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Piwi Protein as a Nucleolus Visitor in *Drosophila melanogaster*

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Abstract—The evolutionarily conserved nuclear Piwi protein of *Drosophila melanogaster* is the defining member of the Argonaute small RNA-binding protein family. Guided by piRNAs, Piwi functions in transposon silencing in somatic and germ cells of gonads. We found that in ovarian somatic and germ cells, as well as in the established ovarian somatic cell line, Piwi accumulates predominantly in the nucleolus, the main nuclear compartment which participates not only in rRNA synthesis, but also in various cell stress responses. We have shown the colocalization of Piwi with the nucleolar marker proteins fibrillarin and Nopp140. The $piwi^{Nt}$ mutation which prevents the transport of Piwi to the nucleus and disrupts transposon silencing led to 6–8 fold upregulation of rRNA gene expression, as assessed by the level of transcripts of transposon insertions in 28S rRNA genes. RNase treatment of live cultured ovarian somatic cells depleted Piwi from the nucleolus. The same effect was observed upon inhibition of the activity of RNA polymerase I, which transcribes rRNA genes, but not RNA polymerase II. In contrast, upon heat shock, Piwi concentrated in the nucleolus and was depleted from the nucleoplasm. These results implicate Piwi in RNA polymerase I activity modulation and stress response in the nucleolus. Possible noncanonical Piwi functions are discussed, which are not related to the role of Piwi in transposon silencing.

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

INTRODUCTION

Drosophila Piwi protein is the founding member of the Piwi subfamily, belonging to a large family of Argonaute proteins, which contains proteins from different organisms that mediate functions of small noncoding RNAs, including microRNAs, small interfering RNAs (siRNA), and piRNA (RNAs which are bound by proteins of the Piwi subfamily) [1, 2]. The *Drosophila melanogaster* Piwi protein is a component of the piRNA silencing system aimed mainly at transposon repression in both the germline cells and the surrounding somatic cells [3]. Short-sized piRNAs (25–35 nucleotides in length) are generated as a result of processing of the transcripts of clusters containing transposon fragments [4, 5]. With the help of Piwi and piRNAs recognition of complementary sequences of a transposon transcript in the nucleus is achieved, which leads to heterochromatinization and transcriptional silencing of the transposon [6–8]. However, the underlying mechanisms of Piwi participation in this process remain obscure. Piwi orthologs in mammals also take part in transposon repression, which is accompanied by DNA methylation [9]. The impaired expression of these orthologous proteins is characteristic of malignant transformation [10, 11]. It is known that certain cellular mRNAs selected by a piRNA-dependent silencing system can be the source of piRNA in *Drosophila*. In this case, similarly to microRNAs, piRNAs can presumably play a role in the regulation of the activity of protein-coding genes [12], becoming the targets of piRNA-Piwi complexes in addition to transposons. For example, in the sea slug *Aplysia*, Piwi orthologs participate in the silencing of a transcription factor-encoding gene in the nervous system [13]. To our knowledge, the characteristics of subnuclear localization have not yet been described for Piwi protein in *D. melanogaster* or for its orthologs in other organisms. In the current work, we detected the predominant localization of Piwi in the nucleolus, an organelle that is essential not only for ribosome biogenesis, but also for a number of other important regulatory functions, e.g. cell-cycle regulation and stress response [14, 15]. It should also be mentioned that in human cells, some proteins of the Argonaute subfamily bind small RNAs that correspond to rRNA nucleotide sequences;, and the obtained data indicate the functionality of these complexes [16]. Interest has currently arisen in investigating the functional role of fragments of the RNAs with known house-keeping cellular functions, namely, mRNA, tRNA, and rRNA, which may represent more than just occasional products of transcript degradation [17, 18], but rather precursors of short RNAs with functional activity. The

demonstration of Piwi localization in the nucleolus opens the door to unveiling new noncanonical functions of this protein.

EXPERIMENTAL

Drosophila Strains

To detect wild-type nuclear-localized Piwi protein via immunostaining, the ovaries of heterozygous $piwi^{Nt}$/+ and $piwi^{2}$/+ flies were used [19]. To assess the effect of the mutated $piwi^{Nt}$ gene, which encodes the Piwi protein incapable of nuclear transport, on the expression of rRNA genes using RT-PCR, the quantity of transcripts of *R1* and *R2* insertions was compared in the ovaries of $piwi^{Nt}/piwi^{2}$ mutants and in the ovary mix of heterozygotes $piwi^{Nt}$/+ and $piwi^{2}$/+ flies that express wild-type Piwi.

Immunostaining

Embryo fixation and staining was performed according to the protocol available at <http://fruitfly4.aecom.yu.edu/labmanual/12.html>.

Ovaries and cultured somatic cells were fixed in phosphate buffered saline (PBS) containing 3.7% formaldehyde, washed with PBS containing 0.3% Triton X-100 and 0.1% Tween 20, frozen at –20°C, thawed, and incubated in PBS containing 0.3% Triton X-100, 0.1% Tween 20 and 0.3% normal goat serum for 1 h. The samples were further incubated with specific antibodies at +4°C overnight and subsequently washed with PBS containing 0.3% Triton X-100 and 0.1% Tween 20, then incubated with secondary antibodies conjugated with fluorescent dyes (Alexa 488, Alexa 546 and Alexa 633 were used for for mouse, rabbit and guinea pig antibodies in different combinations, Invitrogen) and subsequently washed with PBS containing 0.3% Triton X-100 and 0.1% Tween 20. The antibodies used were as follows: rabbit polyclonal (1 : 500, provided by G. Hannon) or mouse monoclonal (serum, 1 : 5, provided by M. Siomi) antibodies against Piwi; rabbit polyclonal (1 : 1000, Abcam) antibodies against fibrillarin; guinea pig polyclonal antibodies (1 : 300, provided by P. DiMario) against Nopp140; rabbit polyclonal (1 : 500, provided by P. A. Fisher) antibodies against lamin.

Ovarian somatic stem cell culture (OSC) was grown as described previously [20] in M3 media with the addition of 10% fetal serum and 10% fly extract. To obtain the fly extract, 1 g of flies 3–7 days after eclosion was homogenized in 6.8 mL of M3 medium and centrifuged for 15 min at 1500 g. Supernatant was heated for 10 min at 60°C and centrifuged, after which the pellet was discarded.

Reverse Transcription with Subsequent Polymerase Chain Reaction

Total RNA from ovaries of flies 0–7 days after eclosion was extracted using Trizol reagent (Invitrogen) and precipitated with 70% ethanol at –20°C. DNA was removed by precipitation with 3.3 M LiCl at –20°C and subsequent DNAse I (Ambion) treatment for 1 h at 37°C. The reverse transcription reaction was performed in triplicate using equal quantities of RNA from normal ovaries (mixture of $piwi^{Nt}$/+ and $piwi^{2}$/+ flies) and mutant ovaries ($piwi^{Nt}/piwi^{2}$ flies), with the oligo-dT primer (Silex) and reverse transcriptase SuperScriptII (Invitrogen) according to the manufacturer's instructions. The quantity of *R1* and *R2* insertion transcripts was estimated by real-time PCR using Hot Start DNA polymerase (SibEnzyme) and SYBR Green I dye on DT-96 real-time DNA amplifier (DNA-Technology). The obtained values were normalized to the quantities of *Adh* gene mRNA. The following primers were used: R1A1 forward AATTCCCGAGCTGTGCTAGA, R1A1 reverse GTCTCAAGGCACCTTTCAGC [21]; R2 element s1 TGCTCCCGAAACAACAAACCAC, R2 element as1 AACAATGACCACGCAGCCTC; Adh_RI_s GCCTGCGTACATAGCCGAGAT, Adh_RI_as GCTCCGTTAGTTGTTGGTTTCC.

RESULTS

Piwi Localizes in Nucleoli in Embryo Cells and in Ovarian Somatic and Germinal Cells

Using the immunostaining technique, we detected the colocalization of Piwi and the nucleolar marker fibrillarin in the nuclei of cells of the early embryo. The same result was obtained with two different antibodies against Piwi. Figure 1a demonstrates the colocalization of these proteins in synchronously dividing nuclei of the syncytium during the early blastoderm stage, when the cell membranes are not yet formed. It can be seen that Piwi does not colocalize with the main regions of chromatin localization. It is well known that fibrillarin is responsible for ribose methylation during pre-rRNA processing and is located in the dense fibrillar component of the nucleolus of mammalian cells, which lies next to the fibrillar component [22]. The transcription of rRNA genes by RNA polymerase I is considered to take place at the border between fibrillar and dense fibrillar components of the nucleolus [23]. It should be noted that the distinction of structurally different intranucleolar compartments peculiar for mammalian nucleoli should not be directly approximated to the nucleoli of other organisms; the structural features of the nucleolus may differ considerably between mammals and insects [23, 24].

During early embryogenesis germline cells are segregated from the rest of the cells of the embryo, being represented at the early stages of embryonic develop-

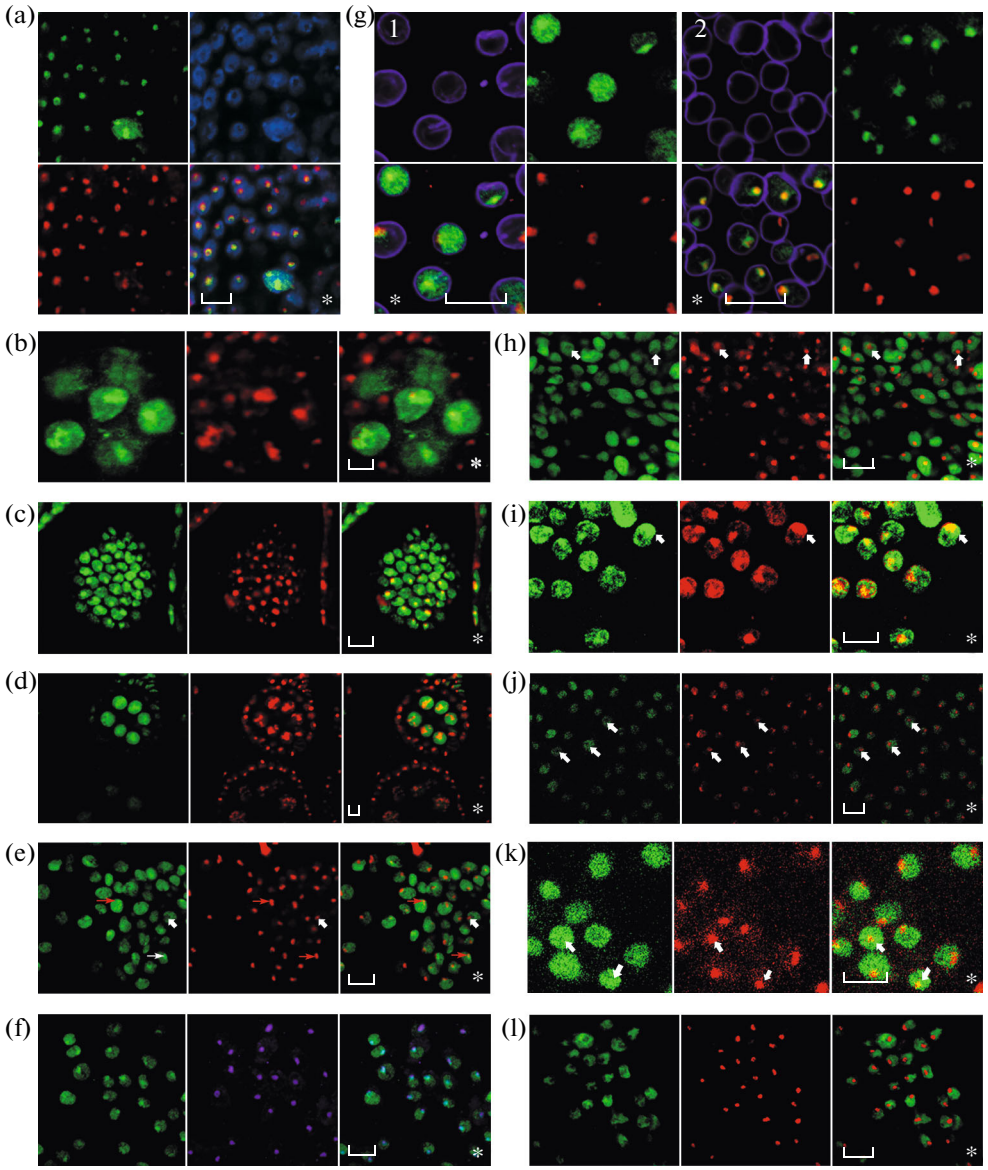

Fig. 1. Piwi protein in the nucleolus detected during early stages of embryonic development, in the cells of a whole *Drosophila* ovary, and in cultured ovarian somatic cells. Asterisks mark the merge images of staining patterns and the scale bar is 10 μm. (a–e) Piwi (green) and fibrillarin (red) colocalization in embryonic and differentiated cells. (a) nuclei of the syncytium at the blastoderm stage, DNA is stained with DAPI (blue); (b) polar germ cells in embryos; (c) ovarian follicle cells; (d) nurse cells of the ovarian egg chamber; (e) cultured OSC cells, thin arrows indicate Piwi and fibrillarin colocalization, thick arrow indicates the absence of colocalization; (f) Piwi (green) and nucleolar protein NOPP140 (purple) colocalization in OSC cells (light-blue when merged); (g) OSC cells under normal conditions (1) and after heat shock (2) at 37°C for 1 h, immunostaining for Piwi (green), Nopp140 (red), and the nuclear envelope marker lamin (purple) is shown. (h–k) Piwi (green) and fibrillarin (red) localization upon treatment of OSC cells with the inhibitors of RNA synthesis. (h) actinomycin D (0.1 μg/mL, 1h), arrows indicate the sites of fibrillarin localization in the nucleus where Piwi is absent; (i) DRB (100 μM, 1h), disruption of fibrillarin compact localization can be observed, arrows indicate Piwi and fibrillarin colocalization (yellow when merged); (j) 9-hydroxiellipticine (6 μM, 45 min), arrows indicate fibrillarin-occupied sites in the nucleus, where Piwi is absent; (k) α-amanitin (20 μg/mL, 2 h), arrows indicate Piwi and fibrillarin colocalization; and (l) live OSC cells treated with RNase A taken up by pinocytosis (1 mg/mL, 1h), Piwi depletion from the nucleolus is observed.

ment by the so-called polar cells. In these precursors of germinal tissue, polar cells, the colocalization of Piwi and fibrillarin can also be traced (Fig. 1b).

In the developing *Drosophila* ovaries, large nurse cells and the oocyte itself form an egg chamber, which is surrounded by small-sized somatic follicle cells, which participate in ovary differentiation and development. Figure 1c depicts the nuclei of the follicle cells from the surface layer that also display the colocalization of Piwi and fibrillarin. The nurse cell nucleolus,

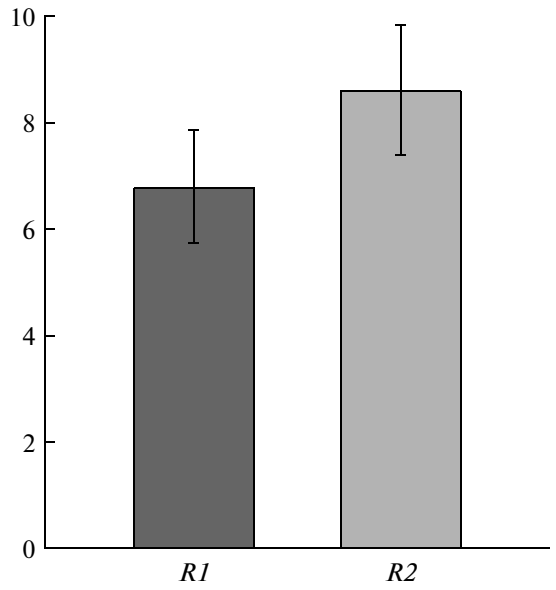

Fig. 2. *piwi*^{Nl} mutation leads to the upregulation of expression of *R1* and *R2* transposons inserted into 28S rRNA genes. Ratios of the transcript abundance of *R1* and *R2* transposons in mutant ovaries to their abundance in heterozygous ovaries are shown as bars. Each transposon transcript level is normalized to the level of *Adh* transcripts in the ovaries of flies of the corresponding genotype, which is not affected by *piwi* gene disruption.

which is known to represent a branched structure that partly adjoins the nuclear membrane [25] instead of being a dense rounded structure, also contains Piwi (Fig. 1d). The presence of Piwi in the nucleus (karyosome) of the oocyte adjacent to the nurse cells, could not be detected at this developmental stage.

The accumulation of Piwi in the nucleoli at the early stages of embryonic development and in the differentiated ovarian cells suggests a possible role of this protein in the functions of the nucleolus both before differentiation, in syncytial nuclei, and in somatic and germinal cells.

Presence of Piwi Protein in Nuclei of Cultured Ovarian Somatic Cells is Determined by the Nucleolar Transcriptome and Active RNA polymerase I

The presence of Piwi protein in cultured ovarian somatic cells, OSC [20], makes it possible to study how various factors, including stress, affect Piwi localization in the nucleolus. In the majority of cells (but not all of them), Piwi is colocalized with fibrillarin (Fig. 1e). The nucleolar localization of Piwi was also revealed when antibodies to another nucleolar protein, Nopp140 [26], were used (Fig. 1f). It should be noted that the nucleolar localization of Piwi varied from cell to cell, which might be accounted for by the dynamics of this localization or the responsiveness to some uncontrollable changes in the physiological state of certain cells in the culture. The hypothesis of Piwi dynamic transition from the nucleoplasm to the nucleolus and back is supported by the results of the experiments that demonstrate changes in the intranuclear localization of the protein under heat shock conditions and the action of transcription inhibitors. Heat shock for 1 h at 37°C led to the accumulation of Piwi in the nucleolus and to considerable decrease in its nucleoplasmic localization (Fig. 1g). It is known that, in mammalian cell culture, heat shock causes immobilization of some proteins in the nucleolus [15]. Under stress conditions, including heat shock, protein immobilization in the nucleolus is mediated in the mammalian cells by noncoding transcripts of rRNA gene intergenic spacers. It can be assumed that Piwi immobilization in the nucleolus is achieved with the participation of small RNAs complementary to the spacer transcripts. The addition of actinomycin D, which inhibits RNA polymerase I and RNA polymerase II activity [27], led to the depletion of Piwi from the nucleolus and the appearance of blank spaces ("holes") that occured in the sites of fibrillarin localization when antibodies against Piwi were used (Fig. 1h, compare to Fig. 1e). It is well known that actinomycin D most effectively inhibits RNA polymerase I [27]. The effect of actinomycin D on the nucleolus in mammals is considered to be a stress stimulus causing the disturbance of nucleolar morphology [28]. In connection with this, it appeared to be of interest to understand if high concentrations of inhibitors of transcription performed by RNA polymerase II would influence Piwi nucleolar localization. Neither α-amanitin, nor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) caused the depletion of Piwi from the nucleolus, at least in majority of cells (Figs. 1k, 1i). It is known that DRB causes visible changes in nucleolus architecture in mammalian cells [23, 29], which we also detected as leakage of fibrillarin from the nucleolus (Fig. 1i). However, DRB did not cause the removal of Piwi from the sites of fibrillarin accumulation. In contrast, the recently described specific RNA polymerase I inhibitor 9-hydroxyellipticine [30], which does not affect the activity of RNA polymerase II and III, effectively depleted Piwi from the nucleolus (Fig. 1j). The obtained results are indicative of Piwi interactions in the nucleolus with RNA polymerase I or transcripts of rRNA and of the rRNA intergenic spacers which are associated with the role of the nucleolus in cellular stress response [15].

The detection of association between the localization of Piwi in the nucleolus and RNA polymerase I-mediated transcription is consistent with Piwi being considered a protein that interacts with newly synthesized RNA [31]. However, the corresponding data are limited and have not yet been confirmed in other published works. It appears that, along with piRNA or other unidentified small RNAs, Piwi may be targeted to the nucleolar transcriptome. In fact, the treatment of live cells with high concentrations of RNase A,

which is taken up by pinocytosis [32], completely depleted Piwi from the nucleolus (Fig. 1l). It can be assumed that Piwi takes part in the modulation of the expression of rRNA genes, a substantial portion of which is known to be repressed. It is generally thought that the cell can meet its needs by transcribing only a portion of rRNA genes [33].

Piwi and Control of rRNA Gene Expression

We hypothesized that the $piwi^{Nt}$ mutation that deprives the Piwi protein of its ability to be transported into the nucleus [19] will lead to enhanced expression of rRNA genes. The level of rRNA gene expression can be estimated by the abundance of transcripts of specific transposons, which insert into a number of rRNA genes and are usually absent in other parts of the genome [34]. 28S rRNA genes of various eukaryotic organisms, including *D. melanogaster*, can contain *R1* and *R2* insertions which are LINE retrotransposons lacking their own promoters [35]. The genes with insertions may be subject to heterochromatin formation and cease producing rRNA [36]. However, some of the insertions may still be transcribed from the rDNA promoter, which drives the synthesis of a cotranscipt of 28S rRNA and R1 or R2 within the rRNA precursor [36], and the insertion can be spliced from the nascent transcript using a ribozyme sequence within the transposon [37]. The level of expression of *R1* and *R2* transposons in normal ovaries of heterozygous flies $piwi^{Nt}/+$ and $piwi^{2}/+$ was compared with that in the trans-heterozygous flies $piwi^{Nt}/piwi^{2}$. The $piwi^{2}$ mutation leads to a complete block of Piwi synthesis [19]. Using RT-PCR, a six- to eightfold increase in the quantity of *R1* and *R2* transcripts was detected in the ovaries of $piwi^{Nt}/piwi^{2}$ flies, where Piwi is not localized in nuclei, compared to the heterozygous flies ($piwi^{Nt}/+$ and $piwi^{2}/+$) (Fig. 2). This result suggests that Piwi participates in silencing rRNA genes with transposon insertions. However, in our previous whole genome ChIP-Seq analysis of the effect of the $piwi^{Nt}$ mutation on chromatin modifications known to correlate with the activation of transposon transcription, *R1* and *R2* insertions were not among the transposons with considerable enrichment with the positive modification (histone H3 lysine 4 dimethylation) [8]. The enrichment with the known repressor modification, histone H3 lysine 9 trimethylation, in the chromatin of *R1* and *R2* was also not altered significantly in the case of the $piwi^{Nt}$ mutation. Thus, it could be assumed that Piwi-mediated heterochromatin formation in rRNA genes containing transposon insertions is achieved via other chromatin modifications that we have not analyzed. Another explanation may be that Piwi regulates nucleolar RNA expression on the post-transcriptional or cotranscriptional level, which does not imply changes at the chromatin level.

DISCUSSION

The involvement of Piwi in silencing rRNA gene-specific transposons gives evidence of its participation in the regulation of rRNA gene-cluster expression. Our data, which indicates that actively functioning nucleolar RNA polymerase I, but not RNA polymerase II, may be a prerequisite for the localization of Piwi in the nucleolus, may suggest the presence of a contact between Piwi and the RNA polymerase I complex or the synthesized rRNA gene transcripts. In mammals, RNA polymerase I transcribes not only ribosomal RNA, but also regulatory noncoding intergenic spacer RNAs, which participate in the silencing and methylation of rRNA gene promoters [38], as well as in binding and immobilizing proteins during cellular stress response [15]. The possibility that Piwi binds small fragments of these noncoding RNAs and rRNAs cannot be excluded. The resulting complexes can interact with nascent rRNA gene transcripts. The binding of rRNA fragments with human Argonaute family proteins has recently been demostrated [16], and these complexes are considered to be candidates for fulfilling important hormone regulatory functions. The regulatory roles of cellular RNA fragments are now being extensively discussed, and these RNAs are no longer regarded as random intermediate products of cellular RNA degradation. In *Neurospora crassa*, a drastic increase in quantities of small rRNA fragments was detected as a result of DNA damage, although the functions of these fragments, which presumably participate in silencing rRNA gene clusters, remain unknown [39].

The detection of Piwi localization in the nucleolus opens the door to further investigations of the functions of this evolutionarily conserved protein both in rRNA gene silencing and in the regulation of function of RNA polymerase I in the nucleolus, which may be mediated by the transcription of intergenic spacers. There is also substantial interest in studying the nucleolus as a multifunctional organelle [14] due to its hypertrophy in cancer cells, which is considered an indicator of malignant transformation [40].

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