piRNAs mediate posttranscriptional retroelement silencing and localization to pi-bodies in the Drosophila germline

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Introduction

The fidelity of genomic information in the germline has to be tightly regulated for accurate transmission to the next generation. In many animal germline cells, Piwi-interacting RNAs (piRNAs) are reported to silence the expression of one class of mobile genetic elements, retroelements, whose transposition may afflict the genome with mutational burden (Lau et al., 2006; Vagin et al., 2006; Aravin et al., 2007; Houwing et al., 2007). In flies and mammals, the Piwi subfamily proteins are engaged in a feed-forward loop to mediate the generation of sense and antisense piRNAs from the active transposons and piRNA clusters (Vagin et al., 2006; Aravin et al., 2007; Brennecke et al., 2007; Houwing et al., 2007). This amplification cycle could contribute to the removal of both strands of retroelement transcripts via a processing mechanism. However, sense piRNAs are present at very low levels in wild-type ovaries (Vagin et al., 2006), and antisense retroelement transcripts are not as abundant as their sense counterparts when processing is compromised in the male and female germlines (Aravin et al., 2001; Savitsky et al., 2006; Chambeyrong et al., 2008; Shpiz et al., 2009). This suggests distinct silencing mechanisms other than the feed-forward processing loop, which could remove the sense strand transcripts. Possible mechanisms include piRNA-mediated transcriptional/cotranscriptional silencing via chromatin modifications and posttranscriptional destabilization of retroelement transcripts.

Cellular mRNA turnover is mediated by conventional mRNA degradation enzymes, such as the decapping enzymes, deadenylases, and exoribonucleases. Some of these enzymes and RNA-binding proteins, such as GW182, are reported to localize to the processing bodies, the putative site where mRNAs are degraded (for review see Anderson, 2005; Eulalio et al., 2007; Parker and Sheth, 2007). Biochemical analyses have demonstrated that the processing body components GW182 and DCP1 interact with the RNA-induced silencing complexes (RISCs) argonaute 1 (AGO1) and AGO2 in mammalian cells (Liu et al., 2005a,b; Behm-Ansmant et al., 2006) and that the Caenorhabditis elegans homologue of GW182, AIN-1, interacts with a putative AGO family protein ALG-1 (Ding et al., 2005). In addition, RISCs have been reported to localize to the nuage, a well-conserved perinuclear organelle found in germline cells, to mediate retroelement repression in Drosophila melanogaster by regulating the production of Piwi-interacting RNAs (piRNAs). In this study, we present evidence that the nuage–piRNA pathway components can be found in cytoplasmic foci that also contain retroelement transcripts, antisense piRNAs, and proteins involved in messenger RNA (mRNA) degradation. These mRNA degradation proteins, decapping protein 1/2 (DCP1/2), Me31B (maternal expression at 31B), and pacman (PCM), are normally thought of as components of processing bodies. In spindle-E (spn-E) and aubergine (aub) mutants that lack piRNA production, piRNA pathway proteins no longer overlap the mRNA degradation proteins. Concomitantly, spn-E and aub mutant ovaries show an accumulation of full-length retroelement transcripts and prolonged stabilization of HeT-A mRNA, supporting the role of piRNAs in mediating posttranscriptional retroelement silencing. HeT-A mRNA is de-repressed in mRNA degradation mutants twin, dcp1, and ski3, indicating that these enzymes also aid in removing full-length transcripts and/or decay intermediates.

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Abbreviations used in this paper: CDS, coding sequence; DIG, digoxigenin; LM, ligation mediated; MCP, MS2 coat protein; PAT, poly(A) tail test; piRNA, Piwi-interacting RNA; RACE, rapid amplification of cDNA ends; RISC, RNA-induced silencing complex; rRNA, ribosomal RNA; UTR, untranslated region.

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processing bodies in human cultured cells (Liu et al., 2005b; Sen and Blau, 2005; Jagannath and Wood, 2009), implying that small RNA-mediated mRNA degradation and/or translational repression could take place in the processing bodies.

Several lines of evidence have implicated the involvement of the nuage, a well-conserved structure in animal germline cells, in posttranscriptional silencing. In Drosophila melanogaster, Dicer-1 and AGO2 are mislocalized in a nuage component mutant maelstrom, suggesting a connection between the nuage and microRNA pathway (Findley et al., 2003). Consistent with this, processing body proteins DCP1a and GW182 and microRNAs let-7 and miR-21 are reported to colocalize with the mouse nuage (Kotaja et al., 2006; Beaudoin et al., 2009). In the suppressor of stellite-deficient testes aubergine (aub), spliced stellite transcript accumulates, and stellite protein is dramatically translated (Kotelnikov et al., 2009), implying that stellite expression is regulated posttranscriptionally. In C. elegans, processing body components PATR-1, CCF-1, DCP2, and CGH-1 have been demonstrated to overlap with P granules (a counterpart of the nuage) in the germline blastomeres (Lall et al., 2005; Gallo et al., 2008). Besides, the presence of abundant processing bodies in Drosophila germline cells (Lin et al., 2008) implies that posttranscriptional regulation is actively taking place and may therefore aid in retroelement decay.

In this study, we show that the piRNA pathway proteins, retroelement transcripts, piRNAs, and mRNA degradation components localize to common cytoplasmic foci. We demonstrate that HeT-A mRNA is stabilized in the piRNA pathway mutant aub and derepressed in the mRNA degradation mutants twin, dcp1, and ski3. These findings suggest that posttranscriptional retroelement silencing is piRNA dependent and that some mRNA degradation enzymes assist in removing the retroelement transcripts. Localization of the piRNA and mRNA degradation components into common foci may reflect the assembly of a macromolecular complex dedicated to the removal of retroelement transcripts.

Results and discussion

Two Piwi subfamily proteins, aubergine (AUB) and AGO3, and a tudor domain protein, KRIMP, have been described previously to localize in perinuclear foci called the nuage in Drosophila germline cells (Snee and Macdonald, 2004; Bremnecke et al., 2007; Lim and Kai, 2007). Interestingly, we observed that these nuage components also existed in cytoplasmic foci that were 0.1–1 μm in diameter (Fig. 1a, arrows; Harris and Macdonald, 2001). These cytoplasmic foci became progressively prominent from stage 4 onwards during oogenesis and were ubiquitously distributed as discrete puncta throughout the nurse cell cytoplasm at stages 4–5 (Fig. 1a). The spatial and temporal distributions of these cytoplasmic foci resemble the processing bodies described in the Drosophila germline (Lin et al., 2008). We costained for the processing body components dDCP1, dDCP2 (Lin et al., 2006), Me31B (a homologue of yeast-decapping activator Dh1p; Coller et al., 2001), and the Drosophila homologue of yeast Xrn1p, pacman (PCM; Till et al., 1998; Barbee et al., 2006; Zabolotskaya et al., 2008). 40–57%, 38–51%, and 31–79% of the processing bodies were found to overlap or dock AUB, AGO3, and KRIMP foci, respectively (Fig. 1, b [arrows], c, and d). This large percentage variation suggests that the association of cytoplasmic nuage with processing bodies is highly dynamic. We also observed processing body foci that lacked the piRNA pathway components (Fig. 1, b and e, arrowheads), suggesting that a subset of processing bodies contains piRNA pathway components, whereas others do not. These observations imply that cytoplasmic foci identifiable as the processing bodies include molecular complexes with distinct functions, as reflected by their different compositions.

Nuage components are reported to mediate retroelement repression in the germline (Lim and Kai, 2007; Pane et al., 2007). To ask whether the cytoplasmic foci containing the nuage and processing body components are involved in retroelement silencing, we looked for the presence of the retroelement transcripts using the MS2 coat protein (MCP)–GFP-labeling system (Forrest and Gavis, 2003). We generated flies harboring two heat shock–inducible transgenes. One contained HeT-A or I-element coding sequences (CDSs), devoid of the 5′ untranslated region (UTR) and promoter regions, and fused to six tandem stem-loop–binding sites for bacteriophage MCP at the 3′ UTR. The other encoded for the fusion protein MCP-GFP. Upon induction, MCP-GFP binds the recognition motif on HeT-A–(ms2)5 or I-element–(ms2)6 transcripts so that these mRNAs can be visualized as GFP signal.

In control (aub or krimp heterozygote) ovaries, GFP signal was found in cytoplasmic foci that were also stained for the 5′ to 3′ exoribonuclease PCM and the piRNA pathway protein KRIMP (Fig. 2a and a′, arrows). These GFP-labeled foci were not detected in the ovary expressing MCP-GFP alone (Fig. 2a), indicating that GFP signals represent full-length HeT-A–(ms2)6 transcripts or the decay intermediates harboring MCP-binding sites. Similarly, we observed the localization of GFP-labeled I-element transcript to KRIMP/PCM foci in the wild-type ovary (Fig. 2a, arrows). Using a nonretroelement control nanos (nos), we did not observe localization of nos–(ms2)6 to distinct cytoplasmic bodies (Fig. 2a′; Forrest and Gavis, 2003), confirming that the localization of the retroelement transcripts is not artifactual. In aub and krimp mutant ovaries, the GFP-labeled HeT-A transcript no longer localized to the cytoplasmic KRIMP foci (Fig. 2a). Instead, it appeared to be diffuse in the cytoplasm and nucleus, indicating that the granular localization of the transcripts observed in the control ovaries depends on AUB and KRIMP functions.

AUB and AGO3 complexes are reported to associate with piRNAs in the germline (Gunawardane et al., 2007; Nishida et al., 2007). In addition, recombinant AUB and AGO3 are competent for cleaving a substrate RNA at an siRNA target site (Gunawardane et al., 2007), indicating that these AGO proteins are potential constituents of germline RISCs. To determine whether the nuage cytoplasmic foci consist of piRNA-loaded germline RISCs, we examined the localization of antisense piRNAs by FISH. The antisense HeT-A piRNA signals colocalized with the GFP–HeT-A foci in the aub control ovary (Fig. 2b, b′ and b′′, arrows), whereas an irrelevant probe hybridizing to antisense 2S ribosomal RNA (rRNA) or a piRNA-unrelated
HeT-A antisense region did not exhibit any significant colocalization (Fig. 2, b and b'; and not depicted). A control staining with secondary antibodies alone did not exhibit nonspecific labeling of the GFP–HeT-A foci (unpublished data), further indicating the specificity of HeT-A piRNA probe. In aub mutants in which the production of piRNAs is impaired (Vagin et al., 2006), the antisense HeT-A piRNA signal was undetectable (Fig. 2 b). Similarly, we observed specific antisense HeT-A piRNA signal in control but not krimp mutant ovaries (unpublished data). The localization of both piRNAs and the target
Figure 2. Retroelement transcripts and antisense piRNAs colocalize with nuage cytoplasmic foci and mRNA degradation proteins (pi-bodies). (a and a') Immunostaining of ovaries expressing MCP-GFP fusion protein and the transgene of retroelements harboring MCP-binding sites (HeT-A-[ms2] or I-element-[ms2]) in control (aub or krimp heterozygotes) and aub or krimp mutants. HeT-A-[ms2] or I-element-[ms2] tagged with GFP colocalizes with the piRNA pathway component KRIMP (magenta) and 5'-3' exoribonuclease PCM (red) in the same cytoplasmic foci (a', arrows). In aub and krimp mutants, GFP-labeled HeT-A mRNA appears to be largely cytoplasmic and nuclear and no longer localizes to the cytoplasmic KRIMP foci. (a') A nonretroelement control nos-[ms2] recapitulates the endogenous mRNA localization at the oocyte posterior, but no localization to distinct cytoplasmic bodies is observed. Bars: (egg chambers) 20 µm; (nurse cells) 10 µm. (b) FISH for antisense HeT-A piRNAs in aub mutant. (b') In the aub control ovary, antisense HeT-A piRNAs colocalize with GFP-labeled HeT-A mRNA in the same cytoplasmic bodies (arrows). FISH with the control probe against antisense 25S rRNA does not colocalize with GFP-labeled HeT-A mRNA. In aub mutant, unique antisense HeT-A piRNA signal is undetectable. Bars, 10 µm.
transcripts to common foci that possibly contain the mRNA degradation proteins reflect the assembly of a macroRNP complex committed to the removal of retroelement transcripts. To distinguish them from conventional processing bodies, we termed these foci pi-bodies.

In spindle-E (spn-E) and aub mutants in which the production of piRNAs is compromised (Vagin et al., 2006; Chen et al., 2007), we did not detect any cytoplasmic KRIMP foci that overlap with dDCP1/2, Me31B, and PCM (Fig. 2 a and Fig. 3), indicating that pi-body assembly is disrupted. This observation suggests that cytoplasmic nuage proteins and the targeted mRNAs are recruited to mRNA degradation components, or vice versa, in a piRNA-dependent manner, forming pi-bodies that can contribute to retroelement silencing.

To exclude the possibility that the failure of pi-body assembly in spn-E and aub mutants impairs the activities of mRNA degradation proteins and results in retroelement silencing, we checked two representative activities of mRNA turnover: deadenylation and decapping of a nonretroelement transcript cyclin B (cycB). The rapid degradation of the cyclin transcripts has been shown to be regulated by deadenylation (Morris et al., 2005). To monitor deadenylation activity, we examined the length of the cycB poly(A) tail in the aub mutant using the poly(A) tail test (PAT; Fig. 4 a; Salles et al., 1999). In comparison with the deadenylase mutant twin in which deadenylation was compromised and longer poly(A) tails were observed, cycB transcript appeared to be efficiently deadenylated in the aub mutant. This indicates that deadenylation is unaffected in the piRNA pathway mutants.

To examine decapping, total RNAs extracted from both control and aub mutant ovaries were treated with Terminator 5'-phosphate–dependent exonuclease and subjected to RT-PCR for cycB. RNA molecules with 5' cap structures are protected from the exonuclease and therefore competent for amplification. Similarly, to the control, cycB transcript was comparably degraded by the exonuclease in the aub mutant (Fig. 4 b), indicating that cycB mRNA is efficiently decapped. Furthermore, we did not detect any changes in the protein expression of four mRNA degradation proteins: PCM, SKI3, Me31B, and dDCP1 in aub or krimp mutants (Fig. 4 c). Collectively, these data show that the retroelement-silencing defect in the piRNA pathway mutants is not caused by impairment of the mRNA degradation machinery.

To determine whether piRNA-mediated retroelement silencing is posttranscriptional in vivo, we investigated retroelement decay in the aub control and mutant ovaries using the inducible HeT-A transgene harboring MS2. After the induction of HeT-A MS2 mRNA by heat shock, ovaries were dissected at time intervals up to 12 h, and Northern blotting was performed with a probe that specifically detects exogenous HeT-A MS2. In the control ovary, HeT-A MS2 mRNA was rapidly degraded, and the level of transcript was undetectable by 100 min after induction (Fig. 5 a). However, in the aub mutant, HeT-A mRNA exhibited prolonged stabilization (Fig. 5, a and a') and remained detectable 2–3 d after induction (not depicted). This suggests
that a prolonged posttranscriptional retroelement silencing is dependent on piRNAs.

spn-E mutation causes down-regulation of the repressive chromatin marks H3K9me2 and H3K9me3 at the retroelement promoters, implying that piRNA-dependent retroelement expression is regulated at the transcriptional level (Klenov et al., 2007). Our data further indicate that piRNA-mediated retroelement silencing is in part posttranscriptional. Collectively, at least two hierarchies of retroelement surveillance appear to function in the fly germline: posttranscriptional regulation in the cytoplasm and transcriptional control in the nucleus. Because our exogenous HeT-A transcript was under the control of an inducible promoter and was efficiently repressed in the control ovary, the contribution of natural promoters or UTRs in mediating silencing is ruled out. This also emphasizes posttranscriptional retroelement silencing by piRNAs in trans. However, it remains possible that piRNAs are targeted to the nascent transcript and silence HeT-A cotranscriptionally.

We observed significant accumulations of endogenous full-length HeT-A and I-element transcripts in spn-E, aub, and krimp mutants (Fig. 5 b). Furthermore, a low level of full-length HeT-A transcript accumulated in the deadenylase mutant twin, indicating the involvement of the mRNA degradation enzymes in the posttranscriptional silencing of retroelements either in a piRNA-dependent or -independent manner. We did not observe either of the full-length retroelements or decay intermediates in dcp1, ski3, or pcm mutants (Fig. 5 b and Fig. S1). However, a more sensitive quantitative assay, rapid amplification of cDNA ends (RACE)–PAT/RT-PCR, detected derepression of the CDS, UTRs, and poly(A) regions of HeT-A mRNA in dcp1 and ski3 mutants (Fig. 5 c), further indicating that removal of the retroelement transcripts and/or the decay intermediates involves mRNA degradation enzymes.

We did not detect an obvious reduction of HeT-A and I-element antisense piRNAs in twin, dcp1, ski3, and pcm mutants as compared with the respective control heterozygotes (Fig. S2 a). Furthermore, we observed normal perinuclear and cytoplasmic localization of proteins involved in piRNA production, AUB, AGO3, and KRIMP in the mRNA degradation mutants (Fig. S2 b). These observations suggest that piRNA biogenesis is unaffected and does not contribute to retroelement derepression in those mutants.

Foregoing work has suggested the nuage as a potential site for RISC-mediated posttranscriptional retroelement silencing (Harris and Macdonald, 2001; Kennerdell et al., 2002; Findley et al., 2003; Brennecke et al., 2007; Li et al., 2009; Malone et al., 2009). The 5′ and 3′ moieties of the decay intermediates generated by RISC-mediated endoribonucleolytic cleavage are removed by the XRN1–exosome and SKI–exosome complexes, respectively, in S2 cells (Orban and Izaurralde, 2005). Using the Drosophila ovary as an in vivo system, we have reported the localization of the germline AGO proteins, AUB and AGO3, and mRNA degradation enzymes in the pi-bodies, implying that the mRNA degradation machinery mediates the posttranscriptional removal of the retroelement transcripts or decay intermediates, possibly upon piRNA-mediated cleavage. The absence of retroelement decay intermediates in vivo in our blotting analyses with single
Figure 5. Posttranscriptional retroelement silencing is piRNA dependent. (a) Time course of HeT-A MS2 mRNA decay in the aub mutant. HeT-A MS2 expression is induced by heat shock, and changes in mRNA abundance are measured over time. A rapid initial degradation of HeT-A MS2 mRNA is observed in both control and aub mutant ovaries. However, aub mutant exhibits prolonged stabilization of the HeT-A MS2 transcript, whereas the exogenous mRNA is undetectable in control ovaries by 100 mpi. (a') Graphical output of normalized HeT-A MS2 transcript against gadph mRNA in control and aub mutant ovaries (n = 3). (b) Northern blotting of endogenous retroelement transcripts in the piRNA pathway and mRNA degradation mutants. Full-length HeT-A and I-element transcripts accumulate in spn-E, aub, and krimp mutants at steady state. A low level of full-length HeT-A mRNA accumulates in the deadenylase mutant twin. No detectable full-length retroelement transcript is observed in the mRNA degradation mutants dcp1, skp3, and pcm. (c) Semiquantitative RACE-PAT and RT-PCR analyses of retroelement expression in the mRNA degradation mutants. The schematic diagram shows primer sets [arrows] that are used to amplify regions on HeT-A, 5' UTR, CDS, 3' UTR, and poly(A). All examined regions are derepressed in dcp1 and skp3 mutants. (c') Quantitative RACE-PAT and RT-PCR of HeT-A mRNA normalized against act5C in dcp1 and skp3 mutants. Error bars indicate the standard deviation between the triplicates of each sample. *, P < 0.05; **, P < 0.01 (n = 3).
mRNA degradation mutants may reflect the redundancy of other enzymes mediating degradation. However, we cannot exclude the possibility that mRNA degradation genes contribute to posttranscriptional silencing of the retroelements via a piRNA-independent pathway. We have also provided evidence that retroelement silencing is regulated posttranscriptionally in a piRNA-dependent manner. This involves contributions from both the nuage and mRNA degradation proteins. In addition to AUB, AGO3, and KRIMP, we also observed the localization of other nuage components to the same cytoplasmic nuage bodies (unpublished data), suggesting that posttranscriptional retroelement silencing utilizes a large RNP complex or subcomplexes. Future understanding of the signaling pathways and players that regulate the assembly of Drosophila pi-bodies will shed new light on the development of host–parasite communities.

Materials and methods

Fly strains and transgenes
For avory staining, y w was used as a wild-type control unless otherwise stated. Mutant alleles and allelic combinations used in this study were 

For analysis of endogenous HeT-A, Helem, and gadph degradation, DIG DNA probes were synthesized with T7 and SP6 primers using pGEM-T Easy MS2 (Table S1). Total RNA was extracted from ovaries with TRIzol reagent according to the manufacturer’s instructions (Roche). For stripping, the blots were incubated with boiling distilled water for 20 min. For hybridization, the blots were washed in 0.1% (wt/vol) Tween 20 and hybridized at 42°C overnight in Hyb buffer. 10 µg total RNA from the ovaries was separated on a 15% polyacrylamide/8 M urea denaturing gel, transferred, and cross-linked as described previously in Gavris et al., 2004). DIG-labeled RNA probes were detected using anti-DIG HRP antibody (1:100; Roche) with fluorescein amplification (tyramide signal amplification kit; PerkinElmer).

Time course of HeT-A M52 mRNA decay
aub control and mutant flies harboring HeT-A–(ms2) were subjected to 2 h of heat shock at 36°C. Ovaries were dissected in cold Grace’s medium at 0, 20, 40, 60, 80, 100, and 120 min and 6 and 12 h after heat shock termination and immediately frozen in TRizol reagent (Invitrogen) at –80°C until RNA extraction. For mRNA abundance measurement, band intensities corresponding to HeT-A M52 were quantified using ImageJ (National Institutes of Health) and normalized to gadph transcript.

Western blotting
Ovary lysates were prepared as described previously in Drummond-Barbosa and Spradling (2004). Rabbit anti-PCM polyclonal, rat anti-SKI3 polyclonal, rabbit anti-DCP1, mouse anti-Mel18, and mouse antiaxin RNA was used as a wild-type control and mutant flies harboring HeT-A–(ms2) were microinjected into embryo using standard excision protocol. One mutant allele, pcm–, was isolated by single-fly PCR using the primers pcmEx forward and pcmEx reverse (Table S1). For ski3, the piggyBac insertion line f03251 exhibited no apparent phenotype and was cleaned up by backcrossing to y w.

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products were visualized on a 3% agarose/1X Tris Boric EDTA gel. All primer sequences can be found in Table S1.

Cap analysis
100 ng total RNA from ovaries was incubated with 1 U Terminator 5′-phosphate–dependent exonuclease [Epicentre] at 30°C for 3 h. Reactions were terminated by adding 0.5 µl 100 mM EDTA, pH 8.0. To check levels of cycB and U1 expression, one-step RT-PCR was performed using diluted or neat total RNA according to the manufacturer’s instructions [Invitrogen]. All primer sequences can be found in Table S1.

RACE-PAT and RT-PCR
1–3 µg total RNA from ovaries was treated with DNaseI [Roche]. Reverse transcription was performed using Oligo(dT)20 primer and avian myeloblastosis virus reverse transcription [Promega] as described previously [Salles et al., 1999]. A mock reaction without reverse transcription was prepared for each RNA sample. The newly synthesized cDNAs were checked for genomic DNA contamination by PCR with actin5C or gapdh primers. PCR was subsequently performed using 1 µl diluted or neat cDNA sample/reaction. For analyses of retroelement derepression, primer sets corresponding to actin5C and HeT-A UTRs, CDS, and poly(A) regions were used. For the examination of mRNA expression in pcm21 and ski203251 mutant alleles, primer sets corresponding to different regions of pcm or ski3 and a neighboring gene of pcm, nat1, were used. All primer sequences can be found in Table S1.

The same cDNAs were used for quantitative PCR in the presence of iQ SYBR Green supermix [Bio-Rad Laboratories]. For amplification and detection, a single-color real-time PCR detection system [MyQ; Bio-Rad Laboratories] was used. Control experiments measuring the change in Ct with template dilution demonstrated that the efficiencies of amplification of the target genes and the control act5C were approximately the same. All the results were normalized with respect to act5C. P-values were measured using one-tailed student’s t-test.

Online supplemental material
Fig. S1 shows that pcm21 and ski3 are loss of function alleles. Fig. S2 shows that piRNA production is unaffected in the mrna degradation mutants. Table S1 shows primer sequences. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200904063/DC1.

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