4-dependent manner. Therefore, these regions, referred to as DART4-dependent piRNA clusters (DART4 clusters), are considered to be primitive clusters driven by the propagation of Rhino initially loaded onto the genome via ADMA-histones.

Results

Rhino localizes to the ends of dual-strand clusters in the OSC genome

To observe how Rhino in OSCs behaves intracellularly, we first performed immunofluorescence in FLAG-Rhino inducible OSCs to examine the subcellular localization of FLAG-Rhino upon Dox induction. This showed that Rhino assembled

nuclear speckles in OSCs similar to Rhino-positive foci in the ovary[5,10] when the cells were treated with Dox at a concentration of 5 ng/mL (Fig. 1A). At lower Dox concentrations and lower levels of Rhino expression, no clear foci appeared (Supplementary Fig. 1A, B), suggesting that Rhino-positive foci form in a manner dependent on the level of Rhino expression.

We next conducted ChIP-seq of FLAG-Rhino under conditions where Rhino assembles nuclear foci as in Fig. 1A. Genome mapping of the ChIP-seq reads showed that Rhino can be found, albeit weak, on dual-strand piRNA clusters, such as 42AB and 38C on chromosome 2, 80F on chromosome 3, and 102F on chromosome 4, all of which are in an active state in piRNA production in the ovary, but the signals seem to be biased toward the ends of the clusters (Fig. 1B and Supplementary Fig. 1C). ChIP-seq of Rhino was also performed in the ovary: Rhino was strongly enriched at highly transcribed piRNA clusters, 42AB, 38C, 80F, and 102F, as previously reported[5,6,7,8,9,10,11,12,13,14] (Fig. 1B and Supplementary Fig. 1C), which suggests that our ChIP-seq procedure is reliable.

We then compared the mode of Rhino genome binding in OSCs with that in the ovary in a more comprehensive manner. The results show that Rhino in OSCs tends to reside in the genome where Rhino binds locally in the ovary (Fig. 1C). The metaplot indicates a tendency for Rhino in OSCs to be toward the cluster ends (Fig. 1D). Rhino also binds to the cluster bodies in OSCs, but the signals were weaker compared to those at the ends. Based on these results, we interpret that OSCs contain a factor(s) that promotes Rhino binding toward the ends of the clusters, but unlike the ovary, they lack a factor(s) that promotes and stabilizes Rhino spreading throughout the clusters.

The terminal localization of Rhino in OSCs does not appear to be due to H3K9me2 (Fig. 1B, E, F): Of the 823 Rhino signals, only 14 signals overlapped with the

H3K9me2 signals (Fig. 1E). The heatmap also showed that H3K9me2 barely accumulated at the Rhino location (Fig. 1F). The positional correlation between Rhino and H3K9me3 is stronger than between Rhino and H3K9me2 (i.e., 77%; 636 in 823 signals) (Fig. 1E). Nonetheless, the overlap between Rhino and H3K9me3 was not very strong according to the heatmap (Fig. 1F, G). Immunofluorescence also showed weak overlap between the prominent H3K9me3 speckles and FLAG-Rhino foci (Fig. 1A). HP1a, another HP1 family member required for constitutive heterochromatin and known to be a general H3K9me3 marker, co-localizes with H3K9me2/3 and is distributed along the entire length of piRNA clusters similarly in both OSCs and ovary (Fig. 1B and Supplementary Fig. 1C). HP1a is present in the broad H3K9me3 peak (Fig. 1G). Therefore, although H3K9me3 and HP1a might be involved in some aspect of the mechanism underlying Rhino localization, they do not contribute to the localization specificity within that mechanism at least.

FLAG-Rhino ChIP-seq was also performed at low Rhino expression levels. The trend toward terminal localization of Rhino at 5 ng/mL Dox was observed at 0.05 ng/mL and 0.5 ng/mL Dox as well (Supplementary Fig. 1D, E). This indicates that Rhino binds to the OSC genome even at low expression levels. However, Rhino foci were not clearly observed with the lower Dox concentrations (Supplementary Fig. 1A). It is interpreted that genome binding occurs first, followed by Rhino foci formation, which is dependent on Rhino expression levels.

The use of anti-FLAG antibodies in Rhino ChIP in OSCs was justified by the high similarity between the signals of anti-FLAG and anti-Rhino antibodies (Fig. 1B and Supplementary Fig. 1C, D).

Rhino co-localizes with asymmetric dimethylarginine histones on the OSC genome

To identify genomic scaffolds that are potentially involved in determining the genomic localization of Rhino in OSCs, we performed ChIP-seq of key histone modifications and compared the data with Rhino ChIP-seq data. Heatmap revealed that specific ADMA-histones, including H3R2me2a, H3R8me2a, H3R17me2a, and H4R3me2a, tend to co-localize with Rhino across the OSC genome (Fig. 2A). Consistent with this, these ADMA-histones showed a tendency to localize to the ends of Rhino-bound dual-strand piRNA clusters (Fig. 2B, C, and Supplementary Fig. 2A). H3K4me1 and H3K27ac have been reported to co-localize with H3R8me2a in mammals[25], which was not observed in OSCs (Fig. 2A, B, and Supplementary Fig. 2A). Co-localization of H3K4me1 and H3K27ac with Rhino was also not observed.

Examination of the correlation coefficients revealed that signal peaks of each ADMA-histone were significantly correlated with those of FLAG-Rhino (Fig. 2D). The ChIP-seq signals of H3R2me2s and H3R8me2s correlate more strongly with FLAG(Dox-) than with FLAG(Dox+), suggesting that these histone modifications may represent background noise (see “background noise” in Fig. 2D). In contrast, ADMA-histones show a higher correlation coefficient with FLAG(Dox+) than with FLAG(Dox-): it is also worth noting that in hierarchical clustering, ADMA-histones form a monophyletic group with FLAG(Dox+) (see “Rhino localization” in Fig. 2D). Histone modifications related to transcription, such as H3K4 and H3K27, as well as constitutive heterochromatin markers like H3K9me2 are not associated with the localization of FLAG-Rhino (see “transcriptional regulation” in Fig. 2D). Involvement of ADMA-histones in the genomic localization of Rhino was implicated.

The overlaps of ChIP-seq peaks were examined. In the diagram shown in Fig. 1E, approximately 23% of Rhino ChIP peaks did not overlap with H3K9me3 (187 in 823 sites), but these Rhino peaks were almost encapsulated by the read sets of ADMA-histones (Fig. 2E). This suggests that ADMA-histones are involved in the localization of Rhino. However, the degree of overlap of those sites is not very high in each case (Supplementary Fig. 2B). It appears that each ADMA-histone contributes differently to Rhino localization. HP1a was found at the dual-strand clusters in OSCs (Fig. 1B). However, unlike Rhino, genomic localization of HP1a does not appear to be determined by ADMA-histones and H3K9me3 because approximately 30% of HP1a ChIP peaks did not overlap with the read sets of ADMA-histones and H3K9me3 (492 in 1,672 sites) (Supplementary Fig. 2C). These results suggest that ADMA-histones, together with H3K9me3, contribute significantly and specifically to the recruitment of Rhino to the ends of dual-strand clusters in OSCs.

Arginine methyltransferases DART1 and DART4 differently regulate Rhino genomic binding

Arginine methylation of histones is catalyzed by protein arginine methyltransferases (PRMTs), and the *Drosophila* genome encodes 11 of these PRMTs, also known as *Drosophila* arginine methyltransferases (DARTs)[26,27]. Of those, DART1, a homolog of mammalian PRMT1, and DART4/CARMER (DART4), a homolog of mammalian PRMT4/CARM1 (CARM1), were reported to be type I PRMTs that catalyze the methylation of monomethylarginine to asymmetric dimethylarginine[28,29,30,31,32,33,34,35]. DART8 is involved in asymmetric dimethylation of H3R2[36]. CG17726 is considered to be a DART member because of its sequence similarity and is expressed in the ovary

(Flybase 2024). However, its function remains unknown. If ADMA-histones play an important role in the local recruitment of Rhino to the OSC genome, it is highly likely that the loss of DARTs responsible for ADMA modification would influence the subcellular localization of Rhino.

A previous study showed that HP1a germline knockdown (GLKD) causes Rhino foci to be more prominent in the ovary and reduces Rhino occupancy at the clusters[37]. Because a similar phenotype was observed for Rhino foci in HP1a-knockdown (KD) OSCs (Fig. 3A, B), we used this phenotype to examine the loss-of-function effects of DARTs. Similar phenotypes were observed in DART1- and DART4-KD OSCs as in HP1a-KD OSCs, suggesting that DART1/4-dependent histone ADMA modification is involved in Rhino recruitment (Fig. 3A, B). Moreover, the localization of Rhino in DART1/4 KD cells closely matches prominent H3K9me3 foci, suggesting that the arginine methylation activity of DART1/4 contributes to the genomic localization of Rhino in OSCs as a genome scaffold beyond H3K9me3 (Supplementary Fig. 3A). In contrast, this phenotype was not observed for HP1a, suggesting that the effects of DART1/4 are specific to Rhino (Supplementary Fig. 3B).

Fluctuation in the localization of Rhino in the DART1- and DART4-KD OSCs was also observed in ChIP experiments. In ChIP-seq, Rhino tended to be decreased in DART1 KD and DART4 KD (Fig. 3C, D), which was confirmed by ChIP-qPCR (Fig. 3E). Among the ADMA-histones that co-localized with Rhino, H3R17me2a decreased in a DART4-dependent manner and H4R3me2a decreased in a DART1-dependent manner (Fig. 3C), consistent with previous findings for human PRMT1 and CARM1[38,39,40,41]. Detailed analysis revealed that the genomic localizations of H4R3me2a lost in DART1 KD (575 sites) and H3R17me2a lost in DART4 KD (653 sites) overlapped at 48 sites

(Fig. 3F). Correspondingly, the genomic localizations of Rhino that decreased in DART1 KD (115 sites) and those that decreased in DART4 KD (207 sites) overlapped at 25 sites (Fig. 3G). These results suggest that DART1 and DART4 are involved in Rhino recruitment at distinct genomic sites through the decreases in ADAM-histones in each of their KD conditions (H4R3me2a and H3R17me2a, respectively).

Among ADMA-histones, only H3R8me2a tended to decrease in both DART1 and DART4 KD (Fig. 3C). To examine the specificity of the regulation of H3R8me2a by DART1 and DART4, the degree of overlap was visualized in Venn diagrams for H3R8me2a, as well as H3R17me2a and H4R3me2a. The genomic localizations of H3R8me2a decreased by DART1 KD (235 sites) and DART4 KD (58 sites) weakly overlapped (8 sites) (Supplementary Fig. 3C). The genomic localizations of H3R8me2a (58 sites) and H3R17me2a (652 sites) that decreased in DART4 KD overlapped only at 7 sites (Supplementary Fig. 3D), as was the case for H3R8me2a (235 sites) and H4R3me2a (575 sites), which were decreased in DART1 KD (Supplementary Fig. 3E). These results suggest that DART1 and DART4 regulate Rhino localization through H3R8me2a at different sites, as well as through H3R17me2a and H4R3me2a.

The level of Rhino expression was unchanged in DART1/4-KD cells (Supplementary Fig. 3F). Rhino and HP1a even in normal OSCs were under detection levels with anti-ADMA antibodies (Supplementary Fig. 3G). Thus, the loss of DART1/4 does not appear to affect Rhino or HP1a function directly through methylation.

ADMA-histones co-localize with Rhino in a manner dependent of DART4 in the ovary

We examined the localization of ADMA-histones in the Oregon-R ovary. In the

wild-type (WT) ovary, we detected Rhino peaks in FLAG-Rhino localization sites in OSCs and ADMA-histones, H3R8me2a, H3R17me2a, and H4R3me2a, co-localized with them (Fig. 4A and Supplementary Fig. 4). Heatmap showed that these ADMA-histones are detected in the narrow Rhino-positive regions as in the case of Rhino in OSCs (Fig. 4B). As well, the metaplot showed that ADMA-histones tend to localize to the ends of piRNA clusters (Fig. 4C). The trend of Rhino genome binding observed in OSCs was also recapitulated in ovaries at corresponding genomic locations.

To determine whether the destabilization of Rhino genome binding observed in OSCs under DART4-KD conditions is also observed in the ovary, we performed GLKD of DART4 using the GAL4-UAS system. Under the DART4-GLKD conditions (Supplementary Fig. 5A), Rhino foci were more prominent in the absence of DART4 (Fig. 5A, B). ChIP-seq experiments showed that Rhino localizes almost the same genomic regions in the ovary as in OSCs, although these localizations are weaker compared to piRNA clusters such as 42AB (Fig. 5C, D). Notably, Rhino levels significantly decreased with the loss of H3R17me2a under DART4-GLKD conditions (Fig. 5C–F, and Supplementary Fig. 5C, D). These results indicate that the relationship between ADMA-histones and Rhino found in OSCs is conserved in the ovary.

Interestingly, Rhino spreading across clusters annotated in the piRNA cluster database[42], such as 42AB, 38C, 80F, and 102F, was little affected by DART4 GLKD (Supplementary Fig. 5E). These results suggest that the weak Rhino localization dependent on ADMA-histones (such as at the terminal sites of piRNA clusters) and the strong Rhino localization within the core regions of piRNA clusters are fundamentally governed by distinct regulatory mechanisms.

Loss of Kdm3 in the ovary (i.e., Kdm3-GLKD ovary) causes an increase in

H3K9me2 and regional expansion, accompanied by Rhino spreading and the appearance of ectopic piRNA clusters[13]. To determine whether ADMA-histones are involved in this process, we examined the localization of ADMA-histones in the ectopic piRNA clusters. ADMA-histones were found near the 5′ and 3′ ends of the Kdm3 GLKD-dependent piRNA clusters, as well as in the authentic piRNA clusters (Supplementary Fig. 5F). Relatively few ADMA-histones were observed inside the clusters, even in the Kdm3 GLKD-dependent piRNA clusters (Supplementary Fig. 5F). This raises the possibility that ADMA-histones may be involved in the Rhino propagation by defining the spreading regions.

Genomic regions where Rhino spreads in a DART4-dependent manner, but not stably anchored, produce some piRNAs

Counting the genomic sites where H3R17me2a was no longer localized in DART4-GLKD ovaries, 3,082 sites were identified. The genomic sites where Rhino was decreased under the same circumstance were also counted (2,078 sites). Of these, 497 sites overlapped in the two groups (Fig. 6A). This indicates that 76.1% of the Rhino genomic localization reduced by DART4 GLKD appears to be H3R17me2a-independent. Forty-three such regions were found in the *Drosophila* genome (Fig. 6B, C). Representative examples are shown in Fig. 6D and Supplementary Fig. 6A: In control KD, these regions have Rhino in their internal regions and H3R17me2a at their ends (and other ADMA-histones at each end in WT Oregon-R strain, albeit at low levels). In DART4 GLKD, H3R17me2a disappeared from the terminal regions as expected. The internal Rhino also disappeared, although the areas did not contain H3R17me2a (Fig. 6D, E). These regions are distinctly different in nature from authentic piRNA clusters,

where Rhino localization remained largely unaffected by DART4 GLKD (Fig. 6B).

Not only H3R17me2a but also other ADMA-histones were found at the ends of these regions, and H3K9me2 accumulated in the internal areas (Supplementary Fig. 6B), consistent with previous studies suggesting H3K9me2 involvement in the Rhino spreading[13]. FLAG-Rhino in OSCs was also localized at the ends of the regions (Fig. 6E). These results support the hypothesis that ADMA-histones are involved in the genomic binding of Rhino both before and after Rhino spreading, resulting in stable genome binding.

We next investigated the potential for piRNA production from the genomic regions where Rhino propagates in a DART4-dependent manner but does not stably anchor. Under control conditions, piRNAs were detected mapping to these regions, albeit at low levels (Fig. 6D, F, and Supplementary Fig. 6A). Under DART4-GLKD conditions, piRNA levels were significantly reduced, suggesting that piRNA production from these regions is dependent on DART4. Although piRNA production was also influenced by Rhino, the reduction observed upon Rhino loss was much less pronounced compared to annotated piRNA clusters (Fig. 6G).

The expression levels of transposons and genes presumed to be targeted by piRNAs originating from genomic regions showed minimal changes under DART4-GLKD conditions (Supplementary Fig. 6C). Similarly, egg hatching rates remained largely unaffected (Supplementary Fig. 6D). These observations could be attributed to the relatively low levels of piRNAs arising from these regions compared to authentic piRNA clusters (Supplementary Fig. 6E, F), as well as their shorter genomic lengths (Supplementary Fig. 6G). This can also be explained by the fact that piRNA production from authentic clusters remained largely unaffected. Notably, even the functional loss of

authentic piRNA clusters in germ cell piRNA production does not usually result in infertility[43]. Based on these findings, which highlight that these genomic regions differ from authentic piRNA clusters in many aspects yet possess the ability to produce piRNAs, we designate them as DART4-dependent piRNA clusters (DART4 clusters).

Kipferl stabilizes the Rhino–genome binding[12]. ChIP-seq using anti-Kipferl antibodies showed that Kipferl localizes to the genomic regions where Rhino spreads but does not remain stable, and decreases in DART4 GLKD like Rhino (Supplementary Fig. 7A, B). This suggests that Kipferl is already present in these regions but is not sufficient to stabilize Rhino–genomic binding after Rhino propagates. Therefore, an unknown factor(s) other than Kipferl is required to convert these regions into “authentic” piRNA clusters that stably produce piRNAs by stabilizing Rhino–genomic binding.

In Kipferl mutants, Rhino localization is biased toward satellite repeats[12]. In DART4 GLKD, there appears to be no significant difference in Rhino localization (Supplementary Fig. 7C), nor does Rhino become enriched at satellite repeats (Supplementary Fig. 7D).

A recent comprehensive annotation of active piRNA clusters across eight highly inbred *Drosophila* strains classified the clusters into eight levels of evolutionary novelty[44]. We subsequently analyzed the enrichment of ADMA-histones, which significantly correlate with Rhino localization in OSCs (Fig. 2A–E) and ovaries (Fig. 4 A–C) (e.g., H3R2me2a, H3R8me2a, H3R17me2a, and H4R3me2a), around these piRNA clusters. Notably, a trend of greater ADMA-histone enrichment was observed at the ends of the newer clusters (Fig. 7A). This trend was also observed in DART4 clusters (Supplementary Fig. 8A). In contrast, such bias was not observed in older clusters or other genomic loci (Fig. 7A, B, Supplementary Fig. 8A). H3R2me2a, H3R8me2a,

H3R17me2a, and H4R3me2a showed a loss of enrichment as the clusters aged (Fig. 7A). The DART4-dependent H3R17me2a differences observed between newer and older clusters were less pronounced in DART4 GLKD (Fig. 7B). We next extracted piRNA clusters where piRNAs were detected in the Oregon-R strain and examined them for ADMA-histone enrichment. The results remained consistent (Supplementary Fig. 8B). A similar analysis using GLKD strains yielded identical findings (Supplementary Fig. 8C). These results suggest that DART4 clusters represent relatively new piRNA clusters.

Based on all these results, we propose that ADMA-histones play a crucial role in determining Rhino's initial genomic binding (Fig. 7C, left). Furthermore, ADMA-histone-dependent Rhino binding to the genome may also contribute to the propagation of Rhino within the piRNA cluster regions (Fig. 7C, center). Our evolutionary analysis suggests that DART4 clusters may eventually develop into *bona fide* piRNA clusters like 42AB (Fig. 7C, right); however, it does not provide direct evidence for this process. Further studies will be necessary to explore this possibility.

Discussion

In this study, we took advantage of cultured OSCs for our analysis and found that chromatin marks (i.e., ADMA-histones) play a crucial role in the loading of Rhino onto the genome. Comparative analysis of OSC and ovarian ChIP-seq data also allowed us to identify "primitive" piRNA clusters, DART4 clusters. Based on these results, DART4 clusters are piRNA clusters where Rhino propagates in a DART4-dependent manner from the H3R17me2a marks at the ends, but this spread is not stable. We propose that the process of piRNA cluster formation begins with the initial loading of Rhino onto bivalent nucleosomes containing H3K9me3 and ADMA-histones (Fig. 7C). In OSCs, the

absence of Kipferl and other necessary factors means that Rhino loading into the genome does not proceed to the next step. In DART4 clusters, Rhino spreads but does not stabilize, indicating that these regions may be nascent piRNA clusters. The evolutionary analysis showing a stronger tendency for ADMA accumulation at the ends of younger piRNA clusters (Fig. 7A) provides indirect evidence that DART4 clusters represent evolutionarily young, nascent piRNA clusters. While DART4 clusters may represent a “later” stage compared to annotated piRNA clusters, the essential role of piRNAs in reproduction and species conservation makes it unlikely that a large number of clusters exist in a degenerate state. Thus, we speculate that these clusters represent an “earlier” stage in piRNA cluster formation.

Whether DART4 clusters evolve into large piRNA clusters such as 42AB and 38C during the course of evolution remains an open question. Although these large clusters were not affected by DART4 GLKD, ADMA-histones were still detectable, albeit at low levels, at the ends of their Rhino peaks (Fig. 2B and 4A). This observation raises the possibility that even large piRNA clusters like 42AB and 38C may have initially formed through a process involving ADMA-histone–mediated Rhino loading followed by propagation, as seen in DART4 clusters. While it remains possible that these clusters formed through a different mechanism without passing through a DART4-cluster-like phase, such a scenario would leave the presence of residual ADMA-histones at their ends unexplained. Nonetheless, evolutionary analyses provide only indirect evidence, and experimental validation will be necessary to determine whether DART4 clusters can indeed mature into larger piRNA clusters.

In general, ADMA-histones are known to function as transcriptional coactivators[45]. In mammalian cells, H3R8me2a does not co-localize with H3K9me3, but

with enhancer modifications such as H3K4me1 and H3K27ac[25]. This may indicate that the function of ADMA-histones has evolved in a *Drosophila*-specific manner, which is not unlikely given that Rhino itself is *Drosophila*-specific. However, there are regions where Rhino does not localize in the read sets of ADMA-histones and H3K9me3, suggesting that an unknown factor may contribute to the specificity of Rhino targeting (Fig. 2E).

This study showed that multiple ADMA-histones, such as H3R17me2a and H4R3me2a, play a role in the initial Rhino loading. This suggests that a co-factor(s) with an affinity for ADMAs might be involved. Tudor domains are known to bind partner proteins through methylation of arginine and lysine residues[46], and Tudor domain-containing protein 3 (TDRD3) was identified as a factor that specifically binds to H3R17me2a and H4R3me2a in humans[47]. Therefore, TDRD protein family members may be involved in Rhino loading via ADMAs. ADMAs of other histone arginine residues such as H3R42 may also be involved in the regulation of Rhino behavior[42]. In this study, ChIP-seq was not performed for these modifications due to the lack of commercially available antibodies.

The mechanism by which ADMA-histones is involved in Rhino propagation remains unclear. Because Rhino propagation has been shown to be correlated with transcription[7,10], one interpretation is that ADMA-histones may affect Rhino spreading via transcriptional activity. Insertion of insulators into piRNA clusters inhibits piRNA production[48], indicating that the three-dimensional (3D) structure of the genome within the piRNA cluster also affects the extent of Rhino spreading. ADMA-histones were found at the ends of piRNA clusters, reminiscent of the boundaries of topologically associated domains. An unknown stabilizing factor(s) suggested by the absence of Kipferl in some DART4 clusters (Supplementary Fig. 7A, B) may be a relevant factor(s) determining such

genomic 3D structures.

Maternally inherited siRNAs initiate piRNA biogenesis from a transgene inserted into the uni-strand piRNA cluster 20A[14]. In this previous report, evidence was presented that siRNAs induce "*de novo"* Rhino loading onto this transgene (20A-X) via H3K9me3. This led us to hypothesize that ADMA-histones should be present in the vicinity of 20A-X. Indeed, we detected ADMA-histones in the region and Rhino, albeit weakly (Supplementary Fig. 5). Thus, this study provided experimental evidence in support of the "*de novo* model" by reinforcing the findings of siRNA induced piRNA biogenesis. This model has been becoming supported by evolutionary analyses[44,48,49], but experimental results had been limited. The evolutionarily young piRNA clusters, which appear in a limited number of strains, may have formed through the spread of Rhino into newly inserted transposons, facilitated by the presence of ADMA-histones in their vicinity (Fig. 7A).

In *Drosophila*, major piRNA clusters such as 42AB and 38C are located at the boundary between heterochromatin and euchromatin[4,51]. It was unclear why these highly transcribed clusters, which are capable of producing substantial piRNAs, tend to appear in regions where two different types of chromatins collide. If DART4 clusters represent the initial stage in the formation of piRNA clusters, our model can answer this question by showing that cluster formation begins with initial Rhino loading by ADMA-histones onto euchromatic regions and then spreads to adjacent regions of H3K9me2-rich heterochromatin (Fig. 7C). Rhino shows a tendency to spread from an DART4 clusters on chromosome 2 to heterochromatin/euchromatin boundary regions where 42AB and 38C are present (Fig. 6C), supporting our hypothesis.

Methods

Establishment and cloning of FLAG-Rhino inducible OSCs

To construct Tet-ON 3G inducible FLAG-Rhino plasmid, cDNA encoding FLAG-Rhino was cloned into pPB-2× Ty1-Tjen-EGFP-P2A-BlastR[52] using In-Fusion cloning (TAKARA), bearing pPB-FLAG-Rhino-Tjen-EGFP-P2A-BlastR. The Actin promoter and *EGFP* cDNA were replaced respectively with the TRE3G promoter and *rtTA* cDNA from inducible Caspex expression plasmid (Addgene). PCR was performed using PrimeSTAR MAX (TAKARA) with primers (Supplementary Table 1). FLAG-Rhino inducible OSCs were established using the piggyBac system as described previously[52]. In brief, 2×10^6 OSCs were transfected with 4 μg of pPB-TRE3G-FLAG-Rhino-Tjen-trTA-P2A-BlastR and 1 μg of pHsp70-Myc-hyPBase using Nucleofector Kit V (Lonza). After incubation in media containing blasticidin (50 μg/mL), colonies were isolated. One of the colonies contained a single FLAG-Rhino copy in the intron of the *jing interacting gene regulatory 1* gene, and this was used in this study.

Cell culture and RNAi

FLAG-Rhino inducible OSCs were cultured in Shields and Sang M3 Insect Medium (Sigma) supplemented with 10% fly extract, 10% fetal bovine serum, 0.6 mg/mL glutathione, and 10 mU/mL insulin[26,53]. To perform RNAi, 400 pmol of siRNA duplex was introduced into 3.0×10^6 cells using the Cell Line 96-well Nucleofector Kit SF (Lonza) with program DG150 of the 96-well Shuttle Device (Lonza). After 48 h incubation, cells were transfected with another 400 pmol of siRNA and incubated further for 48 h. For HP1a KD, RNAi was performed once to avoid cell damage. All siRNAs used are shown in Supplementary Table 2.

Fly stock

Flies were raised in standard Bloomington medium at 26 °C. Oregon-R was employed as a WT strain. For DART4 GLKD, BDSC 4937 (nosGAL4, w[1118]; P{w[+mC]=GAL4::VP16-nanos.UTR}CG6325[MVD1]) and BDSC 36833 (y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.GL01073}attP2) were used as the maternal GAL4 driver and DART4 UAS-RNAi lines, respectively. BDSC 36303 (y[1] v[1]; P{y[+t7.7]=CaryP}attP2) was used as a control (ctrl).

Western blotting

Western blotting was performed as described previously[54]. Antibodies are summarized in Supplementary Table 3. Nuclear extraction and immunoprecipitation were performed as described previously[55].

Immunofluorescence

Immunofluorescence of OSCs was performed essentially as described previously[56]. OSCs were fixed using 4% formaldehyde (Wako) and permeabilized in PBS containing 0.1% Triton X-100 for 24 h at RT. For DAPI staining, VECTASHIELD Mounting Medium with DAPI (Vector Laboratories) was used. Immunofluorescence of ovaries was performed as described previously[10]. All images were obtained using a confocal laser scanning microscope (Olympus FV3000 or Carl Zeiss LSM 710). For image processing, ZEN software (Carl Zeiss) was used. Antibodies used are listed in Supplementary Table 3.

Generation of mouse anti-Rhino monoclonal antibody

Anti-Rhino monoclonal antibody for immunofluorescence in the ovary were raised against N-terminal 200 residues of Rhino using a protocol published previously[57]. The N-terminal 200 residues of Rhino were fused to glutathione S-transferase (GST) and the purified fusion protein was used as antigen to immunize mice. The expression vector was produced by inserting the cDNA encoding the Rhino fragment into pGEX-5X-3 (Cytiva) by Infusion (Takara). The primers used are listed in Supplementary Table 1. The specificity of the antibody was validated using OSCs (Method Fig. A).

RNA extraction and RT-qPCR

Total RNAs were extracted from OSCs or ovaries with the ISOGEN II reagent (Nippon Gene) and treated with DNase (Life Technologies) to eliminate DNA contaminations. The cDNA libraries were prepared using the Transcriptor First Strand cDNA Synthesis Kit (Roche). For RT-qPCR, cDNAs were amplified using the StepOnePlus Real-Time PCR System (Applied Biosystems) with PowerUp SYBR Green Master Mix (Thermo Fisher Scientific). The expression level of target genes and transposons in OSCs and ovary were calculated by ΔΔCt method using *ribosomal protein 32* (*RP49/RpL32*) as an internal control. Data are presented as mean values ± SE. The primers used are listed in Supplementary Table 1.

OSC ChIP

OSCs were fixed in PBS containing 1% formaldehyde for 10 min at RT and glycine was added to 150 mM to stop the fixation. Cells were collected and suspended in swelling buffer [25 mM HEPES-KOH (pH 7.3), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 0.1% NP-

40, supplemented with cOmplete ULTRA Tablets, EDTA-free (Roche)]. The nuclei were collected by centrifugation at 1,000 g for 5 min at 4°C, suspended in sonication buffer [50 mM HEPES-KOH (pH 7.4), 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, supplemented with cOmplete ULTRA Tablets, EDTA-free], and sonicated using a Covaris S220 Focused ultrasonicator for 10 min at 4°C with setting of peak power of 200, duty factor of 10.0, and 200 cycles/burst. The chromatin fraction was obtained by centrifugation at 20,000 g for 15 min at 4°C and diluted with sonication buffer to make the DNA concentration to 0.2 mg/mL. Immunoprecipitation was performed O/N at 4°C using 1mL of chromatin fraction per ChIP. Antibodies used are listed in Supplementary Table 3. The reaction mixture was mixed with 25 μL of Dynabeads ProteinG (Invitrogen) and incubated for 1 h at 4°C. Beads were washed one time each with low-salt buffer [20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 0.1% Triton X-100, 0.1% SDS, and 1 mM phenylmethylsulfonyl fluoride (PMSF)], high-salt buffer [20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 2 mM EDTA, 0.1% Triton X-100, 0.1% SDS, and 1 mM PMSF], and LiCl buffer [20 mM Tris-HCl (pH 8.0), 250 mM LiCl, 2 mM EDTA, 1% NP-40, 1% sodium deoxycholate, and 1 mM PMSF], and then twice with TE buffer [20 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 1 mM PMSF]. The beads were suspended in elution buffer [50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 1% SDS] and incubated at 65°C for 30 min. For de-crosslinking, the supernatant was incubated O/N at 65°C after supplement of NaCl to 200mM. RNAs and proteins were eliminated from the sample by incubating at 37°C for 30 min with 10 mg/mL RNase A and 20 mg/mL proteinase K at 55°C for 1 h. The DNA in the supernatant was purified by EconoSpin column IIa (Funakoshi). 50 μL of nuclease-free water was added to it and the sample was centrifuged at 13,000 rpm for 1 min to obtain ChIP product. The following

experiments were conducted to evaluate the specificity of the antibodies involved in the main claims of this paper in ChIP-seq: H3R8me2a: ChIP-seq using two different anti-H3R8me2a antibodies showed that signal was obtained at the same genomic locations (Method Fig. B). H3R17me2a: A reduction in DART4-KD cells was demonstrated in separate experiments using western blotting (Method Fig. C). H4R3me2a: A histone peptide array kit (Active motif) confirmed that the antibody recognizes the H4R3me2a peptide with high specificity (Method Fig. D). The histone peptide array was performed according to the manufacturer's protocol.

Ovary ChIP

150 ovary pairs were crosslinked in PBS containing 1% formaldehyde for 10 min at RT, quenched with 180 mM glycine, and washed twice in PBS. Ovaries were flash frozen and suspended with swelling buffer (see "OSC ChIP"). Ovaries were disrupted using a Dounce homogenizer and incubated at 4°C for 10 min. The nuclei were collected by centrifugation at 1,000 g for 5 min at 4°C, suspended in sonication buffer, and sonicated (see "OSC ChIP"). The process after this is as described above (see "OSC ChIP"). Antibodies used are listed in Supplementary Table 3.

ChIP-seq library preparation

ChIP-seq libraries were constructed using NEBNext Ultra II FS DNA Library Prep Kit for Illumina (New England BioLabs) using ChIP products from "OSC ChIP" and "Ovary ChIP". In the adaptor ligation step, the original adaptor reagent was diluted 10-fold. Adapter ligated DNAs were PCR-amplified (10–12 cycles) with Illumina primers. The PCR products of 100–1,000 bp (including adaptor and primer sequences) were purified using

AMPure XP beads (Beckman Coulter) and captured on an Illumina flow cell. The libraries were sequenced using the Illumina MiSeq.

Computational analysis of ChIP-seq

Prior to mapping, low-quality reads were removed and adapters were trimmed using Fastp[58]. The filtered reads were mapped to the genome of *Drosophila melanogaster* (dm6) using Bowtie 2[59]. Only uniquely mapped reads were used in the analysis using samtools view command option -q 42[60]. For genome browser view, we mapped these unique reads and the inverse scores of the *Drosophila* genome mappability, a measure of mapping difficulty, calculated using GenMap software with the k-mer size set to 150[61]. We performed ChIP-seq experiment using at least two biological replicates, except for ChIP with anti-FLAG antibody in OSCs under Dox = 0.05 and 0.5ng/mL conditions. The replicates were highly correlated, so we merged them together and used the merged libraries for analyses. Because of the low mapping score, four replicates were prepared for Rhino and three for Kipferl in the GLKD condition. Peakcall was completed with MACS2 with q = 0.01 settings using input ChIP-seq data as control unless otherwise noted in figure legends[62]. For FLAG-Rhino peakcall in OSCs, anti-FLAG ChIP-seq data in Dox = 0 ng/mL condition was used as control. For Rhino peakcall in Oregon-R ovary in Fig. 1C, D and Fig. 4B, and H3K9me3 peakcall in OSCs in Fig. 1F, options --broad --broad-cutoff 0.01 were used. For Fig. 1D, called peak of more than 1.5 kb were extracted to select piRNA clusters where Rhino was localized broadly. For heatmaps and profile plots, ChIP scores were calculated by deeptools[63]. Bigwig files of ChIP were produced by the bamCoverage / bamCompare program of deeptools and matrix data were calculated by the computeMatrix program of deeptools using bigwig files and plotted. For

bamCompare program, anti-FLAG ChIP data with Dox = 0 (ng/mL) was subtracted as control for FLAG-Rhino in OSCs and input for the other data. Mapping file correlation and hierarchical clustering were computed using multibamSummary programs of deeptools. To draw the Venn diagram, the narrowPeak file obtained with Macs2 callpeak was stored in the findOverlapsOfPeaks function in R's ChIPpeakanno library and visualized with makeVennDiagram program[64]. The manorm was used for quantitative comparison of called peaks[65]. As ChIP-seq quality metrics, aligned rates are provided in Supplementary Table 4, while library complexity values and the SSP (strand cross-correlation peak)[66], a signal-to-noise ratio that can be calculated independently of read number or peak shape, are listed in Supplementary Table 5.

ChIP-qPCR

Real-time qPCR was performed using PowerUp SYBR Green Master Mix and StepOnePlus real-time PCR system. Data are presented as mean values ± SE. The primers used are listed in Supplementary Table 2.

Definition of DART4-dependent piRNA clusters

DART4-dependent piRNA clusters were defined using manorm results based on the macs2 callpeak with option p = 0.01 and --broad --broad cutoff 0.1 output files that met the following seven criteria. First, M value > 0.5 is required for Rhino to be predominantly enriched against DART4 GLKD. Second, the sharp peaks of the mononucleosome units were excluded because of their non-spreading initial Rhino localization. Third, peaks overlapped with DART4-dependent H3R17me2a were excluded because these peaks cannot distinguish spread Rhino and initial Rhino loading. Fourth, each Rhino peak was

checked in the genome browser, and if there were consecutive Rhino peaks around it that met the first, second and third criteria, they were grouped together as one peak. Fifth, peaks that overlap with major piRNA clusters such as 42AB, 38C, 80F, and 102F were excluded because they are highly transcribed piRNA clusters. Sixth, peaks on subtelomeres and pericentromeres were excluded because ChIP-seq reads cannot distinguish piRNA clusters or repeats. Finally, peaks whose localization spanned the entire length of a single transposon were excluded because they may reflect genomic differences between ctrl GLKD and DART4 GLKD, such as copy number variation of transposons. The combined peaks that met these seven criteria were defined as single DART4 cluster together with the surrounding contiguous ctrl GLKD-specific Rhino localizations if they were more than 5 kb. The genomic location of DART4 clusters in Fig. 6C was visualized using Phenogram software[67]. The regions of DART4 clusters are listed in Supplementary Table 6. The regions where Rhino localization increased upon DART4 GLKD were identified as regions with an M value of less than -2 and are listed in Supplementary Table 7.

Ovary piRNA extraction

100 ovary pairs were disrupted using a Dounce homogenizer and incubated in lysis buffer [20 mM HEPES-KOH (pH7.3), 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1% NP-40, 40 U/ml RNasin (Promega), 2 μg/ml pepstatin, 2 μg/ml leupeptin, 0.5% aprotinin] at 4°C for 10 min. The homogenized ovaries were centrifuged at 13,500rpm for 15 min at 4°C. The supernatant was then collected and cooled on ice. 10μg of anti-Piwi antibody was immobilized on Dynabeads protein G (Invitrogen) in lysis buffer at 4°C for 5 min. Beads were incubated with ovarian lysates at 4°C for 2 h, and then washed three times

with low-salt wash buffer [20 mM HEPES-KOH (pH7.3), 300 mM NaCl, 1 mM DTT, 0.05% NP-40, 2 μg/ml pepstatin, 2 μg/ml leupeptin, 0.5% aprotinin], three times with high-salt wash buffer [20 mM HEPES-KOH (pH7.4), 500 mM NaCl, 1 mM DTT, 0.05% NP-40, 2 μg/ml pepstatin, 2 μg/ml leupeptin, 0.5% aprotinin]. After wash, Piwi-bound RNAs were extracted from the beads with phenol–chloroform (Ambion) and then precipitated with ethanol. The precipitated RNAs were denatured with Gel Loading Buffer II (Ambion) for 5 min at 95°C, then electrophoresed on 12% urea-PAGE gel. 20-30 nt nucleic acids were gel purified as piRNAs. piRNA-seq library preparation followed a previous study[68].

Computational analysis of piRNA-seq

Raw reads were adapter trimmed and the random 4 bases of each side were removed using cutadapt[69]. Trimmed reads were mapped to the dm6 genome using bowtie with the option -m 1[70]. For read count in Supplementary Fig. 7D, this option was excluded. Read counts for each piRNA cluster were performed using bedtools[71]. For the analysis of piRNA in Rhino GLKD, GSM1580790 and GSM1580793 datasets[72] were used as piRNA-seq data in the ovaries of ctrl GLKD and Rhino GLKD, respectively.

Egg hatching assay

50 males and 50 virgin females were collected and mated in a cage with apple juice agar plate and yeast paste for 2 days at 25°C. After exchanging plates twice in the morning, the eggs on the plates were collected within 3 hours. Two hundred eggs were placed on new apple juice agar plates and incubated at 25°C for 2 days, followed by counting the number of hatched larvae.

Evolutionary analysis of piRNA clusters

The 500 bp around the ends of a novel dataset comprising piRNA clusters categorized into eight levels of evolutionary novelty[44] ("master-list" of Supplementary Table S4 in reference 44) was used for analysis. The ADMA-histones and input ChIP-seq reads mapped to the piRNA cluster regions were counted by bedtools and reads per piRNA cluster were calculated.Genomic regions with RPKM values above 30 in the input were excluded as they were considered ChIP-seq background noise. For Supplemntary Fig. 8B and 8C, only piRNA clusters with non-zero piRNA RPKM were used for analysis in the Oregon-R (Supplemntary Fig. 8B) and ctrl GLKD (Supplemntary Fig. 8C) strains, respectively. SRR4473614 was used as the piRNA of Oregon-R. Extraction of piRNA from SRR4473614 followed the methods of the original paper[50,73].

Data availability

ChIP-seq and piRNA-seq data generated in this study have been deposited in the Sequence Read Archive (SRA) under accession number PRJNAXXXXXXX and the Gene Expression Omnibus (GSE) under accession number GSEXXXXXX. All other data supporting the findings of this study are available from the corresponding authors upon request.

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Author contributions

R.S. and R.H. conducted biochemical experiments. R.S. and Y.N. performed bioinformatic analysis. H.I. produced FLAG-Rhino inducible OSCs. K.M. produced anti-Rhino antibody. R.S. and M.C.S. conceived the project and designed the experiments. R.S. and M.C.S. wrote the manuscript with input from all authors.

Disclosure and competing interest statement

The authors declare that they have no conflicts of interest.

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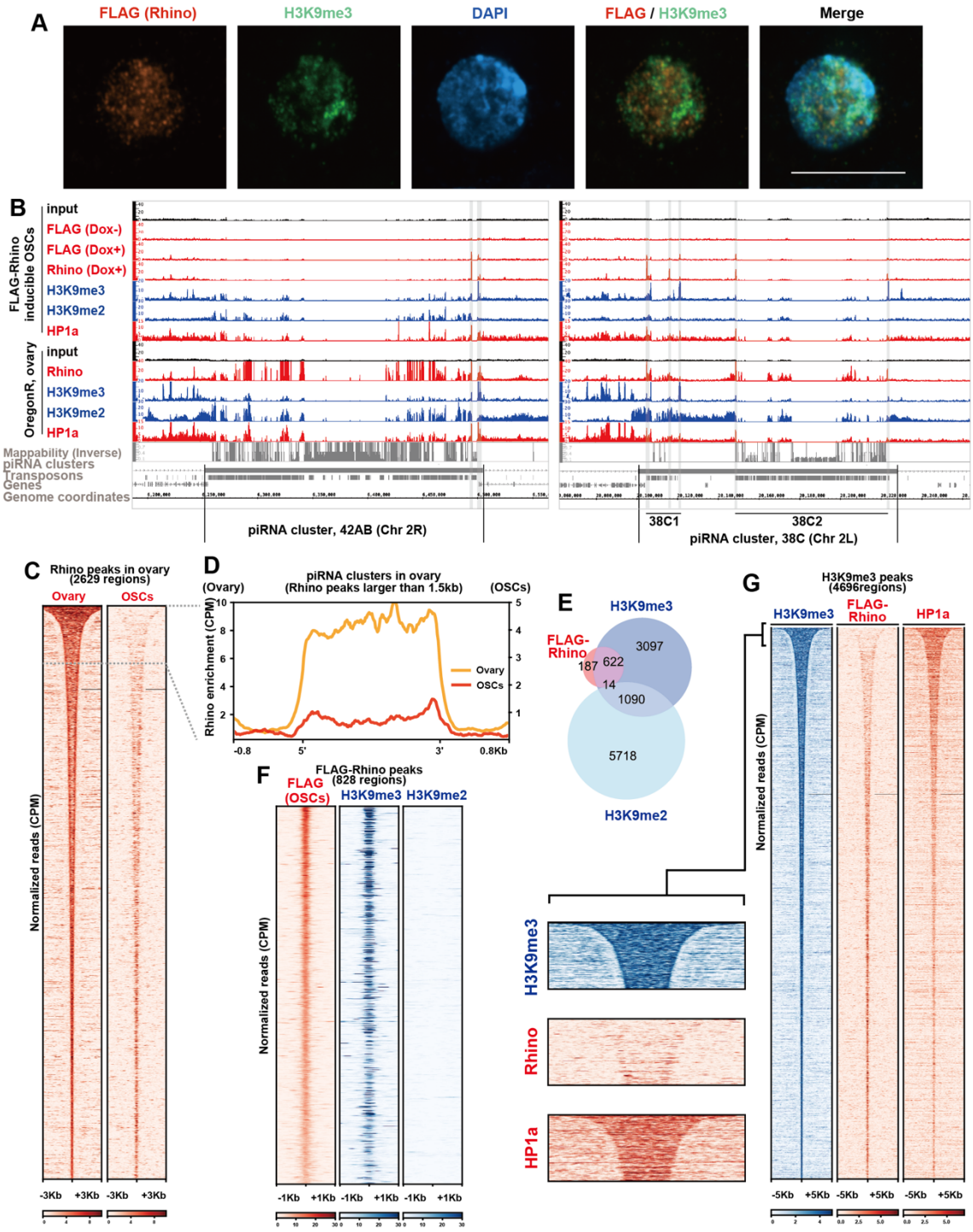


Figure 1. Rhino forms nuclear foci and binds the genome in OSCs

(A) Confocal immunofluorescent images of FLAG-Rhino (red) in FLAG-Rhino inducible OSCs upon Dox induction (5 ng/mL). H3K9me3 and OSC nucleus (DAPI staining) are shown in green and blue. Scale bar represents 10 μm. (B) Genome snapshot showing

ChIP-seq in major dual-strand piRNA clusters (42AB and 38C). The gray-shaded areas indicate the sites at which FLAG-Rhino is localized in each piRNA cluster in OSCs. piRNA cluster 38C has two piRNA-producing regions, labeled 38C1 and 38C2. (C) Heatmap showing FLAG-Rhino localization of OSCs in Rhino peaks of *Drosophila* wild-type Oregon-R ovary. The regions of 6 kb around the Rhino localization site were plotted based on macs2 callpeak results. (D) Metaplot showing the localization of FLAG-Rhino in OSCs at piRNA clusters. For piRNA clusters, broad peaks of Rhino localization in the ovary greater than 1.5 kb were used. (E) Venn diagram showing the overlap of Rhino, H3K9me3, and H3K9me2 peaks in OSCs. (F) Heatmap showing the signals of ChIP-seq using anti-FLAG, anti-H3K9me3, and anti-H3K9me2 in FLAG-Rhino peaks of OSCs. The regions of 2 kb around the FLAG-Rhino localization sites were plotted based on macs2 callpeak results. (G) Heatmap showing the signals of ChIP-seq using anti-H3K9me3, anti-FLAG, anti-HP1a antibodies at H3K9me3 broad peaks of OSCs. The regions of 10 kb around the FLAG-Rhino localization sites were plotted based on macs2 callpeak results.

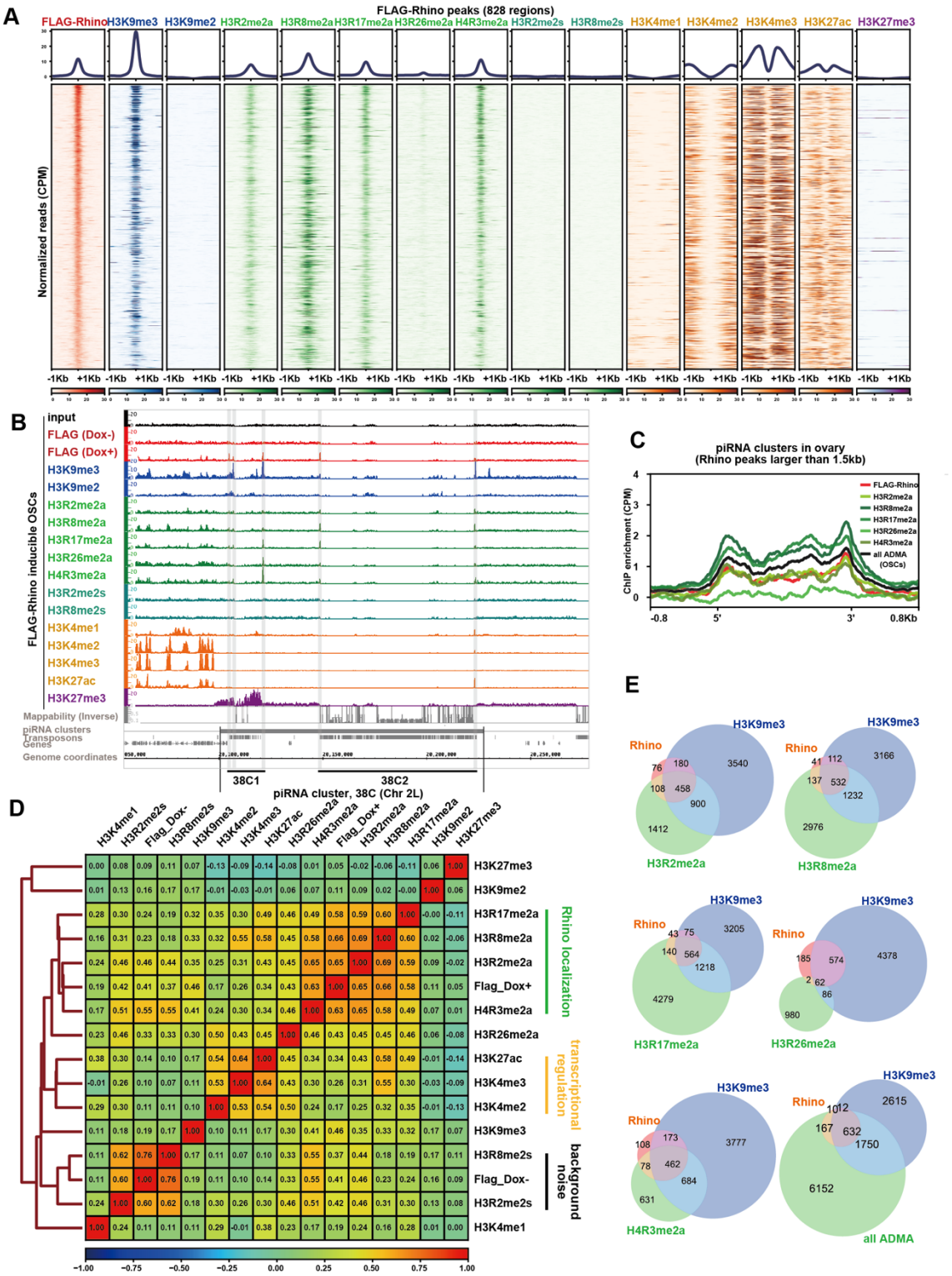


Figure 2. ADMA-histones tend to co-localize with FLAG-Rhino in OSCs

(A) Heatmap showing key histone modifications and HP1a ChIP-seq results at FLAG-Rhino localization sites (828 regions) in OSCs. The regions of 2 kb around the FLAG-

Rhino localization sites were plotted based on macs2 callpeak results. (B) Genome snapshot showing all histone modifications' ChIP-seq data in major dual-strand piRNA clusters (38C). The gray-shaded areas indicate the localization sites of FLAG-Rhino in each piRNA cluster in OSCs. (C) Metaplot showing the localization of ADMA-histones and FLAG-Rhino in OSCs at piRNA clusters. For piRNA clusters, broad peaks of Rhino localization in the ovary greater than 1.5 kb were used. (D) Pearson correlation coefficient comparing the coverage per 100 bp bin size of all ChIP-seq mapping results to the dm6 genome and its hierarchical clustering. (E) Venn diagram showing sites of overlapping localization of FLAG-Rhino, H3K9me3, H3R2me2a, H3R8me2a, H3R17me2a, H3R26me2a, H4R3me2a, and all ADMA-histones in OSCs.

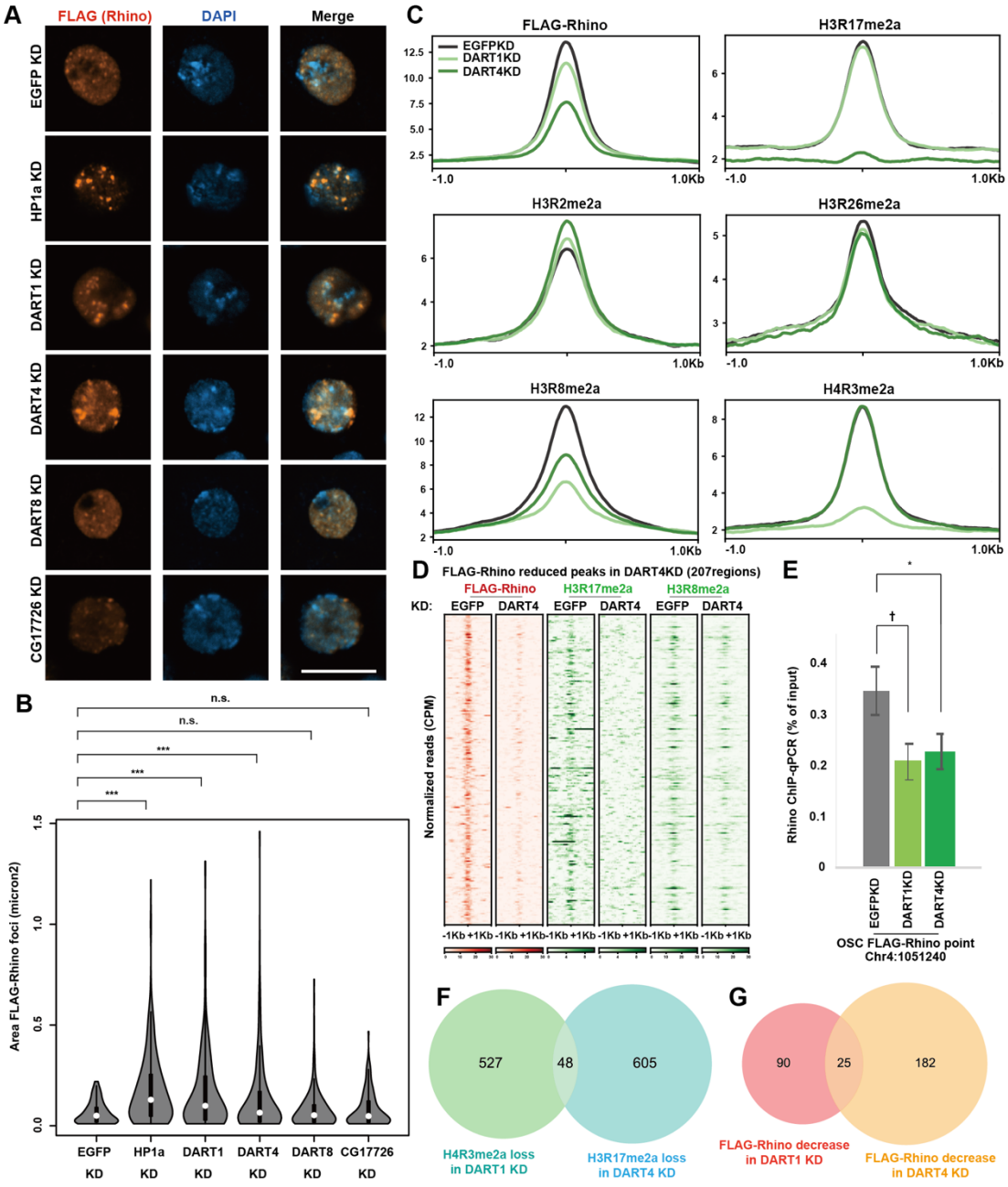


Figure 3. Arginine methyltransferases DART1 and DART4 play a role in Rhino genomic localization and foci formation in OSCs

(A) Confocal immunofluorescent images of FLAG-Rhino (red) and nuclei (blue; DAPI stained) in the EGFP-, HP1a-, DART1-, DART4-, DART8-, and CG17726-KD OSCs. Scale bar represents 10 μm. (B) Comparison of FLAG-Rhino foci sizes among the EGFP-, HP1a-, DART1-, DART4-, DART8-, and CG17726-KD OSCs in (A). Thirty OSCs

from three biological replicates were analyzed. Statistical significance in differences of Rhino foci between the genotypes was examined using Kolmogorov–Smirnov test. *P < 0.05, **P < 0.01, and ***P < 0.001. HP1a KD: ***P = 8.49×10^{-13}, DART1 KD: ***P = 1.83×10^{-7}, DART4 KD: ***P = 2.83×10^{-4}, DART8 KD: n.s. P = 0.655, CG17726 KD: n.s. P = 0.454. The box indicates the lower (25%) and upper (75%) quartiles, and the open circle indicates the median. Whiskers extend to the most extreme data points. (C) ChIP-seq profile showing the localization of FLAG-Rhino, H3R2me2a, H3R8me2a, H3R17me2a, H3R26me2a, and H4R3me2a in EGFP-, DART1-, and DART4-KD OSCs at FLAG-Rhino localization sites. The genomic regions used to draw the profile are the same as in Fig. 1G. (D) Heatmap showing the localization of FLAG-Rhino, H3R17me2a, and H3R8me2a in EGFP- and DART4-KD OSCs at the sites of decreased FLAG-Rhino localization in DART4 KD. The regions with decreased FLAG-Rhino were defined as those with an M value greater than 0.5 by manorm. macs2 callpeak p = 0.05 was used for manorm with input as a control. (E) FLAG-Rhino ChIP-qPCR results of one site at which Rhino initially localized. DART1 KD: †P = 5.88×10^{-2}, DART4 KD: * P = 3.17×10^{-2}. (F) Venn diagram showing the overlap of H3R17me2a localization decreased in DART4 KD and H4R3me2a localization decreased in DART1 KD. The regions with decreased ADMA-histones were defined as those with an M value greater than 1 in manorm. macs2 callpeak p = 0.01 was used for manorm. (G) Venn diagram showing the overlap of Rhino localizations that were decreased by DART1 KD and DART4 KD. The decreased genomic sites are identified as in (F).

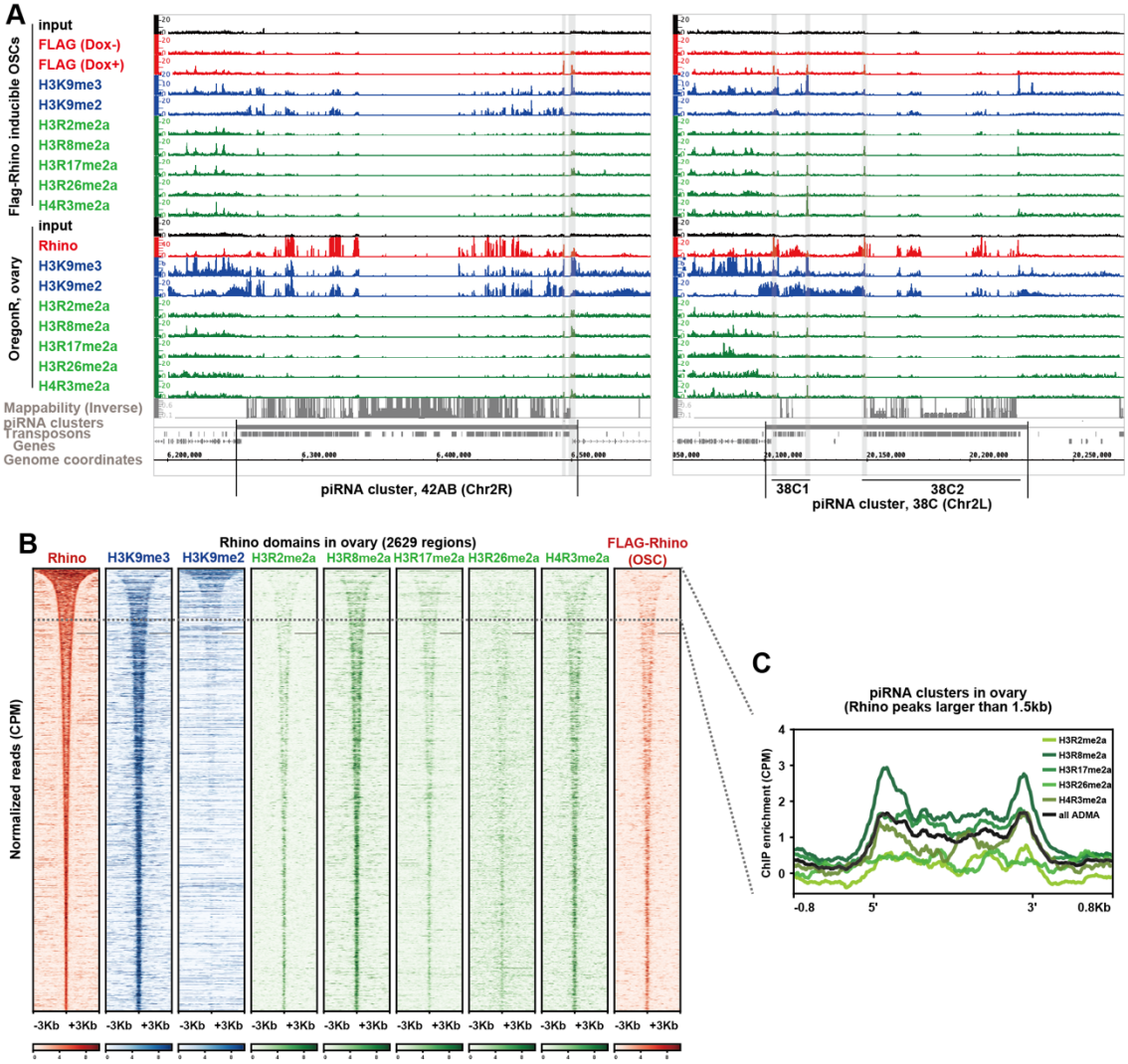


Figure 4. ADMA-histones co-localize with Rhino prior to Rhino spread in the ovary

(A) Genome browser snapshot showing the localization of H3R2me2a, H3R8me2a, H3R17me2a, H3R26me2a, and H4R3me2a in piRNA clusters in OSCs and Oregon-R ovary. The gray-shaded areas indicate the localization sites of FLAG-Rhino in each piRNA cluster in OSCs. (B) Heatmap showing the localization of H3R2me2a, H3R8me2a, H3R17me2a, H3R26me2a, and H4R3me2a at Rhino peaks in Oregon-R ovary. The Rhino peak regions shown in Fig. 1C were used. (C) Metaplot showing the localization of ADMA-histones in the ovary at piRNA clusters. For piRNA clusters, broad peaks of Rhino localization in the ovary greater than 1.5 kb were used.

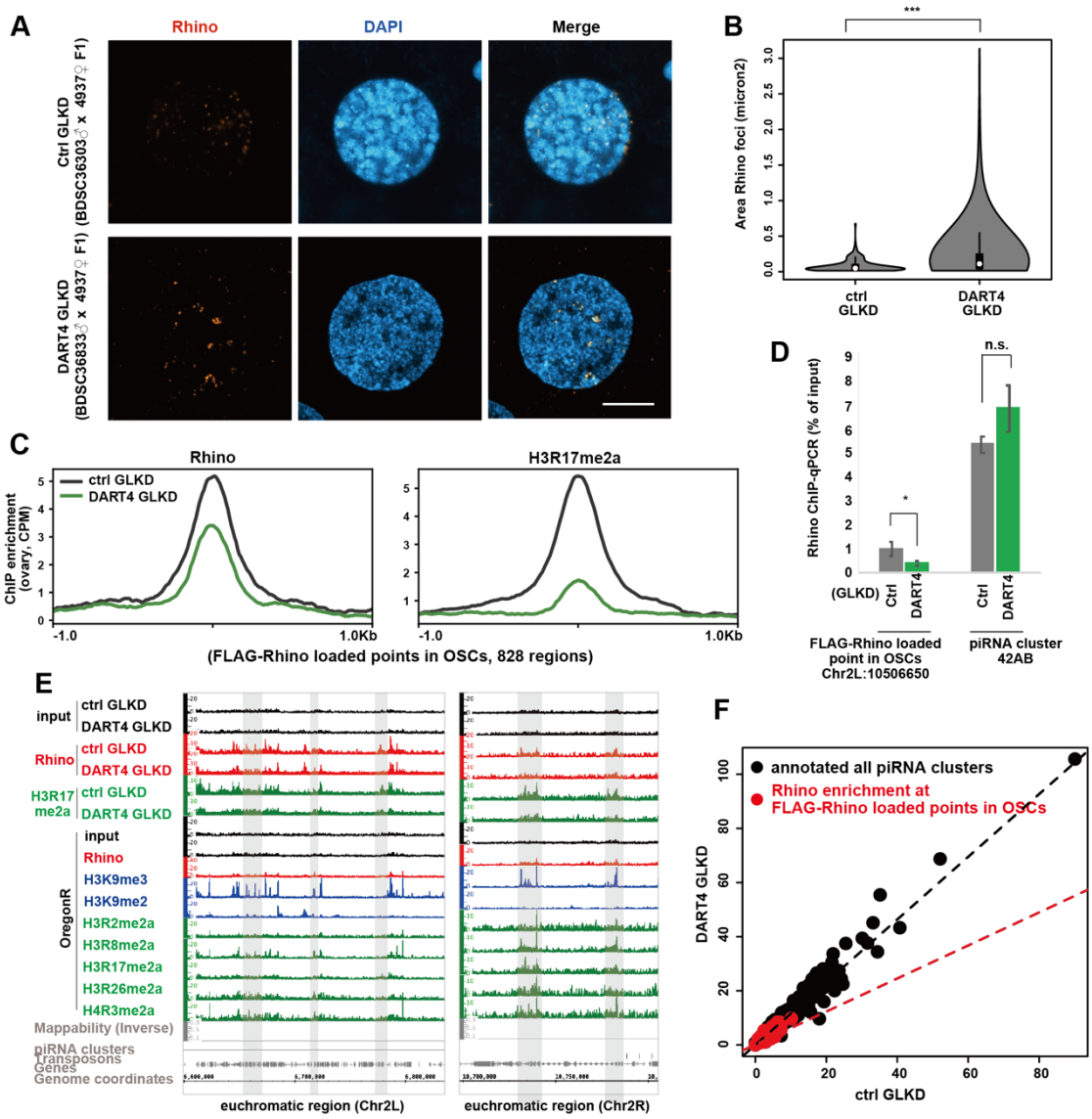
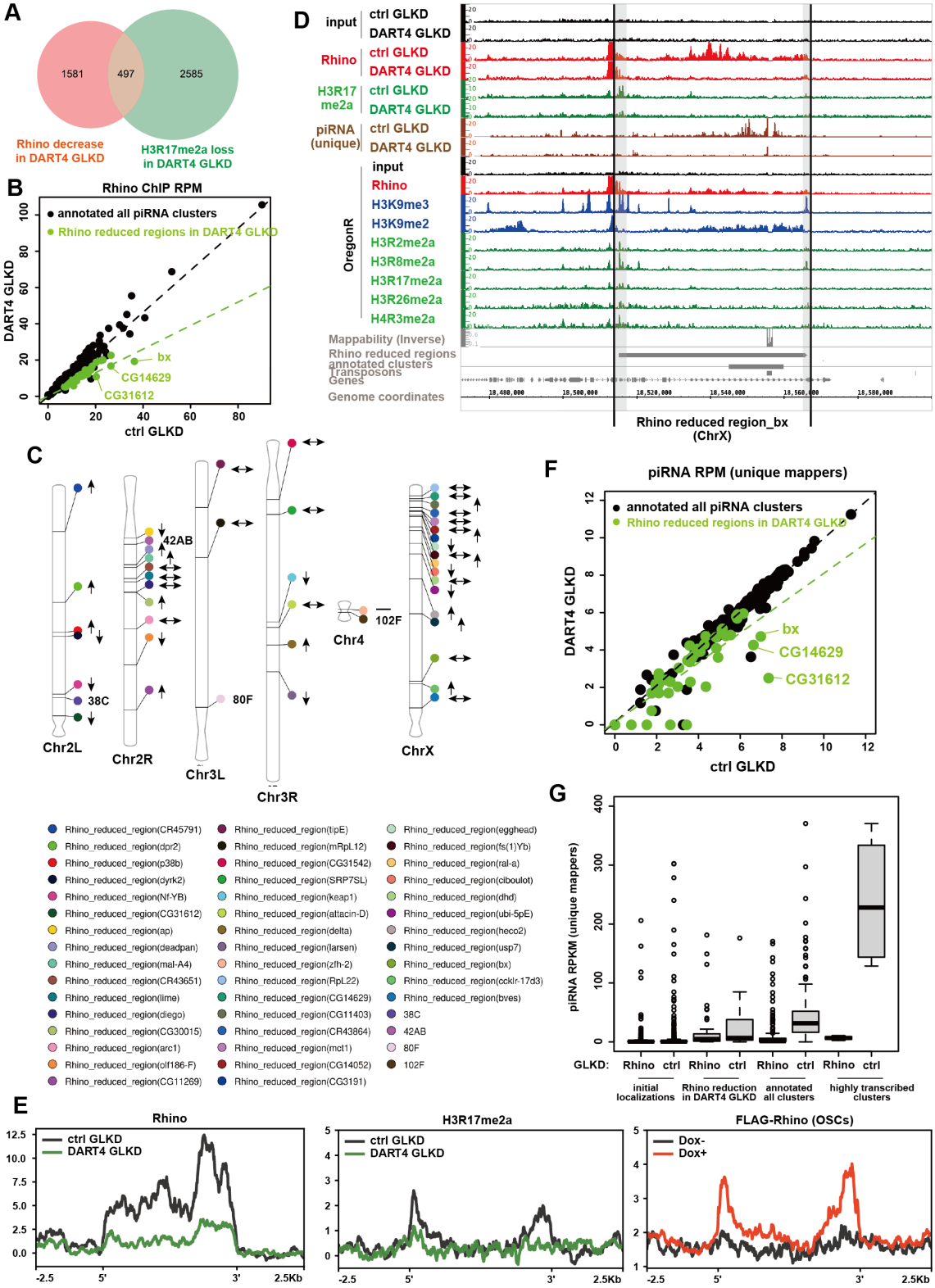


Figure 5. DART4 GLKD has impacts on Rhino foci and Rhino genomic localization in the ovary

(A) Confocal immunostaining images of Rhino (red) in ctrl- and DART4-GLKD ovaries. DAPI shows nuclei (blue). Scale bar represents 10 μm. (B) Comparison of sizes of Rhino foci between the ctrl- and DART4-GLKD ovaries in (A). Ten ovaries from two biological replicates were analyzed. Statistical significance in differences of Rhino foci between the two genotypes was examined as in Fig. 3B. DART4 GLKD: ***$P = 1.26 \times 10^{-10}$. (C) ChIP-seq profile showing the localization of Rhino and H3R17me2a in ctrl- and DART4-GLKD

ovaries at the FLAG-Rhino localization sites in OSCs. The FLAG-Rhino peak regions, as in Fig. 1G, are used. (D) Rhino ChIP-qPCR results of one site at which Rhino initially localized and piRNA cluster 42AB. t-test was used for statistical hypothesis testing. Rhino initial localization point: * $P = 4.03 \times 10^{-2}$, piRNA cluster 42AB: n.s. $P = 0.11$. (E) Genome snapshot of ChIP-seq data in euchromatic regions. The gray-shaded areas indicate genomic locations where Rhino localization is decreased in a H3R17me2a-dependent manner. (F) Scatter plot comparing Rhino enrichment in ChIP-seq between ctrl GLKD and DART4 GLKD. Each dot represents the square root of the read count per million mapped reads (RPM) within the annotated piRNA clusters in proTRAC database (colored in black, 184 clusters, without clusters on chromosome Y and uni-strand piRNA clusters flamenco/20A) and Rhino initial localizations as in Fig. 5C (colored in red, 828 regions). The dotted lines represent an approximation by a linear regression model.



1 **Figure 6. DART4-dependent piRNA clusters (DART4 clusters) are genomic loci**

2 **where Rhino spreads from H3R17me2a**

(A) Venn diagram showing the overlap between sites with reduced Rhino localization and sites where H3R17me2a was lost in DART4 GLKD. The regions where Rhino was decreased were defined as those with an M value greater than 0.5 by manorm, and the regions where H3R17me2a was lost were defined as those with an M value greater than 0. For manorm, the output results of macs2 callpeak p = 0.01 were used with input as a control. (B) Scatter plot comparing Rhino enrichment in ChIP-seq between ctrl GLKD and DART4 GLKD. Each dot represents the square root of the read count per million mapped reads (RPM) within the annotated piRNA clusters in proTRAC database (colored in black, 184 clusters, without clusters on chromosome Y and uni-strand piRNA clusters flamenco/20A) and Rhino reduced regions in DART4 GLKD (colored in green, 43 clusters). The dotted lines represent an approximation by a linear regression model. (C) Chromosomal locations of Rhino reduced regions in DART4 GLKD and the estimated direction of Rhino spread based on initial localization in OSCs. Depressed areas indicate pericentromeres. Arrows show the spread direction based on ADMA-histones. Bidirectional arrows indicate that ADMA-histones are present at both ends and the direction of spread cannot be determined. Horizontal bars indicate clusters without ADMA-histones at both ends. (D) Genome snapshot showing Rhino reduced regions in DART4 GLKD around the *bx* gene. Black lines mark Rhino reduced regions boundaries, and gray-shaded areas highlight potential ADMA-histone localization at cluster ends. (E) ChIP-seq profiles of Rhino and H3R17me2a in ctrl GLKD and DART4 GLKD across all 43 Rhino reduced regions in DART4 GLKD, and Rhino in OSCs. (F) Scatter plot comparing piRNA enrichment between ctrl GLKD and DART4 GLKD. Each dot represents the logged RPM value within piRNA clusters (black: 184 annotated clusters; green: 43 Rhino reduced regions in DART4 GLKD). Dotted lines indicate linear

regression. (G) Box plot showing RPKM of piRNAs uniquely mapped to Rhino sites in OSCs, Rhino reduced regions in DART4 GLKD, all proTRAC-annotated clusters, and highly transcribed clusters (42AB, 38C, 80F, 102F). Clusters with RPKM below 400 are shown.

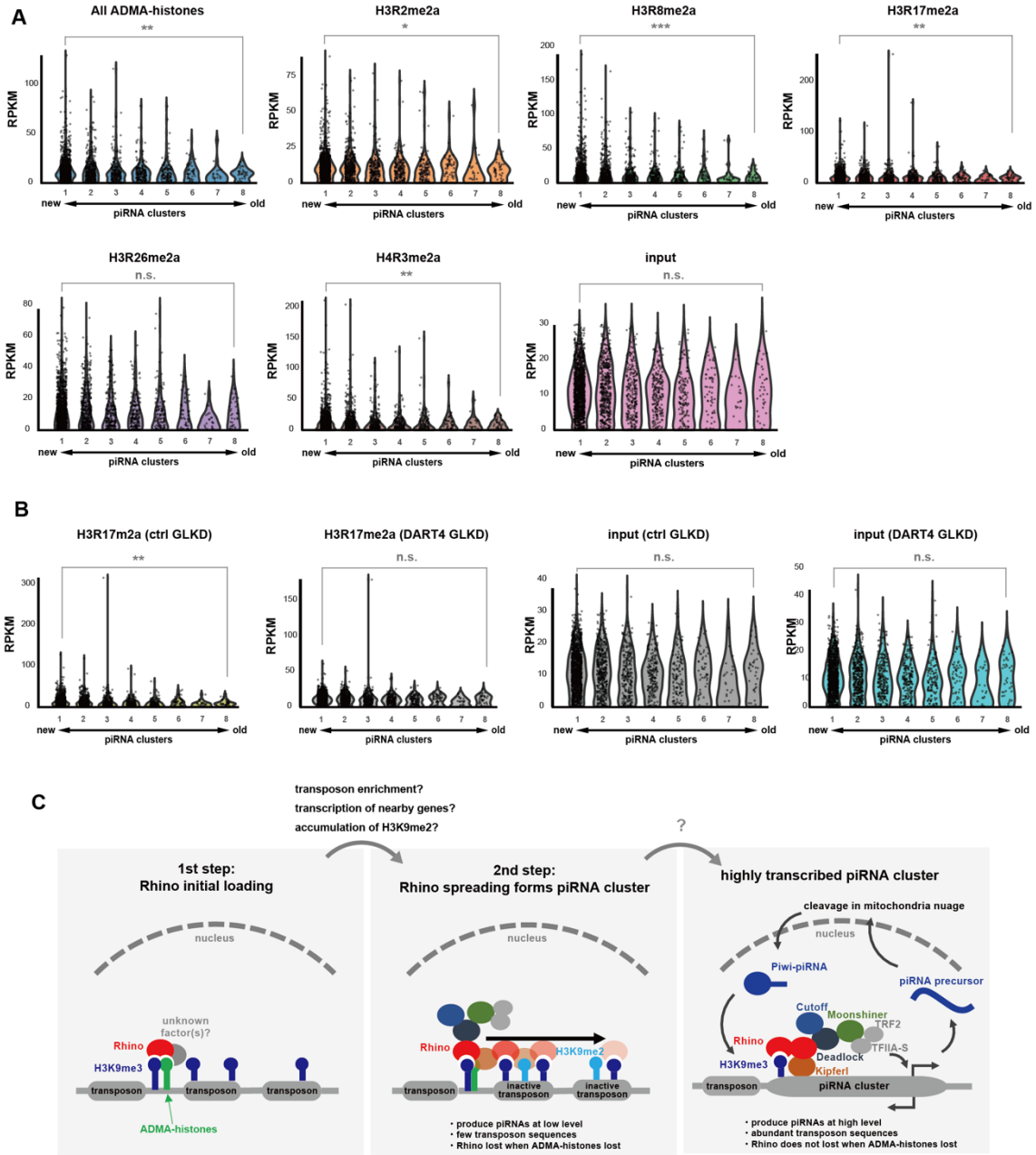


Figure 7. ADMA-histones are enriched at the ends of evolutionarily young piRNA clusters

(A) RPKM of each ChIP-seq dataset (Oregon-R) in the piRNA cluster dataset with eight levels of evolutionary novelty. RPKM was calculated for the 500 bp region surrounding the ends of the piRNA clusters. t-test was used for statistical hypothesis testing between clusters found only in 1 strain (cluster 1, newly emerged) and clusters found in all 8

strains (cluster 8, evolutionarily conserved). All ADMA-histones: ** $P = 1.11 \times 10^{-3}$, H3R2me2a * $P = 1.45 \times 10^{-2}$, H3R8me2a: *** $P = 1.27 \times 10^{-4}$, H3R17me2a: ** $P = 7.28 \times 10^{-3}$, H3R26me2a: n.s. $P = 0.23$, H4R3me2a: ** $P = 1.91 \times 10^{-3}$, input: * $P = 0.53$. (B) Same analysis of Fig. 7A for each ChIP-seq dataset (ctrl GLKD and DART4 GLKD). H3R17me2a (ctrl GLKD): ** $P = 2.77 \times 10^{-3}$, H3R17me2a (DART4 GLKD): n.s. $P = 0.24$, input (ctrl GLKD): n.s. $P = 0.42$, input (DART4 GLKD): n.s. $P = 0.47$. (C) Model for the piRNA cluster formation. In the cell conditions lacking Kipferl such as in OSCs, Rhino initially localizes to bivalent nucleosomes containing H3K9me3 and ADMA-histones. In the second step, Rhino spreading occurs, leading to the formation of DART4 clusters. Transcription of adjacent regions and/or H3K9me2 enrichment may play a role in this process. Rhino in DART4 clusters is lost if ADMA-histones are lost. It remains unclear whether DART4 clusters can eventually develop into highly transcribed piRNA clusters, such as 42AB, over the course of evolutionary time.