Cell Stress and Chaperones (2019) 24:1127–1136
https://doi.org/10.1007/s12192-019-01035-6

ORIGINAL PAPER

MAPKAP kinase 2–mediated phosphorylation of HspA1L protects male germ cells from heat stress–induced apoptosis

Patrick A. Williams[1] · Heather E. Kobilnyk[1] · Emily A. McMillan[1] · Todd I. Strochlic[1]

Received: 8 July 2019 / Revised: 29 August 2019 / Accepted: 12 September 2019 / Published online: 22 October 2019

Abstract

Developing male germ cells are extremely sensitive to heat stress; consequently, anatomic and physiologic adaptations have evolved to maintain proper thermoregulation during mammalian spermatogenesis. At the cellular level, increased expression and activity of HSP70 family members occur in response to heat stress in order to refold partially denatured proteins and restore function. In addition, several kinase-mediated signaling pathways are activated in the testis upon hyperthermia. The p38 MAP kinase (MAPK) pathway plays an important role in mitigating heat stress, and recent findings have implicated the downstream p38 substrate, MAPKAP kinase 2 (MK2), in this process. However, the precise function that this kinase plays in spermatogenesis is not completely understood. Using a proteomics-based screen, we identified and subsequently validated that the testis-enriched HSP70 family member, HspA1L, is a novel substrate of MK2. We demonstrate that MK2 phosphorylates HspA1L solely on Ser241, a residue within the N-terminal nucleotide-binding domain of the enzyme. This phosphorylation event enhances the chaperone activity of HspA1L in vitro and renders male germ cells more resistant to heat stress–induced apoptosis. Taken together, these findings illustrate a novel stress-induced signaling cascade that promotes the chaperone activity of HspA1L with implications for understanding male reproductive biology.

Keywords HspA1L · MAPKAP kinase 2 · MK2 · Spermatogenesis · Heat shock protein · Stress signaling

Introduction

Spermatogenesis is a highly orchestrated process that occurs within the seminiferous tubules of the testes and culminates in the production of mature sperm. The underlying developmental program is sensitive to increases in temperature of even 2–3 °C. Adaptations present in most male mammals therefore include maintenance of the testes outside of the body cavity at a temperature of a few degrees below that of core body temperature and a venous countercurrent exchange mechanism within the spermatic cord that serves to cool blood in arteries supplying the gonads.

The most relevant consequence of heat stress on the testis is death of germ cells via apoptosis (Yin et al. 1997). Like other cell types, male germ cells respond to environmental cues and external stress by activating intracellular signaling pathways that ultimately determine whether the cell will live or die (Baum et al. 2005). Of particular importance among these signaling cascades is the p38 MAPK pathway. This pathway regulates both apoptosis and spermatocyte differentiation, and several studies have demonstrated a role for this pathway in male germ cell biology (Almog and Naor 2008; Lizama et al. 2009; Ewen et al. 2010). Upon activation, p38 phosphorylates a downstream effector kinase, the serine/threonine kinase MAPKAP kinase 2 (MK2). Direct p38-mediated phosphorylation of MK2 on two threonine residues (T222 and T334) results in its activation (Ben-Levy et al. 1995) leading to phosphorylation of downstream substrate proteins. A role for MK2 in spermatogenesis was recently reported (Williams et al. 2016), demonstrating that this kinase is an emerging enzyme in male germ cell development and suggests that MK2 may have additional functions in gametogenesis that have yet to be identified.

In response to heat stress, most cell types also induce expression of a group of enzymes known as heat shock proteins (HSPs), molecular chaperones that recognize damaged or misfolded client proteins and attempt to refold them in an

✉ Todd I. Strochlic
tis35@drexel.edu

[1] Department of Biochemistry and Molecular Biology, Drexel University College of Medicine, 245 N. 15th St., MS 497, Philadelphia, PA 19102, USA

effort to restore function, inhibit proteotoxicity, and prevent apoptosis (Lanneau et al. 2008). The largest group of heat shock proteins in mammals is the HSP70 (HSPA) family, comprised of 13 members in humans (Radons 2016). Interestingly, two of these family members, HspA2 and HspA1L, are highly expressed in the male germ line (Vydra et al. 2006), and multiple studies have established HspA2 as a critical regulator of male meiotic progression and sperm function (Scieglinska and Krawczyk 2015). Even so, male germ cells are still more sensitive to hyperthermia than somatic cells despite the expression of these two additional HSP70 family members, suggesting that other factors and/or pathways are involved in regulating their activities.

The purpose of this study was to gain additional insight into the role of the p38-MK2 signaling pathway in spermatogenesis. Toward that end, we demonstrate that the testis-enriched HSP70 family member HspA1L is a substrate of the stress-activated protein kinase MK2. We show that MK2-mediated phosphorylation of HspA1L increases its chaperone activity in vitro and renders male germ cells more resistant to heat stress–induced apoptosis. Collectively, these results describe a novel signaling pathway by which HspA1L activity is enhanced in response to hyperthermia during spermatogenesis.

Materials and methods

Antibodies and reagents

Anti-HspA1L (C-6; Cat. no. sc-393297), anti-GAPDH (6C5; Cat. no. sc-32233), and anti-GFP (B-2; Cat. no. sc-9996) antibodies were purchased from Santa Cruz Biotechnology. Anti-MK2 (Cat. no. 12155), anti-phospho-MK2-T334 (Cat. no. 3007), anti-p38 MAPK (Cat. no. 8690), anti-phospho-p38 MAPK-T180/Y182 (Cat. no. 4511), anti-cleaved caspase 3 (Cat. no. 9664), anti-PARP (Cat. no. 9532), and anti-cleaved PARP (Cat. no. 94885) antibodies were from Cell Signaling Technology. Anti-thiophosphate ester antibody (51-8; Cat. no. ab92570) and PNBM (*p*-nitrobenzyl mesylate) were from Abcam. ATPγS (adenosine 5′-[3-thiotriphosphate]) was purchased from Sigma Aldrich.

Plasmid construction

pcDNA5/FRT/TO-GFP-HSPA1L was a gift from Harm Kampinga (Addgene plasmid no. 19484). To generate a construct for bacterial expression of His_6-tagged HspA1L, the *HSPA1L* coding sequence was amplified as a *Bam*HI-*Eco*RI restriction fragment and cloned into the corresponding sites of vector pET28a (Novagen). Generation of the S241A, S241D, and K73E mutations was accomplished by site-directed mutagenesis using the QuikChange kit (Agilent Technologies). To generate constructs encoding N-terminal GFP-tagged HspA1L (and mutants thereof), restriction fragments were excised from the respective pET28a-HspA1L plasmids and subcloned into the *Bgl*II-*Eco*RI sites in vector pEGFP-C1 (Clontech). All constructs were fully sequenced. Primer sequences are available upon request.

Cell culture

GC-2spd cells were purchased from ATCC and grown in DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (5 μg/mL). Cells were grown at 37 °C in 5% CO_2. Cells were transfected using Lipofectamine 2000 (Life Technologies) according to the manufacturer's recommended protocol. For the generation of MK2-knockdown GC-2spd cells, retroviral constructs targeting murine MK2 and a scrambled control shRNA construct were purchased from OriGene. Stably transfected GC-2spd cells were selected with puromycin (3 μg/mL) and were maintained in medium containing the same antibiotic (1 μg/mL). Reduced expression of MK2 was confirmed by Western blotting.

Protein expression and purification

BL21 (DE3) cells (Novagen) were transformed with plasmids encoding His_6-HspA1L, His-HspA1L S241A, His-HspA1L S241D, or His-HspA1L K73E. Protein expression was induced at 18 °C for 12 h with 1 mM isopropyl-β-D-thiogalactoside (IPTG). After centrifugation, the pellet was resuspended in 15 mL of lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM DTT, and 1 mM EDTA with protease inhibitors [Roche]). The lysate was sonicated on ice and centrifuged at 20,000×*g* for 20 min at 4 °C. The supernatant was added to 0.5 mL (packed volume) of Ni-NTA agarose beads (Gold Biotechnology) and incubated at 4 °C for 12 h with end-over-end tumbling. The slurry was transferred to a column and washed extensively with wash buffer. His_6-tagged fusion proteins were eluted with excess imidazole in lysis buffer (pH 7.4), and appropriate fractions were pooled. The protein was dialyzed overnight at 4 °C into dialysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM DTT). Glycerol was added to a final concentration of 10%, and the proteins were snap-frozen and stored at − 80 °C.

In vitro kinase assays

Active recombinant MK2 (residues 40–400; SignalChem) was incubated with 20 μg of His-HspA1L-WT, His-HspA1L-S241A, or His-HspA1L-K73E in kinase reaction buffer (50 mM HEPES, pH 7.5, 0.65 mM $MgCl_2$, 0.65 mM $MnCl_2$, 12.5 mM NaCl) with 500 μM ATPγS. Kinase reactions were incubated at 30 °C for 30 min. Following incubation, PNBM (dissolved in dimethyl sulfoxide [DMSO]) was added to a final concentration of 2.5 mM, and reactions were

incubated at room temperature for 1 h, followed by the addition of 6X boiling sample buffer to stop the reaction. Reactions were then resolved by SDS-PAGE followed by immunoblotting with the indicated antibodies.

SDS-PAGE and Western blotting

Proteins were resolved on 10% or 12.5% polyacrylamide gels and transferred to nitrocellulose membranes (GE Amersham) using the Pierce G2 Fast Blotter (Thermo Scientific). Membranes were blocked in 5% non-fat milk in Tris-buffered saline (TBS) followed by overnight incubation with the indicated primary antibody in the same buffer. Membranes were washed three times the following day in TBS + 0.5% Tween (TBS-T), incubated for 1 h with the appropriate HRP-conjugated secondary antibody (Cell Signaling Technology), washed three times again in TBS-T, and processed for signal detection using enhanced chemiluminescence (Santa Cruz Biotechnology). For detection of proteins from the same reaction or lysate that migrate at similar molecular weights (i.e., phosphorylated and non-phosphorylated forms of the same protein), samples were split and run on separate gels.

Luciferase refolding assay

This assay is based on a published protocol (Lu and Cyr 1998). Briefly, QuantiLum Recombinant Luciferase (Promega) was diluted in denaturing buffer (25 mM HEPES pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, 6 M guanidine HCl, 5 mM DTT) to a final concentration of 2.48 μM and incubated at 25 °C for 40 min. Denatured luciferase (2 μL) was then added to 125 μL refolding buffer (25 mM HEPES pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, 1 mM ATP) supplemented with 4 μg of the indicated recombinant His-tagged HspA1L protein in dialysis buffer (20 mM Tris pH 7.4, 150 mM NaCl, 1 mM DTT) or dialysis buffer alone in a 96-well plate. Refolding was performed for the indicated times at 25 °C. After refolding, 20 μL of Luciferase Assay Reagent (Promega) diluted 1:1 in refolding buffer was added to each well, and the plate was analyzed using a 1450 MicroBeta JET Microplate Scintillation and Luminescence Counter (Wallac).

Immunofluorescence microscopy

GC-2spd cells were seeded on glass coverslips and transfected for 24 h with the indicated constructs. Cells were then exposed to heat stress for 3 h in a 43 °C incubator. After a recovery period of 12 h at 37 °C, cells were fixed in 4% formaldehyde and processed for immunofluorescence microscopy. Coverslips were mounted on glass slides with Vectashield mounting medium (Vector Laboratories), and cells were imaged using an EVOS FL Auto microscope (ThermoFisher Scientific) with a × 40 objective.

Statistical analysis

Quantitative results were obtained from at least three independent experiments and are presented as the average ± standard deviation. Where indicated, *p* values were calculated using one-way ANOVA (analysis of variance).

Results

HspA1L is a novel substrate of MAPKAP kinase 2

To gain insight into the biological functions of MK2, a proteomics-based screen was conducted to identify novel substrates of this kinase (Williams et al. 2016). Using this approach, the heat shock protein HspA1L was identified as a putative substrate. HspA1L (heat shock protein 70 kDa 1-like, also known as HSP70-hom and Hsc70t) is highly expressed in spermatids in the mammalian testis (Ito et al. 1998; Tsunekawa et al. 1999) and is a member of the HSP70 family of chaperone proteins. In response to heat stress, heat shock proteins of the HSP70 family mediate cytoprotective effects and prevent nonspecific aggregation and thermal denaturation of cellular proteins (Mayer and Bukau 2005; Daugaard et al. 2007). These molecular chaperones function by virtue of their ability to selectively recognize and associate with hydrophobic regions that are transiently exposed in their respective client proteins (Schlecht et al. 2011). Via an ATP-dependent cycle of release and rebinding, they assist in the proper refolding of proteins (Radons 2016).

Due to their vital functions in mediating resistance to heat stress and given the sensitivity of developing sperm to heat, HSPs have garnered interest in the field of male germ cell research (Dix 1997; Eddy 1999; Naaby-Hansen and Herr 2010; Dun et al. 2012). In addition to other HSP70 family proteins, male germ cells uniquely express two additional members of this family, HspA2 and HspA1L. Targeted deletion of *HSPA2* in mice results in meiotic failure and germ cell apoptosis (Dix et al. 1996; Dix et al. 1997), and decreased mRNA and protein expression of this chaperone has been observed in human males with azoospermia (Son et al. 2000; Feng et al. 2001). HspA2 is expressed on the surface of mature sperm (Lima et al. 2006; Naaby-Hansen and Herr 2010) where it plays an important role in promoting the binding of sperm to the zona pellucida of the egg (Nixon et al. 2015). HspA2 is thus important both for meiotic progression during spermatogenesis and for successful fertilization (Eddy 1999). However, in contrast to HspA2, surprisingly little is known about the biological function(s) of the other testis-enriched heat shock protein HspA1L.

To validate HspA1L as a substrate of MK2, His_6-tagged HspA1L was expressed and purified from bacteria. This fusion protein was used as a substrate in an in vitro kinase assay with ATPγS (adenosine 5′-[3-thiotriphoshate]) in the presence or absence of constitutively active MK2. Following incubation with the alkylating agent PNBM (*p*-nitrobenzyl mesylate) that generates a thiophosphate ester moiety on thiophosphorylated proteins, Western blotting of the kinase reactions using an anti-thiophosphate ester antibody demonstrated a signal only in the presence of both MK2 and HspA1L (Fig. 1a). These results demonstrate that MK2 phosphorylates HspA1L in vitro.

HspA1L is comprised of 641 amino acids and contains several functional domains (Fig. 1b): a highly conserved N-terminal nucleotide-binding domain that hydrolyzes ATP, a substrate binding domain, and an EEVD motif that mediates association with co-chaperones (Erbse et al. 2004). To determine the specific residue(s) of HspA1L that are phosphorylated by MK2, we used a bioinformatics approach, leveraging publicly available data regarding the consensus motif for MK2-mediated phosphorylation (www.phosphosite.org). This motif is defined by the presence of an arginine residue at the −3 position (i.e., three residues N-terminal to the phospho-acceptor site) and hydrophobic residues at the −5

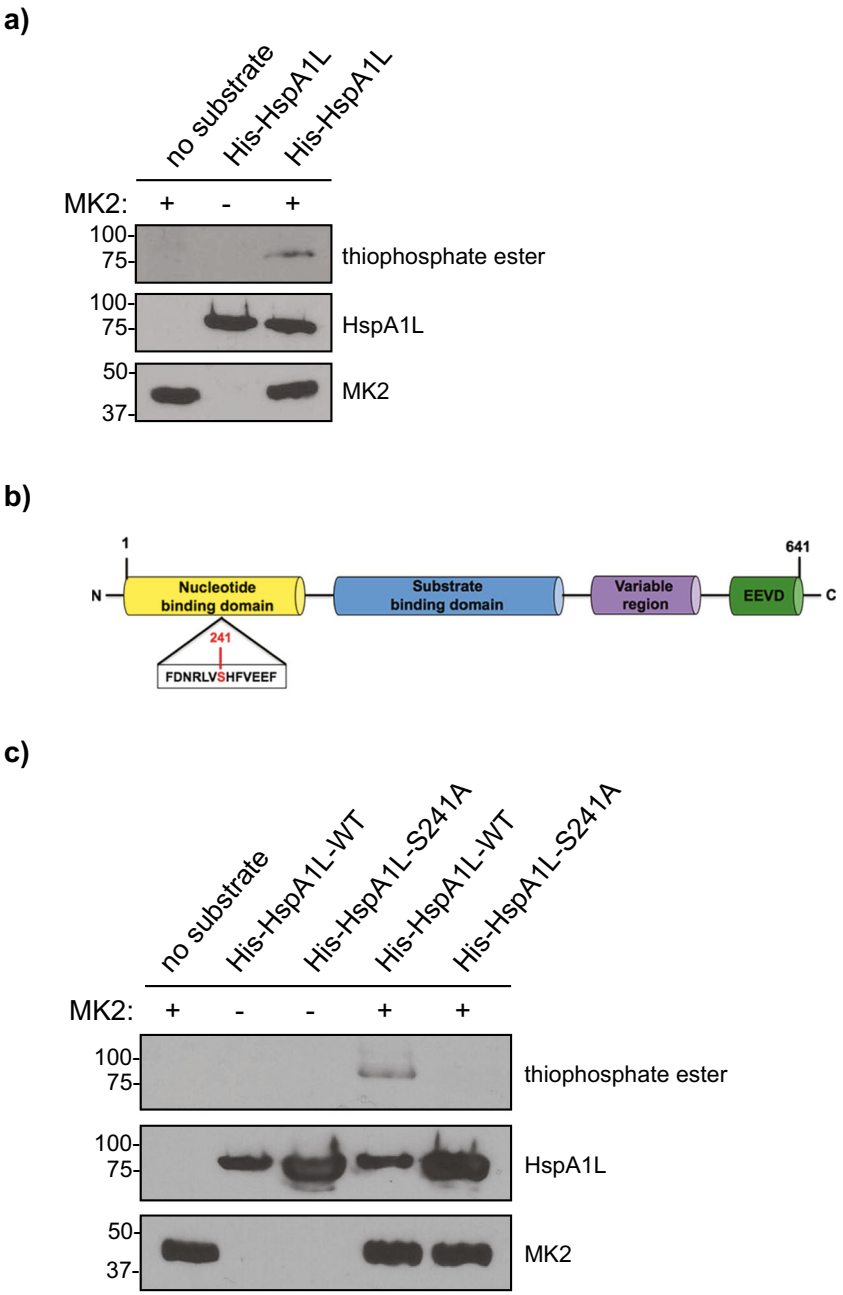

Fig. 1 MK2 phosphorylates the heat shock protein HspA1L at S241. **a** In vitro kinase assay using His-tagged HspA1L and MK2. His_6-HspA1L (or no substrate) was incubated in the presence or absence of recombinant active MK2 and ATPγS followed by treatment with PNBM. Kinase reactions were resolved by SDS-PAGE followed by immunoblotting with the indicated antibodies. Molecular weight markers (in kDa) are indicated. Western blots shown are representative of three independent experiments. **b** Domain architecture of human HspA1L. The putative MK2 phosphorylation site (S241) is indicated in red. **c** MK2-mediated phosphorylation of HspA1L at S241. His-tagged HspA1L or HspA1L-S241A proteins were incubated with active recombinant MK2 and ATPγS followed by incubation with PNBM. Kinase reactions were resolved by SDS-PAGE followed by immunoblotting with the indicated antibodies. Molecular weight markers (in kDa) are indicated. Western blots shown are representative of three independent experiments

and − 6 positions (Stokoe et al. 1993). Analysis of the primary sequence of HspA1L revealed a serine residue (S241) in the N-terminal ATPase domain located within a potential consensus motif for MK2 phosphorylation. This site is evolutionarily conserved and is also present in HspA2 (Radons 2016; Wisniewska et al. 2010). To test if this residue is phosphorylated by MK2 in vitro, we used site-directed mutagenesis to generate a mutant in which this serine was replaced with non-phosphorylatable alanine (S241A). This mutant was expressed and purified from bacteria as a His-tagged fusion protein as was done with the wild-type protein and used as a substrate in an in vitro kinase assay along with wild-type HspA1L as a control (Fig. 1c). Mutation of this residue abrogated the signal in the in vitro kinase assay, indicating that S241 is the sole site of MK2-mediated phosphorylation. Together, these data demonstrate that HspA1L is a *bona fide* substrate of MK2 and that HspA1L is phosphorylated by this kinase solely at S241.

MK2-mediated phosphorylation of S241 enhances HspA1L chaperone activity in vitro

Given the location of the phosphorylated residue in the HspA1L ATP-binding domain, we hypothesized that phosphorylation by MK2 may affect the catalytic activity of the enzyme. To test if MK2-mediated phosphorylation of HspA1L at S241 affects its chaperone activity, an in vitro luciferase refolding assay was conducted (Lu and Cyr 1998). In this experiment, purified luciferase was incubated in denaturing buffer resulting in unfolding of the enzyme. Denatured luciferase was then added to a fixed concentration of either wild-type HspA1L or a phosphomimetic form of the enzyme, HspA1L-S241D, and luciferase activity was measured following addition of luciferin substrate. As shown, wild-type HspA1L displayed little refolding ability over time and was only slightly higher than the control (Fig. 2a). Strikingly, however, the refolding of luciferase was significantly enhanced upon incubation with HspA1L-S241D. To confirm this result via a strategy that avoids the use of mutant proteins (as phosphomimetic mutations often do not faithfully recapitulate phosphorylation), we coupled the previously described kinase assay with the luciferase refolding assay (Fig. 2b). When wild-type HspA1L was pre-incubated with MK2 and phosphorylated, the refolding activity was increased over non-phosphorylated HspA1L and was comparable to that observed with HspA1L-S241D (Fig. 2a). Furthermore, when the luciferase refolding assay was performed using a catalytically dead mutant, HspA1L-K73E (Rajapandi et al. 1998; Hasson et al. 2013), the refolding activity was comparable to control (Fig. 2a), despite MK2-mediated phosphorylation of this mutant (Fig. 2b). Collectively, these results demonstrate that phosphorylation of HspA1L by MK2 at S241 enhances its chaperone activity in vitro.

MK2-HspA1L signaling renders male germ cells more resistant to heat stress

We then sought to determine whether this post-translational modification is physiologically relevant. To address this in an appropriate model system, we used murine GC-2spd cells that display spermatid-like features and that have been used to study various aspects of male germ cell biology (Zhang et al. 2012). Western blot analysis of GC-2spd cell lysates indicated that these cells express endogenous HspA1L and relevant components of the p38-MK2 signaling pathway (Fig. 3a). Moreover, the p38-MK2 signaling axis is activated in response to a transient supraphysiologic (i.e., higher than core body temperature) heat stress in this cell type (Fig. 3a), conditions known to impair mammalian spermatogenesis in vivo (Paul et al. 2008).

To determine if MK2 plays a role in mediating resistance to heat stress–induced apoptosis in GC-2spd cells, expression of MK2 in these cells was reduced by shRNA-mediated knockdown. Successful knockdown of MK2 was confirmed by Western blotting while levels of endogenous HspA1L remained unaltered compared with cells expressing control shRNA (Fig. 3b). These cells were then exposed or not to heat stress and apoptosis was assessed by analyzing levels of cleaved poly-ADP ribose polymerase (PARP), an established marker of apoptosis. The results indicate that knockdown of MK2 sensitizes GC-2spd cells to heat stress–induced apoptosis as elevated levels of cleaved PARP were detected in the MK2 knockdown cells relative to control (Fig. 3c), suggesting that MK2 plays a critical role in mediating the response to heat stress.

We then tested if we could rescue the sensitivity of MK2 knockdown cells to heat-induced apoptosis by overexpression of HspA1L or mutants thereof. For this experiment, constructs encoding GFP-tagged wild-type HspA1L, HspA1L-S241A, or HspA1L-S241D were transfected into MK2-knockdown GC-2spd cells. Subsequent immunoblotting of whole cell lysates demonstrated equal expression of the wild-type and mutant proteins (Fig. 4a). These cells were then exposed or not to heat stress, and the percentage of GFP-positive cells that were also positive for the apoptotic marker cleaved caspase-3 was determined by immunofluorescence microscopy (Fig. 4b). As expected, untransfected cells displayed a substantial amount of cell death upon exposure to heat that was slightly reduced by overexpression of wild-type HspA1L. However, overexpression of the phosphomimetic mutant (HspA1L-S241D) significantly enhanced cell survival while cells overexpressing the non-phosphorylatable mutant (HspA1L-S241A) did not (Fig. 4b). Collectively, these data indicate that MK2-mediated phosphorylation of HspA1L promotes the survival of male germ cells exposed to heat stress.

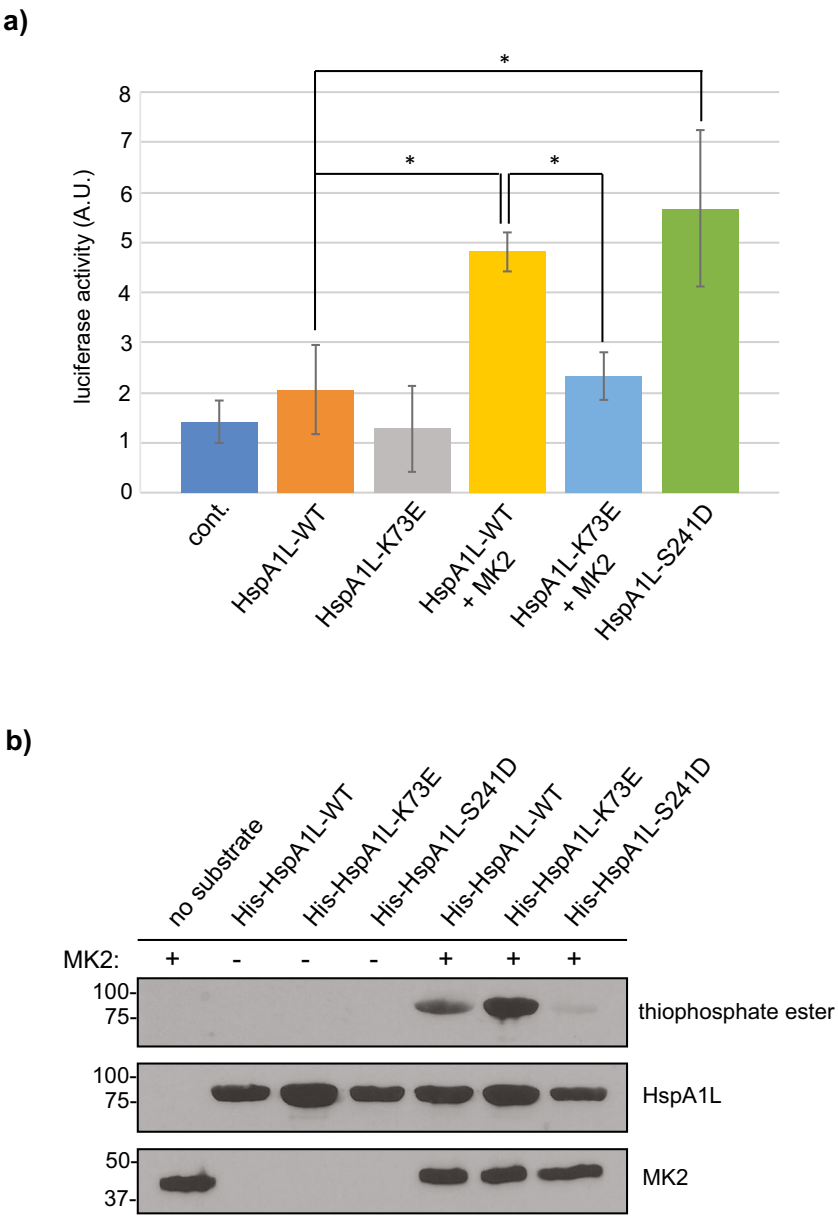

Fig. 2 MK2-mediated phosphorylation of HspA1L enhances its chaperone activity in vitro. **a** HspA1L chaperone activity as measured by a luciferase refolding assay. Denatured luciferase was incubated in the presence or absence (cont.) of 4 μg of purified recombinant wild-type HspA1L, HspA1L-K73E, or HspA1L-S241D, phosphorylated or not by MK2, as indicated. Following the addition of luciferin, luciferase activity was measured at 15 min using a luminometer. *p value < 0.05; A.U. = arbitrary units. **b** Western blot analysis of the kinase reactions in **a** prior to performing the luciferase refolding assays. His-tagged HspA1L, HspA1L-S241D, or HspA1L-K73E proteins were incubated or not with active recombinant MK2 and ATPγS followed by incubation with PNBM after a sample was removed for use in the luciferase refolding assay. Kinase reactions were resolved by SDS-PAGE followed by immunoblotting with the indicated antibodies. Molecular weight markers (in kDa) are indicated. Western blots shown are representative of three independent experiments

Discussion

This study demonstrates novel site-specific phosphorylation of the testis-enriched chaperone HspA1L by the stress-activated protein kinase MK2. Phosphorylation of HspA1L at serine 241 results in enhanced enzymatic activity in an in vitro refolding assay and protects male germ cells from heat stress–induced apoptosis. Thus, activation of the p38-MK2 stress signaling axis coupled with subsequent phosphorylation and stimulation of HspA1L activity is a mechanism for these cells to mitigate heat stress. Moreover, as HspA1L is constitutively expressed and is not transcriptionally induced by heat, these results describe a pathway for rapid upregulation of HspA1L enzymatic activity driven by post-translational modification. Interestingly, a rare single nucleotide polymorphism in *HSPA1L* associated with spontaneous preterm birth results in a substitution of alanine at residue 268 with threonine and thus introduces a potential disease-associated phosphorylation site in close proximity to serine 241 (Huusko et al. 2018). However, whether this site is phosphorylated in vivo is not known.

HSP70 directly binds to MK2 during myoblast differentiation and modulates the stability of p38 by regulating the p38-MK2 interaction (Fan et al. 2018) but whether MK2 directly phosphorylates HSP70 was not investigated in this study. Along these lines, whether HspA2 is also phosphorylated and regulated by MK2 remains to be tested, although given the conservation of this serine residue and the surrounding sequence (Wisniewska et al. 2010), we predict that this is likely to be the case.

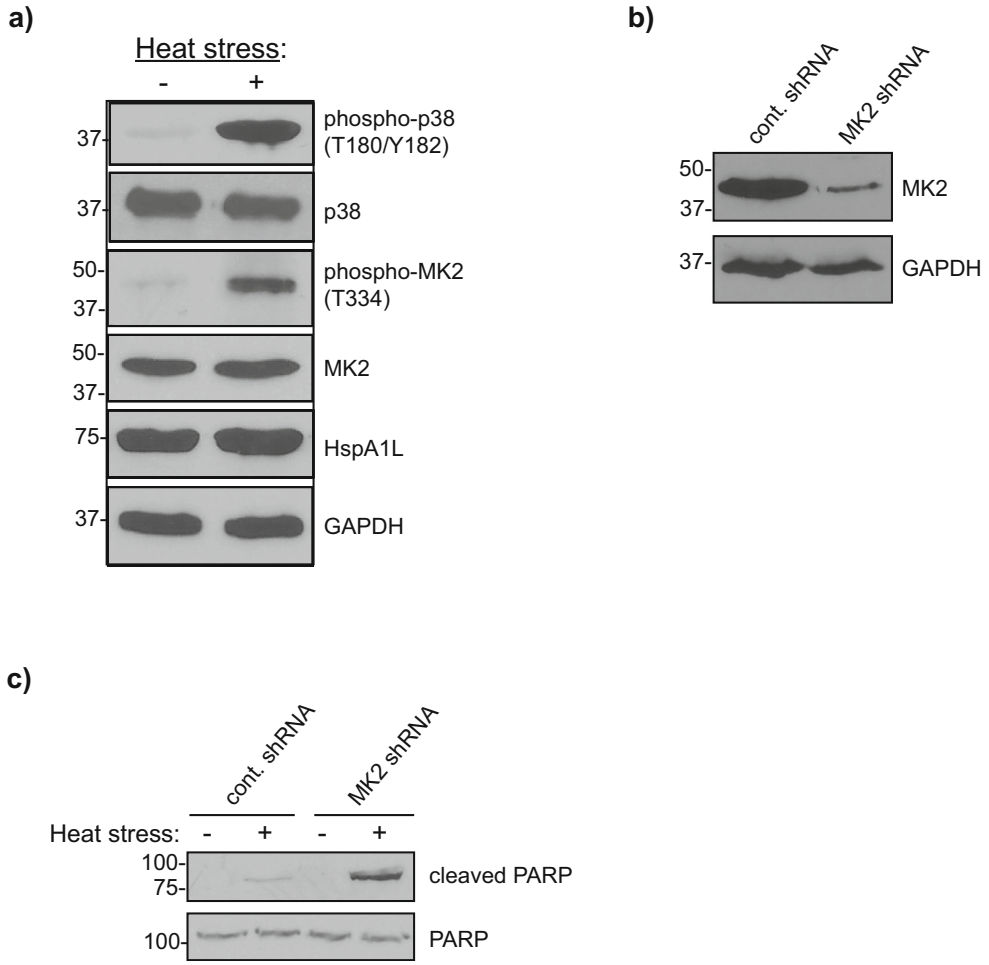

Fig. 3 Knockdown of MK2 sensitizes male germ cells to heat stress–induced apoptosis. **a** Activation of the p38-MK2 signaling pathway in GC-2spd cells exposed to heat stress. Lysates prepared from cells exposed or not to heat stress (43 °C for 3 h) were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. Molecular weight markers (in kDa) are indicated. Western blots shown are representative of three independent experiments. **b** Generation of GC-2spd MK2-knockdown cells. Whole cell lysates from GC-2spd cells stably expressing either control shRNA or MK2 shRNA were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. Molecular weight markers (in kDa) are indicated. Western blots shown are representative of three independent experiments. **c** Increased apoptosis in male germ cells with reduced expression of MK2 in response to heat stress. GC-2spd cells stably expressing either control shRNA or MK2 shRNA were exposed to heat stress (43 °C for 3 h followed by a 12-h recovery at 37 °C). Cell lysates were prepared, resolved by SDS-PAGE, and immunoblotted with the indicated antibodies. Molecular weight markers (in kDa) are indicated. Western blots shown are representative of three independent experiments

Interestingly, one of the first identified substrates of MK2 is another heat shock protein, the small heat shock protein Hsp27 (Freshney et al. 1994; Engel et al. 1995). Hsp27 forms multimers in the non-phosphorylated state but dissociates into monomers when phosphorylated by MK2 (Lambert et al. 1999; Kato et al. 2001). Monomeric Hsp27 functions in the de-capping of actin polymers, resulting in cytoskeletal rearrangement and increased cell migration (Doshi et al. 2009). It is intriguing, although perhaps not surprising, that MK2 phosphorylates members of multiples families of heat shock proteins, given the central role of MK2 in regulating the cellular stress response. The findings described here thus expand the repertoire of heat shock proteins whose activities are regulated in an MK2-dependent manner.

During spermatogenesis, MK2 phosphorylates the RNA-binding protein Dazl (deleted in azoospermia-like) (Williams et al. 2016). This post-translational modification negatively regulates the function of Dazl and inhibits mRNA translation resulting in increased germ cell death. These results are, at first glance, paradoxical in terms of understanding how the same kinase can promote both apoptosis and cell survival in response to stress in the same cell type. However, this apparent issue can be reconciled by examining the temporal expression of these two MK2 substrates during spermatogenesis. While Dazl is primarily expressed during early germ cell development in primordial germ cells (Niederberger et al. 1997), HspA1L expression is detected later on during spermatogenesis, specifically during spermiogenesis in early and late

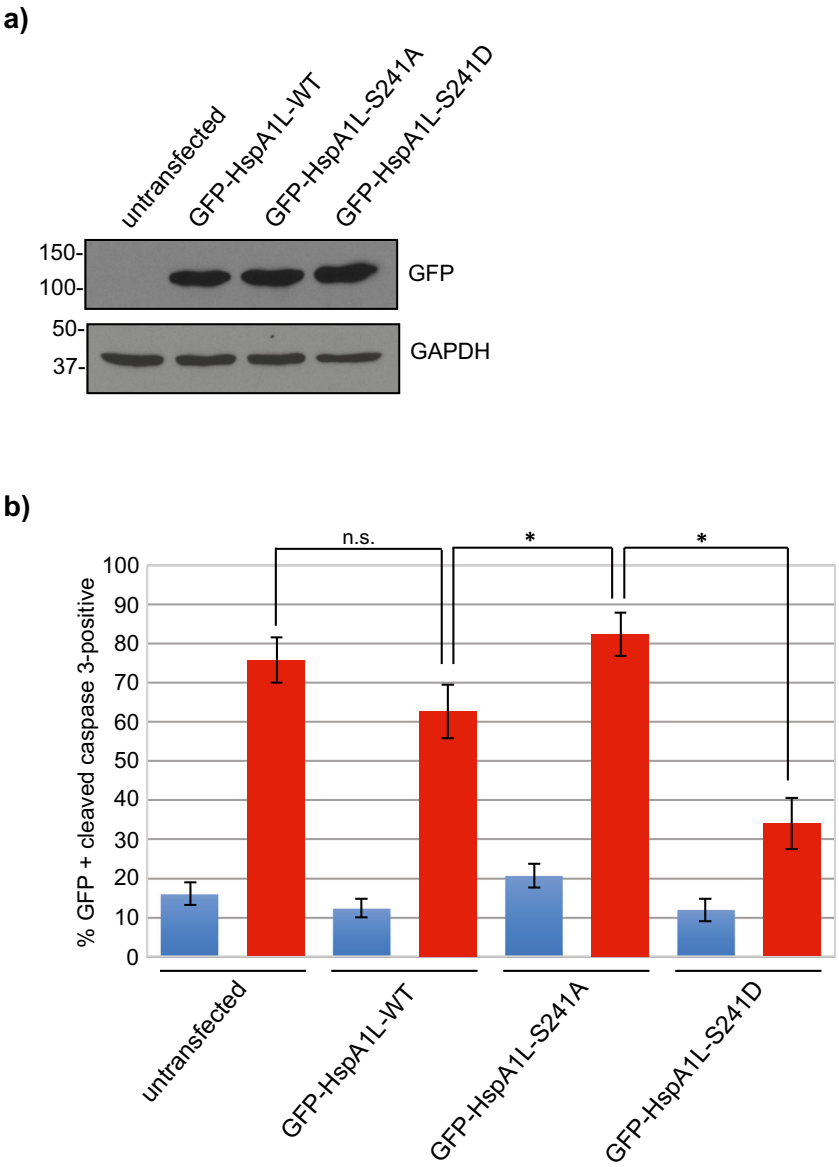

Fig. 4 Overexpression of phosphomimetic HspA1L in MK2-knockdown cells rescues sensitivity to heat stress–induced apoptosis. **a** Expression of GFP-tagged wild-type and mutant HspA1L proteins. Whole cell lysates from MK2-knockdown GC-2spd cells transfected with the indicated constructs were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. Molecular weight markers (in kDa) are indicated. Western blots shown are representative of three independent experiments. **b** Reduced apoptosis in cells overexpressing HspA1L-S241D but not HspA1L-S241A in response to heat stress. MK2-knockdown GC-2spd cells expressing the indicated constructs were exposed (red bars) or not (blue bars) to heat stress (43 °C for 3 h followed by a 12-h recovery at 37 °C). Cells were then fixed and stained with anti-cleaved caspase 3 antibody, and the percentage of GFP-positive cells that were also positive for cleaved caspase 3 was assessed by immunofluorescence microscopy. A total of 300 cells was analyzed per experimental condition. *p value < 0.05; n.s. = not significant

spermatids (Tsunekawa et al. 1999). Thus, due to the differential expression patterns of Dazl and HspA1L during spermatogenesis, the activation of MK2 may result in different cellular outcomes in different stages of sperm development.

In addition to its classical role in mitigating heat stress, HspA1L has moonlighting functions such as promoting Parkin translocation to mitochondria during mitophagy (Hasson et al. 2013) and stabilizing the prion protein PrP^c in colorectal cancer cells (Lee et al. 2017). Moreover, mutations in *HSPA1L* are associated with the development of inflammatory bowel disease (Takahashi et al. 2017), and increased levels of *HSPA1L* mRNA have been observed in graft vs. host disease (Atarod et al. 2015). HspA1L is therefore likely involved in modulating the stress response in a variety of disease states, and whether MK2 regulates HspA1L activity in these pathological contexts has yet to be determined.

Acknowledgments We thank M. Krug for the generation of MK2-knockdown GC-2spd cells.

References

Almog T, Naor Z (2008) Mitogen activated protein kinases (MAPKs) as regulators of spermatogenesis and spermatozoa functions. Mol Cell Endocrinol 282(1–2):39–44

Atarod S, Turner B, Pearce KF, Ahmed SS, Norden J, Bogunia-Kubik K, Wang XN, Collin M, Dickinson AM (2015) Elevated level of HSPA1L mRNA correlates with graft-versus-host disease. Transpl Immunol 32(3):188–194

Baum JS, George JPS, McCall K (2005) Programmed cell death in the germline. Semin Cell Dev Biol 16(2):245–259

Ben-Levy R, Leighton IA, Doza YN, Attwood P, Morrice N, Marshall CJ, Cohen P (1995) Identification of novel phosphorylation sites required for activation of MAPKAP kinase-2. EMBO J 14(23):5920–5930

Daugaard M, Rohde M, Jaattela M (2007) The heat shock protein 70 family: highly homologous proteins with overlapping and distinct functions. FEBS Lett 581(19):3702–3710

Dix DJ (1997) Hsp70 expression and function during gametogenesis. Cell Stress Chaperones 2(2):73–77

Dix DJ, Allen JW, Collins BW, Mori C, Nakamura N, Poorman-Allen P, Goulding EH, Eddy EM (1996) Targeted gene disruption of Hsp70-2 results in failed meiosis, germ cell apoptosis, and male infertility. Proc Natl Acad Sci U S A 93(8):3264–3268

Dix DJ, Allen JW, Collins BW, Poorman-Allen P, Mori C, Blizard DR, Brown PR, Goulding EH, Strong BD, Eddy EM (1997) HSP70-2 is required for desynapsis of synaptonemal complexes during meiotic prophase in juvenile and adult mouse spermatocytes. Development 124(22):4595–4603

Doshi BM, Hightower LE, Lee J (2009) The role of Hsp27 and actin in the regulation of movement in human cancer cells responding to heat shock. Cell Stress Chaperones 14(5):445–457

Dun MD, Aitken RJ, Nixon B (2012) The role of molecular chaperones in spermatogenesis and the post-testicular maturation of mammalian spermatozoa. Hum Reprod Update 18(4):420–435

Eddy EM (1999) Role of heat shock protein HSP70-2 in spermatogenesis. Rev Reprod 4(1):23–30

Engel K, Ahlers A, Brach MA, Herrmann F, Gaestel M (1995) MAPKAP kinase 2 is activated by heat shock and TNF-alpha: in vivo phosphorylation of small heat shock protein results from stimulation of the MAP kinase cascade. J Cell Biochem 57(2):321–330

Erbse A, Mayer MP, Bukau B (2004) Mechanism of substrate recognition by Hsp70 chaperones. Biochem Soc Trans 32(Pt 4):617–621

Ewen K, Jackson A, Wilhelm D, Koopman P (2010) A male-specific role for p38 mitogen-activated protein kinase in germ cell sex differentiation in mice. Biol Reprod 83(6):1005–1014

Fan W, Gao XK, Rao XS, Shi YP, Liu XC, Wang FY, Liu YF, Cong XX, He MY, Xu SB, Shen WL, Shen Y, Yan SG, Luo Y, Low BC, Ouyang H, Bao Z, Zheng LL, Zhou YT (2018) Hsp70 interacts with mitogen-activated protein kinase (MAPK)-activated protein kinase 2 to regulate p38MAPK stability and myoblast differentiation during skeletal muscle regeneration. Mol Cell Biol 38(24)

Feng HL, Sandlow JI, Sparks AE (2001) Decreased expression of the heat shock protein hsp70-2 is associated with the pathogenesis of male infertility. Fertil Steril 76(6):1136–1139

Freshney NW, Rawlinson L, Guesdon F, Jones E, Cowley S, Hsuan J, Saklatvala J (1994) Interleukin-1 activates a novel protein kinase cascade that results in the phosphorylation of Hsp27. Cell 78(6): 1039–1049

Hasson SA, Kane LA, Yamano K, Huang CH, Sliter DA, Buehler E, Wang C, Heman-Ackah SM, Hessa T, Guha R, Martin SE, Youle RJ (2013) High-content genome-wide RNAi screens identify regulators of Parkin upstream of mitophagy. Nature 504(7479):291–295

Huusko JM, Karjalainen MK, Graham BE, Zhang G, Farrow EG, Miller NA, Jacobsson B, Eidem HR, Murray JC, Bedell B, Breheny P, Brown NW, Bodker FL, Litterman NK, Jiang PP, Russell L, Hinds DA, Hu Y, 23andMe Research, Rokas A, Teramo K, Christensen K, Williams SM, Ramet M, Kingsmore SF, Ryckman KK, Hallman M, Muglia LJ (2018) Whole exome sequencing reveals HSPA1L as a genetic risk factor for spontaneous preterm birth. PLoS Genet 14(7): e1007394

Ito Y, Ando A, Ando H, Ando J, Saijoh Y, Inoko H, Fujimoto H (1998) Genomic structure of the spermatid-specific hsp70 homolog gene located in the class III region of the major histocompatibility complex of mouse and man. J Biochem 124(2):347–353

Kato K, Ito H, Iwamoto I, Lida K, Inaguma Y (2001) Protein kinase inhibitors can suppress stress-induced dissociation of Hsp27. Cell Stress Chaperones 6(1):16–20

Lambert H, Charette SJ, Bernier AF, Guimond A, Landry J (1999) HSP27 multimerization mediated by phosphorylation-sensitive intermolecular interactions at the amino terminus. J Biol Chem 274(14):9378–9385

Lanneau D, Brunet M, Frisan E, Solary E, Fontenay M, Garrido C (2008) Heat shock proteins: essential proteins for apoptosis regulation. J Cell Mol Med 12(3):743–761

Lee JH, Han YS, Yoon YM, Yun CW, Yun SP, Kim SM, Kwon HY, Jeong D, Baek MJ, Lee HJ, Lee SJ, Han HJ, Lee SH (2017) Role of HSPA1L as a cellular prion protein stabilizer in tumor progression via HIF-1alpha/GP78 axis. Oncogene 36(47):6555–6567

Lima SB, Cenedeze MA, Bertolla RP, Filho PA, Oehninger S, Cedenho AP (2006) Expression of the HSPA2 gene in ejaculated spermatozoa from adolescents with and without varicocele. Fertil Steril 86(6): 1659–1663

Lizama C, Lagos CF, Lagos-Cabre R, Cantuarias L, Rivera F, Huenchunir P, Perez-Acle T, Carrion F, Moreno RD (2009) Calpain inhibitors prevent p38 MAPK activation and germ cell apoptosis after heat stress in pubertal rat testes. J Cell Physiol 221(2):296–305

Lu Z, Cyr DM (1998) Protein folding activity of Hsp70 is modified differentially by the hsp40 co-chaperones Sis1 and Ydj1. J Biol Chem 273(43):27824–27830

Mayer MP, Bukau B (2005) Hsp70 chaperones: cellular functions and molecular mechanism. Cell Mol Life Sci 62(6):670–684

Naaby-Hansen S, Herr JC (2010) Heat shock proteins on the human sperm surface. J Reprod Immunol 84(1):32–40

Niederberger C, Agulnik AI, Cho Y, Lamb D, Bishop CE (1997) In situ hybridization shows that Dazla expression in mouse testis is restricted to premeiotic stages IV-VI of spermatogenesis. Mamm Genome 8(4):277–278

Nixon B, Bromfield EG, Dun MD, Redgrove KA, McLaughlin EA, Aitken RJ (2015) The role of the molecular chaperone heat shock protein A2 (HSPA2) in regulating human sperm-egg recognition. Asian J Androl 17(4):568–573

Paul C, Murray AA, Spears N, Saunders PT (2008) A single, mild, transient scrotal heat stress causes DNA damage, subfertility and impairs formation of blastocysts in mice. Reproduction 136(1):73–84

Radons J (2016) The human HSP70 family of chaperones: where do we stand? Cell Stress Chaperones 21(3):379–404

Rajapandi T, Wu C, Eisenberg E, Greene L (1998) Characterization of D10S and K71E mutants of human cytosolic hsp70. Biochemistry 37(20):7244–7250

Schlecht R, Erbse AH, Bukau B, Mayer MP (2011) Mechanics of Hsp70 chaperones enables differential interaction with client proteins. Nat Struct Mol Biol 18(3):345–351

Scieglinska D, Krawczyk Z (2015) Expression, function, and regulation of the testis-enriched heat shock HSPA2 gene in rodents and humans. Cell Stress Chaperones 20(2):221–235

Son WY, Han CT, Hwang SH, Lee JH, Kim S, Kim YC (2000) Repression of hspA2 messenger RNA in human testes with abnormal spermatogenesis. Fertil Steril 73(6):1138–1144

Stokoe D, Caudwell B, Cohen PT, Cohen P (1993) The substrate specificity and structure of mitogen-activated protein (MAP) kinase-activated protein kinase-2. Biochem J 296(Pt 3):843–849

Takahashi S, Andreoletti G, Chen R, Munehira Y, Batra A, Afzal NA, Beattie RM, Bernstein JA, Ennis S, Snyder M (2017) De novo and rare mutations in the HSPA1L heat shock gene associated with inflammatory bowel disease. Genome Med 9(1):8

Tsunekawa N, Matsumoto M, Tone S, Nishida T, Fujimoto H (1999) The Hsp70 homolog gene, Hsc70t, is expressed under translational control during mouse spermiogenesis. Mol Reprod Dev 52(4):383–391

Vydra N, Malusecka E, Jarzab M, Lisowska K, Glowala-Kosinska M, Benedyk K, Widlak P, Krawczyk Z, Widlak W (2006) Spermatocyte-specific expression of constitutively active heat shock factor 1 induces HSP70i-resistant apoptosis in male germ cells. Cell Death Differ 13(2):212–222

Williams PA, Krug MS, McMillan EA, Peake JD, Davis TL, Cocklin S, Strochlic TI (2016) Phosphorylation of the RNA-binding protein

Dazl by MAPKAP kinase 2 regulates spermatogenesis. Mol Biol Cell 27(15):2341–2350

Wisniewska M, Karlberg T, Lehtio L, Johansson I, Kotenyova T, Moche M, Schuler H (2010) Crystal structures of the ATPase domains of four human Hsp70 isoforms: HSPA1L/Hsp70-hom, HSPA2/Hsp70-2, HSPA6/Hsp70B′, and HSPA5/BiP/GRP78. PLoS One 5(1):e8625

Yin Y, Hawkins KL, DeWolf WC, Morgentaler A (1997) Heat stress causes testicular germ cell apoptosis in adult mice. J Androl 18(2): 159–165

Zhang M, Jiang M, Bi Y, Zhu H, Zhou Z, Sha J (2012) Autophagy and apoptosis act as partners to induce germ cell death after heat stress in mice. PLoS One 7(7):e41412