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Telomeric repeat silencing in germ cells is essential for early development in *Drosophila*

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ABSTRACT

The germline-specific role of telomeres consists of chromosome end elongation and proper chromosome segregation during early developmental stages. Despite the crucial role of telomeres in germ cells, little is known about telomere biology in the germline. We analyzed telomere homeostasis in the *Drosophila* female germline and early embryos. A novel germline-specific function of deadenylase complex Ccr4-Not in the telomeric transcript surveillance mechanism is reported. Depletion of Ccr4-Not complex components causes strong derepression of the telomeric retroelement *HeT-A* in the germ cells, accompanied by elongation of the *HeT-A* poly(A) tail. Dysfunction of transcription factors Woc and Trf2, as well as RNA-binding protein Ars2, also results in the accumulation of excessively polyadenylated *HeT-A* transcripts in ovaries. Germline knockdowns of Ccr4-Not components, Woc, Trf2 and Ars2, lead to abnormal mitosis in early embryos, characterized by chromosome missegregation, centrosome dysfunction and spindle multipolarity. Moreover, the observed phenotype is accompanied by the accumulation of *HeT-A* transcripts around the centrosomes in early embryos, suggesting the putative relationship between overexpression of telomeric transcripts and mitotic defects. Our data demonstrate that Ccr4-Not, Woc, Trf2 and Ars2, components of different regulatory pathways, are required for telomere protection in the germline in order to guarantee normal development.

INTRODUCTION

Telomeres are nucleoprotein complexes at the ends of linear chromosomes that protect chromosome ends from fusion and degradation owing to the end under-replication process (1). Protection of chromosome ends is essential in all cell types to prevent chromosome end-to-end fusion. At the same time, loss of the terminal DNA is repaired only in germ and stem cells by the synthesis of tandem telomeric repeats (2,3). Elongation of chromosome ends in proliferating somatic cells is believed to lead to cancer (4). In germ cells, a special mechanism of telomere length maintenance is present, providing replicative potential for the shortened telomeres in normal terminally differentiated somatic cells (5). Telomere length control is an essential germline-specific function implying that telomere organization in the germ cells has distinctive characteristics. However, the basic aspects of telomere biology in the germline and in early development are far from being understood.

In most eukaryotres, telomeric DNA is maintained through telomerase action, which is responsible for the synthesis of 6–9 nucleotide repeats using its RNA component as a template (6). The telomeres of *Drosophila* are maintained as a result of the retrotransposition of specialized telomeric non-long terminal repeat retrotransposons (7–11). In all cases, telomere elongation utilizes reverse transcription, i.e. synthesis of DNA on the RNA template. Also, telomeric DNA serves as a platform for the binding of telomeric proteins, required for the protection of chromosome termini and the maintenance of specific chromatin structure. In particular, heterochromatic protein 1 (HP1) and the DNA repair complex provide protection against telomere fusions in both *Drosophila* and humans (12,13), demonstrating strong similarities in the telomere protection pathways in different organisms.

Recent data show that the transcription of telomeric DNA and the requirement for RNA-interference (RNAi)

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machinery in telomere function are also conserved among eukaryotes, irrespective of the mode of telomere elongation. Telomeres in organisms that use telomerase are transcribed into telomeric repeat-containing RNA (TERRA) (14,15), which serves as a precursor for small RNA production. Telomere-specific piRNA-like small RNAs have been detected in mouse embryonic stem cells (16) and in *Arabidopsis* (17). These small RNA species strongly resemble telomeric transposon-derived small RNA in *Drosophila* (18,19). In the *Drosophila* germline telomeric retrotransposons are silenced by the PIWI-interacting RNA (piRNA) pathway (18,19). Moreover, the increased telomeric transposon expression in flies with a disrupted piRNA pathway is correlated with an increased frequency of telomeric element transposition and, therefore, with telomere elongation (18). Recent studies have provided strong evidence that telomeric piRNAs impact the transcriptional status of telomeric retroelements (19,20) as well as formation of the telomere protection complex (21). Most likely, it is the piRNA pathway proteins that provide germline-specific peculiarities to telomeric complex formation. However, possible piRNA pathway partners in the germline telomeric protein complex are currently unknown.

HeT-A telomeric element is the main structural component of *Drosophila* telomeres present at all chromosome ends. In contrast, the other two telomere retrotransposons, *TART* and *TAHRE*, are represented by only a few copies (7,22). In order to discover new components of the *HeT-A* silencing complex, we performed a selective RNAi-based screen in the *Drosophila* female germline, using endogenous *HeT-A* expression as a read-out. We found that germline knockdown of the components of the Ccr4-Not deadenylase complex as well as the transcription factors Woc (without children) and Trf2 (TATA box-binding protein (TBP)-related factor 2) caused a strong derepression of *HeT-A* retrotransposon in ovaries. The Ccr4-Not complex is a conserved multi-subunit complex that regulates gene expression at different levels, including mRNA deadenylation, transcription, transport and degradation (23,24). In *Drosophila*, the Ccr4-Not deadenylase complex regulates the expression of specific germline mRNAs by controlling their poly(A)-tail length (25). Here, we provide evidence for the essential role of the Ccr4-Not complex in telomeric transposon *HeT-A* transcript adenylation status in the germ cells.

Germline knockdowns of factors affecting the *HeT-A* RNA level, such as Ccr4-Not components, the transcription factors Woc and Trf2 and RNA-binding protein Ars2 cause mitotic defects during the early stages of embryonic development. Our findings suggest that these factors act cooperatively to regulate telomeric repeat expression and telomere protection in the germline, providing genome integrity during early development.

MATERIALS AND METHODS

Drosophila strains

KD (from 'knockdown') flies were F1 of the cross of two strains bearing construct with short hairpin RNA (shRNA) (Supplementary Table S1) and, unless otherwise specified, strain #25751 ($P\{UAS\text{-}Dcr\text{-}2.D\}1$, w^{1118}, $P\{GAL4\text{-}nos.NGT\}40$, Bloomington Stock Center) providing GAL4 expression under the control of the germline-specific promoter of the *nanos (nos)* gene. An additional Gal4 driver strain was #4937 (w^{1118}; P{GAL4::VP16-nos.UTR}CG6325^{MVD1}, Bloomington Stock Center). Control flies were F1 of the crossing of the *white* hairpin construct and #25751 (Bloomington Stock Center). The $Trf2^{p1}$ allele was described in (26). Strains bearing *spindle-E* (*spn-E*) mutations were $ru^1\ st^1\ spn\text{-}E^1\ e^1\ ca^1/TM3$, $Sb^1\ e^s$ and $ru^1\ st^1\ spn\text{-}E^{hls3987}\ e^1\ ca^1/TM3$, $Sb^1\ e^s$. *twin* mutants were $twin^{DG24102}/Df(3R)\ crb87\text{-}4$, $twin^{S1}/Df(3R)\ crb87\text{-}4$ and $twin^{S3}/Df(3R)\ crb87\text{-}4$. *Not1* mutant was $Not1^{MI07631}/Not1^{G5743}$. Polymerase chain reaction (PCR) with genomic DNAs using *Wolbachia*-specific primers demonstrated that there was no contamination with *Wolbachia* for the strains used in the study.

In situ RNA hybridization and immunochemistry

In situ RNA analysis was carried out according to the previously described procedure (27) using digoxigenin (DIG)-labeled strand-specific *HeT-A, TART* or *TAHRE* riboprobes and alkaline phosphatase (AP)-conjugated anti-DIG antibodies (Roche, diluted 1/2000). The *HeT-A* antisense probe contained a fragment of the ORF (nucleotides 4330–4690 of GenBank sequence DMU06920); the *TART* antisense probe contained a fragment corresponding to nucleotides 2377–2888 of GenBank sequence DMU02279. The *TAHRE*-specific probe contained a fragment corresponding to nucleotides 5717–6295 of GenBank sequence AJ542581.

Combined protein and RNA localization was done as described (28). Embryos were collected and fixed in methanol as described (29). The RNA fluorescence *in situ* hybridization (FISH) was done with DIG-labeled strand-specific riboprobes. For hybridization signal enhancement, we used incubation with anti-DIG- fluorescein (FITC) antibodies (Roche, diluted 1/500), followed by incubation with anti-FITC Alexa Fluor 488 antibodies (Life Technologies, diluted 1/500). Tyramide signal amplification was done as an alternative method of signal enhancement (30). Immunostaining was done using anti-γ-tubulin (Sigma), anti-α-tubulin (Sigma), anti-mouse-Alexa 546 (Life Technologies) and anti-rabbit-Alexa 546 (Life Technologies). Embryos were stained with 4′,6-diamidino-2-phenylindole (DAPI) and mounted in ProLong Gold antifade reagent (Life Technologies). Images were captured using a Zeiss LSM 510 Meta confocal microscope. Embryonic chromosome spreading was done as described (31).

RTqPCR analysis

cDNA was synthesized using random hexamer or oligo(dT) primers and SuperScriptII reverse transcriptase (Life Technologies). cDNA samples were analyzed by real-time quantitative PCR using SYTO-13 dye. qPCR was performed on DT-96, DNA Technology, Russian Federation. Eight serial three-fold dilutions of cDNA or DNA were amplified in duplicate with each primer pair to build standard curves. Values were averaged and normalized to the expression level of ribosomal protein gene *rp49*. Normalization of RT-qPCR

data to the retrotransposon copy number in each strain was performed; however, no significant differences in copy number were detected. All experiments were performed with at least two independent RNA samples, and each sample was analyzed in triplicate. The primers used are listed in Supplementary Materials and Methods. Nuclear run-on (NRO) assay was performed as described previously (19).

Polyadenylation assay

Poly(A) tail length was analyzed using a poly(A) tail (PAT) assay as previously described (32) with an oligo (dT)-anchor primer and specific primers (Supplementary Materials and Methods). PCR was carried out in the presence of αdATP-P^{32}, and the products were separated in a 6% non-denaturing polyacylamide gel. A polyadenylation assay based on RNA ligation-coupled RT-PCR was done as described (33) using the Illumina 3′linker RA3.

RESULTS

Trancription factors Woc and Trf2 suppress telomeric repeat HeT-A transcription in ovaries

In order to detect germline components contributing to telomeric homeostasis, we performed a selective RNAi screen for factors that downregulate *HeT-A* expression in *Drosophila* ovaries. This approach was based on the existing data that in somatic tissues certain factors of the telomeric complex are required for the silencing of telomeric repeats (34–36). As reported earlier, HP1, encoded by *Su(var)205* gene, a component of the telomere protection complex (37,38), elicits transcriptional repression of telomeric retrotransposons both in soma (34) and in the germline (39,40). At the same time, transcription of telomeric and non-telomeric transposons in the germline is controlled by the piRNA system, supporting the idea that the telomeric complex in the germline consists of both germline-specific and ubiquitous components. To investigate the structure of telomeric chromatin in the germline, we examined *HeT-A* expression in the ovaries of flies depleted for known components of the somatic telomeric complex as well as factors involved in RNA surveillance. Here, we describe genes exerting the greatest effect on telomeric repeat expression in ovaries. Germline knockdown of several genes, previously implicated in telomere functioning in soma, did not noticeably affect *HeT-A* expression in our screen and were not included in further analysis (Supplementary Table S2).

In parallel to an independent study that describes Trf2 as a negative regulator of telomeric transposon expression in somatic tissues (41), we demonstrated that Trf2 is associated with the polytene chromosome telomeres of salivary glands (Supplementary Figure S1) and discovered a role of Trf2 in telomere functioning in the germline. RT-qPCR analysis of telomeric and non-telomeric retrotransposon expression demonstrated increased levels of *HeT-A* and *TAHRE* transcripts in ovaries upon germline knockdown of *Trf2* (*Trf2*KD^{nos}; nos designates the promoter of the germline-specific gene *nanos* that was used to express the Gal4 driver) (Figure 1A). Given that the *HeT-A* and *TAHRE* untranslated regions (UTR) are highly homologous (7), we suggest that Trf2 recognizes a similar sequence motif in the promoter regions of both elements.

In order to determine if Trf2 functions in the piRNA pathway together with *spindle-E* (encoding DEAD box RNA helicase SpnE) to silence *HeT-A*, we applied a double mutant analysis. We used a viable $Trf2^{p1}$ mutation (26) to generate *Trf2/Trf2; spnE/spnE* double mutants and compared *HeT-A* transcript abundance in their ovaries to that in ovaries of single *Trf2/Trf2* or *spnE/spnE* mutants. *HeT-A* expression in the double mutants was higher than in *Trf2* single mutants, but not in *spnE* single mutants (Figure 1B, Supplementary Figure S2). This could mean that in the absence of piRNAs, inhibitory complex containing Trf2 cannot be recruited to the *HeT-A* promoter. Northern blotting of short RNA did not reveal changes in the abundance of *HeT-A*-specific piRNA in ovaries of *Trf2* mutants and *Trf2*KD^{nos} flies (Supplementary Figure S3A). These data suggest that Trf2 may act downstream of piRNA processing to establish transcriptional silencing of *HeT-A*.

The zinc finger transcription factor Woc has been shown to bind *Drosophila* telomeres, exerting telomere-capping functions (42). *woc*KD^{nos} causes a strong derepression of *HeT-A* and *TAHRE* but not *TART* and other TEs (Figure 1A), suggesting sequence-specific binding of this transcription factor to telomeric repeats in ovaries.

We extended the analysis of the telomeric protein complex by including an RNA binding protein, Ars2, involved in mi- and siRNA biogenesis (43), which was identified in an RNAi screen as a negative regulator of *HeT-A* expression (44). Particular interest in this protein was due to the fact that Ars2 downregulates the expression of human TERRA (45). RT-qPCR analysis revealed a strong derepression of *HeT-A/TAHRE* in ovaries of *Ars2*KD^{nos} flies (Figure 1A). To address the question about the involvement of Trf2, Woc and Ars2 in the transcriptional regulation of *HeT-A,* we conducted an NRO assay, which allows estimating the level of nascent *HeT-A* transcripts (46). NRO on ovaries with *Trf2*, *woc* and *Ars2* knockdowns demonstrates that all these factors provide transcriptional silencing of the telomeric retrotransposon *HeT-A* (Figure 1C).

In situ RNA hybridization analysis was performed to examine the effect of Trf2, Woc and Ars2 depletion on telomeric retroelement transcript distribution in ovaries (Figure 1D, Supplementary Figure S4). Knockdowns of Trf2, Woc and Ars2 exerted the most pronounced effect on *HeT-A* and *TAHRE* expression, resulting in the accumulation of their transcripts in nurse cells. The RNA FISH analysis on ovaries depleted for Trf2, Woc and Ars2 revealed *HeT-A* RNA signals not only in the cytoplasm, but also in the nuclei of nurse cells, where *HeT-A* transcripts were accumulated in distinct foci (Figure 1E). Similar nuclear foci were observed in *spnE*KD ovaries (Figure 1E). Previously, using combined DNA/RNA FISH analysis, it was shown that both *HeT-A* RNA and telomeric DNA were co-localized in nurse cell nuclei in *spnE* mutants (19). Most likely, we observed the accumulation of nascent transcripts at sites of transcription upon Trf2, Woc and Ars2 knockdown, in accordance with the result of NRO.

Taking into account the *HeT-A/TAHRE*-specific effects of Woc and Trf2, it is unlikely that these factors participate in piRNA biogenesis. However, it is possible that DNA-

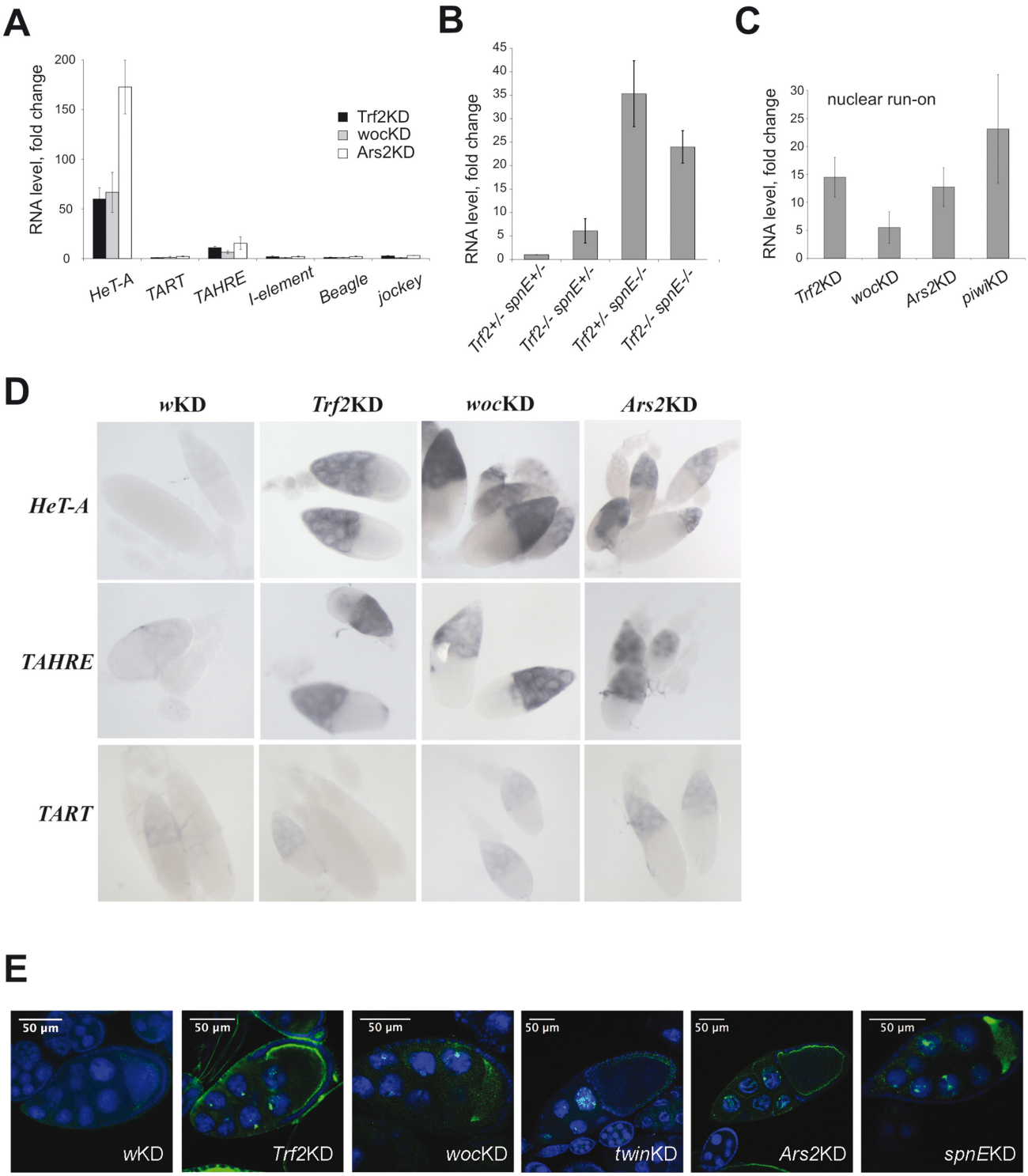

Figure 1. Telomeric element *HeT-A* expression is downregulated in ovaries by Trf2, Woc and Ars2. (**A**) RT-qPCR analysis of TE expression in ovaries of *Trf2*KD^{nos}, *woc*KD^{nos} and *Ars2*KD^{nos}. (**B**) *HeT-A* transcript abundance in ovaries of *Trf2/Trf2, spnE/spnE* and *Trf2,spnE* double mutants. The fold change in the *HeT-A* RNA level is shown. (**C**) NRO analysis of *HeT-A* transcription in ovaries of *Trf2*KD^{nos}, *woc*KD^{nos}, *Ars2*KD^{nos} and *piwi*KD^{nos} flies. The RNA level was normalized to *rp49*. Mean values (±SD) for three biological samples normalized to control knockdown (*white*(*w*)KD^{nos}) are shown. (**D**) *In situ* RNA hybridization of *HeT-A, TART* or *TAHRE* antisense probes with ovaries of the indicated genotypes (*GAL4-nos* driver was used). (**E**) RNA FISH of *HeT-A* antisense probe with ovaries of the indicated genotypes. The accumulation of *HeT-A* transcripts (green) in nuclei of nurse cells was observed. DNA was stained with DAPI (blue). *GAL4-nos* driver was used.

binding factors Woc and Trf2 are involved in the piRNA-mediated transcriptional silencing mechanism that is specific for telomeric repeats.

Ccr4-Not deadenylase complex provides telomeric element HeT-A silencing in the germline

mRNA turnover enzymes have been proposed to be involved in the piRNA-dependent degradation of transposon transcripts in cytoplasmic bodies (47). We observed a strong derepression of *HeT-A* upon germline knockdowns of *twin* (encoding Ccr4), *Not1* and *Pop2,* which encode components of the *Drosophila* Ccr4-Not deadenylase complex (48) (Figure 2, Supplementary Figure S5). RT-qPCR analysis showed that changes in *HeT-A* expression level were considerably higher in comparison to other TEs (Figure 2A). We also examined additional mutant strains and shRNAs targeting Ccr4 and Not1 (Supplementary Figure S4, S5). Although the level of derepression varied, we always observed *HeT-A* RNA accumulation as a result of Ccr4-Not depletion. This indicates a specificity of the observed effects. piRNA pathway gene germline knockdowns exhibit a wide range of *HeT-A* upregulation comparable to the effects of Ccr4-Not knockdowns (Supplementary Figure S5). Increased *HeT-A* expression was detected in ovaries but not in the carcasses of *twin* mutants (Figure 2B), indicating that the Ccr4-Not complex represses *HeT-A* expression specifically in gonads. Northern blotting of short RNA did not show changes in *HeT-A*- and *I-element*-specific piRNA abundance in the ovaries of *twin*KD^{nos} and *Not1*KD^{nos} flies (Supplementary Figure S3B,C). Thus, *HeT-A* transcripts accumulated upon Ccr4-Not knockdown avoid piRNA-mediated degradation. *In situ* RNA hybridization on Ccr4, Pop2 and Not1 KD ovaries revealed *HeT-A* transcripts in nurse cells (Figure 2C). Importantly, RNA FISH revealed *HeT-A* RNA foci in the nuclei of *twin*KD^{nos} nurse cells (Figure 1E) as well as upon Trf2, Woc, Ars2 and SpnE knockdown, suggesting that all these components regulate the telomeric transcript level at the site of transcription.

Polyadenylation state of HeT-A telomeric transposon transcripts

The Ccr4-Not complex is responsible for the cytoplasmic deadenylase activity in *Drosophila* ovaries and embryos (48–52). To verify whether stabilization of *HeT-A* transcripts upon germline knockdowns of Ccr4-Not components is due to increased polyadenylation, the poly(A) tail length of the *HeT-A* transcripts was measured in a PAT assay (32). Indeed, *twin* knockdown led to the appearance of additional PAT products with higher molecular weights (Figure 3A). To confirm that these products corresponded to *HeT-A* transcripts with longer poly(A) tails, but were not the result of alternative polyadenylation, the top and bottom PAT products were cloned and sequenced. These products corresponded to *HeT-A* transcripts with poly(A) tail lengths of 50–65 nt and 7–11 nt, respectively (Figure 3B). At the same time, the poly(A) tail length of *TART* was not affected by Ccr4 knockdown (Figure 3A). To confirm this result, we compared the data from the RT-qPCR analysis of TE expression obtained with oligo(dT) or random primers used in reverse transcription. Using oligo(dT) for reverse transcription priming allows for the estimation of the polyadenylation status of transcripts. We observed that the ratio of polyadenylated to total *HeT-A* transcripts increased upon Ccr4 knockdown. This ratio remained unaffected for *TART* transcripts (Supplementary Figure S6).

Given the accumulation of *HeT-A* transcripts in the nuclei upon Ccr4 depletion (Figure 1E), we investigated the poly(A) tail length of the *HeT-A* RNA in the nuclear fraction of ovarian RNA upon germline knockdown of Ccr4. The PAT assay demonstrated that in the nuclei of control ovaries, *HeT-A* transcripts were not adenylated, while Ccr4 KD led to an increase in *HeT-A* poly(A) length in the nucleus (Figure 3C). In yeast, the Ccr4-Not complex has been reported to have both cytoplasmic and nuclear localization and activity (24). Previously, it was shown by immunostaining that Pop2 (CAF1), Ccr4 and Not1 were localized mostly in the cytoplasm in *Drosophila* ovaries and embryos (48,53). To address the question about the presence of Ccr4-Not in the nuclei of *Drosophila* ovaries, we performed cellular fractionation and showed that both Ccr4 and Not1 are present not only in the cytoplasm but also in the nucleus (Figure 3D). Next, we co-immunoprecipitated Ccr4 from the ovarian nuclear extract using Not1 antibody (Figure 3E). Thus, our data show that the Ccr4-Not complex negatively regulates the poly(A) tail length of *HeT-A* transcripts. Taking into account the nuclear localization of the Ccr4-Not complex, we suggest that this process takes place in the nucleus; however, we cannot exclude the possibility that deadenylated *HeT-A* RNA could be imported back into the nucleus from the cytoplasm.

To explore a potential relationship of different factors affecting the *HeT-A* transcript level, we analyzed the poly(A) tail length of *HeT-A* transcripts in ovaries upon knockdown of Woc, Trf2, Ars2 and Piwi, factors that regulate *HeT-A* at a transcriptional level. We applied direct ligation of a 3′ adapter to RNA, followed by a PCR analysis of cDNA, since the oligo(dT)-based protocol of the PAT assay introduces a strong bias against transcripts with short poly(A) tails. Germline knockdowns of Woc, Trf2, Ars2 and Piwi caused the appearance of additional PCR products with higher molecular weights (Figure 3F) corresponding to *HeT-A* transcripts with longer poly(A) tails, as verified by sequencing. These results suggest that all these components act in a common pathway to regulate the life cycle of the telomeric transcripts, and that this process most likely occurs within the telomeric compartment in the nucleus.

Germline knockdowns of Ccr4, Not1, Woc and Trf2 cause abnormal mitosis in early stages of development.

In order to determine whether Trf2, Woc, Ars2 and Ccr4-Not depletion in the germline affects early stages of embryogenesis, we analyzed chromosome segregation in 0–2-h-old embryos during mitotic divisions. Maternally deposited RNA and proteins govern this stage until the onset of zygotic transcription, which occurs after two hours of development. Germline knockdowns of the *Trf2, woc, twin* and *Ars2* genes led to asynchronous divisions during the syncytial stage of embryogenesis (Supplementary Figure S7), as well as a high lethality of embryos (Supplemen-

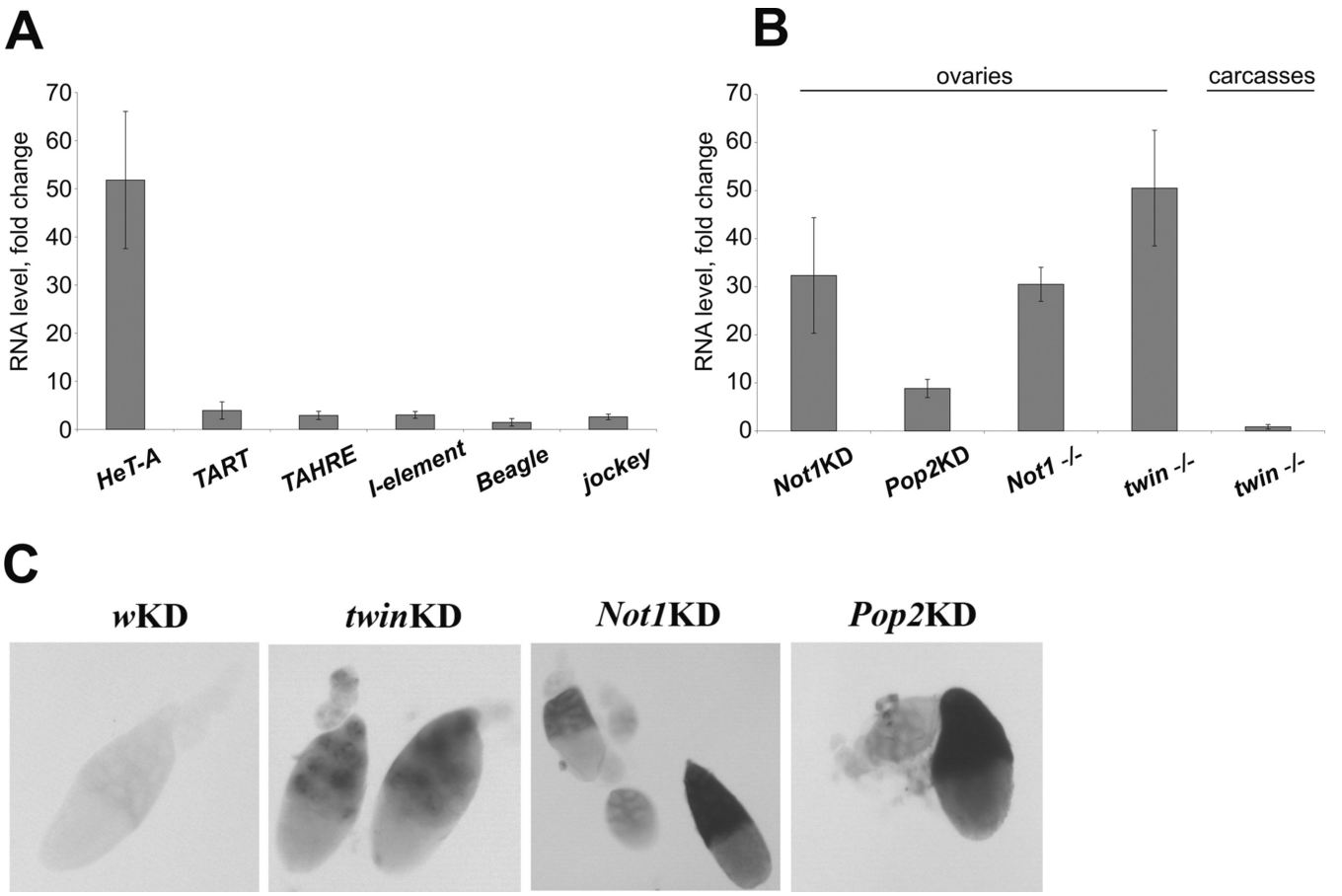

Figure 2. Ccr4-Not deadenylase complex suppresses telomeric element *HeT-A* expression in the germline. (**A**) RT-qPCR analysis of TE expression in ovaries of *twin*KD^{nos}. (**B**) Changes in *HeT-A* steady state RNA levels in ovaries and carcasses of flies with indicated genotypes. *twin* mutant (*twin*-/-) is a trans-heterozygote *twin*^{DG24102}/*Df(3R) crb87-4*. Error bars indicate SD. (**C**) *In situ* RNA hybridization of *HeT-A* antisense probes with ovaries of the indicated genotypes. *GAL4-nos* driver was used.

tary Table S3). Such embryos have extensive regions devoid of nuclei (Supplementary Figure S7) and exhibit various mitotic defects. Most frequently, we observed chromosome bridges, abnormal mitotic chromosome figures and multipolar spindles (Figure 4A,B, Supplementary Table S4). Surface optical sections of *Trf2*KD^{nos}, *woc*KD^{nos}, *Ars2*KD^{nos} and *twin*KD^{nos} embryos revealed free centrosomes that remained at the cortex while the nuclei sank into the interior of the embryo. Detached centrosomes are positive for γ-tubulin, the major centrosome component (Figure 4C, Table 1). Configurations of mitotic chromosomes in *Trf2*-/-, *woc*KD^{nos}, *Ars2*KD^{nos} and *twin*KD^{nos} embryos were visualized using an embryonic squashing technique (31). We observed frequent chromosome end-to-end fusions, ring chromosomes and anaphase bridges, hallmarks of telomere dysfunction (Figure 4D, Supplementary Figure S8A). We performed DNA FISH on *Trf2* mutant embryos with the *HeT-A* probe and showed that the *HeT-A* signal is present at the site of anaphase chromosome bridges (Supplementary Figure S8B). These data indicate that the mitotic bridges are caused by telomere fusions. Therefore, maternal depletion of Trf2, Woc, Ars2 and Ccr4 leads to *HeT-A* overexpression, which compromises the telomere protection complex (or vice versa), resulting in chromosome segregation defects.

Table 1. Quantification of free centrosomes at the late stages of syncytial development after migration of nuclei to the cortex

Genotype	Nuclei/centrosomes[a]	Free centrosomes associated with *HeT-A* RNA[b]
*w*KD^{nos}	46/93	0
Trf2^{p1}	69/246	26
*woc*KD^{nos}	42/156	53
*Ars2*KD^{nos}	78/232	36
*twin*KD^{nos}	71/206	41

[a]The number of nuclei/centrosomes was counted per optical field of samples immunostained with anti-γ-tubulin antibodies and hybridized with *HeT-A* antisense probe.
[b]Only *HeT-A* signals that overlapped with γ-tubulin staining (or located at a distance less than one centrosome diameter away from it) were quantified.

At later stages of syncytial development of such embryos, centrosomes frequently detach from the mitotic nuclei resulting in the accumulation of abundant γ-tubulin positive free centrosomes at the cortex while the defective nuclei are eliminated.

To ask whether hyperexpression of *HeT-A* in ovaries leads to *HeT-A* transcript accumulation in early embryos, we examined *HeT-A* RNA abundance in 0–2-h-old

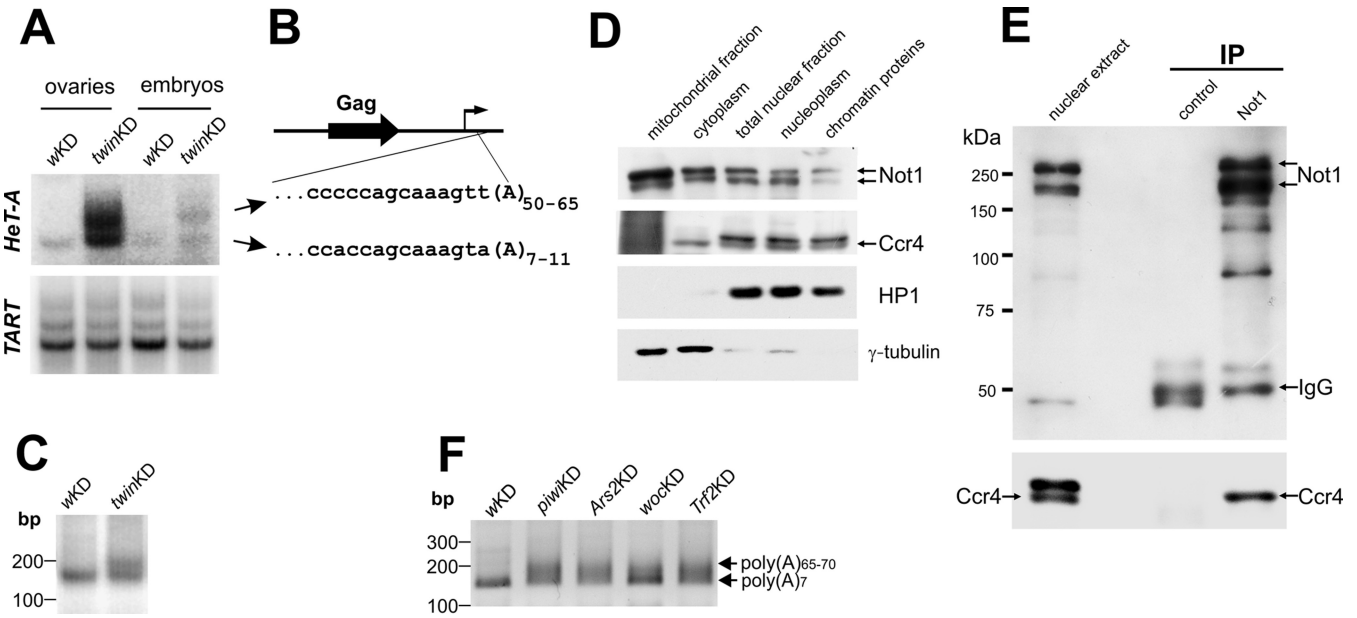

Figure 3. Polyadenylation state of *HeT-A* telomeric transposon transcripts. (**A**) PAT assay of *HeT-A* and *TART* RNA in ovaries and embryos of *twin*KD^{nos} flies. (**B**) Sequencing of the top and bottom PCR products (three clones for each band) shows elongation of the poly(A) tail of *HeT-A* transcripts in ovaries of *twin*KD^{nos}. The diagram on the top shows a scheme of *HeT-A*: open reading frame (black arrow), transcription start site (thin arrow) and the fragments of the sequenced regions. (**C**) PAT assay of *HeT-A* transcripts in the nuclear RNA fraction of control and *twin*KD^{nos} flies. (**D**) Considerable amount of both Ccr4 and Not1 localize in ovarian nuclei. Western blot of w^{1118} ovary extract fractions probed with antibodies against Not1, Ccr4, HP1 and γ-tubulin. (**E**) Ccr4 was coimmunoprecipitated with Not1 in the nuclear extract from ovaries of w^{1118}. Immunoprecipitation was performed with anti-Not1 antibodies or normal mouse serum (control IP). A Western blot was probed with anti-Not1 (top) or anti-Ccr4 (bottom). (**F**) Analysis of poly(A) tail length of *HeT-A* RNA using the 3′ ligation–PCR approach in ovaries upon *white, Trf2, woc, Ars2* and *piwi* germline knockdown.

Trf2-/-, *woc*KD^{nos}, *Ars2*KD^{nos} and *twin*KD^{nos} embryos by RT-qPCR. We observed an accumulation of *HeT-A* transcripts in the embryos (Supplementary Figure S9), indicating that maternal *HeT-A* transcripts are effectively loaded in the embryo. Next, we studied the localization of *HeT-A* transcripts using fluorescence *in situ* RNA hybridization on 0–2-h-old embryos combined with immunostaining of γ-tubulin. *HeT-A* transcripts accumulated around the centrosomes at different stages of mitosis in the embryos upon germline knockdown of Trf2, Woc, Ccr4 and Ars2 (Figure 5AB, Supplementary Figure S10). Similar distribution of *HeT-A* RNA was observed in the embryos upon knockdown of the piRNA pathway genes *spnE* and *rhino* (*rhi*), which encodes HP1 family protein Rhino (Figure 5B). By contrast, transcripts of retrotransposons *I-element*, *TART* and *jockey*, which are overexpressed upon *spnE* and *rhi* depletion, were not detected around centrosomes (not shown). *HeT-A* RNA foci, associated with free centrosomes, were also visible in the cortex of *woc*KD^{nos}, *twin*KD^{nos}, *Ars2*KD^{nos}, *Trf2*-/- and *spnE*KD^{nos} embryos (Figure 5C, Supplementary Figure S11, Table 1). To detect RNA, we applied a tyramide signal amplification method (30) (Figure 5), as well as a traditional method using a DIG-labeled probe and anti-DIG antibodies (Supplementary Figures S10, S11). Similar patterns of *HeT-A* transcript localization revealed by both methods indicate that the observed signals were not artifacts of tyramide amplification. This was confirmed by the detection of *oskar* mRNA in the pole plasm using the tyramide amplification method (Supplementary Figure S12) (54). *TART* transcripts were not detected in the embryos upon germline knockdown of Trf2, Woc, Ccr4 and Ars2 (not shown).

DISCUSSION

In this study, we investigated the properties of *Drosophila* telomeric transcriptome in the germline, as well as the impact of the telomere repeat overexpression in the germline on the early development. We have shown that different components exert quantity and quality control of the telomeric transcriptome, resulting in the silencing of telomeric retroelements. The main and specific target of the silencing system is telomeric retrotransposon *HeT-A*, which is a major component of *Drosophila* telomeres. In addition to the piRNA system, transcriptional factors Woc and Trf2 are involved in the silencing of *HeT-A* in the germline. Expression of the major telomeric retrotransposon *HeT-A* and related telomeric element *TAHRE,* but not *TART,* is downregulated by Trf2 and Woc, indicating a specific interaction of these transcription factors with distinct sequences present in the *HeT-A/TAHRE* elements. This fact allows suggesting the *HeT-A* sequence as a possible platform for binding of specific telomeric proteins. In fission yeast, telomere-specific proteins Ccq1/Taz1 and the RNAi factors are involved in the recruitment of a higher-order chromatin complex at the telomeres (55). Assembly of the telomeric complex in the *Drosophila* germline, involving cooperation of DNA-binding proteins and piRNA pathway, resembles a mechanism operating at fission yeast telomeres.

Our data suggest an existence of an additional layer of control of the telomeric transcriptome. Deadenylase com-

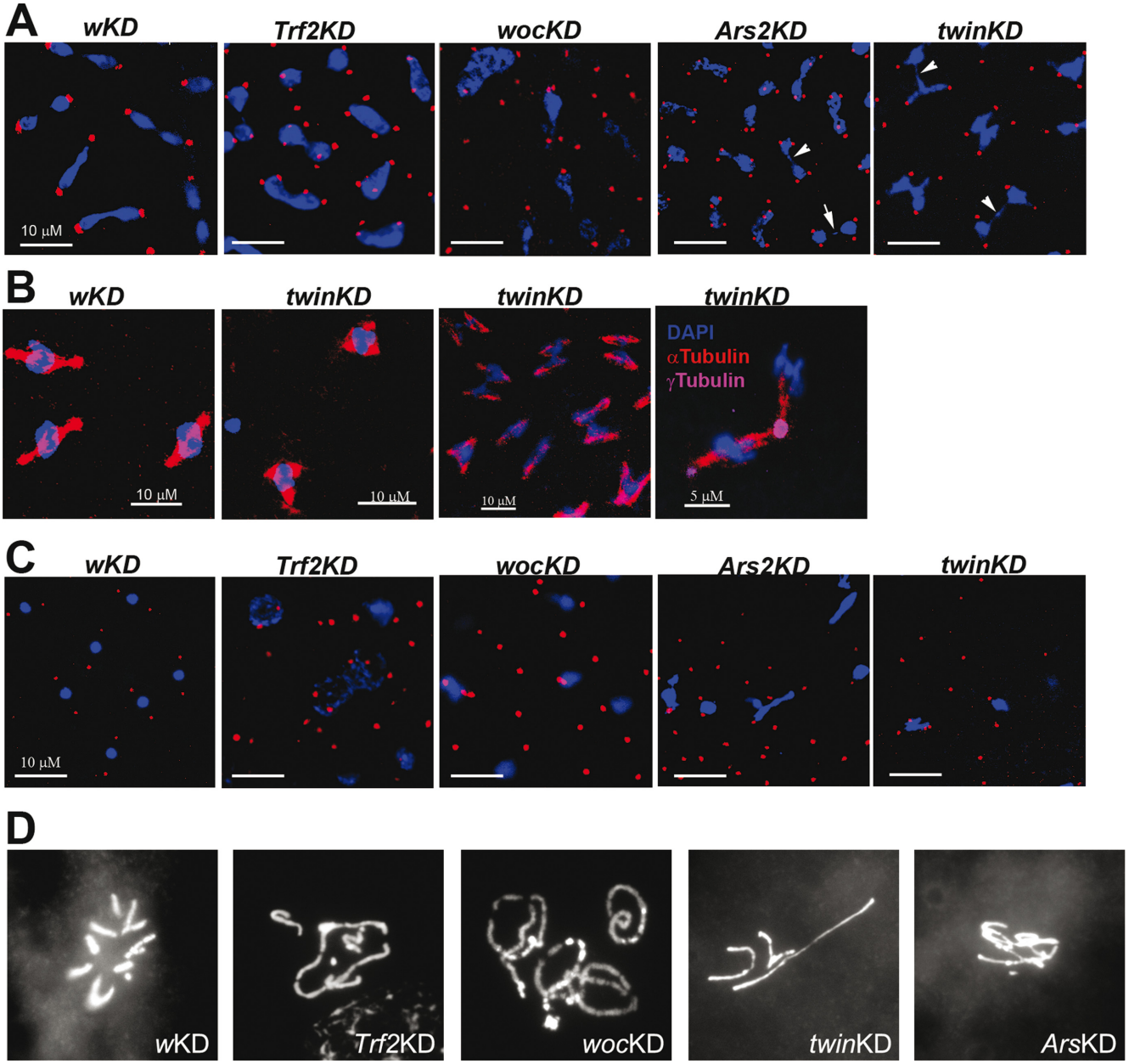

Figure 4. Mitotic defects observed in syncytial embryos from *woc*KD^{nos}, *Not1*KD^{nos}, *twin*KD^{nos}, *Ars2*KD^{nos} females. (**A**) Confocal images of embryos with the indicated genotypes. Abnormal chromatin figures, chromatin bridges (arrows) and free centrosomes were observed. Red: γ-tubulin; blue: DNA. Bar: 10 μm. (**B**) Multipolar spindles observed in syncytial embryos from *twin*KD^{nos}. Red: α-tubulin; magenta: γ-tubulin; blue: DNA. (**C**) Cortical areas of embryos with the indicated genotypes. Abundant free centrosomes were observed. Nuclei fallen into the interior of the embryo are visible. Red: γ-tubulin; blue: DNA. Bar: 10 μm. (**D**) Mitotic chromosomes from embryos. Chromosome fusions were observed in *Trf2*KD^{nos}, *woc*KD^{nos}, *Ars2*KD^{nos} and *twin*KD^{nos} embryos.

plex Ccr4-Not provides the deadenylated state of *HeT-A* transcripts in normal conditions. We do not know whether Ccr4-Not exerts *HeT-A* RNA deadenylation or prevents the polyadenylation of the nascent *HeT-A* transcripts. Moreover, depletion of different components affecting *HeT-A* silencing, such as Trf2, Woc and Ars2, leads to the accumulation of polyadenylated telomeric transcripts in ovaries. These data suggest that all of these components act cooperatively to regulate the life cycle of telomeric transcripts. Despite the fact that piRNA production was not affected by Trf2 and Ccr4-Not depletion, abundant *HeT-A* transcripts were accumulated upon knockdown of these factors.

It is noteworthy that only a minor fraction of human and yeast TERRA molecules are polyadenylated (15,56). Human poly(A)-negative TERRA is chromatin-associated, suggesting its role in telomeric heterochromatin assembly (57). Ars2, which was previously identified as a regulator of human TERRA levels (45), is implicated in the regulation of *HeT-A* expression in *Drosophila* (present study), indicating a conservative mechanism of telomeric transcript turnover.

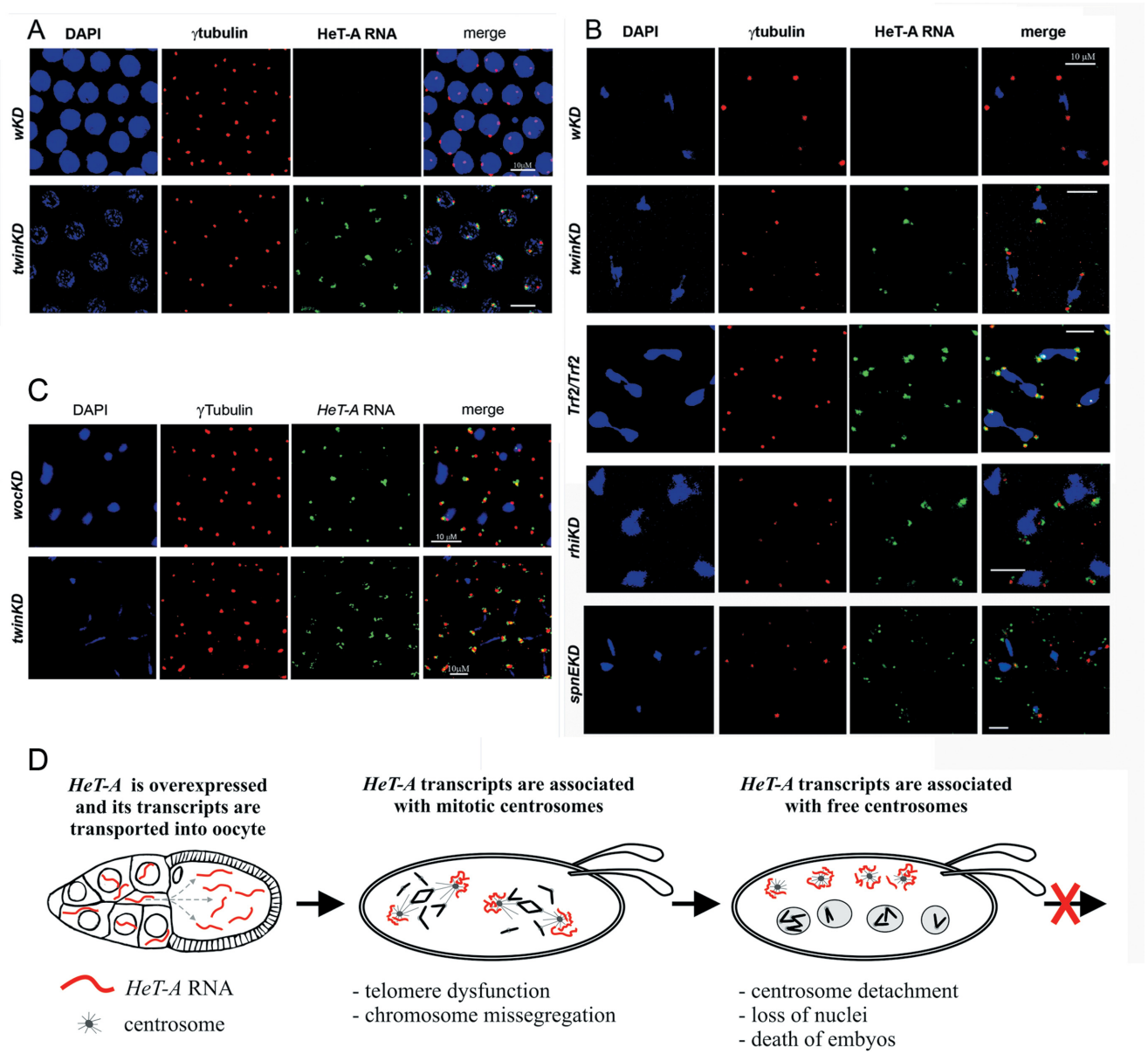

Figure 5. Telomeric transcripts are localized around centrosome in syncytial embryos. Confocal images of 0–2-h-old embryos with the indicated genotypes. RNA FISH of *HeT-A* antisense probe revealed *HeT-A* RNA (green) near centrosomes in the prophase of *twin*KD^{nos} embryos (**A**), during metaphase-anaphase in *twin*KD^{nos}, *Trf2/Trf2, rhi*KD^{nos} and *spnE*KD^{nos} embryos (**B**) and in the cortex of *woc*KD^{nos} and *twin*KD^{nos} embryos (**C**). Red: γ-tubulin; blue: DNA. Bar: 10 μm. (**D**) The scheme depicts mitotic defects in early developmental stages as a result of *HeT-A* derepression in the germline and the accumulation of abundant *HeT-A* transcripts around centrosomes in embryos.

In zebrafish and frog early embryos, mRNA poly(A) tail length correlated strongly with the translation efficiency (58). *HeT-A*-encoded Gag protein has been shown to localize at the telomeres in somatic tissues (36) and bind *HeT-A* transcripts (59). It is likely that polyadenylated *HeT-A* mRNA is effectively translated to produce abundant Gag protein that may be involved in telomere protein complex destabilization.

Our data show that the mechanisms of telomeric repeat silencing and assembly of the telomere protection complex are directly connected (Figure 5D). The germline knockdown of distinct factors affecting the *HeT-A* RNA level, such as Woc, Trf2, Ars2, Ccr4 and Not1, caused severe mitotic abnormalities during early development. Most likely, depletion of telomere silencing complex components in the germline causes a loss of telomere protection in the early embryos which leads to the occurrence of telomere fusions, chromosome-segregation failures and eventually to a 'mitotic catastrophe'. Damaged nuclei sank to the interior of the embryo and free centrosomes were left in the cortex. DNA damage and replication stress have been shown to cause centrosome inactivation and elimination of the defective nuclei from the embryonic blastoderm (60,61). In those cases, detached centrosomes lacked γ-tubulin while the free centrosomes observed after Trf2, Woc, Ars2 and Ccr4 depletion were positive for γ-tubulin. Thus, the mechanisms

of centrosome detachment caused by DNA damage and disruption of telomere silencing complex appear to be different. In latter case, an additional component, namely, *HeT-A* RNA, is accumulated as a result of loss of telomere protection.

We found that the *Drosophila HeT-A* RNA overexpressed in the germline was transported to the early embryos, where it was concentrated around the centrosomes. The association of telomeric transcripts with centrosomes is a novel observation. It is tempting to speculate that the localization of the *HeT-A* transcripts around the centrosome, per se, may be related to mitotic defects. Detached centrosomes present in the cortex of embryos with knockdowns of the telomeric silencing factors were frequently associated with *HeT-A* transcripts. *HeT-A* RNA and its encoded Gag protein form spherical ribonucleoprotein particles that are targeted to chromosome ends by a telomeric protein Verocchio (59). It remains to be tested whether *HeT-A* RNA in embryos can interact with certain proteins and how it may impact the observed mitotic defects. We do not exclude that *HeT-A* overexpression plays an important if not the critical role in the early developmental defects linked to disruption of the piRNA pathway. Indeed, biochemical data show that interplay between telomere and mitotic machinery does exist. In mammals, the telomere-associated proteins tankyrase 1 and telomere repeat binding factor 1 (TERF1) not only localize to telomeres but are also translocated to the spindle poles and have been proposed to mediate proper kinetochore-microtubule attachment during metaphase-anaphase progression (62–65). In *Drosophila*, BubR1, a component of kinetochore, was shown to bind unprotected telomeres, resulting in mitotic arrest (66). Although the functional significance of this dual distribution of telomere and mitotic control factors is not yet clear, these data suggest the existence of a new level of cell cycle control. Likely, it is the telomeric transcripts that provide the information link between telomeres and the mitotic spindle checkpoint components. Intriguingly, TERRA levels are elevated in cancer cells (67), which are characterized by centrosome amplification, aneuploidy and chromosome instability (68). We speculate that TERRA could mediate missegregation defects in cancer cells. Although speculative, this hypothesis opens up the possibility for better understanding the function of the telomeric transcriptome and the potential pathological significance of the telomeric repeat overexpression in cancer.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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