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Crosstalk between HSF1 and HSF2 during the heat shock response in mouse testes

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ABSTRACT

Heat Shock Factor 1 (HSF1) is the primary transcription factor responsible for the response to cellular stress, while HSF2 becomes activated during development and differentiation, including spermatogenesis. Although both factors are indispensable for proper spermatogenesis, activation of HSF1 by heat shock initiates apoptosis of spermatogenic cells leading to infertility of males. To characterize mechanisms assisting such heat induced apoptosis we studied how HSF1 and HSF2 cooperate during the heat shock response. For this purpose we used chromatin immunoprecipitation and the proximity ligation approaches. We looked for co-occupation of binding sites by HSF1 and HSF2 in untreated (32 °C) or heat shocked (at 38 °C or 43 °C) spermatocytes, which are cells the most sensitive to hyperthermia. At the physiological temperature or after mild hyperthermia at 38 °C, the sharing of binding sites for both HSFs was observed mainly in promoters of *Hsp* genes and other stress-related genes. Strong hyperthermia at 43 °C resulted in an increased binding of HSF1 and releasing of HSF2, hence co-occupation of promoter regions was not detected any more. The close proximity of HSF1 and HSF2 (and/or existence of HSF1/HSF2 complexes) was frequent at the physiological temperature. Temperature elevation resulted in a decreased number of such complexes and they were barely detected after strong hyperthermia at 43 °C. We have concluded that at the physiological temperature HSF1 and HSF2 cooperate in spermatogenic cells. However, temperature elevation causes remodeling of chromatin binding and interactions between HSFs are disrupted. This potentially affects the regulation of stress response and contributes to the heat sensitivity of these cells.

1. Introduction

Proteotoxic stress, e.g. induced by hyperthermia, provokes a rapid response to maintain homeostasis, so called heat shock response (HSR). This stress-induced response is executed by heat shock proteins (HSPs), which are major molecular chaperones contributing to protein repair and degradation in stress conditions, but also assisting protein folding during biosynthesis. Mammalian HSPs are classified according to molecular weight into several families: HSPH (HSP110), HSPC (HSP90), HSPA (HSP70), DNAJ (HSP40), HSPB (small HSPs, sHSPs), and two chaperonin families: HSPD/E (HSP60/HSP10) and CCT (TRiC) (Kampinga et al., 2009). Each HSP family includes members that are either inducible by stress (e.g. HSPA1), constitutively expressed, or both (e.g. HSPH1, HSPA8, HSP90AA1). Expression of some HSPs is developmentally regulated or restricted to specific cells (Rupik et al., 2011; Ji et al., 2012). The HSR is regulated by Heat Shock Factors (HSFs), which are major transcriptional activators of *HSP* genes. Several members of the HSF family have been found in vertebrates (Vydra et al., 2014). Once

Abbreviations: ChIP-Seq, chromatin immunoprecipitation combined with high-throughput sequencing; HSF, heat shock factor; HSP, heat shock protein; HSE, heat shock element; HSR, heat shock response; PLA, proximity ligation assay.

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activated they form trimers and bind specifically to Heat Shock Elements (HSEs) throughout the genome. In mammals, HSF1 is the primary transcription factor responsible for the response to different forms of cellular stress, while HSF2 becomes activated during development and differentiation (e.g. during spermatogenesis). Nevertheless, both HSF1 and HSF2 can form heterotrimers and cooperate either during stress or under physiological conditions (Mathew et al., 2001; He et al., 2003; Ostling et al., 2007; Shinkawa et al., 2011).

Despite the high degree of conservation of the HSR, different cells vary in their ability to induce HSPs synthesis, and consequently in sensitivity to harmful conditions. Interestingly, some types of cells, e.g. spermatocytes, lack the typical HSR and are hypersensitive to elevated temperatures (Yin et al., 1997). In the majority of mammals, male gonads are located outside the main body cavity to provide the lower testicular temperature required for correct spermatogenesis and fertility. Increasing the temperature of testis up to the body temperature (or above it) leads to the activation of HSF1. However, inducible *Hspa1* (*Hsp70i*) genes expression is blocked in heat shocked murine spermatocytes (Izu et al., 2004; Vydra et al., 2006), although HSF1 binds to their promoters (Kus-Liśkiewicz et al., 2013). Moreover, the constitutively expressed testis-specific variant of HSP70 (HSPA2) is depleted after HSF1 activation (Widlak et al., 2007). Hence, an over-expression of constitutively active HSF1 in mice leads to the apoptotic death of spermatocytes and male infertility (Nakai et al., 2000; Widłak et al., 2003; Vydra et al., 2006). On the other hand, endogenous HSF1 appears to be important for spermatogenesis because *Hsf1* null males, although fertile, produce less sperm than wild type mice (Salmand et al., 2008). It has been shown that HSF1 is required for the transcriptional regulation of sex chromosomal multicopy genes during postmeiotic repression (Akerfelt et al., 2010). Reduction of fertility was also observed in *Hsf2* null males (Wang et al., 2003). Moreover, double *Hsf1* and *Hsf2* knockout causes male sterility and a complete lack of mature sperm in mice (Wang et al., 2004). It has been shown that both HSF1 and HSF2 are required for correct chromatin organization during normal spermatogenesis (Akerfelt et al., 2008, 2010), and that both factors can form heterotrimers at the chromatin (Sandqvist et al., 2009). These findings indicate functional crosstalk between HSF1 and HSF2 during spermatogenesis in normal conditions. However, the interplay between both factors during the heat shock response in testis has never been studied. Aiming to elucidate the mechanisms of such interaction, here we studied the chromatin binding of HSF1 and HSF2 in mouse spermatocytes subjected to hyperthermia.

2. Materials and methods

2.1. Isolation of spermatocytes

Adult (10–16-week-old), inbred FVB/N male mice were used for spermatocytes isolation (20 males per one isolation) by unit gravity sedimentation in linear BSA gradient as described earlier (Kus-Liśkiewicz et al., 2013). Isolated fraction contained up to 80% of spermatocytes and was contaminated mainly by round spermatids. After each isolation cells were equally divided for three groups: control (cultured at 32 °C), and heat shocked at 38 °C or at 43 °C.

2.2. Hyperthermia treatment

For ChIP experiments heat shock was performed as described in details elsewhere (Kus-Liśkiewicz et al., 2013): an equal volume (10 ml) of CO_2 saturated, pre-heated media (to 53 °C or 60 °C) were added to the cell suspension, which immediately raised the temperature of the media from 32 °C to 38 °C or 43 °C, respectively. The tubes were submerged in a water bath at the appropriate temperature for an additional 5, 10, or 20 min (these samples were pooled and treated as heat shocked sample). Immediately after heat shock cells were fixed for 10 min by adding formaldehyde to final concentration 1%, while the cell medium was quickly cooled to room temperature. The whole-body hyperthermia was performed *in vivo* in a water bath at 38 °C or 43 °C as described earlier (Widlak et al., 2007). The animal experiments were carried out according to Polish legislation, and were approved by the Local Committee of Ethics and Animal Experimentation at the Medical University of Silesia in Katowice, Poland (Decision No 82/2009) and by the institutional animal care policy of the Cancer Center and Institute of Oncology (Gliwice, Poland).

2.3. Chromatin immunoprecipitation (ChIP)

For analyses of HSF1 and HSF2 binding, the ChIP assay was carried out according to the protocol of ChIP kit of Upstate Biotechnology (Lake Placid, NY) using protein A-sepharose beads (Amersham). For 30 μg of chromatin sonicated to 100–500 bp fragments, 3 μg of rabbit anti-HSF1 (cat. no ADI-SPA-901, Enzo Life Sciences), or 5 μg of goat anti-HSF2 (cat. no AF5227, R&D Systems, USA) polyclonal antibodies were used. For negative controls chromatin samples were proceeded without antibody, with anti-TetR rabbit polyclonal antibody (Abcam), with IgG from rabbit serum, or with normal goat serum; all such controls generated similar results. Immunoprecipitated DNA was analyzed by PCR (ChIP-PCR) to assess quality of preparation before sequencing and to validate ChIP-Seq results. Primers characteristics used in analyses are presented in Suppl. Table 1.

2.4. High-throughput sequencing, data analysis and functional annotation

In each experimental point two PCR-verified ChIP replicates were collected and combined in one sample before DNA sequencing. Sequencing libraries were generated using ChIP-Seq Sample Prep Kit (Illumina). Template amplification and cluster generation were performed using the cBot and TruSeq SR Cluster Kit v2 cBot-GA, and 80 nucleotides were sequenced with Illumina Genome Analyzer IIx using TruSeq SBS Kit v5 reagents. After quality filtering (average phred > 30) and removal of duplicates, reads were mapped to the mouse genome (mm10) with Bowtie2 (Langmead et al., 2009). A minimum fold enrichment of five times over negative control was set as a cutoff criterion for target sites. The peaks were called with Model-based Analysis of ChIP-Seq (MACS) 1.4.2 (Feng et al., 2012). HSF1 and HSF2 target sites were annotated to genomic regions using HOMER software (Heinz et al., 2010). Fifty percent of peak length was centered on the summit point, and peaks that fell on exon-intron boundaries are indicated as exons. The density signals of HSF1 and HSF2 on the mouse genome were visualized with the Integrative Genomics Viewer version 2.2.1 (Thorvaldsdóttir et al., 2013). The consensus DNA sequences for HSF1 and HSF2 were identified *in silica* by motif analysis of large DNA datasets (MEME-ChIP Version 4.9.1) (Bailey, 2011; Machanick and Bailey, 2011) using a 120-bp region centered on the summit point. Biological processes associated with HSF1 or HSF2 bound genes were analyzed with NucleoAnnot application created within the confines of the GENEPI Low-RT project (FP6-036452; available on Silesian Bioinformatic Platform: http://cellab.polsl.pl/index.php/software/standalone-app, August, 2014). The hypergeometric test was applied for calculation of the P value for enriched gene ontology terms.

2.5. Proximity ligation assay

To detect the HSF1/HSF2 interactions the DuoLink in situ Proximity Ligation Assay (PLA) (Olink Bioscience, Uppsala, Sweden) was used according to the manufacturer's protocol. Reactions were performed on sections (8 μm) of formalin-fixed (4% in PBS, overnight at 4 °C) and paraffin-embedded mouse testes. For each experimental point (control, heat shocked for 15 min, for 30 min, and for 30 min with 2 h recovery) three males were used. An antigen retrieval step in 0.01 M citrate buffer pH 6.0 was performed before the procedure. Sections were washed in PBS (3 × 5 min), incubated in Blocking Solution (Olink Bioscience), and immunolabeled with primary antibodies. We used the same antibodies as for ChIP (1:90 dilution, 1% BSA in PBS, overnight, 4 °C); negative controls were proceeded without one primary antibody or both giving similar results. Then the secondary antibodies with attached PLA probes (PLA Probe anti-Rabbit PLUS and PLA Probe anti-Goat MINUS; supplied in the Duolink kit) were used. Signals of analyzed complexes were observed by confocal microscopy at ×600 magnification or by fluorescent microscopy at ×1008 magnification; red fluorescence signal indicated close proximity (<40 nm) of proteins recognized by both antibodies (Fredriksson et al., 2002). Images from confocal microscopy were taken in 25 focal planes (every 340 nm) and combined for one image. Images from fluorescent microscopy were taken in two–three focal planes. The same setting (acquisition time) was used for all the images. At least seven images were chosen with tubules in different developmental stages and all spots inside tubules were counted.

3. Results

3.1. Genome-wide identification of HSF1 and HSF2 chromatin binding sites in mouse spermatocytes

We used the ChIP-Seq approach to characterize actual binding sites for HSF1 and HSF2 in isolated mouse spermatocytes that were either untreated or heat-shocked for 5–20 min at 38 °C or at 43 °C. Duration of the heat shock was previously established as optimal for HSF1 activation (Kus-Liśkiewicz et al., 2013). We used a spermatocyte-enriched fraction of testicular cells because these cells are the most sensitive to damage at elevated temperatures (Yin et al., 1997); this approach also allows the avoiding of possible interference of somatic testicular cells (Kus-Liśkiewicz et al., 2013). The ChIP-Seq provided high-resolution maps of HSF1 and HSF2 target sites in the mouse genome (Suppl. Dataset 1; Gene Expression Omnibus accession no. GSE56735). Under physiological temperature (that is 32–33 °C for mouse testes), 1,562 binding sites were identified for HSF1 and 1,284 for HSF2. An elevation of the temperature caused changes in the HSF1 and HSF2 chromatin binding. The number of binding sites of HSF1, after an initial decrease at 38 °C, reached its maximum at 43 °C, while the binding of HSF2 gradually decreased with temperature elevation. A similar profile was observed in case of both global genome binding and binding at promoter regions (Fig. 1). In both control and heat-shocked cells either transcription factor occupied chromatin primarily in intergenic sequences (44–61%) and introns (32–40%), while 5-12% of all HSF1-binding sites and 1.5–4% of all HSF2-binding sites were located in promoter regions (Fig. 2). Either HSF1 or HSF2 binding sites were detected in promoters of 60 genes encoding for HSPs and other stress-related proteins (Suppl. Table 2). The *Gene to GO BP* (Gene Ontology Biological Process) analyses revealed that among genes occupied in promoters by both HSF1 and HSF2 in all conditions (except HSF2 binding at 43 °C, which is minimal) these associated with classical HSF-related functions (e.g., genes involved in response to stress and in protein folding) are over-represented

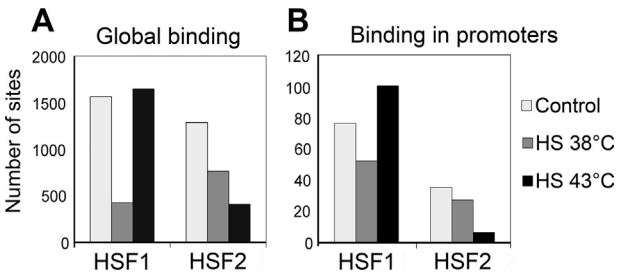

Fig. 1. The number of HSF1 and HSF2 binding sites in mouse spermatocytes: control and heat shocked (HS) for 5–20 min at 38 °C and 43 °C. Promoters were defined as the region −1000 bp, +100 bp around the transcription start site of Refseq genes.

(Suppl. Dataset 2). GO analyses of HSFs targets in non-promoter regions (including exons and introns) showed that in all conditions genes involved in DNA-dependent (regulation of) transcription, in transport, and in (protein) phosphorylation are over-represented.

3.2. Shared binding of HSF1 and HSF2 at promoters of stress-related genes was remodeled at an elevated temperature

We observed that several binding sites were shared by both transcription factors, which was characteristic for promoters of *Hsps* and other stress-related genes. The ChIP-Seq analysis revealed that HSF1 and HSF2 could simultaneously occupy gene promoters at the physiological temperature and/or following "mild" hyperthermia at 38 °C, but not at 43 °C. In general, for 159 genes which promoter regions bound either HSF at the physiological temperature or at 38 °C, promoters of 12 genes (~8%) were simultaneously occupied by both factors. Importantly, for 12 chaperone and co-chaperone genes that bound either HSF in these conditions there were 9 genes, which promoters were co-occupied by HSF1 and HSF2 at 33 °C or 38 °C (Table 1; Suppl. Tables 3A and B). In marked contrast, for about 3,300 binding sites for either factor detected outside promoter regions at the physiological temperature or at 38 °C, only ~0.4% (12 sites) were shared by HSF1 and HSF2. After temperature elevation up to 43 °C, remodeling of HSFs binding to gene promoters was observed: binding of HSF1 and releasing of HSF2. Such temperature-related changes were observed in promoters of chaperone and co-chaperone genes (*Hspa8*, *Hsp90aa1*, *Hsp90ab1*, *Hspd1*, *Hspe1*, *Dnaja1*, *Hsph1*, *Cct6a*, *Stip1*, *St13*) (Fig. 3), genes coding for proteins involved in ubiquitination (*Ube2g2*, *Ubqln1*, *Uspl1*), and some other genes (*Acot7*, *Aldh1a2*, *Ccdc117*, *Rsrp1*, *Gm10069*, *Hnrnpa2b1*, *Ptges3*, *Setx*, *Slc35e2*, *Spo11*) (Fig. 4; Table 1; Suppl. Tables 2 and 3). As a result, target sites shared by both HSF1 and HSF2 were not detected in promoter regions in cells subjected to heat shock at 43 °C (only three such sites were detected outside promoter regions; Suppl. Table 3C). Consequently, looking for DNA motifs enriched in the ChIP-Seq datasets we found

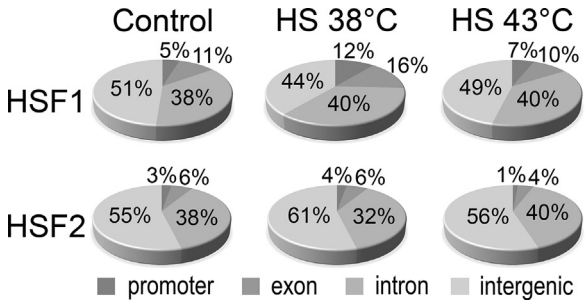

Fig. 2. Distribution of HSF1 and HSF2 binding sites within different genomic regions in control and heat shocked spermatocytes. Exons and introns are from RefSeq; promoter region: −1000 bp to +100 bp from the transcription start site.

Table 1
Shared binding of HSF1 and HSF2 to promoters of selected genes in mouse spermatocytes, control and heat shocked for 5–20 min at 38 °C or at 43 °C (for more details see Suppl. Table 2).

Gene Name (Entrez ID)	Full name	Peak Score					
		HSF1			HSF2		
		Control	HS 38 °C	HS 43 °C	Control	HS 38 °C	HS 43 °C
Stress-related genes							
Hspa8 (15481)	Heat shock protein 8	118.46	98.22	705.02	115.80	116.27	–
Hsp90aa1 (15519)	Heat shock protein 90, alpha (cytosolic), class A member 1	402.16	597.48	358.41	232.67	129.05	–
Hsp90ab1 (15516)	Heat shock protein 90, alpha (cytosolic), class B member 1	–	300.93	1747.56	105.83	69.18	–
Dnaja1 (15502)	DnaJ (Hsp40) homolog, subfamily A, member 1	–	99.29	388.24	83.21	–	–
Hspd1/Hspe1[a] (15510/15528)	Heat shock protein 1 (chaperonin)/heat shock protein 1 (chaperonin 10)	100.35	227.90	1176.39	74.60	216.35	–
Hsph1 (15505)	Heat shock 105 kDa/110 kDa protein 1	–	59.20	272.90	–	129.88	–
Cct6a(12466)	Chaperonin containing Tcp1, subunit 6a (zeta)	–	–	55.11	83.35	–	–
Ube2g2 (22213)	Ubiquitin-conjugating enzyme E2G 2	138.33	70.81	177.11	69.51	–	–
Ubqln1 (56085)	Ubiquilin 1	–	–	276.22	51.95	–	–
Uspl1 (231915)	Ubiquitin specific peptidase like 1	104.37	311.85	792.74	157.96	143.70	–
St13 (70356)	Suppression of tumorigenicity 13	104.37	280.31	254.59	211.47	92.66	–
Stip1 (20867)	Stress-induced phosphoprotein 1	65.91	184.28	708.82	105.83	–	–
Other genes							
Acot7 (70025)	Acyl-CoA thioesterase 7	276.39	183.61	222.30	82.43	–	–
Aldh1a2 (19378)	Aldehyde dehydrogenase family 1, subfamily A2	148.23	–	118.88	–	129.88	–
Ccdc117 (104479)	Coiled-coil domain containing 117	–	55.64	80.19	56.78	–	–
Rsrp1 (27981)	Arginine/serine rich protein 1	–	–	109.51	199.39	–	–
Gm10069 (791299)	Predicted gene 10069	–	57.95	214.29	–	109.02	–
Hnrnpa2b1 (53379)	Heterogeneous nuclear Ribonucleoprotein A2/B1	–	–	364.14	–	112.15	–
Ptges3 (56351)	Prostaglandin E synthase 3 (cytosolic)	–	–	135.92	–	66.52	–
Setx (269254)	Senataxin	–	53.35	156.13	71.16	–	–
Slc35e2 (320541)	Solute carrier family 35, member E2	89.68	151.70	112.08	–	107.82	–
Spo11 (26972)	SPO11 meiotic protein covalently bound to DSB homolog (*S. cerevisiae*)	–	157.82	192.04	85.43	–	–

[a] Genes oriented "head-to-head".

classical HSE motifs in promoters targeted by HSF1 and HSF2 at the physiological temperature and at 38 °C, while at 43 °C HSE motifs were found only in the HSF1-IP sample (Suppl. Table 4). Using the gene-specific ChIP-PCR approach we validated such temperature-induced remodeling of HSF1 and HSF2 binding in promoters of selected genes (*Hspa8, Hspe1, Hsph1, Spo11, Stip1, St13, Uspl1*). The obtained results confirmed that an elevation of the temperature resulted in a gradual increase in the binding of HSF1 and a decrease in the binding of HSF2 to target HSE motifs (Suppl. Fig. 1).

3.3. Hyperthermia caused disruption of HSF1/HSF2 interactions in mouse testes

Using the proximity ligation assay (PLA) we studied direct interactions between HSF1 and HSF2 (which potentially included heterotrimers) in mouse testes at the physiological temperature (32–33 °C), and following heat shock at 38 °C or 43 °C (Fig. 5). The HSF1/HSF2 complexes were clearly detected in spermatogonia, spermatocytes and spermatids of control untreated animals. Many complexes were located on the boundary between the nucleus and cytoplasm, which suggests HSF1-HSF2 interactions in the dense (sex) body (Table 2). Complexes localized in the cytoplasm of elongating spermatids near the luminal center of the cross-sections were also abundant (Fig. 5C). Importantly, the number of HSF1/HSF2 complexes dramatically decreased following the heat shock, which was the most striking after 15 minutes of hyperthermia at 43 °C (Fig. 5A and G). After "mild" hyperthermia, performed at 38 °C, the changes were smaller and appeared gradually. After two hours of recovery at the physiological temperature the number of detected HSF1/HSF2 complexes started to rise from the minimum. This indicates a reconstitution of direct interaction between both transcription factors disrupted after exposure to elevated temperatures.

Table 2
Intracellular distribution of HSF1/HSF2 complexes detected *in situ* in mouse testes at physiological conditions.

	Nucleus	Boundary	Cytoplasm
Spermatogonia	10%	35%	55%
Spermatocytes	38%	36%	26%
Round spermatids	25%	45%	30%

4. Discussion

In the present work genomic binding sites for HSF1 and HSF2 transcription factors were characterized in mouse spermatocytes using chromatin immunoprecipitation combined with next generation sequencing (ChIP-Seq). Several hundred actual binding sites were detected at the physiological temperature, which is in agreement with previous reports describing the important role of HSF1 or HSF2 in mouse testes (Akerfelt et al., 2008, 2010). In fact, many less HSF1 binding sites were found at the physiological temperature using ChIP combined with promoter tiling arrays (Kus-Liśkiewicz et al., 2013). We suppose that such variability between both technologies can be observed when the binding is weaker, which is the case for HSF1 binding at the physiological temperature. When the binding is stronger (e.g. following heat shock), it could be easily detected using either method. ChIP-Seq allowed us to identify the majority (~90%) of HSFs binding sites in introns and intergenic regions. We have evidence that the binding of HSF1 to some introns could have functional importance, for both suppression or activation of gene expression (unpublished), yet the role of HSFs binding outside promoters is mostly speculative at the moment. It has been suggested that the location of the HSF1 binding (promoter versus distal regions) could be connected with the mode of regulation (positive versus negative, respectively) (Mendillo

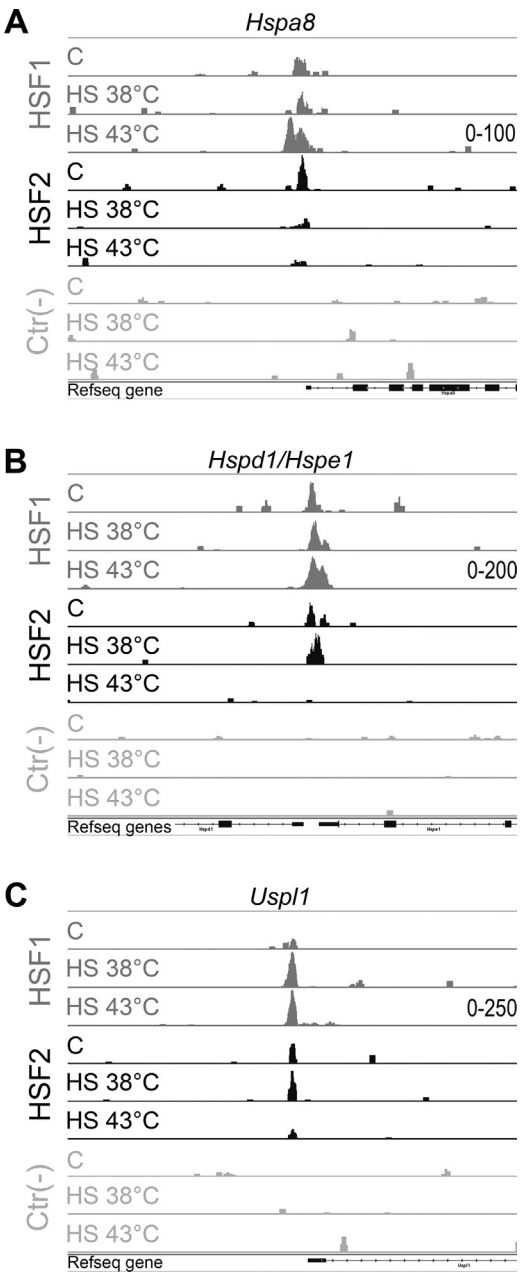

Fig. 3. Remodeling of HSF1 and HSF2 binding in promoter regions of selected genes coding for HSPs or proteins involved in ubiquitination, estimated by the ChIP-Seq approach. HSFs binding at the physiological temperature (C) and after heat shock at 38 °C and 43 °C is visualized by peaks built with the Integrative Genomics Viewer above the scheme of the gene organization (lines – introns, boxes – exons). Approximately 6 kb is shown. The scale for each sample is set to 0–50 (except a highly enriched HSF1 binding at 43 °C, which is shown individually in the figure on the right) to better visualize differences in samples with lower binding. Ctr(−), negative control without specific antibody.

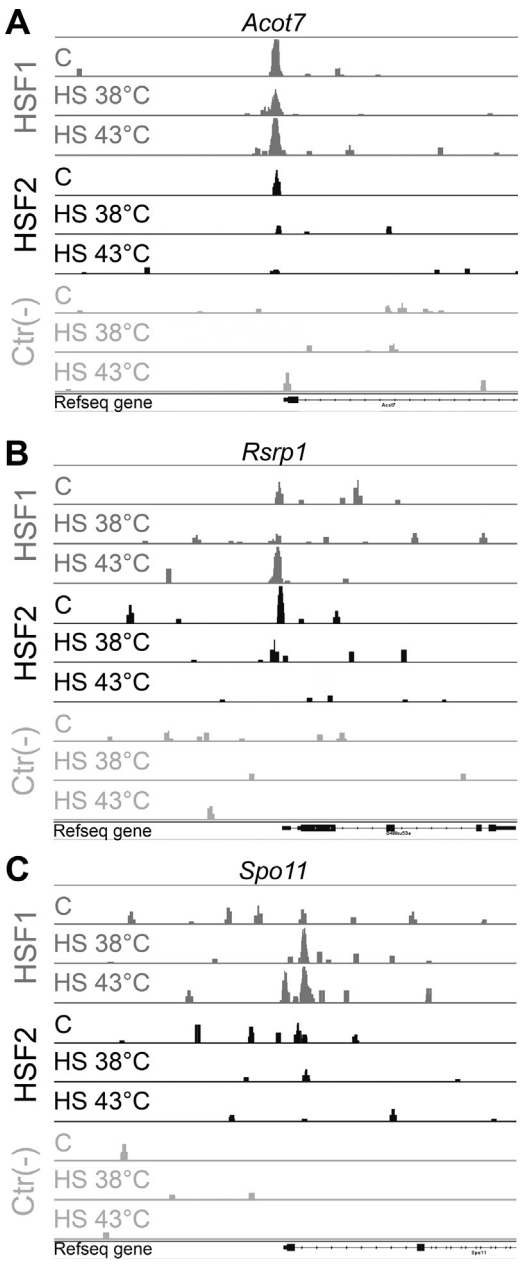

Fig. 4. Remodeling of HSF1 and HSF2 binding in promoters of some non-*Hsp* genes. HSFs binding at the physiological temperature (C) and after heat shock at 38 °C and 43 °C is visualized by peaks built with the Integrative Genomics Viewer above the scheme of the gene organization (lines – introns, boxes – exons). Approximately 7 kb is shown. The scale for *Acot7* is set to 0–50, for *Rsrp1* and *Spo11*, 0–30. Ctr(−), negative control without specific antibody.

et al., 2012). Furthermore, since HSFs are known to initiate chromatin remodeling (Sullivan et al., 2001; Jolly et al., 2004; Xing et al., 2005), it is possible that their binding to DNA in intragenic and intergenic regions could have an influence on the transcription of noncoding RNAs, which has been shown for somatic cells (i.e. at Sat III) (Jolly et al., 2004). It is assumed that 70–90% of the mammalian genome is transcribed in some contexts as long non-coding RNAs (lncRNAs), and that transcription of the genome is substantially more widespread in the testis (where extensive chromatin remodeling occurs) compared to somatic tissues (Soumillon et al., 2013). Therefore, it is possible that in spermatocytes, the binding of HSFs detected outside the classical promoter region reflects the regulation of non-coding RNAs. Furthermore, some identified binding sites could result from a transient sequence-independent chromatin binding corresponding to the HSFs search for more specific targets, which mechanism was recently suggested for HSF1 by Herbomel et al. (Herbomel et al., 2013).

We found that in spermatocytes at the physiological temperature, the promoters of several genes are co-occupied by both HSF1 and HSF2, which indicated the importance of HSF1-HSF2 crosstalk during spermatogenesis. In agreement with this observation, the short-distance proximity (<40 nm) of both transcription factors was detected in spermatogenic cells *in situ* at the physiological temperature using the proximity ligation assay. In this assay the signal observed in nuclei might possibly correspond also to HSF1 and HSF2

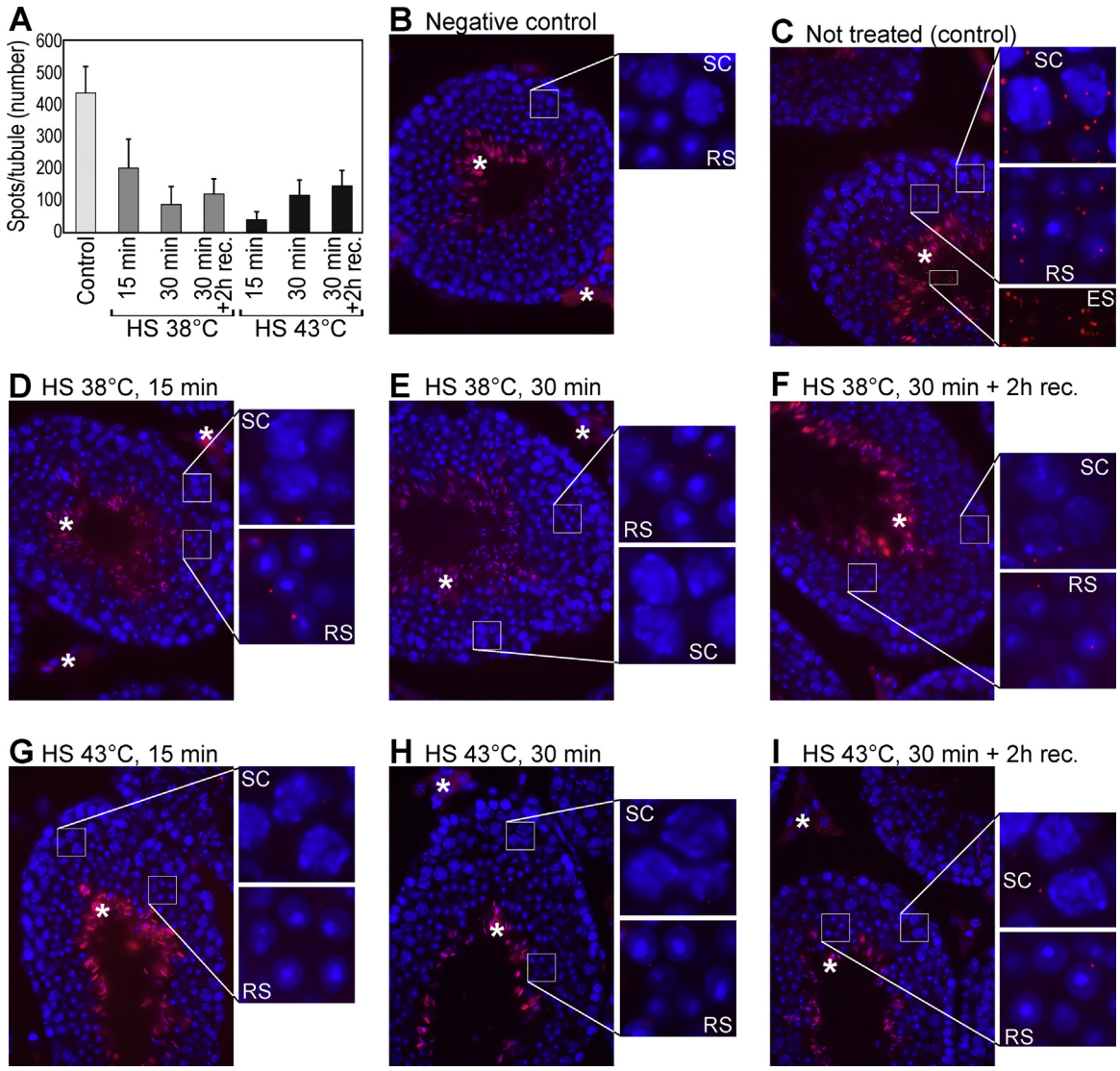

Fig. 5. HSF1/HSF2 complexes in mouse spermatogenic cells assessed by Proximity Ligation Assay. (A) The number of all HSF1/HSF2 complexes per seminiferous tubule cross-section. Mean values ± SD from at least seven sections from two-three testes are presented. (B–I) Tubule cross-sections; HSF1/HSF2 complexes are visible as red spots (asterisks indicate unspecific fluorescence), nuclei are stained in blue. (B) Negative control without primary antibodies. (C–I) Testes of not treated or heat-shocked animals. SC – spermatocytes, RS – round spermatids, ES – cytoplasm of elongating spermatids; insets show blow-ups of selected regions.

homotrimers bound to DNA separately at a distance smaller than ~110–120 bp (or kept in close proximity by chromatin looping). However, the signals observed in cytoplasm indicate an existence of direct HSF1/HSF2 interactions, most possibly in the form of heterotrimers (or heterodimers). Putative HSF1/HSF2 complexes were observed in the nucleus (presumably bound to DNA) and in the cytoplasm. They were also found on the boundary between nucleus and cytoplasm, which suggests their localization in the dense (sex) bodies, structures associated with synapsis and the formation of the XY body during meiosis. Therefore, our observation is in agreement with the previous finding that both HSF1 and HSF2 occupy sex chromatin during meiotic repression (Akerfelt et al., 2008, 2010). Interestingly, many HSF1/HSF2 complexes were localized near the luminal center of the cross-sections of the testicular tubules, in the cytoplasm of elongating spermatids. Such cytoplasmic complexes could form before both factors gain the competence to bind DNA and/or represent complexes released from DNA. The existence of HSF1/HSF2 complexes during mouse spermatogenesis at the physiological temperature has been already shown by Sandqvist et al. (Sandqvist et al., 2009). Additionally, co-localization of HSF1 and HSF2, most probably in the form of heterotrimers, was shown in the nuclear stress granules/bodies (nSBs), which were formed in response to heat shock in human cells (Alastalo et al., 2003). It was also demonstrated that HSF1-dependent transcription could be modulated by the HSF1/HSF2 ratio, both at the physiological temperature and during stress (He et al., 2003; Loison et al., 2006; Ostling et al., 2007; Sandqvist et al., 2009). This suggests cooperation of HSF1 and HSF2 not only during "normal" processes at the physiological temperature, but also during response to stress. Although nSBs are not formed in rodent cells, one should consider the cooperation of HSF1 and HSF2 in the regulation of stress response in mouse spermatogenic cells.

Genes whose promoters are co-occupied by both HSF1 and HSF2 in mouse spermatocytes encode mainly for chaperones and co-chaperones that facilitate protein folding. Thus, both factors could participate in the regulation of the basal level of transcription of these genes at the physiological temperature. Some of these genes could be still co-regulated by both factors during mild hyperthermia at 38 °C, yet stronger hyperthermia at 43 °C caused complete remodeling of HSFs binding. Most strikingly, temperature elevation to 43 °C resulted in an increased binding of HSF1 to promoters of chaperones and other stress-related genes, while HSF2 was almost

completely released from the promoters of such genes; to note, such increased HSF1 binding was not associated with activation of transcription (Kus-Liśkiewicz et al., 2013). In agreement with this observation, the number of HSF1/HSF2 complexes detected in spermatogenic cells *in situ* by the proximity ligation assay was markedly (~10-fold) reduced at 43 °C. Temperature-related changes in the DNA-binding ability of HSF1 and HSF2 have been previously observed using *in vitro* experimental model. Recombinant HSF1 acquired HSE-binding ability (examined by gel-shift assay) at a temperature above 39 °C (with maximum at 42–43 °C), while HSF2 lost HSE-binding starting from 39 °C, up to a complete loss at 43 °C (Sarge et al., 1991). However, later *in vivo* studies using different somatic cells revealed that following heat shock, at least in some *HSPs* promoters, both HSFs could bind at the same time (Trinklein et al., 2004; Ostling et al., 2007; Ahlskog et al., 2010; Shinkawa et al., 2011). More recently, a ChIP-Seq study performed in human K562 erythroleukemia cells also showed the involvement of both HSF1 and HSF2 in the regulation of genes coding for chaperones and co-chaperones, where the total number of HSF1 and HSF2 target loci was increased after temperature elevation (Vihervaara et al., 2013). Here we showed different involvement of HSF1 and HSF2 in the response to thermal stress, apparently specific for spermatogenic cells. Hence, both factors play a different role in somatic and spermatogenic cells at normal, physiological conditions, but also behave differently at an elevated temperature. It has been shown that overexpression of HSF1 is sufficient to trigger apoptosis in spermatogenic cells in the absence of activation of *HSP* genes (Vydra et al., 2006; Widlak et al., 2007). Thus, one should assume that disturbances in HSF1/HSF2 interactions and their chromatin binding observed in spermatogenic cells subjected to strong hyperthermia have an apparent impact on the viability of these cells in stress conditions.

5. Conclusion

During heat shock in mouse testes interactions between HSF1 and HSF2 are disrupted and their binding to chromatin is remodeled. This could contribute to the heat sensitivity of spermatogenic cells, since the cooperation of both factors is required for correct spermatogenesis.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2014.10.006.

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