**Supplementary materials for**

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

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**Materials and Methods**

*Primers used in this study (5’-to-3’)*

**Primers for RT-PCR:**
- **rp49**
  5’-ATGACCATCCGCCAGCATAC-3’ and 5’-GCTTAGCATATCGATCCGACTGG-3’
- **HeT-A**
  5’-GGAGTGATGAGCGGCGGAAA-3’ and 5’-CCAGGCAAGCGGACAAACGA-3’
- **TART-A1**
  5’-ATCTGTCTACTGTCCGCCTCGCTA-3’
- **TAHRE**
  5’-GATAAGGAGGTCATATATTAAGGGG-3’ and 5’-CATCAGACGAATCATAACGC-3’
- **HMS Beagle**
  5’-CGGGCTGCTGACAAAGTG-3’ and 5’-CGGCTGCTGCTGCTAGTAC-3’
- **I-element**
  5’-ACAAAATCACTTCAAAACATACATCCC-3’ and 5’-GCATCCCCTTACCTCTCCACAG-3’
- **jockey**
  5’-AAATGAGGAAAGGTGTATTCAATTGAAGC-3’ and 5’-CGCAGATTGAGTGGCATCCTCC-3’

**Primers for poly(A) tail analysis:**
- anchor primer 5’-GCCTGCCCACCTCC(T)12-3’
- **PAT-rp49** 5’-GTCGCTGCTGCTCTTCAAG-3’
- **PAT-HeT-A** 5’-TGCAATGTTAAACTCTAAACTCAAATT-3’
- **PAT-TART-A** 5’-GCACATATCTCGGCCCAAG-3’
- Illumina 3’linker 5’-P-TGGAATTCTCGGGTGCCAACTCddC-3’
Wolbachia-specific primers
5'-CCTGATTCGCGTTACGGCAGTG-3' and 5'-GGTACTTGATAAGTTGCACCACC-3'.

Subcellular fractionation and immunoprecipitation

Fractionation was done as described (1) with modifications. 550 pairs of ovaries of strain w1118 were dissected in PBS on ice, and homogenized with a Dounce homogenizer in cold buffer A containing 10 mM Hepes (pH 7.9), 10 mM KCl, 3 mM MgCl2, 340 mM sucrose, 10% glycerol, 1 mM DTT, and 1% protease and phosphatase inhibitor cocktails (Sigma). The lysate was filtered through Miracloth (Calbiochem) and the nuclear fraction was precipitated at 1800 g for 10 min at 4°C. The supernatant (crude cytoplasm) was separated by centrifugation at 16,000 g for 20 min at 4°C into supernatant (cytoplasm) and pellet (mitochondrial fraction). Nuclei were washed with buffer A, resuspended in buffer B (3mM EDTA, 0.2mM EGTA, 1 mM DTT, and 1% protease and phosphatase inhibitors), and incubated on a rotator at 4°C for 20 min. Soluble (nucleoplasm) and insoluble (chromatin) fractions were obtained by centrifugation at 1700 g for 10 min at 4°C. The pellet was resuspended in the digestion buffer (20 mM Hepes (pH 7.9), 10 mM NaCl, 1 mM MgCl2, 1% protease and phosphatase inhibitor cocktails (Sigma), and chromatin was digested with 30 U/ml benzonase (Sigma) for 10 min at room temperature on a rotator. The reaction was stopped in 2mM EDTA and EGTA. Then, chromatin-associated proteins were extracted with 300 mM NaCl and 0.3% NP-40. After extensive mixing, the samples were centrifuged at 1700 g for 10 min at 4°C and the supernatant (chromatin proteins) was collected. All fractions were analyzed by SDS-PAGE and Western blotting; equal protein amounts from each fraction (40 µg) were loaded on the gel. For the Western blot analysis, the following antibodies were used: murine monoclonal anti-Not1 2G5 antibody (2), 1:250; rabbit polyclonal anti-Ccr4 antibodies (3), 1:300; murine monoclonal anti-HP1 C1A9 antibody (Developmental Studies Hybridoma Bank, University of Iowa (DSHB)), 1:500; murine monoclonal anti-γ-tubulin GTU-88 antibody (Sigma), 1:500.

For immunoprecipitation with anti-Not1 2G5 antibody, we used a total nuclear fraction (mix of nucleoplasm and chromatin proteins). Non-immune mouse serum was used as a negative control. 75 µl Dynabeads M-280 sheep anti-mouse IgG (Invitrogen) was equilibrated in PBST and incubated with 15 µl Not1 antibody or 0.17 µl non-immune mouse serum for 15 min at room temperature on a rotator. Unbound antibodies were removed by washing with PBST. Beads were incubated with 200 µl nuclear extract in IP-buffer (10 mM Hepes (pH 7.9), 150 mM NaCl, 2 mM MgCl2, 0.15% NP-40, 5% glycerol, 0.1 mM DTT and protease and phosphatase inhibitor cocktails (Sigma)) on a rotator at room temperature for 40 min. The supernatants were removed and the beads were washed five times with 800 µl IP-buffer. Bound proteins were eluted from the beads by boiling in 30 µl 2xsample buffer containing 0.2 M DTT. Samples were resolved by 9% SDS-PAGE and blotted onto PVDF membrane Immobilon-P (Sigma). Blots were analyzed with anti-Not1 and Ccr4 antibodies and developed using the Immun-Star AP detection system (Bio-Rad Laboratories), in accordance with the manufacturer’s recommendations.

DNA FISH

DNA FISH on squashed embryos was done as previously described with some modifications (4). Before hybridization, slides were incubated for 1 hour at 55°C in air thermostat. Then, DNA denaturation was performed in 70% formamide, 2xSSC at 70°C for 2 minutes. For detection, FITC-conjugated anti-DIG antibodies (Roche) diluted at 1:200 were used. The FISH signal was amplified using anti-FITC Alexa Fluor 488 antibodies (Life Technologies, diluted at 1/500). The probe used for DNA FISH analysis was a fragment of
HeT-A ORF, corresponding to 1746 to 4421 nucleotides in GenBank sequence DMU 06920. The labeling was done using DIG DNA labeling kit (Roche).

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Supplementary Fig. 1 Trf2 is present at somatic telomeres. Immunostaining of polytene chromosomes of salivary glands with anti-Trf2 antibodies (5) was performed as described (4). Chromosome ends of different chromosomes are shown. Trf2 is associated with telomeres. In addition, Trf2 is present in multiple loci on Drosophila chromosome arms (6). Trf2 is in red. Chromosomes are stained with DAPI (blue).
Supplementary Fig. 2 HeT-A expression in spnE/Trf2 double mutants. *in situ* RNA hybridization of the HeT-A antisense probe with ovaries of Trf2^po1/+; spnE^fl1/+ (Trf2^+/- spnE^+/+), Trf2^po1/Trf2^po1; spnE^fl1/+ (Trf2^-/- spnE^+/+), Trf2^po1/+; spnE^fl1/spnE^del3987^ (Trf2^+/- spnE^-/-) and Trf2^po1/Trf2^po1; spnE^fl1/spnE^del3987^ (Trf2^-/- spnE^-/-).
Supplementary Fig. 3 Abundance of HeT-A small RNA was not affected by Trf2 and Ccr4-Not depletion. Northern analysis of short RNAs was done as previously described (7). (A) Northern analysis of the RNA isolated from ovaries of Trf2<sup>+/+</sup>, Trf2<sup>+/−</sup>, Trf2<sup>KD</sup>, w<sup>KD</sup> and piwi<sup>KD</sup>. No difference in HeT-A small RNA abundance was observed between Trf2 hetero- and homozygous mutants as well as between Trf2<sup>KD</sup> and w<sup>KD</sup>. (B) Northern analysis of the RNA isolated from ovaries of w<sup>KD</sup>, twin<sup>KD</sup> and Not1<sup>KD</sup>. Hybridization was done with HeT-A sense riboprobe. (C) Northern analysis of the RNA isolated from ovaries of w<sup>KD</sup>, twin<sup>KD</sup> and Not1<sup>KD</sup>. Hybridization was done with the I-element sense riboprobe corresponding to nucleotides 2109-2481 of GenBank sequence M14954. Lower panels represent hybridization with oligonucleotide 5’-ACTCGTCAAAATGGCTGTGATA-3’complementary to the mir-13b<sub>1</sub> microRNA. P<sup>32</sup>-labeled RNA oligonucleotides were used as size markers.
Supplementary Fig. 4 Localization of *HeT-A* transcripts in ovaries. *in situ* RNA hybridization of the *HeT-A* antisense probe with ovaries of the indicated genotypes. Collection numbers of RNAi strains (see Table S1) used for knockdown are indicated. nosGal4 driver strain was #25751 (Bloomington Stock Center) except for Not1KD (32836).
Supplementary Fig. 5 *HeT-A* derepression as a result of depletion of Ccr4-Not and piRNA pathway components. RT-qPCR analysis of *HeT-A* expression in ovaries of several independent RNAi and mutant lines. Collection numbers of RNAi strains used for knockdown are indicated. nosGal4 driver strain was #25751 (Bloomington Stock Center). Error bars indicate SD.
Supplementary Fig. 6 The ratio of polyadenylated to total *Het-A* and *TART* transcripts. Normalized ratio of *Het-A* and *TART* RNA abundance (RT-qPCR) obtained with oligo(dT) for reverse transcription to that obtained with random primers in ovaries with the indicated genotypes (*GAL4-nos* driver was used), normalized to the value in *wKDnos*. SD is not indicated when one biological replicate was analyzed.
Supplementary Fig. 7 Mitotic defects observed in 0–2-hour-old embryos from females with Trf2, Woc, Ars2, Ccr4 and Not1 germline knockdown. DAPI staining of syncytial blastoderm embryos is shown. Nuclear cleavages were synchronous in embryos of wKD<sup>nos</sup> (magnification is x10), whereas asynchronous mitotic divisions were observed in embryos from Trf2KD<sup>nos</sup>, wocKD<sup>nos</sup>, Ars2KD<sup>nos</sup>, twinKD<sup>nos</sup> and Not1KD<sup>nos</sup> females (x20 magnification).
Supplementary Fig. 8 Mitotic defects in Trf2<sup>pl</sup>/Trf2<sup>pl</sup>, wocKD, Ars2KD and twinKD early embryos. (A) Anaphase bridges observed on mitotic chromosome preparations from 0–2-hour-old embryos from Trf2<sup>pl</sup>/Trf2<sup>pl</sup>, wocKD<sup>dnos</sup>, Ars2KD<sup>dnos</sup> and twinKD<sup>dnos</sup> females. (B) DNA FISH with HeT-A probe on mitotic chromosomes showing that telomeres are fused in Trf2<sup>pl</sup> mutants.
Supplementary Fig. 9 Overexpression of HeT-A in the germline led to accumulation of HeT-A transcripts in 0–2-hour-old embryos. RT-qPCR analysis of HeT-A expression in embryos from Trf2<sup>p1</sup>/Trf2<sup>p1</sup>, wocKD<sup>nos</sup>, Not1KD<sup>nos</sup>, twinKD<sup>nos</sup>, Ars2KD<sup>nos</sup> females. The RNA level was normalized to rp49. Mean values (±SD) for two biological samples normalized to control knockdown (vKD<sup>nos</sup>) are shown.
Supplementary Fig. 10 Telomeric transcripts are localized around centrosomes. Confocal images of embryos with indicated genotypes. GAL4-nos driver was used for germline knockdown. Trf2-/- were homozygous Trf2p1/Trf2p1 mutants. HeT-A transcripts (green) were detected by in situ RNA hybridization with DIG-labeled probe followed by staining with anti-DIG FITC-conjugated antibodies. Red, γ-tubulin; blue, DNA stained with DAPI. Bar, 10 μm.
Supplementary Fig. 11 *HeT-A* RNA foci localize around free centrosomes in the embryonic cortex. Surface confocal images of embryos with the indicated genotypes. *GAL4-nos* driver was used for germline knockdown. *Trf2*-/- were homozygous *Trf2*^{+/+}/*Trf2*^{+/+} mutants. Green, *HeT-A* transcripts; red, γ-tubulin; blue, DNA, DAPI staining.
Supplementary Fig. 12 oskar RNA in situ hybridization. Oskar mRNA was detected by the tyramide amplification method at the posterior pole of twinKDnos embryos (A) but is not detected at the anterior pole (B) and near centrosomes (C). Red, $\gamma$-tubulin; green, oskar mRNA; blue, DNA. The template for the oskar antisense riboprobe synthesis was a PCR fragment amplified using primers 5'-acgggcatacccttgcttt-3' and 5'-taatacgactcactatagggaatcttt-3'.
Supplementary Tables

**Supplementary Table 1** RNAi strains used in the study

| gene      | strain # | collection        |
|-----------|----------|-------------------|
| *Trf2*    | 10443    | VDRC              |
| woc*      | 20994    | VDRC              |
| twin*     | 13365    | VDRC              |
| twin      | 32490    | Bloomington       |
| *Not1*    | 45463    | VDRC              |
| Not1      | 13740    | VDRC              |
| Not1      | 32836    | Bloomington       |
| *Pop2*    | 28396    | VDRC              |
| white*    | 33623    | Bloomington       |
| Su(var)205| 31994    | VDRC              |
| *Ars2*    | 106344   | VDRC              |
| rhi*      | 101620   | VDRC              |
| *spnE*    | 21376    | VDRC              |
| spnE      | 34808    | Bloomington       |
| aub       | 35201    | Bloomington       |
| aub       | 33728    | Bloomington       |
| Ago3      | 34815    | Bloomington       |
| *piwi*    | 101658   | VDRC              |

* indicated strains, unless otherwise specified, were used to obtain germline knockdown by crossing with #25751 Gal4 driver strain (Bloomington Stock Center, \(P\{\text{UAS-Dcr-2.D}1, w^{1118}, P\{\text{GAL4-nos.NGT}\}40\)).
**Supplementary Table 2** Effects of germline knockdown of telomere components on *HeT-A* expression in ovaries

| Gene, reference | strain # (collection) | GAL4-nos driver, strain # (Bloomington)* | *HeT-A in situ* RNA hybridization on ovaries | RT-qPCR (HeT-A RNA fold change+/−SD) |
|-----------------|-----------------------|------------------------------------------|---------------------------------------------|-------------------------------------|
| Z4 (pzg) (8,9)  | 35448 (Bloomington)   | 25751, 4937                              | rudimentary ovaries                         | ND                                 |
| Z4 (pzg)        | 25541 (VDRC)          | 4937                                     | ND                                          | 1.3 (±0.3)                         |
| JIL-1 (8,9)     | 42571 (VDRC)          | 25751                                    | unchanged                                  | 0.7 (±0.2)                         |
| JIL-1           | 107001 (VDRC)         | 25751                                    | unchanged                                  | 0.6 (±0.1)                         |
| Chriz (Chro) (10)| 101663 (VDRC)         | 25751                                    | rudimentary ovaries                         | ND                                 |
| Chriz (Chro)    | 36084 (Bloomington)   | 25751                                    | unchanged                                  | 1.0 (±0.7)                         |
| Dref (10)       | 35692 (Bloomington)   | 25751, 4937                              | rudimentary ovaries                         | ND                                 |
| Dref            | 22209 (VDRC)          | 4937                                     | unchanged                                  | 1.3 (±0.7)                         |
| Dref            | 22209 (VDRC)          | 25751                                    | ND                                          | 1.2 (±0.2)                         |
| caravaggio (cav) (11)| 15021 (VDRC)   | 25751                                    | modest activation at later stages of oogenesis | 1.8 (±0.1) |
| verrocchio (ver) (12) | 17404 (VDRC) | 25751                                    | unchanged                                  | 1.0 (±0.5)                         |
| ver             | 110243 (VDRC)         | 25751                                    | unchanged                                  | 1.4 (±0.5)                         |

* #25751 (P{UAS-Dcr-2.D}1, w^{1118}, P{GAL4-nos.NGT}40); #4937 (w^{1118}, P{GAL4::VP16-nos.UTR}CG6325MV01). ND – not determined.
### Supplementary Table 3 Embryonic lethality

| genotype* | % dead embryos** | n, embryos |
|-----------|------------------|------------|
| wKDnos    | 2 (±1)           | 332        |
| Trf2p1    | 28 (±7)          | 859        |
| wocKDnos  | 43 (±14)         | 304        |
| Ars2KDnos | 15 (±5)          | 583        |
| twinKDnos | 15 (±4)          | 653        |
| Not1KDnos | 41 (±3)          | 152        |

* Gal4 driver strain #25751 (Bloomington Stock Center, (P\{UAS-Dcr-2.D\}1, w1118, P\{GAL4-nos.NGT\}40) was used to obtain germline knockdown.  
** mean value (±SD) for three biological replicas.

### Supplementary Table 4 Mitotic defects observed in 0–2-hour-old embryos

| genotype | N, nuclei scored** | Multipolar spindles (%) | Monopolar spindles (%) | Chromosome bridges (%) |
|----------|--------------------|-------------------------|------------------------|------------------------|
| wKDnos   | 464                | 0                       | 0                      | 0.9 (±0.1)             |
| Not1KDnos* | 364            | 19.3 (±8.5)             | 3.5 (±0.4)             | 14.7 (±5.1)            |
| Trf2KDnos* | 570            | 10.1 (±5.2)             | 2.1 (±0.5)             | 12.6 (±4.8)            |
| Ars2KDnos | 559                | 18.6 (±3.5)             | 2.9 (±0.2)             | 13.8 (±4.8)            |
| wocKDnos | 347                | 12.4 (±1.8)             | 5.4 (±1.2)             | 32.5 (±6.1)            |
| twinKDnos | 557                | 21.0 (±4.4)             | 5.4 (±1.0)             | 17.3 (±6.6)            |

* Gal4 driver strain was #4937 (Bloomington Stock Center, w1118, P\{GAL4::VP16-nos.UTR\}CG6325MVDI). In other cases, Gal4 driver strain was #25751 (Bloomington Stock Center, (P\{UAS-Dcr-2.D\}1, w1118, P\{GAL4-nos.NGT\}40).

** Mitotic defects were scored using immunostaining of α–tubulin and γ–tubulin and DNA staining with DAPI. Three independent samples were analyzed (±SD).