The peculiarities of piRNA expression upon heat shock exposure in *Drosophila melanogaster*

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Abbreviations: ME, mobile genetic element; *Hsp(s)*, heat shock protein(s); *Hsp*, heat shock protein genes; HS, heat shock; piRNAs, small RNAs associated with PIWI-protein

Different types of stress including heat shock may induce genomic instability, due to the derepression and amplification of mobile elements (MEs). It remains unclear, however, whether piRNA-machinery regulating ME expression functions normally under stressful conditions. The aim of this study was to explore the features of piRNA expression after heat shock (HS) exposure in *Drosophila melanogaster*. We also evaluated functioning of piRNA-machinery in the absence of major stress protein *Hsp70* in this species. We analyzed the deep sequence data of piRNA expression after HS treatment and demonstrated that it modulates the expression of certain double-stranded germinal piRNA-clusters. Notable, we demonstrated significant changes in piRNA levels targeting a group of MEs after HS only in the strain containing normal set of *hsp70* genes. Surprisingly, we failed to detect any correlation between the levels of piRNAs and the transcription of complementary MEs in the studied strains. We propose that modulation of certain piRNA-clusters expression upon HS exposure in *D. melanogaster* occurs due to HS-induced altering of chromatin state at certain chromosome regions.

Introduction

Mobile genetic elements (MEs) occupy significant part of eukaryotic organism genome. Thus, ME fraction reaches 12–15% of various *Drosophila* species genomes, 45% in humans and up to 90% in certain plant species.^{1–3}

It is evident that ME activation and amplification in the host genomes should have deleterious consequences due to resulted high frequency of chromosomal and point mutations.⁴ On the other hand, the emerging polymorphism provide abundant material for natural selection and, hence, microevolution.^{4,5} The accumulated data on the distribution of MEs in close species and geographical populations as well as the discovery of epigenetic mechanisms regulating the expression of ME clearly demonstrate important role of different ME classes in the evolution of all eukaryotic organisms.^{6,7}

At the present time multiple instances of spontaneous ME activation as well as amplification induced by crosses or various abiotic stimuli have been described in plants and animals.^{8–13} Specifically, there is some evidence that “heavy heat shock” may increase transcription level and lead to transposition “bursts” of certain MEs in *Drosophila* and other organisms.^{14–17}

However, the exact molecular mechanisms underlying this effect were not revealed in most of such cases. The investigation of piRNA-system functioning under various stressful stimuli may

shed light on the mechanisms underlying the observed effects of HS and other challenges on ME activity and amplification.

RNA interference and, in particular, piRNA-machinery represents deeply conserved and powerful mechanisms to combat parasitic nucleic acids including various transposons.⁵ In this system silencing of ME expression is exercised by small RNAs 23–29 nt in length, the so called “piRNAs” (Piwi-interacting RNAs), that are synthesized predominantly in germinal tissues and are complementary to different MEs.¹⁸ Long, single-stranded, continuous precursors for most piRNAs are synthesized at certain genome regions called piRNA-clusters or “master-loci.”¹⁹ In *Drosophila* ovaries piRNAs in the master-loci may map either on 2 strands which is typical for germ cells (e.g. nurse cells) or on only single strand typical for somatic follicle cells.¹⁹ The mechanisms of primary and secondary piRNAs processing are tissue specific. Thus, primary piRNAs processing from the long precursor transcript takes place in both somatic and germ cells, while secondary processing of piRNAs by cycles of “ping-pong amplification” occurs only in nurse cells.^{18,19}

Canalization or developmental robustness provides the ability of organisms to produce the same phenotypes regardless of variability of genotype or environmental influences.²⁰ This implies the existence of special systems that prevent deleterious expression of various mutations and modifications under normal conditions and, in particular, after various stressful stimuli.

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Multiple data indicate that activity of various stress genes usually termed “heat shock genes” (*hsp*s) represents such “buffering” system in all organisms studied so far.^{21–23} Specifically, it was shown that mutations in *hsp83* gene lead to the manifestation of various phenotypic traits that were not expressed when normal *hsp83* allele was present.^{21,24} It was also shown that in *D. melanogaster* mutation of *hsp83* leads to mobilization of several MEs and strongly affects piRNA biogenesis in the germline cells.^{25,26} It is of note, that besides *hsp83* which is expressed under normal temperature, other *hsp*s genes (i.e. *hsp22*, *hsp67* u *hsp70*) are also apparently involved in the control of normal morphogenesis and developmental stability.^{27–29} Therefore, the accumulated data implicated *hsp*s genes not only as components of powerful anti-stress system enabling organism to survive various challenges but also as agents necessary for normal development providing genome stability under various conditions.

It is not quite clear whether HS system as a whole and individual *Hsps* may interact with components of piRNA-system and modify MEs activity under normal conditions and after stress. Therefore, the comparison of piRNAs patterns under normal conditions and after HS should provide valuable information on the mechanisms underlying HS influence on ME expression and

transposition. To our knowledge such comparative analysis of piRNAs biogenesis and the levels of correspondent MEs transcription under normal conditions and after temperature challenge was not yet performed in *Drosophila*.

Herein, we analyzed the deep sequence data of piRNAs isolated from *D. melanogaster* females under normal physiological conditions and after HS. Besides, we determined expression level of a set of MEs to investigate a possible effect of HS on their mRNA level.

It is well known that *hsp*s expression is dramatically induced after HS and proteins belonging to *Hsp70* family in most organisms play dominant role in basic and induced thermotolerance and recovery of cellular homeostasis after termination of stressful stimuli.^{30,31} Therefore, we specifically studied a possible role of *Hsp70* in functioning of the piRNA-machinery after HS. To reach this goal we explored *D. melanogaster* strain with a deletion of all 6 *hsp70* genes (*hsp70-* strain).³² In these experiments we also used as a control *w¹¹¹⁸* strain which was used to develop *hsp70-* strain and contains a normal set of *hsp70* genes.³²

Results

Heat shock induces quantitative modulation of piRNAs targeting specific set of MEs

Analysis of deep sequence data revealed the expression of piRNAs complementary to more than 100 ME in the investigated strains. As expected, the repertoire of these MEs in these strains was rather similar. We also noted several differences between the small RNA libraries from the 2 strains. In particular, sequences homologous to piRNAs to *Tirant* were present in strain *w¹¹¹⁸* but absent in *hsp70-* strain.

Analysis of differential expression indicated significant quantitative differences in piRNAs expression targeting a group of MEs after one hour after HS only in *w¹¹¹⁸* strain while in *hsp70-* strain differentially expressed piRNAs were not detected (Fig. 1A; only 2-fold and higher changes were taken into consideration, $P_{adj} \leq 0.05$).

Our analysis confirmed that the analyzed small RNAs do have specific characteristics of piRNAs. It is known that piRNAs can be divided into primary and secondary species

Figure 1. piRNA expression after HS treatment. (A) Analysis of piRNA expression changes after HS in *w¹¹¹⁸* strain (We did not observe any changes in piRNA expression pattern in *hsp70-* strain). (B) Characterization of HS-modulated piRNAs in *w¹¹¹⁸* strain under normal conditions. The investigated MEs include: *Accord2*, *Ivk*, *Invader3*, *Tirant* u *TART-B*. At the left (1) – length distribution of small RNAs mapped to the corresponding TE sequence. The frequency of occurrence of reads having 1U and 10A biases are indicated for sense [+] and antisense [-] strand (only 24–29-nt reads were considered). At the middle (2) – The relative frequencies of 5'-overlap for sense and antisense piRNAs to the corresponding TE sequence. At the right (3) – Normalized small RNA distribution to the corresponding full-length TE sequence. (C) Immunostaining of *Drosophila* ovary with Vasa protein in control conditions and after HS. Arrows indicate a perinuclear organelle *nuage*. Scale bar: 10 micron.

depending upon their biogenesis mechanism. Primary piRNAs have a strong bias for 5'U for antisense piRNAs, and this characteristic is seen in most sequenced piRNAs 25 to 27 nt in length (Fig. 1B). Characteristically, the majority of piRNAs matching to 14 MEs that was affected by HS (Fig. 1A) are probably transcribed in the germline nurse cells basing on ping-pong signature which includes not only strong bias for 5'U in antisense piRNAs but also adenine at position 10 from 5' of sense piRNAs (Fig. 1B, S1). However, there are 3 other ME (*Accord2*, *Invader1* and *Hopper2*), belonging to a group of "strand-reversed elements" that are targeted in both germline and somatic follicle cells of *Drosophila* ovary, according to the list of AGO3-dependent MEs.^{19,33,34}

It is known that HS severely inhibits activities of various ribonucleoprotein-complexes in the cell.³⁵ It was of significant interest to find out whether HS affects *nuage*, a unique germline perinuclear organelle associated with piRNA secondary processing (i.e., ping-pong cycle) and repression of MEs in germline cells.^{36,18} Immunostaining of *Drosophila* ovaries with Vasa, the core protein of *nuage*, demonstrated rather similar localization pattern in the control ovaries and ovaries from HS-treated females in both studied strains (Fig. 1C).³⁶ This observation implies, that possibly the functioning of *nuage* is preserved after HS treatment used. However, additional histological and biochemical approach should be used to determine the state and localization of other major components of *nuage* after HS.

Changes in the levels of piRNAs do not correlate with the expression of the targeted MEs after HS

We applied Southern blot hybridization to estimate polymorphism in terms of the presence and copy number of MEs exhibiting characteristic modulation in piRNA levels after HS only in one strain (*w¹¹¹⁸*). This group of responsive MEs includes *Accord2*, *Ivk*, *Invader3*, *Tirant* and *TART-B*. Our analysis indicated that although both investigated strains apparently have very similar sets of these MEs (Fig. 2A) there are a few different restriction fragments that may be explained either by the presence of 1–2 extra copies of certain ME in one of the strains or by restriction sites polymorphism (Fig. 2A). In accordance with our analysis of piRNAs levels, ME *Tirant* is represented by lower number of probably inactivated copies in *hsp70*-strain in comparison with *w¹¹¹⁸* strain (Fig. 2A).

Exploring qPCR analysis we tried to correlate the determined levels of piRNAs complementary to the selected set of MEs and their transcription after HS. The performed analysis did not reveal pronounced differences (>2-3-fold) in the studied MEs expression levels after HS (Fig. 2B). Notably, both strains respond similarly in terms of certain MEs transcription modulation after HS. A slight increase (1.5-fold) in ME *Accord2* expression was observed in both studied strains while HS-induced modulation of complementary piRNAs has been detected only in *w¹¹¹⁸* strain. As expected basing on Southern data, the transcription of ME *Tirant* was detected only in strain *w¹¹¹⁸* (Fig. 2B).

We also investigated expression level of retroelement *Dm412* under normal conditions and after HS because previously it was demonstrated by another group that heavy HS induced high

Figure 2. (A) Southern-blot analysis of genomic DNA of *w¹¹¹⁸* and *hsp70*-flies. The following restriction enzymes were used for the analysis: *Accord2* and *Invader3* – Sall; *Ivk* – HindIII; *Tirant* – SacI; *TART* – XhoI. Arrows indicate differences in the restriction patterns of studied MEs between the compared strains. **(B)** Relative expression level of MEs mRNA after HS. Resulting expression value represents average of the results of 3 biological replicas. n/d – expression level was not determined because of absence of PCR product or its very low expression level.

frequency of this ME transpositions.¹⁶ However, in our experiment we did not observe up-regulation of *Dm412* transcription.

Interestingly, HS treatment used results in significant decrease of ME *TART* expression level (~2-3-fold) as well as other telomere-associated ME such as *HeT-A* and *TAHRE* (Fig. 2B). However, we did not detect *TART* transcription in strain *w¹¹¹⁸*, in spite of the presence of *TART* copies revealed by Southern analysis (Fig. 2A). These results correlate with deep sequence data which demonstrated that piRNAs targeting *TART* are mapped not to the whole sequence of this ME but to only its terminal regions in strain *w¹¹¹⁸* (Fig. 1B). It is of note, that in *Drosophila* there are 3 subfamilies of *TART* MEs including *TART-A*, *TART-B* and *TART-C*. These subfamilies probably have similar functions and differ only by untranslated regions (UTRs).³⁷ Deep sequence data as well as the results of Southern-blot analysis suggest that genomes of the compared strains are polymorphic in terms of *TART* copy number and/or structure (Fig. 2A). Therefore, to monitor *TART* expression we designed primers targeting all 3 *TART* subfamilies. Nevertheless, qPCR analysis exploring poly (A) RNA fraction failed to reveal transcription of *TART* in *w¹¹¹⁸* implying the absence of active *TART* copies in this strain.

Overall, we may conclude that the observed modulation of piRNAs expression detected in this strain for MEs *Accord2*, *Ivk*, *Invader3*, *Tirant*, *TART* induced by HS does not correlate with expression levels of the complementary MEs.

Heat shock modulates the expression of several piRNA-clusters

Most part of piRNAs is generated from piRNA-clusters, that are often highly enriched for multiple transposon fragments.¹⁹

As we mentioned above piRNAs that can be modulated by HS in strain w^{1118} , are probably predominantly generated in the ovarian germ cells.³⁴ Since we did not demonstrate any correlation between MEs transcription and levels of complementary piRNAs we speculated that HS somehow affected the expression of certain clusters enriched for the sequences homologous to these ME.

To check this hypothesis we carried out the analysis of differential expression of piRNA-clusters under normal conditions and after HS basing on expression of unique piRNAs that are mapped only once on the genome using all piRNA clusters described in *D. melanogaster*.¹⁹ The analysis demonstrated that HS results in

statistically significant modulation of master-loci expression (only 2-fold and higher changes were taken into consideration, $P_{adj} \leq 0,05$) (Fig. 3A). Importantly, the changes of clusters expression as well as modulations in the total pool of piRNAs were detected only in w^{1118} , but not in the strain lacking *hsp70* genes (*hsp70*- strain). The performed analysis revealed significant correlation between the expression of total pool of piRNAs and piRNAs generated by master-loci for several MEs including *Invader3*, *Ivk*, *Helena*, *Transib2*, *TART-B*, *Invader1*, *Gypsy12* and *G3*, that belong, therefore, to HS-regulated clusters: #5, #10, #12, #14, #32 и #68 (Table 1). Figure 3B shows distribution of total and uniquely mapped

small RNAs on sense and antisense strand of 38C locus (chromosome 2L), representing HS-modulated piRNA-cluster #5 (according to Brennecke et al., 2007; Fig. 3B). It can be seen that all regions with uniquely mapped piRNAs demonstrate down-regulation after HS and include MEs with HS-modulated levels of corresponding piRNAs observed in the analysis of total piRNA expression (Fig. 3B). Therefore, we conclude that apparently piRNAs modulated by HS in strain w^{1118} originated from such piRNA clusters.

Notably, there are no similar correlations between total piRNAs and piRNAs produced by piRNA-clusters for the investigated MEs for many other large clusters (Table 1). The lack of such correlation and certain inconsistencies with the data obtained by Brennecke et al. using other *D. melanogaster* strains may be explained by significant polymorphism which exists in terms of qualitative and quantitative content of the piRNA-clusters when different *Drosophila* strains are compared.^{19,7}

It is also clear that the applied approach to monitor the expression of piRNA-clusters basing on unique piRNAs has clear-cut limitations. Such approach requires the existence of unique and specific for a given cluster piRNAs that may be produced by microdeletions, insertions or substitutions while the proportion of such unique piRNAs is rather low by

Figure 3. piRNA-clusters expression after HS. (A) Analysis of piRNA-clusters expression changes after HS in w^{1118} and *hsp70*- strains. (B) Normalized distribution of total and uniquely mapped small RNAs on sense and antisense strand under normal conditions and after HS to chromosome 2L locus 38C, containing piRNA-cluster #5 (according to Brennecke et al., 2007). The dotted line highlights the regions of uniquely mapped reads. Transposons content of these regions are shown. Bold print indicates TEs that match with HS-modulated expression of total piRNAs. (C) Analysis of all unique cluster-derived piRNAs expression changes after HS in w^{1118} and *hsp70*- strains.

Table 1. Transposon content of HS-modulated piRNA-clusters. Genomic localization, length and transposon content taken from Brennecke et al. 2007. Quantity of total and unique reads corresponding to control sample (strain w^{1118}). Bold letters indicate TEs that match with HS-modulated expression of all piRNAs.

Cluster ID	Genomic localization	Length (bp)	Transposon content (according to Brennecke et al., 2007)	All mapped reads		Uniquely mapped reads	
				Sense	Antisense	Sense	Antisense
5	chr2L: 20148259-20227581	79323	G2; G; Protap; Hobo; Invader3_LTR ; Copia; Pogo; Helena ; Copia_LTR; Transib2 ; Gtwin_LTR; Invader4_LTR ; Ivk ; Transib1 ; S2; Invader4 ; M4; Gtwin; S; Invader3 ; MDG3_LTR; G2; Invader2_LTR ; Transib5 ; DNAREP1	~7000	~3500	~950	~400
10	chrU: 5766708-5772171	5464	TART-B				
12	chr3LHet: 1402377-1557939	155563	Circe; G3 ; Diver2; Gypsy8; Gypsy8_LTR; Protap; Doc4; DMCR1A; DNAREP1; DMRT1C; Doc3; Micropia; Doc; Fw3; BS2; BS3; DMRT1B; Transib5 ; Gypsy_LTR; Batumi; Stalker4; DM297; Gypsy; Fw; Invader6 ; Stalker2; Ninja_LTR; Invader6_LTR ; Invader1 ; Gypsy12_LTR ; Gypsy4_LTR; Gypsy4; DM176; Invader3	~220	~400	~30	~60
				~8000	~4000	~80	~80
14	chrU: 7542733-7545114	2382	TART-B				
32	chr3L: 23452699-23482444	29746	DMRT1A; Fw; DMCR1A; G3 ; Gypsy8; LineJ1; Mariner2; DMRT1B; Stalker4; Diver2; Protap; Doc3; Gypsy3; DNAREP1; G; Bel; Gypsy12_LTR ; Invader3	~1000	~350	~75	~10
33	chr2L: 2499-11131	8633	DNAREP1; Idefix_LTR	~1800	~1500	~70	~50
34	chr3LHet: 2528-2011	21484	Gypsy3_LTR; DNAREP1; Gypsy8; Max; Protap; Roo; Gypsy; Bari1; Gypsy3; Fw; Idefix; Idefix_LTR	~100	~200	~80	~130
				~200	~1300	~10	~20
48	chr3L: 19841657-19861753	20097	DMRT1B; Roo; Roo_LTR; S; DMCR1A	~6000	~2500	~35	~5
58	chr3L: 24467284-24502317	35034	DMRT1B; Gypsy6; Idefix; Gypsy6_LTR; Idefix_LTR; Protap; Rover_LTR; Gtwin_LTR; DM176; DNAREP1; Gypsy8; DM297_LTR; I; DM297; Rover; Gypsy8_LTR; Invader6 ; MDG1; BS2; DMCR1A; Doc5	~2700	~1600	~40	~30
61	chrU: 5466328-5474996	8669	Fw3; Tc1-2; Nomad; Stalker2; Stalker4	~400	~70	~40	~15
68	chrU: 3523650-3541455	17806	Protap; Max_LTR; DMRT1B; DMCR1A; Invader4_LTR ; Tom1_LTR; DM176_LTR; Gypsy12_LTR ; Zam_LTR; Nomad; Max; Invader4	~2000	~1000	~20	~7
71	chr2R: 1220249-1225114	4866	DMRT1B; Doc3	~80	~180	~3	~20
78	chr4: 1020285-1025978	5694	DNAREP1; R1	~1000	~250	~10	~15
97	chr2R: 21139959-21146679	6721	DNAREP1; Protap	~200	~250	~5	~75
100	chr3LHet: 2180368-2230834	50467	DNAREP1; G2; G; FB4; Protap; S; BS2; DM176; DMCR1A; Gypsy8; DM1731; DMRT1C; Gypsy8_LTR; HMS-Beagle; Bagginis1; G5A; Stalker4_LTR; Doc2; Gypsy6_LTR; Stalker4	~2000	~3000	~20	~20

definition. Furthermore, such approach is not always productive because it requires the majority of fragments comprising a cluster be represented by MEs with modified piRNAs expression and these fragments should contain unique piRNAs. Therefore, in our case we analyzed the expression of a pool of unique cluster-derived piRNAs and this approach strongly suggests that HS modulates expression of certain limited set of piRNA clusters.

We conducted such comparison and determined that for 5 MEs (*Gypsy12*, *Accord2*, *Invader3*, *Tart-B*, and *Transib2*) HS-induced expression changes of uniquely mapped reads fully coincide with the data obtained in the analysis of total piRNA expression (Fig. 3C; only 2-fold and higher changes were taken into consideration, $P_{adj} \leq 0.05$). Thus, HS results in the alteration of expression of certain piRNA-clusters indeed.

Discussion

There were multiple attempts to mobilize MEs in plants and animals using heat shock treatment.^{8-12,38,39} At this end, in *Drosophila* several groups reported activation and amplification of certain ME families after various stress stimuli (e.g., *Copia*, *Dm412*, *Roo*). On the other hand, the results of such experiments are not always unambiguous and reproducible even under the same stress conditions.^{14-17,40,41}

At the present time we do not understand the molecular mechanisms underlying HS response in MEs. In our experiments we used severe heat shock which causes misfolding and aggregation of proteins and inhibits translation and storage of mRNAs.⁴² Previously heavy heat shock was successfully used to mobilize certain MEs, although the conditions of stress were different.¹⁶

Small RNAs, in particular, piRNAs represent the major mechanism controlling activity of MEs at the co-transcriptional and post-transcriptional levels.^{43,44} In our experiment we focused on functioning of piRNA-machinery after HS. Furthermore, we made use of *hsp70*- strain which lacks all *hsp70* genes and, hence, represents a convenient tool to reveal the role of this major stress protein in piRNA biogenesis under normal conditions and after HS.

Our investigation demonstrated that apparently piRNA-machinery in *Drosophila* ovary represents rather robust system which is not considerably modulated by HS since we did not observe dramatic changes in piRNA expression pattern after HS. It is also possible that severe HS applied may interfere with piRNAs biogenesis by inhibiting the activity of specific proteins comprising *nuage* such as AGO3 and AUB and, hence, disturbing ping-pong cycle.^{19,35} However, our analysis did not reveal significant differences in the spectrum of sense and anti-sense piRNAs under normal conditions and after HS (Figure S2). Furthermore, piRNAs targeting *G3*, *TAHRE* exhibit clear-cut characteristics of secondary piRNA processing in particular after HS, e.g. appropriate nucleotide bias and ping-pong signature. Furthermore, *nuage* structure is preserved after HS (Fig. 1C).

In our study we observed significant changes in the levels of piRNAs targeting a group of MEs. We tried to find out a

correlation between the MEs transcription efficiency and the level of complementary piRNAs targeting these transposons. Negative correlation between these parameters has been previously demonstrated for most studied MEs.^{43,44} However, if some ME regulatory region comprises a specific regulatory motif - HSE (heat shock elements) necessary for heat shock factor (HSF) binding and HS induction, one may expect a positive correlation after temperature elevation when most piRNAs are originated from correspondent ME's mRNAs due to ping-pong cycle.^{30,45} Increased transcription level of MEs will stimulate piRNA system response and lead to upregulation of piRNAs expression targeting corresponding MEs. Notably, activation of HSF in *Drosophila* ovary was shown previously.⁴⁶ Interestingly, such system involving stress-activation of retrotransposon *Osen* has been recently described in *Arabidopsis thaliana*.¹² We searched for HSE motifs in all ME exhibiting up-regulation of homologues piRNAs after HS using MatInspector (GENOMATIX Software) and did not find any functional HSE sites (data not shown). In addition, quantitative analysis (RT-PCR) of mRNA of *Accord2* and *Ivk* did not show considerable induction after HS in spite of upregulation of homologues piRNAs (Fig. 2B). Thus, up-regulation of piRNAs after HS due to corresponding MEs induction in our experiment is unlikely.

Interestingly, the analysis performed demonstrated the decrease in the expression level of telomere-associated MEs (*TART*, *HeT-A* and *TAHRE*). It is known that telomere formation is of great importance in the process of cell cycle and, hence, temporal inhibition of S-phase by HS may correlate with the observed decrease of telomere-associated MEs expression.^{47,48} The absence of *TART* transcripts and piRNAs complementary to full body of *TART* in *w¹¹¹⁸* strain suggest that there are no active copies of *TART* in this strain. Since *HeT-A* elements do not have their own reverse transcriptase and used either *TART* or *TAHRE* enzyme, probably in strain *w¹¹¹⁸* *HeT-A* elements explored only *TAHRE* reverse transcriptase for their transpositions and telomere formation.^{49,50}

Since the expression of examined MEs in most cases was not changed significantly after HS and there was no correlation between their expression levels and the content of complementary piRNAs, we suggest that HS-modulating piRNAs are probably produced from piRNA-clusters. The performed analysis of piRNA clusters corroborated this speculation and demonstrated that piRNAs affected by HS are probably originated from several definite piRNA-clusters that are modulated by HS in germ cells.

It is known that HS results in dramatic modulations imposed on chromatin structure and different chromatin regions may be either repressed or activated and open for RNA PolII activity.⁵¹ Therefore, piRNA-clusters expression in *Drosophila* ovary may be either induced or suppressed by HS depending on their localization in terms of HS-activated or repressed chromatin areas. Further analysis on the chromatin modifications of piRNA containing regions in the nurse cells after HS will shed light on the exact mechanisms underlining the response of piRNA system to the challenge.

In our investigation we focused on the role of major stress-regulated gene (*hsp70*) in the response of piRNA system to severe

HS. It was previously demonstrated by several groups that *hsp83* is involved in piRNAs biogenesis and its mutation may induce mobilization of certain MEs.^{25,26} However, the role of *Hsp70* in piRNA system response to HS was not clear. Herein, we used a strain lacking all *hsp70* genes and compared its response to HS with a control strain containing normal set of *hsp70* genes. To our surprise we failed to detect any changes in piRNAs levels in the strain with deletion, implying some role of Hsp70 in piRNA biogenesis after HS.

However, although the involvement of Hsp70 in piRNAs biogenesis after HS is our preferred hypothesis we cannot make unambiguous conclusion yet. The thing is that strain *w¹¹¹⁸* represents only one of several strains used to develop a strain with deletion of *hsp70* genes and, hence, the observed difference in the response to HS may be due to interstrain polymorphism in the structure of piRNA-clusters or other components of piRNA-machinery. It is well-known that piRNA-clusters exhibit structural polymorphism in different *D. melanogaster* strains.⁵² In order to directly demonstrate the role of *Hsp70* in the response of piRNA system to HS it is necessary to introduce one or several *hsp70* genes into the *hsp70*- strain or alternatively introduce interference constructs inhibiting *hsp70* genes into *w¹¹¹⁸* strain (work in progress).

Materials and Methods

Drosophila melanogaster strains and HS treatment conditions

Drosophila melanogaster strain *w¹¹¹⁸*: (df(3R)Hsp70A, df(3R)Hsp70B), referred as *hsp70*-, in which all *hsp70* genes were deleted, were obtained from Bloomington Drosophila Stock Center. Strain *w¹¹¹⁸* was used as a control. It should be noted that *w¹¹¹⁸* is one of the ancestor strains, explored in the genetic experiments conducted to obtain *hsp70*- strain. All flies were reared at 25°C on standard sugar-yeast-agar medium.

For HS response study adult 3–5 d old females were used. HS treatment was carried out in a circulating water bath at 38,5°C for 30 min. After 1 hour of recovery after HS at 25°C flies were used for experiments.

Small RNA libraries preparation

Total RNA was isolated from adult 3–5 d old females using Tri-reagent (Sigma-Aldrich, USA). Twenty-five mkg of total RNA were separated in 15% polyacrylamide gel electrophoresis, containing 8M of urea. After incubation in ethidium bromide solution (0,5 mkg/ml) gel fragments corresponding to small RNAs fraction, were cut out. We used chemically synthesized RNAs (Syntol, Russia) corresponding to 21 and 27 nts as size markers.

Cloning of small RNA libraries were conducted using Illumina TruSeq Small RNA Prep Kit (Illumina, USA) according to the manufacturer's protocol. Each sample was made in 2 biological replicas. Sequencing of small RNAs was performed on Illumina HiSeq 2000 (Illumina, USA).

Bioinformatic analysis

As a result of deep sequencing we have obtained 7–15 million reads of small RNAs for each library. After 3'-adapter clipping, the reads were filtered by their length (>18 nt) and quality (80% of nt should have at least 20 Phred quality). The selected reads were mapped to the *Drosophila* genome assembly (dm3) by using bowtie with requiring of perfect match and annotated to UCSC genes by using BEDtools 2.^{53,54} In the order to identify piRNAs, the sequenced small RNAs were mapped to the canonical sequences of transposable elements (http://www.fruitfly.org/p_disrupt/TE.html). piRNA size distributions, ping-pong signature and nucleotide biases were analyzed by using custom scripts written in Perl and R. The analysis of differential expression of piRNAs from TE and master-loci was performed by using edgeR package of R/Bioconductor.⁵⁵

Southern blotting

Genomic DNA was isolated by standard method.⁵⁶ After restriction digestion DNA samples were separated in 0,75% agarose gel electrophoresis and transferred onto nylon membrane Hybond-XL (Amersham Biosciences, USA). Hybridization was carried out at 65°C in solution, containing 6xSSC, 10 mM EDTA, 0,5% SDS, 50 mkg/ml of denatured DNA from salmon sperm and 5x Denhardt's solution, overnight.

Matrices for probe preparation were prepared by PCR (sequences of used primers are shown in Table S1) of genomic DNA of *w¹¹¹⁸* strain. Labeling of probe with (³²P)-dATP was carried out using Decalabel (ThermoScientific, USA). All MEs sequences were obtained from Berkeley Drosophila Genome Project (<http://www.fruitfly.org>).

Quantitative PCR

Total RNA was isolated from ovaries of adult 3–5 d old females using TRI-reagent (Sigma-Aldrich, USA). cDNA was prepared from DNase 1 (Sigma-Aldrich, USA) treated total RNA using oligo(dT) primer and MMLV reverse transcriptase (Evrogen, Russia). Experiment was performed on ABI PRISM[®]7500 Sequence Detection System (Applied Biosystems, USA). Detection of amplification products was carried out using SYBR Green 1 with the presence of ROX reference dye (Evrogen, Russia) in accordance with the manufacturer's protocol. Quantification was normalized to ubiquitously expressed *rp49* and *αTub* genes and calculation of relative expression levels was done using the equation 2^{-ddCt} . The resulting value of the expression was determined basing on 3 biological replicates. Sequences of used primers are shown in Table S2.

Immunostaining

Dissected ovaries were fixed in 4% paraformaldehyde solution for 20 minutes. Permeabilization and washing was carried out in 1x PBS in the presence of 0.3% Triton X-100. Blocking and antibody incubation was performed with the presence of 3% goat serum. Following antibodies were used: anti-*vasa* (Developmental Studies Hybridoma Bank, University of Iowa) at 1/100; anti-mouse Alexa-fluor 488 (Invitrogen, USA) at 1/1000 dilution. Preparations were mounted in vectashield mounting medium

with DAPI (Vector Labs, USA). Images obtained by confocal microscope Leica TCS SP8 (Germany).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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