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Participation of the piRNA Pathway in Recruiting a Component of RNA Polymerase I Transcription Initiation Complex to Germline Cell Nucleoli

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Abstract—Proteins of the Piwi family and short Piwi-interacting RNAs (piRNAs) ensure the protection of the genome from transposable elements. We have previously shown that nuclear Piwi protein tends to concentrate in the nucleoli of the cells of *Drosophila melanogaster* ovaries. It could be hypothesized that the function of Piwi in the nucleolus is associated with the repression of R1 and R2 retrotransposons inserted into the rDNA cluster. Here, we show that Piwi participates in recruiting Udd protein to nucleoli. Udd is a component of the conserved Selectivity Factor I-like (SL1-like) complex, which is required for transcription initiation by RNA polymerase I. We found that Udd localization depends on Piwi in germline cells, but not in somatic cells of the ovaries. In contrast, knockdowns of the SL1-like components (Udd or TAF1b) do not disrupt Piwi localization. We also observed that the absence of Udd or TAF1b in germline cells, as well as the impairment of Piwi nuclear localization lead to the accumulation of late stage egg chambers in the ovaries, which could be explained by reduced rRNA transcription. These results allow us to propose for the first time a role for Piwi in the nucleolus that is not directly associated with transposable element repression.

Keywords: nucleolus, Piwi protein, rDNA, RNA polymerase I, rRNA

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INTRODUCTION

Proteins of the Piwi family form complexes with small piRNAs (Piwi-interacting RNAs), which inhibit transcription or cause degradation of transcripts of transposable elements [1, 2]. The most-studied function of the piRNA pathway is to protect the genome of germline cells from transpositions, which can cause genomic instability, mutations or impairment of gene function. In various organisms, this system was also adapted during evolution to participate in processes, such as regulation of expression and stability of cellular mRNAs, induction of genome rearrangements, gene imprinting, and others (for review, see [3]). The piRNA pathway in *Drosophila melanogaster* operates mainly in the cells of testes and ovaries, as well as at early stages of embryonic development. *Drosophila* ovaries contain germline cells, which are precursors of oocytes, along with somatic cells surrounding them. The piRNA pathway is present in both germline and somatic ovarian cells; however, the proteins of the Piwi family and auxiliary proteins necessary for the formation and functioning of piRNAs differ in these two types of cells [1, 4]. In somatic cells of the ovaries, Piwi is the only one piRNA-interacting protein expressed, whereas germline cells in addition contain two other proteins of this family, Aub and Ago3. In contrast to cytoplasmic Aub and Ago3 proteins, Piwi is a nuclear protein, which carries out the repression of transposable elements at the level of transcription with the help of chromatin-modifying factors [1, 4–6].

We have shown previously that, in the cells of ovaries and embryos of *Drosophila*, Piwi protein is localized predominantly in nucleoli, and in cultured ovarian somatic cells its nucleolar localization is dependent on RNA polymerase I transcription [7]. Piwi is required to repress the transcription of retrotransposons R1 and R2 [7], which are inserted into strictly defined sites in 28S rDNA [8]. We hypothesized that, in addition to the repression of the nucleolar transposons, Piwi may be involved in rRNA biogenesis.

In a recently published study [9] the modulation of rRNA synthesis in the nucleolus has been shown to be associated with the differentiation, growth, and proliferation of germline stem cells of the *Drosophila* ovary, i.e., the processes in which Piwi protein is also involved [10–12]. The protein complex Selectivity Factor I like (SL1-like) of *Drosophila*, which is homologous to the human SL1 complex [13–15], is required for the initiation of RNA polymerase I transcription at rDNA promoters [9]. TAF1B, TAF1C-like and Udd (underdeveloped) proteins are components of the SL1-like complex, the lack of which in the ovaries

leads to a decline in transcription in the nucleolus causing oogenesis defects [9]. It has been shown that a very high level of rDNA transcription is characteristic of germinal stem cells and is essential to prevent their premature differentiation [9]. However, in another study, it was reported that a decrease in the transcription of rRNA genes may cause the process of the differentiation of germinal cells to slow down [16].

In this work, we investigated a possible role of Piwi in regulating the localization of Udd protein, a component of the SL1-like complex, in the ovaries of *Drosophila*. We were able to show that Piwi influenced the recruitment of Udd to the nucleolus, but the reciprocal dependence of the localization of Piwi on the SL1-like complex components was not revealed. We also found that the involvement of the piRNA pathway in recruiting the SL1-like complex to nucleoli is only typical of germline cells.

EXPERIMENTAL

Strains of *Drosophila melanogaster*. The following fly strains were used in this study: $piwi^{Nt}$ mutation, which disrupts the nuclear localization of Piwi protein, and $piwi^{2}$ and $piwi^{3}$ null mutations. To analyze the $piwi^{Nt}$ phenotype, transheterozygous $piwi^{Nt}/piwi^{2}$ females and their sisters (a mixture of $piwi^{Nt}/+$ and $piwi^{2}/+$) which are progeny of the same cross were compared. To obtain tissue-specific knockdowns of genes, animals carrying transgenic constructs expressing double-stranded RNA (hairpin RNA) to *piwi, udd,* and *Taf1b* under the control of the UAS promoter were crossed with flies expressing Gal4 protein in somatic or germline cells of the ovary using driver constructs *tj-Gal4* and *nos-Gal4*, respectively.

Immunostaining. Immunostaining of the ovaries was performed according to [7]. The following primary antibodies were used: mouse monoclonal antibodies to Piwi (1 : 200, from M. Siomi); rabbit polyclonal antibodies to fibrillarin (1 : 1000, Abcam, United Kingdom); Guinea pig polyclonal antibodies to Udd (1 : 800, from M. Buszczak). Then, secondary antibodies were used conjugated with the following fluorescent dyes: Alexa 633 for mouse antibodies, Alexa 546 for rabbit antibodies, and Alexa 488 for Guinea pig antibodies (Invitrogen). Confocal microscopy was carried out using the LSM 510 META system (Carl Zeiss, Germany).

Estimation of the number of egg chambers at late stages of oogenesis. Late stages were defined as those following stage 10 of oogenesis characterized by opaque egg chambers, including mature oocytes.

Western-blot analysis was performed according to [17] using Guinea pig polyclonal antibodies to Udd (from M. Buszczak) and mouse monoclonal antibody to β-actin (ab8224, Abcam).

Northern-blot hybridization. Total RNA was extracted from ovaries of 0–8 day old flies, transheterozygous $piwi^{Nt}/piwi^{2}$ and their sisters $piwi^{Nt}/+$ and $piwi^{2}/+$ (control), using Trizol reagent (Invitrogen). Various amounts of total RNA (1, 2, and 4 μg) were separated by electrophoresis in a denaturing formaldehyde gel. Northern-blot analysis was carried out in accordance with the guidelines [18] with upward capillary transfer under alkaline conditions to the Hybond-N+ membrane (Amersham) with subsequent fixation of RNA under UV. Hybridization was performed with the DNA oligonucleotide complementary to 28S rRNA: GTAACTAGCGCGGCATCAGGTGATCGAAGATCCTCCC (28Sb) [19], labeled with [γ-^{32}P] UTP. Visualization was performed using Typhoon FLA9500 (GE Healthcare) scanner.

RESULTS

Piwi Mutations Impair the Localization of Udd in the Nucleoli of Germline Cells of the Ovaries

To study the interactions of Piwi and the Udd transcription factor, a component of the SL1-like protein complex [9], we determined the localization of Udd in the ovaries of *piwi* mutants. The ovaries of *Drosophila* consist of ovarioles, chains of egg chambers at different stages of development. Each egg chamber contains a developing oocyte and 15 germline nurse cells, which are covered by a layer of somatic follicle cells. In the ovaries of wild-type and heterozygous control (*piwi*/+) flies, Udd protein colocalized with fibrillarin, a marker of the nucleolus, both in nurse and follicle cells (Fig. 1a).

It should be noted that the nucleolus of a polyploid nurse cell is a branched structure that is adjacent to the nuclear envelope [20]. As previously shown, Piwi protein is concentrated in nucleoli of both somatic and germline ovarian cells [7]. However, further studies showed that the degree of colocalization of Piwi with the nucleolus in germline cells of different lines of *Drosophila* varies considerably. For example, in the egg chamber in Fig. 1a, the areas of distribution of Piwi protein and fibrillarin overlap only partially. Mutation $piwi^{Nt}$, which prevents Piwi nuclear import [21], does not cause a visible change in the structure of the nucleolus and the localization of fibrillarin, but leads to a significant reduction in the amount of Udd in the nucleoli of germline, but not somatic cells (Fig. 1b). Western blotting showed that the amount of Udd protein in the ovaries of $piwi^{Nt}$ mutants was not decreased compared to the control (Fig. 2a). This suggests that Piwi only affects Udd protein localization in the nucleoli, but not its expression. The apparent disappearance of the protein from the nuclei of germline cells of *piwi* mutants detected by confocal microscopy (Fig. 1b) can be explained by the fact that the protein ceases to form local clusters in the nucleolus and is uniformly distributed in the nucleoplasm. In the case of *piwi* null mutations ($piwi^{2}/piwi^{3}$ transheterozygotes), Udd also disappears from the nucleoli of germ-

line, but not somatic cells (Fig. 1c). Thus, Udd accumulation in nucleoli is reduced both in case of disruption of Piwi nuclear localization and in case of total lack of Piwi. It was of interest to find out whether Piwi knockdown in germline cells would lead to Udd delocalization from the nucleolus. The expression of the *UAS-piwi-RNAi* construct was induced exclusively in germline cells using the *nos-Gal4* driver (*nos-Gal4>UAS-piwi-RNAi*). In this case, Piwi protein is not detected in germline cells, however, Udd localization in the nucleoli remains unaffected (Fig. 1d). The lack of the effect of *piwi* knockdown on the localization of Udd might be explained by incomplete removal of Piwi in the knockdown in comparison to the mutation at early development stages of *Drosophila* germline, which may be critical for establishing epigenetic marks. In a number of studies, gaps in expression of the *nos-Gal4* driver in germline cells at certain stages of embryonic development were noted [22, 23]. At the same time, it is known that the rDNA clusters are extremely polymorphic and can vary in the numbers of copies of rDNA and of insertions of ribosomal transposons [8]. In this regard, it cannot be excluded that the fly stocks bearing *piwi* mutations and the stocks used to produce knockdowns contain different sets of rDNA, the transcriptional activity of which may be sensitive to the absence of Piwi to a different degree. Since in $piwi^{Nt}/piwi^{2}$ and $piwi^{2}/piwi^{3}$ mutants the functioning of Piwi is impaired both in germinal and somatic cells, we tested the assumption whether Udd localization in germline cells may depend on the presence of Piwi in somatic cells. To this end, females with knockdown of Piwi in somatic cells of the ovaries were obtained using the *tj-Gal4 driver*; however, in this case, the distribution of Udd was not affected as well (data not shown).

We tested whether Udd protein and another component of the SL1-like complex, TAF1b protein, can influence Piwi localization in the nucleolus. The colocalization of Piwi with fibrillarin in ovaries of *udd* and *Taf1b* knockdowns in somatic or germline cells of the ovaries was assessed by immunostaining. In these knockdowns, Udd protein in the nucleoli was missing or its content was significantly reduced; however, the localization of Piwi was not changed (Fig. 1e, other data not shown).

Piwi Mutation Leads to a Decrease in the Level of Mature rRNA in Ovaries and to Oogenesis Defects Similar to Those in the Absence of Components of the SL1-Like Complex

Upon elimination of Udd protein, the synthesis of rRNA in the cells of the ovaries drops dramatically [9]. We estimated the amount of rRNA in the ovaries of $piwi^{Nt}/piwi^{2}$ mutants by Northern blot hybridization using a probe detecting 28S rRNA. The amount of mature 28S rRNA in the mutants decreased by approximately two-fold compared with the heterozygotes (Fig. 2b) when normalized to the amount of total RNA. As rRNA is the major constituent of total RNA in the cell, this result means that, in the total RNA of *piwi* mutants, the representation of other types of RNA or the number of rRNA fragments significantly increases. Since according to the immunostaining data, the amount of Udd is only reduced in germline, but not somatic cells of *piwi* mutants and the total RNA from whole ovaries is used in the Northern hybridization, it can be assumed that the level of rRNA in germline cells of *piwi* mutants decreases more than two-fold. It should also be noted that a decrease in rRNA synthesis may lead to delayed development of germline cells and cell divisions can be delayed until the required number of ribosomes is accumulated. Thus, the actual decrease in the level of transcription of rRNA may be more significant than that deduced from the degree of reduction in the amount of mature rRNA detected using Northern hybridization.

It was of interest to find out how the impact of Piwi on Udd protein localization and the reduction of the amount of rRNA in germline cells may affect oogenesis. It is known that *piwi* mutations lead to various defects in maintenance, self-renewal, and differentiation of germline stem cells, which may be caused by the upregulation of transposable elements [10–12]. We previously found that, in addition to the germline stem cell defects, $piwi^{Nt}/piwi^{2}$ females exhibit abnormal accumulation of egg chambers at late stages of ovarian development (after stage 10, including mature oocytes) [12]. A similar effect was found in the ovaries of females with *udd* knockdown (*nos-Gal4>UAS-udd RNAi*) and *Taf1b* (*nos-Gal4>UAS-Taf1b-RNAi*) in germline cells (Fig. 2c). The arrest at the terminal stages of oogenesis can be caused by a decrease in the activity of RNA polymerase I in the nuclei of nurse cells, as intense transcription of rDNA in these cells is important for the accumulation of numerous proteins that are transported from the nurse cells to the developing oocyte.

DISCUSSION

We found that the localization of Udd protein, a component of the SL1-like complex, in the nucleoli of germline cells is affected by *piwi* mutations. This effect can be explained by direct interaction of Piwi protein with components of this complex and by the influence of the piRNA system on the epigenetic state of chromatin of the rDNA cluster. The latter assumption allows to explain the absence of influence of germline *piwi* knockdown on the localization of Udd by the fact that *piwi* knockdown by the *nos-Gal4* driver only occurs in the germline cells of adult ovaries, whereas the formation of a specific structure of rDNA chromatin that recruits Udd and is later inherited epigenetically, occurs at early stages of ovary development. The interaction of Piwi and the SL1-like complex is only revealed in germline, but not somatic cells. This tissue

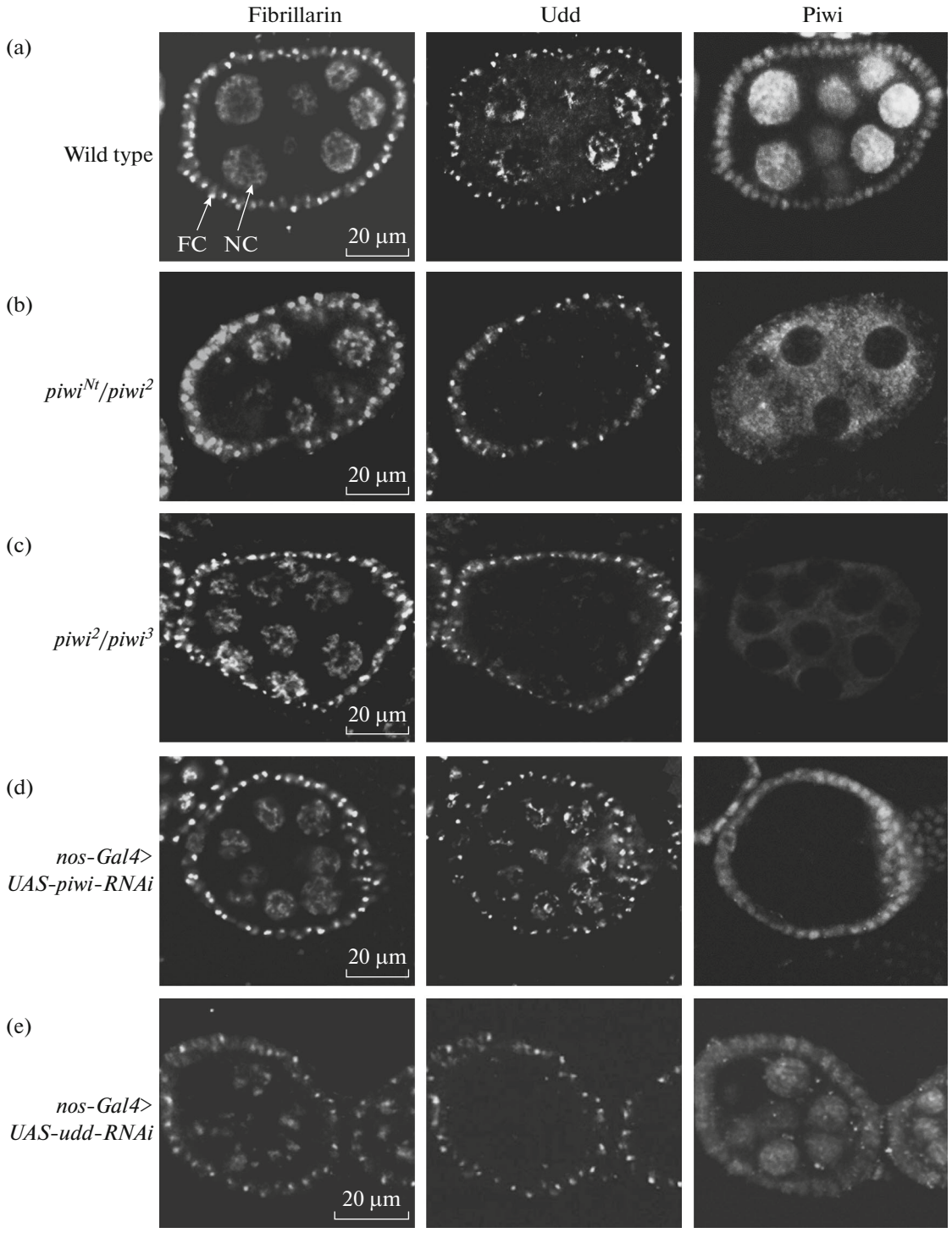

Fig. 1. Immunostaining of ovarian egg chambers with antibodies to fibrillarin, Udd, and Piwi. (a) In wild type ovaries, Udd is located in the nucleoli of germline nurse cells (NC) and somatic follicle cells (FC). In both cell types, Udd is colocalized with fibrillarin, the marker of the nucleolus. Piwi is present in nuclei of germline and somatic cells, and partially colocalizes with the nucleolus. (b) In $piwi^{Nt}/piwi^{2}$ mutants (transheterozygotes that carry the chromosome with the $piwi^{Nt}$ mutation and a homologous chromosome with the null $piwi^{2}$ mutation), nuclear localization of Piwi is disrupted in all types of cells. Udd disappears from nucleoli of germline cells, but is retained in nucleoli of somatic cells. (c) In *piwi* null mutants ($piwi^{2}/piwi^{3}$), Udd disappears from nucleoli of germline, but not somatic cells. (d) In germline *piwi* knockdowns induced by the *nos-Gal4* driver, Piwi protein is not detected in germline cells, but the localization of Udd in nucleoli remains unaffected. (e) In germline *udd* knockdowns, Udd protein is not detected in germline cells, while the localization of Piwi is unaffected.

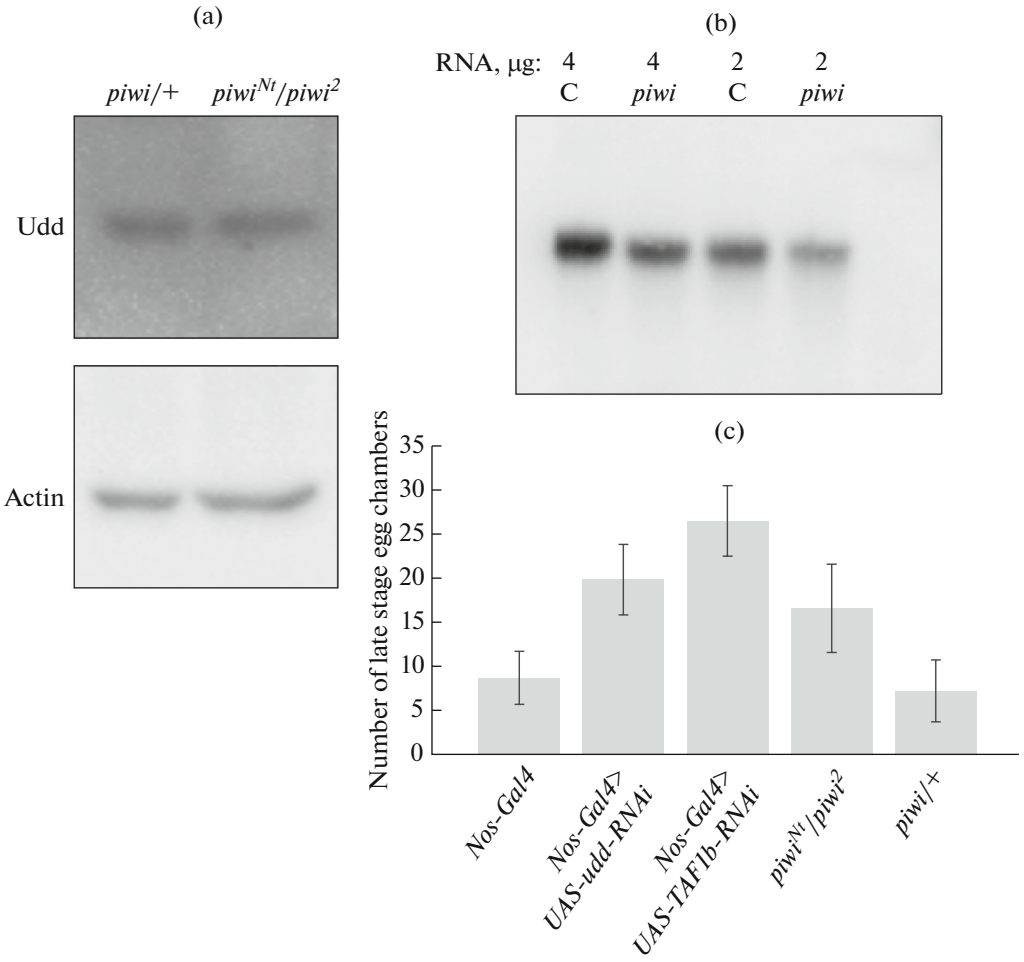

Fig. 2. (a) Western-blot analysis of Udd protein in ovaries of $piwi^{Nt}/piwi^{2}$ mutants and control heterozygotes (*piwi*/+). Antibodies to actin were used for loading control. (b) Northern blot hybridization with a probe to 28S rRNA in $piwi^{Nt}/piwi^{2}$ mutants (*piwi*) and control heterozygotes (C); numbers at the top indicate the amount (μg) of total RNA per track. In *piwi* mutants the amount of mature 28S rRNA is reduced. (c) Number of egg chambers at late oogenesis stages (after stage 10) per ovary in seven day old females: controls (flies with the *nos-Gal4* driver and *piwi*/+ heterozygotes); *udd* (*nos-Gal4>UAS-udd RNAi*) or *Taf1b* (*nos-Gal4>UAS-Taf1b-RNAi*) knockdowns in germline cells; disrupted nuclear localization of Piwi ($piwi^{Nt}/piwi^{2}$).

specificity of the influence of Piwi is also consistent with the hypothesis about the epigenetic role of Piwi in the regulation of rDNA clusters, rather than with the assumption about the direct involvement of Piwi in recruiting the factors of the SL1-like complex. The different effect of *piwi* mutations on the presence of Udd in the nucleoli of somatic and germline cells are also indicative of marked differences in epigenetic characteristics of rDNA clusters in these two cell types.

Overall, the results obtained previously [7] and in this work show that Piwi performs a dual role in the regulation of transcription of rRNA genes by suppressing rDNA clusters with insertions of R1 and R2 transposons and by providing a high level of transcription of rDNA copies that do not contain the insertions. The possible mechanism of Piwi-dependent regulation of transcription of both rDNA units that do and do not contain the transposon insertions, could be the change of chromatin state. It is known that maintenance of the wild type structure of the nucleolus and rRNA gene transcription in somatic tissues of *D. melanogaster* requires the presence of the H3K9me3 repressor modification of histone H3, heterochromatin protein HP1a [24] and the linker histone H1 [25]. It has also been shown that Piwi may affect the distribution of these chromatin proteins in the chromatin of transposable elements [5, 6, 26]. However, the impact of the piRNA system on the composition of the chromatin of rDNA clusters in ovaries has not been studied. Presumably, the recruitment of Piwi to certain parts of the rDNA cluster may occur due to short piRNAs complementary to the ribosomal transposons and possibly due to short RNAs that correspond to rRNA itself.

Our results, for the first time, show the role of the piRNA pathway in the regulation of transcription by

RNA polymerase I, but the molecular mechanisms of this process remain unknown and will be investigated in the future.

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