Expression of Stress Inducible Protein 1 (Stip1) in the Mouse Testis

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ABSTRACT Phthalate esters are considered endocrine disruptors that interfere with the endocrine balance and development of the mammalian testis. Mono-2-ethylhexyl phthalate (MEHP), the active metabolite of the ubiquitously used plasticizer di-2-ethylhexyl phthalate (DEHP), acts upon Sertoli cells as initial target. By subtractive cDNA libraries we identified genes deregulated as response to MEHP in primary cultures of mouse Sertoli cells. The expression of mouse stress inducible protein 1 (Stip1) was detected as upregulated as a result of MEHP exposure. Stip1 is a cochaperone protein that is homologous to the human heat shock cognate protein 70 (hsc70)/heat shock protein 90 (hsp90)—organizing protein (Hop). To assess the presence and localization of Stip1 in mouse testis and its potential role in stress defense, we studied the expression pattern of the Stip1 protein by immunohistochemistry and of the mRNA by in situ hybridization. Both the protein and the mRNA of Stip1 were mainly found in the cytoplasm of all types of spermatogonia and spermatocytes up till zygotene, the expression decreased during late pachytene and was very weak in diplotene spermatocytes and round spermatids. Interestingly, this expression pattern resembled the pattern of stress sensitivity of spermatogenic cells in that the most sensitive cell types show the weakest expression of Stip1. This suggests an important role for Stip1 in the ability of germ cells to survive in stress conditions including high temperatures. *Mol. Reprod. Dev. 73: 1361–1366, 2006.*

Key Words: endocrine disrupters; testis; stress response; spermatogenesis; Sertoli cells

INTRODUCTION

Spermatogenesis is a complex and well-organized developmental process, with germ cells in mitotic cycles, meiosis, and post-meiotic cells undergoing transformation to become spermatozoa (de Rooij and Grootegoed, 1998). The differentiation of germ cells from spermatogonia to spermatozoa occurs in seminiferous tubules under the control of specialized somatic cells: the Sertoli cells. Different types of damage to the spermatogenic process including heat, endocrine disruptors (EDs) such as MEHP, radiation and other toxicants can generate an apoptotic response in germ cells directly or indirectly via Sertoli cells (Potten, 1987). Heat is a widely studied stress agent, mainly in the testis where a temperature of about 5–78C below body temperature is necessary for normal mammalian spermatogenesis. Cells exposed to elevated temperatures or other stress factors respond by synthesizing heat shock proteins (Hsps) (Lindquist, 1986). Hsps as stress proteins (chaperones) and associated proteins (cochaperones) show enhanced synthesis under conditions leading to protein misfolding, preventing protein aggregation of damaged proteins and facilitating refolding (Voellmy, 2004).

Upon heat stress induction, the expression of Hsps is induced by the transient activation of heat shock transcription factor 1 (HSF1) (Morimoto, 1998). In circumstances of no stress, HSF1 is in a transcriptionally inactive non-DNA-binding monomer form in the cytoplasm. After stress induction, it undergoes a transition from a monomer to a homo-dimer form and translocates to the nucleus where it acquires a DNA-binding and transactivation activity (Mosser et al., 1993; Zuo et al., 1994; Wu, 1995). The DNA-binding activity of HSF1 is regulated by hsp70 (Mosser et al., 1993). Since HSF1 induces Hsp70 expression in response to stress, it is considered that Hsp70 functions as a negative feedback mechanism for HSF1 (Abravaya et al., 1992; Mosser et al., 1993; Rabindran et al., 1994; Shi et al., 1998). Another member of the Hsp70 protein family is called heat shock cognate 70 (Hsc70) (Mosser et al., 1993; Nunes and Calderwood, 1995). The function of Hsc70 is to keep HSF1 in the inactive monomeric state (Mosser et al., 1993; Nunes and Calderwood, 1995; Ahn et al., 2005). There is a major difference between the expression patterns of Hsp70 and Hsc70, as

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Hsp70 expression is induced by stress whereas Hsc70 is constitutively expressed (Nunes and Calderwood, 1995). HSF1 directly interacts with Hsc70 regardless of stress conditions (Ahn et al., 2005).

There is another chaperone involved in the modulation of HSF1, Hsp90, a protein from the same heat shock family, which is expressed at high levels even under nonstress conditions and is required for cell viability in eukaryotes (Jakob and Buchner, 1994; Pratt and Toft, 1997). In eukaryotes, Hsp90 fulfills its chaperone function in a complex with a large number of cofactors (Wegele et al., 2003). Primordial germ cells express high levels of Hsp90 as well as both male and female premeiotic germ cells suggesting that Hsp90 may be essential for these cells (Ohsako et al., 1995). Biggiogera et al. (1996) showed that Hsp90 is present in the cytoplasm of all male germ cell types during mouse spermatogenesis.

Stress inducible protein 1 (Stip1) has a role in a dynamic heterocomplex chaperoning system involving Hsps (Smith, 1993). Stip1 interacts with Hsc70 at its N-terminus and with Hsp90 at its C-terminus, to link Hsc70 and Hsp90 (van der Spuy et al., 2001). Whereas Stip1 has also direct chaperone activities on Hsp70 and Hsp90 in vivo (Odunuga et al., 2004), it has no measurable chaperone activity in vitro (Bose et al., 1996; Freeman et al., 1996). Stip1 can indepen- dently regulate individual functions of Hsp70 and Hsp90 (Song and Masison, 2005) and it might move between cytoplasm and nucleus under certain cell cycle conditions (Longshaw et al., 2004).

Steroid receptors are highly dependent on Hsp90 and Hsp70 activity (Pratt and Toft, 1997; Morishima et al., 2000) and for the receptor heterocomplex assembly cochaperones are important (Young et al., 2001; Pratt and Toft, 2003). Molecular chaperones facilitate the folding of other proteins under physiological and stress conditions (Odunuga et al., 2004). The importance of Stip1 in the chaperoning activity of hsp90 of glucocorti- coid and progesterone receptors (PR) has been shown (Kosano et al., 1998; Morishima et al., 2000). Besides being a cochaperone for other proteins, Stip1 also has a role in PR formation (Chen and Smith, 1998). When the hormone-binding activity of PR is lost by heat induction or drug (geldanamycin) treatment, it can be recovered substantially by treatment with Stip1, Hsp70, or Hsp90 in vitro (Dittmar and Pratt, 1997; Dittmar et al., 1997, 1998; Kosano et al., 1998).

Stip1 has been detected in all major mouse organs such as lung, liver, heart, skeletal muscle, spleen, kidney, brain, and various mouse cell lines (Blatch et al., 1997). Furthermore Stip1 expression has been shown in monkey, human, and mouse cell lines, but not in bovine cell lines (Blatch et al., 1997). However, the expression of Stip1 in the testis has not yet been studied.

EDs are synthetic and natural compounds that have the potential to interfere with the normal function of endocrine systems. Some environmental contaminants such as phthalates have been considered as EDs affecting normal testis development and function (Sharpe, 2001; Li and Kim, 2003). Both di-2-ethylhexyl phthalate (DEHP) and its active metabolite mono- 2-ethylhexyl phthalate (MEHP) (Sjoberg et al., 1986) have been shown to interfere with Sertoli cell function (Li et al., 2000; Kavlock et al., 2002). In a search for genes deregulated by MEHP in testicular tubule cells, we found indications that Stip1 becomes upregulated in Sertoli cells cultured in presence of MEHP (data not shown). Hence, we studied the expression pattern of Stip1 in seminiferous tubule cells both at the mRNA and protein levels to correlate its accumulation with the stress sensitivity of the different cell types.

MATERIALS AND METHODS

Experimental Animals

Adult CD-1 male mice were obtained from Charles-River (Sulzfeld, Germany). The experiments were approved by the committee on ethics of animal experimentation Utrecht University. The mice were sacrificed by CO_2 asphyxiation and the testes were removed for Sertoli cell culture or fixed in RNase free Bouin's solution for histology, immunohistochemistry, and in situ hybridization purposes.

Cell Culture

SK49 (Walther et al., 1996) and TM4 (Mather, 1980) Sertoli cell lines were grown at 378C in a humidified incubator under 5%CO_2, 95% air in MEM supplemented with 2.5% FBS, in culture flasks. Culture medium was replaced twice a week. At confluency, cells were passaged following trypsinization with 0.25% trypsin–etylene diamine tetra-acetic acid (EDTA) solution (Invitrogen, Life Tech, Breda, The Netherlands).

Histology and Immunohistochemistry

Pairs of Bouin's fixed 5-mm-thick consecutive sections were used of which one was stained with periodic acid schiff (PAS)-hematoxylin to determine cell types and epithelial stages.

For immunolocalization of Stip1 5-mm paraffin sections were mounted on 3-aminopropyl triethoxysilane (TESPA, Sigma, St. Louis, MO)-coated glass slides and dried overnight at 378C. A three times 10 min antigen retrieval (0.1 mM sodium citrate buffer, pH ¼ 6) step was applied at 700 W in a microwave oven (Polaron H2500 Microwave Processor, BioRad, Hertfordshire, UK). Endogenous peroxidase was blocked with 0.35% H_2O_2 in PBS for 15 min. After blocking in 5% normal horse serum (Vector Laboratories, Burlingame, CA), slides were consecutively incubated with avidin and biotin blocking solutions (Vector Laboratories) then 1 hr at 48C with 1:100 Stip1 monoclonal antibody (BD Biosciences, San Jose, CA) in 1% BSA in PBS (Sigma). Secondary biotinylated horse anti-mouse antibody (BA-2001, Vector Laboratories) was used 1:200 in 1% BSA in PBS for 1 hr at room temperature. Horseradish peroxidase avidin–biotin complex reaction was per- formed according to the manufacturer's protocol (Vector Laboratories). Antibody was finally detected by

diaminobenzidine (DAB; Sigma) in 50 mM Tris-HCl, pH 7.6. Sections were counterstained with hematoxylin, dehydrated, and mounted with Pertex (Cellpath Ltd., Hemel Hempstead, UK).

Mouse IgG (SC-2025, Santa Cruz, CA) was used to replace the primary antibody in immunohistochemistry to check the specificity of the staining.

Reverse Transcription-Polymerase Chain Reaction

RNA was extracted from mouse testis and mouse-derived Sertoli cell lines SK49 (Walther et al., 1996) and TM4 (Mather, 1980) using the FastRNA Pro Green kit (Qbiogene, Illkirch Cedex, France), according to the manufacturer's instructions. Two micrograms of each total RNA fraction was reverse transcribed in a 20 ml volume, using random hexamers and the Superscript II pre-amplification system (Invitrogen), according to the manufacturer's instructions. Reverse transcription reactions were performed with (þRT) and without (−RT) Superscript II reverse transcriptase. Each type of cDNA was used as template for PCR amplification using the Stip1-specific forward (5⁰-CCAGGCTCT-GAGCGAACACT-3⁰) and reverse (5⁰-TCTCATTGGC-GAAGGGAAGA-3⁰) primers. PCRs were carried out in 50 ml volumes, each containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl2, 0.01% gelatin, 200 mM each dNTP, 50 pmol primer 1665, pmol primer 1666, 1 ml cDNA, and 1 U Super*Taq* (HT Biotechnologies Ltd, Cambridge, UK). Stip1-specific PCR products of ~125 bp were visualized on 2% agarose gels.

Preparation of RNA Probes

A ~350 bp mouse Stip1 cDNA insert, cloned in the vector pGEM-T-Easy (Promega, Madison, WA), was linearized by *Pst* I and *Sph* I, and in vitro transcribed to generate antisense and sense DIG-labeled cRNAs using DIG-RNA labeling mix (Roche, Penzberg, Germany), and T7 RNA polymerase and SP6 RNA polymerase, respectively, according to the manufacturer's instructions.

In Situ Hybridization

After deparaffinization of the sections, a post-fixation step was applied with 4% paraformaldehyde (PFA) solution for 15 min at room temperature. A second 4% PFA post-fixation step was applied after proteinase K treatment at a concentration of 10 mg/ml. After the second 4% PFA post-fixation step, DNase (Roche, RNase free, 100 U in PBS) control was introduced for 1 hr at 378C. Sections were hybridized overnight at 558C with 200 ng/ml of mSTI1 digoxigenin-labeled RNA probes in the hybridization solution (10% dextran sulfate, 4x SSC (1 x SSC ¼ 150 mM NaCl, 15 mM sodium citrate, pH 7.2), 50% formamide, 10 mM dithiothreitol (DTT), 0.25 mg/ml poly A, 0.25 mg/ml of denatured and sheared herring sperm DNA, and 1x Denhardt's buffer), following 2 hr of pre-hybridization step 558C. After hybridization, sections were quickly washed with 1 x SSC (containing 10 mM DTT) at room temperature, two successive washes of 15 min were done with 1 x SSC (containing 10 mM DTT) at 558C, with further two washes of 15 min with 0.5 x SSC (containing 10 mM DTT) also at 558C. RNase treatment was introduced immediately after the stringency washes. The slides were then washed three times with Tris-buffered saline (TBS) and incubated 30 min in a blocking solution (TBS þ0.3% Triton X-100 þ2% normal sheep serum). The RNA probes were detected using a monoclonal anti-digoxigenin antibody (Roche) in a 1:100 dilution in the blocking solution. After overnight incubation at 48C, the slides were washed with TBS four times (10 min each) and incubated with the biotinilated anti-sheep secondary antibody (Vector Laboratories) 1/100 dilution in PBS for 1 hr at room temperature. Horseradish peroxidase avidin–biotin complex reaction was performed according to the manufacturer's protocol (Vector Laboratories). Finally detection was done by DAB (Sigma) in 50 mM Tris-HCl, pH 7.6. Sections were counterstained with hematoxylin, dehydrated, and mounted with Pertex (Cellpath Ltd.).

SDS–PAGE and Western Blotting

Protein lysates from testis, liver, spleen, brain tissue homogenates were prepared in RIPA buffer (PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) including 1 mm phenylmethylsulfonylfluoride. Of each sample, 50 mg were separated on a 12% SDS-polyacrylamide gel and blotted onto a polyvinylidene fluoride membrane (Millipore Corp., Bedford, MA). Western blot was blocked using Blotto-A, containing 5% Protifar (Nutricia, Zoetermeer, The Netherlands) in TBS (10 mm Tris; 150 mm NaCl, pH 7.6), including 0.05% Tween-20. Mouse monoclonal anti-Stip1 antibody was diluted 1:500 in Blotto-A and incubated overnight at 48C. Blot was washed with TBS including 0.05% Tween-20. After incubation with rabbit anti-mouse-HRP (1:1,000, P-0260, DAKO Cytomation, Glosturp, Denmark) sec- ondary antibody for 1 hr, blots were incubated with ECL and exposed to X-ray film (RX-omat, Kodak, Chalone/ Saone, France).

RESULTS

By both approaches: immunohistochemistry and in situ hybridization of mRNA we could not clearly detect expression of Stip1 in Sertoli cells. A weak signal was detected in the irregular shape of these cells in the seminiferous epithelium. A low expression of Stip1 in Sertoli cells was also observed by dot blot analysis of RNAs from cultured Sertoli cells (data not shown). However, other tubule cell types displayed a strong expression of Stip1.

Immunohistochemistry revealed the localization of the Stip1 protein in the cytoplasm of A spermatogonia, intermediate (In) spermatogonia, B spermatogonia and spermatocytes. A particularly strong staining was observed in the cytoplasm of pre-leptotene and leptotene spermatocytes while the staining intensity was decreasing in the cytoplasm of pachytene spermatocytes (Fig. 1A). In epithelial stage XI, in the cytoplasm of

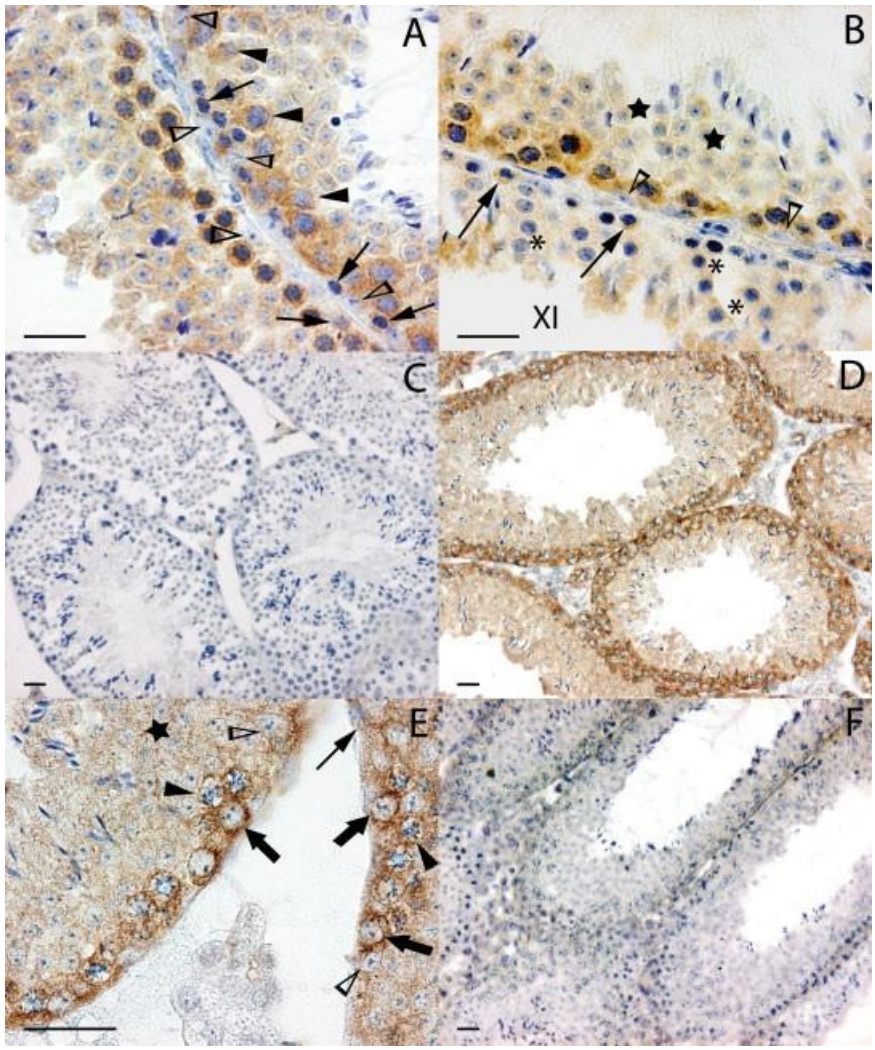

Fig. 1. A: Immunohistochemistry for Stip1 staining was found in the cytoplasm of spermatogonia (thin arrow), a decreasing staining in pachytene spermatocytes (arrow head), and in Sertoli cell nuclei (hollow arrow head) B: Immunohistochemistry, spermatogonia (thin arrow), and weakly stained diplotene spermatocytes (asterisk) in seminiferous tubule Stage XI. Weakly stained round spermatids (star), Sertoli cell nuclei (hollow arrow head) C: Immunohistochemistry, negative control slide, D: In situ hybridization for Stip1 mRNA. Antisense probe hybridized slide, staining in the cytoplasm of spermatogonia (thin arrow), pre-leptotene spermatocytes (thick arrow), pachytene spermatocytes (arrow head), decreasing staining in round spermatids (star) and Sertoli cell nuclei (hollow arrow head) E: In situ hybridization, DNase-, and Rnase-treated control slide. F: ISH, sense probe hybridized slide. Bars represent 20 mm.

diplotene spermatocytes the protein expression was very weak (Fig. 1B). The staining intensity further decreased in round and elongating spermatids. As negative controls for the immunohistochemistry we applied mouse IgG in a concentration equal to the IgG concentration of the applied specific antibody. We did not detect any staining in the negative control slides (Fig. 1C).

In situ hybridization analysis showed that the Stip1 mRNA was localized in early germ cells in the seminiferous epithelium in all epithelial stages (Fig. 1D). The expression of Stip1 mRNA was clearly evident in the cytoplasm of all spermatogonial cell types as well as pre-leptotene and leptotene spermatocytes. The signal began to diminish in pachytene spermato- cytes reaching a weak level at diplotene spermatocytes. Round and elongated spermatids showed very low expression of Stip1 mRNA. DNase and RNase treatments were applied to see whether the signal was specific. After treatment of DNase and RNase the signal did not diminish (Fig. 1E). Also, as a control, the DIG-labeled sense riboprobe was applied in the same concentration as the antisense probe, and no signal was detected (Fig. 1F).

The immunohistochemical and in situ hybridization experiments did not reveal any staining in the interstitial tissue.

In addition, to confirm the presence of Stip1 in Sertoli cells we performed RT-PCR on mouse derived Sertoli cell lines (SK49 and TM4) and on whole mouse testis. We observed the expression of Stip1 in SK49 and TM4 Sertoli cell lines and in mouse testis (Fig. 2). We further

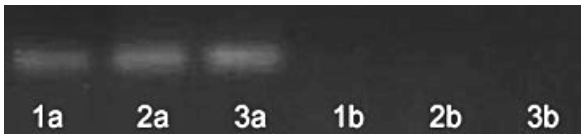

Fig. 2. RT-PCR results showing the presence of Stip1 mRNA in Sertoli cell lines and mouse testis. 1a: (þ) RT in SK49 cell line; 2a, (þ) RT in TM4 cell line; 3a, (þ) RT in mouse testis; 1b, (−) RT in SK49 cell line; 2b, (−) RT in TM4 cell line; 3b, (−) RT in mouse testis.

confirmed the expression of Stip1 in mouse testis by Western Blot technique (Fig. 3).

DISCUSSION

The Stip1 mRNA was found in the cytoplasm of all types of spermatogonia, pre-leptotene, leptotene, and zygotene spermatocytes, began to fade in pachytene and diplotene spermatocytes and the signal reached the weakest level in round and elongating spermatids. The presence of the Stip1 protein completely mirrored that of its mRNA showing exactly the same localization.

Comparing this localization to that of other components of the Hsp system: Hsp90 is present in all types of germ cells and Sertoli cells in the testis (Ohsako et al., 1995; Biggiogera et al., 1996; Ogi et al., 1999; Huang et al., 2005). Two members of the hsp70 family are expressed during spermatogenesis, one is spermatocyte-specific Hsp70 (hsp70-2) expressed from zygotene through diplotene, but especially at high levels in pachytene spermatocytes (Dix et al., 1997; Zhu et al., 1997). The other member, testis specific Hsp70 (hsc70-t) (Maekawa et al., 1989; Matsumoto and Fujimoto, 1990) first appears in spermatids. While the mRNA is expressed in round spermatids it translates effectively until these cells progress to the transcriptionally inactive stage coinciding with chromatin condensation (Tsunekawa et al., 1999). HSF1 is expressed in all germ cells in the testis (Nakai et al., 2000). Mice over-expressing an active form of HSF1 in the testis are reported to be infertile due to an arrest in late spermatocytes and apoptosis of these cells (Nakai et al., 2000; Widlak et al., 2003). The figures in the article by Nakai et al. (2000) suggest that diplotene spermatocytes are the ones that enter apoptosis.

Spermatogenesis is sensitive to elevated temperatures. Cryptorchidism, leads to germ cell degeneration by hyperthermia, causing infertility (Chowdhury and Steinberger, 1970). Short-term experimental cryptorchidsm or experiments of heat stress in testis revealed spermatocytes and round spermatids as the most sensitive cell types (Henriksen et al., 1995; Yin et al., 1997; Wang et al., 1998; Tomomasa et al., 2002).

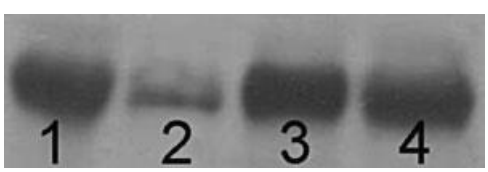

Fig. 3. Western Blot results showing the expression of Stip1 protein in mouse testis as well as mouse liver, brain, and spleen. Starting from left side; lane 1 liver, lane 2 brain, lane 3 spleen, and lane 4 testis.

It is intriguing that in the seminiferous epithelium the cell types showing the highest vulnerability to stress conditions coincide with low amounts of Stip1 in these cells. Stip1 expression decreased in pachytene spermatocytes and was hardly detectable from diplotene onwards. To explain this, it should be taken into account that HSF1 overexpression induces apoptosis of late spermatocytes and spermatids. Apparently high levels of active HSF1 are deleterious to cells. Hsc70 and Hsp90 serve to keep HSF1 in the inactive state and the functions of Hsc70 and Hsp90 in turn are regulated by Stip1. The present results suggest that in the absence of Stip1, high levels of HSF1 as induced by increased temperatures or other stressing factors may be fatal to spermatogenic cells.

In conclusion, for the first time we show that Stip1 is present in low amounts in Sertoli cells and is mainly expressed in all types of spermatogonia, pre-leptotene, leptotene, and zygotene spermatocytes. Then Stip1 expression starts to decrease in pachytene spermatocytes and diminishes further in diplotene spermatocytes and in spermatids. Interestingly, the correlation between low accumulation of Stip1 and higher sensitivity to stress suggests a crucial role for Stip1 in the defense against stress conditions in the seminiferous epithelial cells.

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