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Interaction of Telomeric Retroelement *HeT-A* Transcripts and Their Protein Product Gag in Early Embryogenesis of *Drosophila*

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Abstract—The telomere is a nucleoprotein complex at the ends of linear chromosomes that protects them from fusion and degradation. The telomere consists of telomeric DNA, a protective protein complex and telomeric RNA. Biogenesis of telomeric transcripts in development is still far from being understood. *Drosophila* telomeres are elongated by a transposition of specialized telomeric retrotransposons that encode proteins. Using transgenic constructs encoding tagged telomeric protein, we found that transcripts of *Drosophila* telomeric element *HeT-A* bind Gag-HeT-A protein encoded by these transcripts. Maternal *HeT-A* transcripts and Gag-HeT-A form ribonucleoprotein granules around centrosomes, centers of microtubule organization, during blastoderm formation, upon disruption of telomere silencing during oogenesis. The specific localization of *HeT-A* RNA is dependent on microtubules since disruption of microtubules caused delocalization of *HeT-A* transcripts. This transgenic system is a valuable model for the study of telomeric RNA biogenesis.

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Key words: telomere, development, retrotransposon *HeT-A*, *Drosophila*, telomeric RNA

Telomeres are nucleoprotein complexes that protect the ends of linear chromosomes of eukaryotes. Telomeres comprise telomeric DNA and associated proteins. Recently, a telomeric RNA transcribed from telomeric repeats was discovered. Transcription of telomeric repeats is an evolutionary conserved property. However, its role remains obscure. In mammals, telomeric repeats, generated by telomerase, are transcribed into telomeric RNAs, TERRAs (telomeric repeat-containing RNAs) that play an important role in formation of telomeric chromatin [1]. Most of the data on TERRA biogenesis were obtained *in vitro* or in tumor cell cultures, where high levels of TERRA were observed. Recent data suggest that TERRA transcription activity is directly associated with the telomere complex status, while telomeric transcripts are able to bind telomeric proteins, telomerase, and replication-related factors [1]. TERRAs are also observed in human oocytes [2]. This indicates that telomeric RNAs play a conserved role in oogenesis and early development.

A distinctive feature of *Drosophila* telomeric repeats is that they are represented by LINE-type (long interspersed nuclear elements) retrotransposons *HeT-A*, *TART*, and *TAHRE*, of which *HeT-A* is the most abundant [3]. The complex of *Drosophila* telomeric proteins, which is a functional analog of shelterin, protects the ends of *Drosophila* chromosomes against degradation and end-to-end fusion [4]. The telomeric transcriptome of *Drosophila* consists of both long transcripts, which correspond to sense and antisense strands of telomeric repeats, and short RNAs, which are generated by the RNA interference system (RNAi) [5, 6]. Telomere transposon transcripts serve as templates for reverse transcription that leads to telomere elongation. An RNA-binding Gag-HeT-A, which participates in *HeT-A* RNA transport to the telomere, is encoded by the *HeT-A* mRNA [7]. Gag-HeT-A in a complex with *HeT-A* transcript was shown to be localized on the telomere [8]. It interacts with one of the telomeric complex proteins, Ver, an ortholog of the yeast telomeric protein Stn1, which is an inhibitor of

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; FISH, fluorescence *in situ* hybridization; HA, hemagglutinin; piRNA, Piwi interacting RNA; RIP, RNA-binding protein immunoprecipitation; RNAi, RNA interference; RNP, ribonucleoprotein; TERRA, telomeric repeat-containing RNA.

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telomerase [7]. Knockdown of Gag-HeT-A in cell culture caused chromosome fusions, which is an indication of telomere dysfunction [9]. These data suggest that Gag-HeT-A is a specific component of telomeric chromatin. Moreover, it is able to bind *HeT-A* RNA, which makes this protein an attractive target for studying the *Drosophila* telomeric complex.

Despite the obvious structural difference between mammalian and *Drosophila* telomeres, their core components are functionally equivalent. In both cases elongation occurs predominantly in germ cells with participation of a reverse transcriptase using an RNA template. Moreover, telomerase is phylogenetically and functionally related to retrotransposase of mobile elements [10, 11]. In mammals, LINE-1 mobile elements are able to transpose onto telomeres, which is similar to *HeT-A* transposition in *Drosophila* [12]. LINE-1 is also required for telomere elongation in cancer cells [13]. In some insects, e.g. in the silkworm *Bombyx mori*, both telomeric and retrotransposon repeats are present, which represents an intermediate stage between the two interchangeable telomere elongation mechanisms [14]. Telomere transcription and formation of short telomeric RNAs are also observed in both taxa and apparently play similar roles [5, 15-18]. Speaking of telomeric protein factors, there are many similarities between mammals and *Drosophila* [4]. Considering this resemblance, we used *Drosophila* as a powerful model system for dissecting telomeric RNAs in female germline and in early development.

While studying telomeres in ovaries and early development in *Drosophila*, we have shown that telomeric transcription is closely associated with formation of telomere protection complex [19]. Normally, telomeric repeat expression is strongly repressed by different silencing mechanisms, including a system involving Piwi-interacting RNA (piRNA) and assembly of telomere-specific repressive chromatin. Knockdown of factors that inhibit expression of *Drosophila* telomeric repeat *HeT-A* in ovaries caused high embryo lethality that is accompanied by mitotic aberrations, namely formation of telomeric bridges, multipolar spindles and free centrosomes [19]. It was demonstrated that maternal telomeric RNAs that were transcribed in the ovarian nurse cells with telomere dysfunction are transported into the oocyte and appear in embryos, where they accumulate around centrosomes. We suggest that telomeric transcripts may play a special role during early development upon blastoderm formation. In human cells, the colocalization of centrosomes

Primers used in the study

Target	Primer name	Primer sequence
pUASTattB-HeT-A-HA-FLAG	20v	GATCAATCTAGAAACTTTGCTGGTGGAGGTACGG
	24v	CCTTTATTTATGGGCCATCG
	26v	CTTGTCATCGTCGTCCTTGTAGTCAGCGTAATCGGGCACGTCATAAG-GGTAGTTGGATGTATCCATGTCAAG
	34v	GATCAAAGATCTCGTTCGCTTGCCAAAGACTCT
	36v	TTCTGACGATGAGGTACTTTCA
	37v	CTCTATTAGCTAAGCTTGTTGTG
	39v	GACTACAAGGACGACGATGACAAGTAATCTTACAACTACTTATATATTC
	HeTA_CHIP_ORFs	CCAGGCAAGCGGACAAACGA
rp49	rp49-f rp49-r	ATGACCATCCGCCCAGCATAC GCTTAGCATATCGATCCGACTGG
HeT-A ORF	HeT-A-ORF-f HeT-A-ORF-r	GGAGTGATGAGCGGCGGAAA CCAGGCAAGCGGACAAACGA
HeT-A-HA	HeT-A-HA-f HeT-A-HA-r	AATCCCAACTCACAAAAAGGCC GTCCTTGTAGTCAGCGTAATCG
TART-A	TART-A-f TART-A-r	AATGAACTTTGTCTGCCCTCCCA ATCTGTCTACTGTCCGCCTTCGCTA
I-element	I-el-f I-el-r	ACAAAATCACTTCAAAAACATACCAATCCC GCATCCCTCAACTTCTCCTCCACAG

with certain telomeric proteins, such as TRF1 and tankyrase, is observed [20, 21]. This may suggest functional interaction between the mitotic apparatus and telomeres. However, the mechanism of such interaction is still not clear.

Present work is aimed at dissection of a phenomenon of maternal telomeric RNA recruitment to centrosomes and development of a system that would allow detection of protein partners that interact with telomeric RNAs in early embryogenesis.

MATERIALS AND METHODS

Creation of pUASTattB-HeT-A-HA-FLAG construct. To create pUASTattB-HeT-A construct, PCR products 34v-37v and 36v-20v were amplified using genomic DNA of y^1; cn^1 bw^1 sp^1 strain as a template, cut with the restriction endonucleases Bgl II, Xma I and Xma I, Xba I, respectively, and ligated simultaneously between Bgl II and Xba I sites of pUASTattB vector. Sequences encoding HA and FLAG were inserted in the plasmid at the 3′-terminus of the Gag protein open reading frame as follows: PCR products HeTA_CHIP_ORFS – 26v and 39v-24v amplified on pUASTattB-HeT-A template were mixed and used in overlap extension PCR with primers HeTA_CHIP_ORFS and 24v. The resulting amplicon was cut with BstAP I restriction endonuclease and ligated with pUAST-HeTAΔBstAPI,BstAPI. The primer sequences are given in the table.

Cell culture and *Drosophila* strains. *Drosophila* embryonic cells Schneider 2 (S2) were transfected using FuGENE 6 reagent (Roche, Switzerland). Strain 24862 (Bloomington Drosophila Stock Center) was used for transgenesis. To induce transcription in S2 cells, expression plasmid pUASTattB-HeT-A-HA-FLAG was co-transfected along with the driver plasmid pAC-GAL4. To induce transcription of HeT-A-HA-FLAG in the flies, a construction that bears GAL4 (w^{1118}, P{GAL4-nos.NGT}40, Bloomington Stock Center) expression inducer was genetically introduced into the genome of transgenic strain. To perform germline knockdown of the *spnE* gene, gene-specific dsRNA was used (strain 103913, Vienna Drosophila Resource Center).

Western blot analysis, immunostaining combined with RNA detection. For Western blot analysis, whole ovarian extracts were separated in 8% polyacrylamide gel and transferred onto a nitrocellulose membrane (GE Healthcare, USA). Signal was visualized using the Immun-Star AP detection system (Bio-Rad, USA). Immunostaining combined with RNA FISH (fluorescence *in situ* hybridization) was carried out as described earlier [19]. Antibodies used in this work were anti-FLAG (Sigma, USA), anti-HA (Cell Signaling, USA), anti-α-tubulin (Sigma), anti-γ-tubulin (Sigma), anti-mouse-Alexa 488 (Life Technologies, USA), anti-mouse-Alexa 546 (Life Technologies), anti-rabbit-Alexa 546 and 633 (Life Technologies). DNA was stained with DAPI (4′,6-diamidino-2-phenylindole). Imaging was performed using a confocal microscope Zeiss LSM 510 Meta (Zeiss, Germany).

RNA quantification. RIP (RNA-binding protein immunoprecipitation). Total RNA was isolated from ovaries of 3-day-old flies using TRIzol reagent (Life Technologies). Reverse transcription (RT) was performed with a random hexanucleotide primer and SuperScriptII reverse transcriptase (Life Technologies) according to the manufacturer's instructions. Gene-specific primers used for quantitative PCR (RT-qPCR) are shown in the table.

For RIP, the whole lysates of S2 cells after cotransfection with the plasmids bearing HeT-A-HA-FLAG and GAL4 transactivator under the control of actin gene promoter were used. Untransfected S2 cells were used as a control. Control and experiment lysates were incubated with anti-HA magnetic particles (Pierce, USA). Immunoprecipitation with subsequent RNA isolation and RT-PCR was performed as described [22]. Reverse transcription was performed with a random hexanucleotide primer and SuperScriptIV reverse transcriptase (Life Technologies) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Expression of transgenic Gag-HeT-A telomeric protein. To study the localization and look for functional partners of telomeric RNAs and proteins, a construct that contained the full-size retrotransposon *HeT-A* encoding a tagged Gag-HeT-A protein was created. To do so, a full-size genome copy of *HeT-A* was cloned in the pUASTattB plasmid carrying a yeast UAS promoter that can be induced by the tissue-specific expression of the GAL4-VP16 transactivator. In this construct, Gag protein contains C-terminal peptides HA (hemagglutinin) and FLAG (HeT-A-HA-FLAG construct; Fig. 1a). Transfection of *Drosophila* S2 with this construct and driver plasmid followed by Western blot analysis revealed the Gag-HeT-A-HA-FLAG protein of expected size (Fig. 1b). This construct was also used for site-specific fly transgenesis using φC31 integrase.

Expression of the transgenic copy of *HeT-A* in germ cells was verified by RT-qPCR with total RNA isolated from ovaries of the transgenic strains under normal conditions and upon RNAi-mediated knockdown of RNA-helicase Spindle-E (SpnE), a component of the piRNA-mediated silencing system. It is known that the piRNA pathway inhibits transcription of retrotransposons (including telomeric ones) in *Drosophila* ovaries [5, 23]. Using primers specific for a transgenic copy and genomic copies of *HeT-A* we demonstrate that their expression rates are similar, indicating that *spnE* knockdown causes

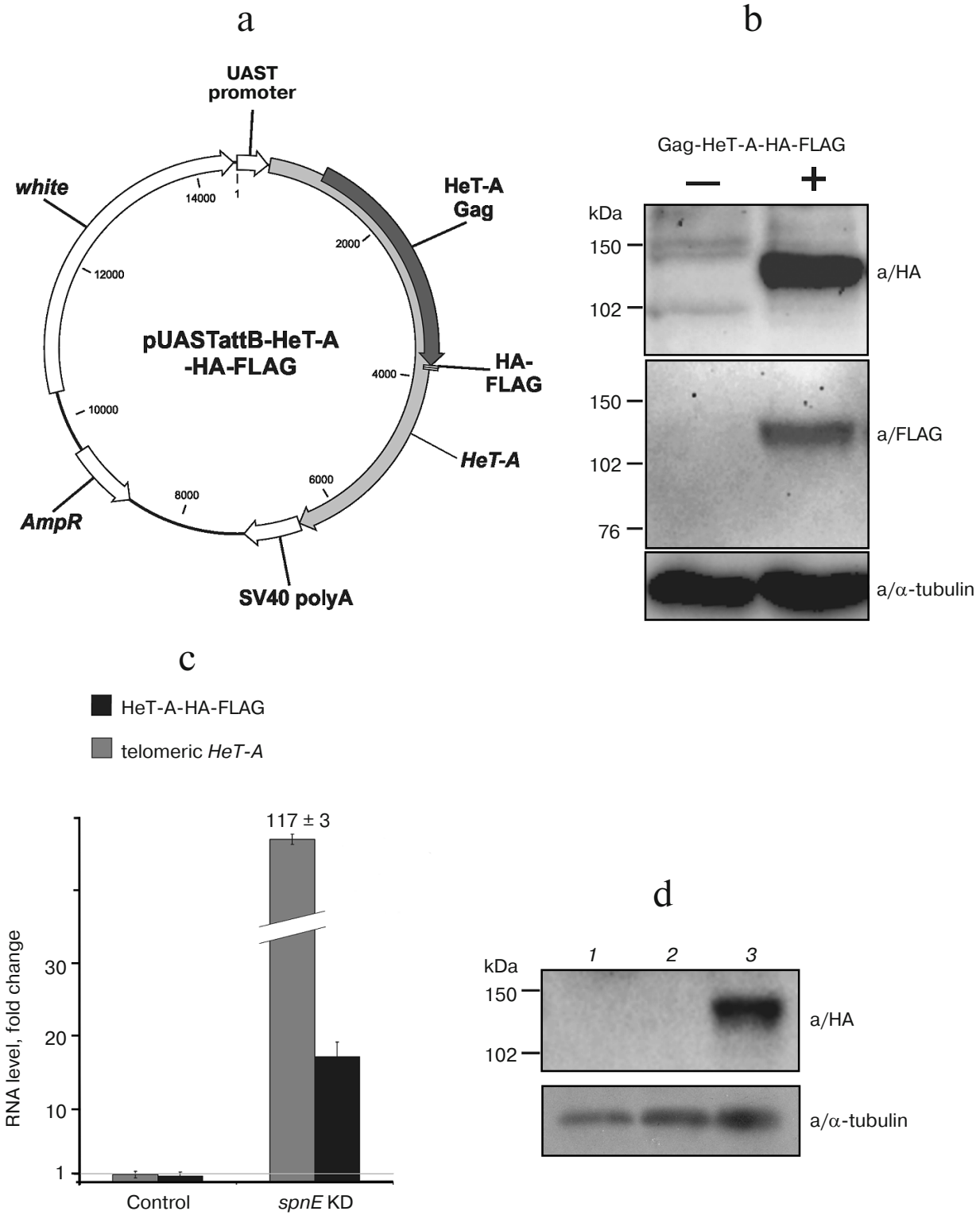

Fig. 1. Expression of transgenic *HeT-A* in ovaries of *Drosophila*. a) Diagram of the pUASTattB-HeT-A-HA-FLAG vector. b) Western blot analysis of extracts of untransfected S2 cell culture (–) and cells after transfection with a plasmid expressing Gag-HeT-A-HA-FLAG (+), using antibodies specific to epitopes HA, FLAG, and α-tubulin as a loading control. Molecular weight markers are indicated on the left in kDa. c) Comparison of abundance of endogenous and transgenic *HeT-A* transcripts by RT-qPCR on total RNA extracted from ovaries of transgenic strain under normal conditions (control) and upon *spnE* germline knockdown (*spnE* KD). d) Western blot analysis of ovarian extracts from control strain *yw* (*1*) and transgenic strain expressing HeT-A-HA-FLAG under normal conditions (*2*) and upon *spnE* germline knockdown (*3*), using anti-HA and anti-α-tubulin antibodies. Loading controls are indicated below.

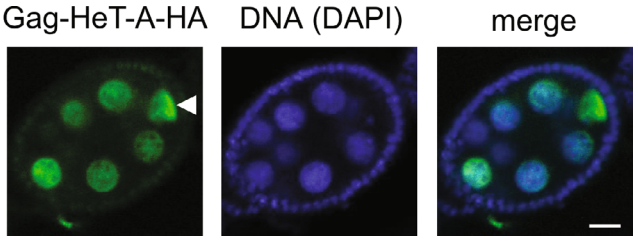

Fig. 2. Immunostaining of ovaries of transgenic flies carrying HeT-A-HA-FLAG upon *spnE* knockdown using anti-HA antibody. Gag-HeT-A-HA is detected in nurse cell nuclei and in the cytoplasm of the oocyte (arrowhead). Scale bar, 10 μm.

derepression of both endogenous and transgenic copies of *HeT-A* (Fig. 1c). The recombinant Gag-HeT-A-HA-FLAG protein is only detected by western blot in ovaries of transgenic flies upon RNAi-mediated SpnE knockdown (Fig. 1d). In this case immunostaining of transgenic fly ovaries revealed accumulation of Gag-HeT-A-HA protein in both nurse cells and oocyte. This indicates that either the protein or a complex of *HeT-A* RNA with the protein is transported from nurse cells to the oocyte (Fig. 2). Such localization was demonstrated earlier for the Gag-HeT-A protein, which is encoded by endogenous repeats of *HeT-A* [7, 24]. Thus, a transgenic construct containing a full-size telomeric *HeT-A* repeat has an expression pattern similar to endogenous telomeric *HeT-A*.

***HeT-A* mRNA interacts with Gag-HeT-A protein.** It was shown earlier that *HeT-A* is over-expressed in the ovaries of the piRNA pathway mutants. As a result, *HeT-A* transcripts and Gag-HeT-A protein were accumulated in the oocyte [7, 24, 25]. Colocalization of *HeT-A* transcripts with the Gag-HeT-A protein in the vicinity of telomeres was observed in proliferating somatic cells [7]. Gag is an RNA-binding protein, and it interacts with its mRNA during translation according to the so-called "*cis* preference" principle [26, 27]. Using RIP, we tested whether the interaction of *HeT-A* RNA with the protein that it encoded occurs. To do so, we performed immunoprecipitation of lysates of the control and HeT-A-HA-FLAG-expressing S2 cells using immobilized anti-HA antibody (Fig. 3a). Then, RNA that coprecipitated with proteins was purified and analyzed by RT-PCR (Fig. 3b). The precipitates were not significantly enriched with transcripts of retrotransposon *I*-element and *TART*. At the same time, the precipitates from Gag-HA expressing cells were significantly enriched with *HeT-A* RNA. This indicates that the *HeT-A* RNA binds to Gag-HeT-A protein in a specific manner.

Localization of *HeT-A* RNA in the vicinity of centrosomes depends on microtubules. *HeT-A* RNA was observed close to centrosomes in syncytial cytoplasm in embryos at the stage that preceded onset of zygotic transcription, upon disruption of telomeric repeat silencing in ovarian germ cells [19]. Therefore, maternal telomeric RNAs are transmitted to progeny, but their role here is unknown. The mechanism of *HeT-A* transcript attraction to centrosomes during early embryogenesis is also unknown. During mitosis, centrosomes nucleate microtubule polymerization. They are required for proper segregation of chromosomes. Minus ends of microtubules are associated with the centrosome. Specific markers for microtubules and centrosomes are α- and γ-tubulin, respectively. We suggested that the *HeT-A* RNA localization close to centrosomes depends on microtubules. Indeed, in embryos treated with colcemid, a reagent that depolymerizes microtubules, disappearance of *HeT-A*

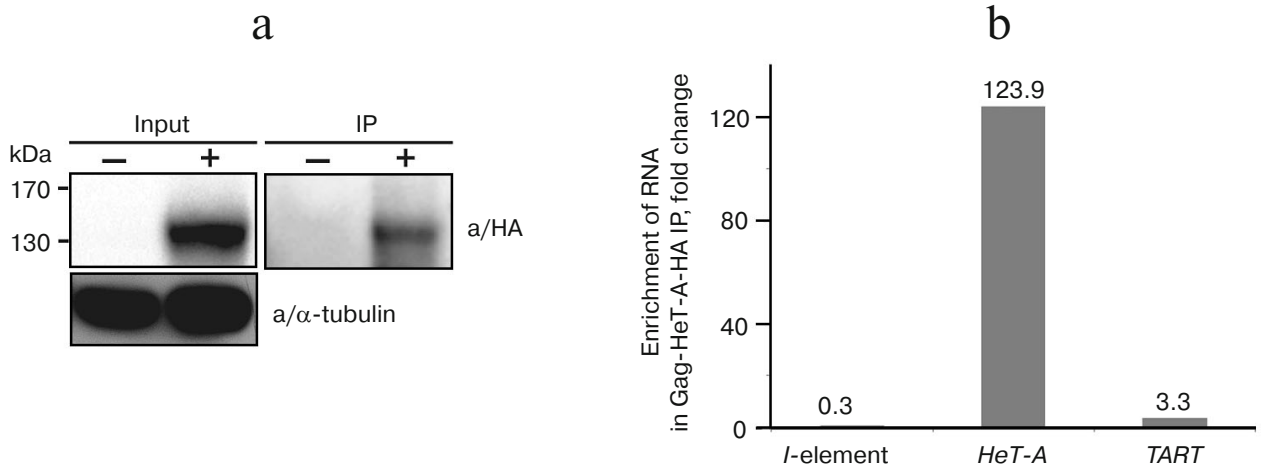

Fig. 3. *HeT-A* mRNA interacts with its encoded Gag-HeT-A protein. a) Western blot of immunoprecipitates from untransfected cultured cells S2 (–) and cells after transfection with plasmid expressing Gag-HeT-A-HA-FLAG (+) using anti-HA antibody (α-tubulin was used as a loading control). b) RT-qPCR of RNA extracted from anti-HA immunoprecipitates (IP) from untransfected (control) and transfected (experiment) S2 cells. The experiment-to-control ratio of immunoprecipitated RNA abundance of *I*-element, *TART*, and *HeT-A*, normalized to control RNA *rp49*, is shown.

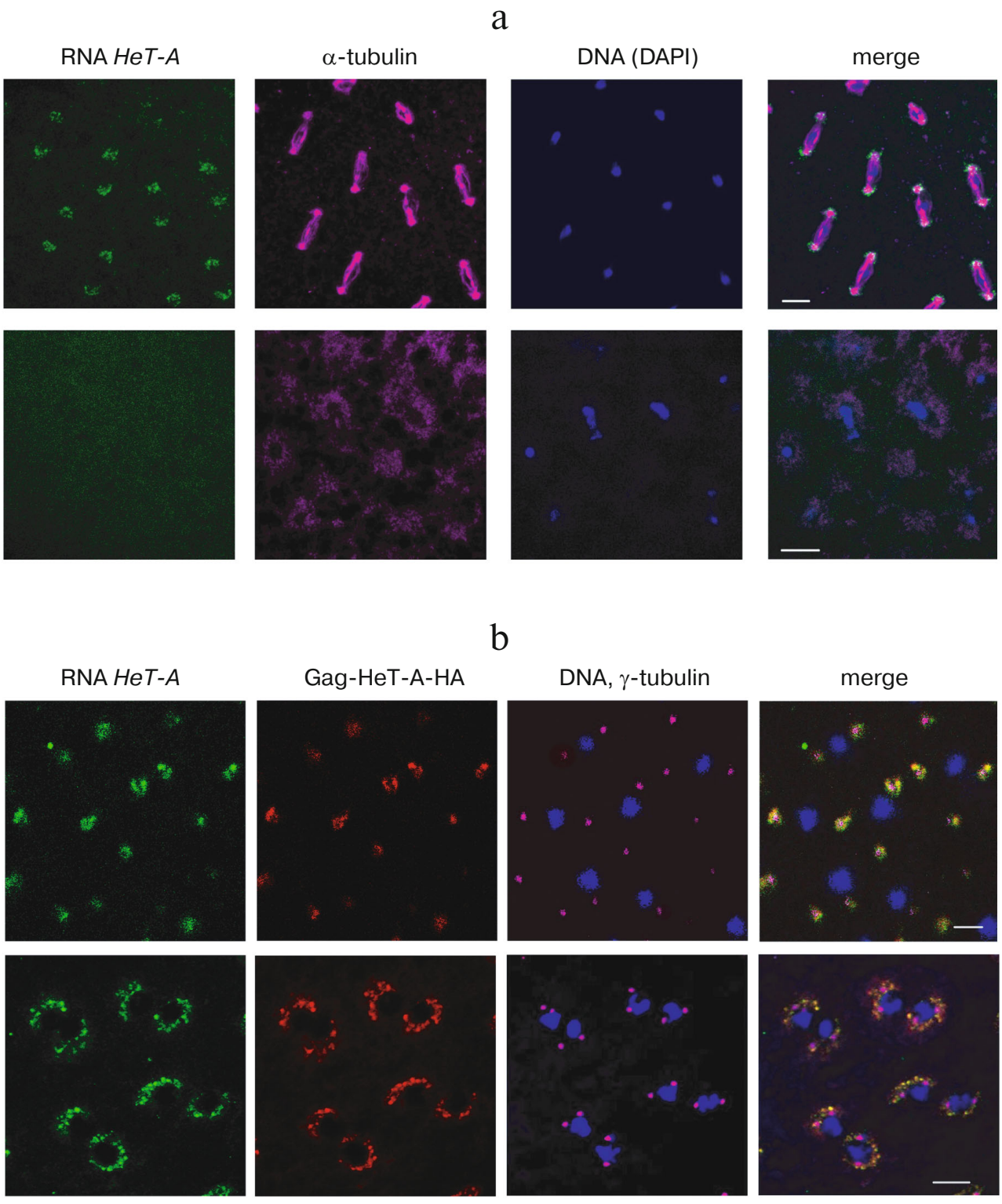

Fig. 4. Localization of *HeT-A* transcripts and Gag-HeT-A protein at the blastoderm formation stage. a) Localization of *HeT-A* transcripts around centrosomes is dependent on microtubules. Immunostaining of α-tubulin, the central component of microtubules (magenta), is combined with *HeT-A* RNA FISH (green) in 0-2-h-old embryos after germline knockdown of the piRNA system component, *spnE*; untreated (top) and colcemid-treated (0.1 mg/ml) (bottom). DNA was stained with DAPI (blue). Scale bars, 5 μm. b) *HeT-A* transcripts and Gag-HeT-A protein form RNP granules around the centrosomes. Immunostaining of 0-2-h-old embryos of transgenic flies carrying HeT-A-HA-FLAG upon *spnE* knockdown, using anti-HA antibody (red). The top panel shows the metaphase stage, bottom – late anaphase. *HeT-A* transcripts (green) were detected by FISH. DNA was stained with DAPI (blue). γ-tubulin (red) was stained with specific antibodies. Scale bars, 5 μm.

RNA granules around centrosomes was observed (Fig. 4a). This suggests that *HeT-A* RNA is transported to the centrosome using microtubules.

***HeT-A* RNA and Gag-HeT-A protein colocalize around centrosomes in early embryos.** Overexpression of telomeric transcripts that occurs upon disruption of telomeric complex in ovaries is accompanied by the accumulation of *HeT-A* transcripts as large granules around centrosomes at the blastoderm formation stage [19]. On the basis of our data on *HeT-A* RNA–Gag-HeT-A interaction, we assumed that these granules represent ribonucleoprotein (RNP) particles that include *HeT-A* RNA and Gag-HeT-A protein. We performed immunostaining using anti-HA antibody combined with RNA-FISH (*HeT-A* probe) in 0-2-h-old transgenic embryos carrying Gag-HeT-A-HA-FLAG upon *spnE* knockdown. We found that at the early syncytium stage the RNA of *HeT-A* retroelement colocalizes with Gag-HeT-A protein and forms multiple RNP granules around centrosomes (Fig. 4b) without overlapping with the centrosome component γ-tubulin. At the metaphase stage, these granules are dense and localize close to the centrosome, while at the anaphase stage they become looser and move away from the centrosomes. We suggest that complex of the protein and *HeT-A* RNA is transported along astral microtubules (not connected to kinetochore) toward their minus end, which is directed to the centrosome. Such a transport mechanism is suggested by dynamic character of the *HeT-A* RNA and protein signals. They look like dense particles that colocalize with the centrosome at the metaphase stage, and like flares at the anaphase stage, indicating their radial movement off the centrosome. Possibly, such dynamic behavior of *HeT-A* RNP particles is associated with assembly–disassembly of microtubules at different stages of the cell cycle.

While studying localization of telomeric RNA during early development of *Drosophila*, we have discovered that maternal transcripts of telomeric *HeT-A* repeats in a complex with Gag-HeT-A protein, encoded by these repeats, are attracted to the centrosomes via microtubules at the blastoderm formation stage. Disruptions of telomere functioning, which accompany piRNA silencing disorder, may cause aberrant chromosome segregation. Perhaps, transport of telomeric transcripts and proteins that they encode, that are overproduced in this case and directed to blastoderm of the embryo, is one of the signals that trigger elimination of defective nuclei, a crucial process that ensures normal development. As we did not observe the association of telomeric transcripts and proteins with telomeres in the blastoderm mitotic nuclei, it is possible that their function here is not directly associated with telomeres. The full-size transgenic *HeT-A* copy used in this work as well as the protein tagged with artificial epitopes, encoded by this copy, are promising molecular tools for studying partners of telomeric RNA during early embryogenesis both under normal conditions and upon telomere dysfunction.

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