

# Expression pattern of HSFY in the mouse testis and epididymis with and without heat stress

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**Abstract** Heat shock factors (HSFs) are critical regulators of spermatogenesis. However, heat shock responses, the associated components and the underlying functional mechanisms remain to be elucidated. Here, we characterize the expression pattern of HSFY, a member of the HSF family in the testis and epididymis. Its expression in testis and epididymis was initially identified by western blots. Immunofluorescence staining demonstrated that HSFY was confined to the cytoplasm of late spermatocytes and spermatids in adult testes, gonocytes in newborn testes and undifferentiated spermatogonia in 7 days post-parturition testes. In the epididymis, HSFY was predominantly expressed in principal cells. Furthermore, a single transient scrotal heat stress did not change HSFY protein expression in the testes or epididymis, either on the expressional level or in cellular localization. In summary, this study detected the expression pattern of HSFY in the testes and epididymis and demonstrated that its expression was not regulated by transient elevated temperature.

**Keywords** HSFY · Epididymis · Expression · Heat stress · Testis

## Introduction

Heat shock factors on chromosome Y (HSFY) are involved in spermatogenesis regulation and are potentially associated with

maturation arrest in human non-obstructive azoospermia (Stahl et al. 2012). HSFY is a member of the heat shock transcriptional factor (HSFs) family, which bind heat shock elements on heat shock protein (HSP) genes to regulate HSPs gene expression (Pirkkala et al. 2001). HSPs are important mediators of stress and act as molecular chaperones to regulate cellular homeostasis and protect cells from death (Hartl 1996). HSFY has been postulated to be functionally different from other classical HSFs, because its DNA-binding domain (DBD) shows only 30 % homology to the classic HSF DBD (Shinka et al. 2004). In addition, it is not clear if HSFY is regulated by high temperature like other HSFs.

HSFY's expression is restricted to the testis (Tessari et al. 2004). It is expressed in Sertoli and spermatogenic cells in humans (Shinka et al. 2004). In mouse testes, *in situ* hybridization analysis has shown that HSFY mRNA is predominantly expressed in round spermatids but not in Sertoli, Leydig, or myoid cells (Kinoshita et al. 2006). These conflicting reports need to be clarified. Although HSFY has been regarded as testis-specific among multiple tested tissues, the epididymis, which is very important for sperm mobility and motility, was not included in their tissue list (Tessari et al. 2004).

Thus, the purpose of this study was to determine the exact expression pattern of HSFY in the testis and epididymis and to evaluate its expression before and after scrotal heat treatment.

## Materials and methods

### Ethics statement

The present study was approved by the Institutional Ethics Committee of China Agricultural University. All experiments with mice were conducted ethically according to the

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guidelines of the National Institutes of Health for the Care and Use of Laboratory Animals.

### Induction of transient heat stress and tissue collection

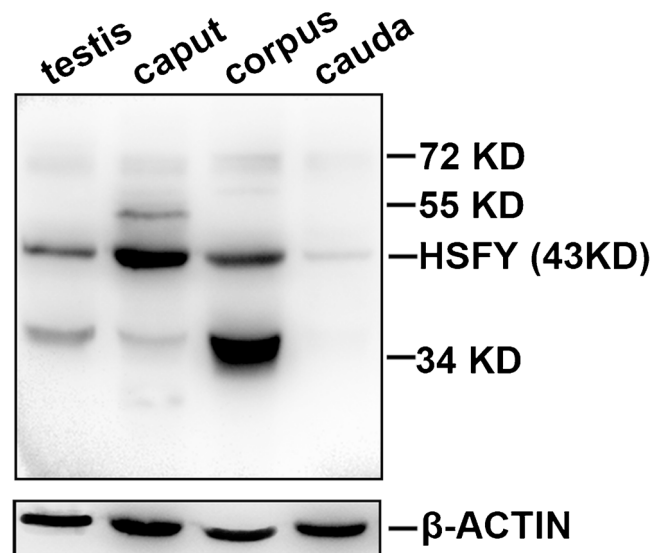
Transient heat stress treatment of mice was conducted as previously reported (Paul et al. 2009). Briefly, adult male C57BL/6 mice were anaesthetized and the lower third of their body (hind legs, tail, and scrotum) was submerged in a water bath of 43 °C for 15 min, 30 min, 1 h, and 2 h, respectively. After treatment, each animal was dried and returned to their cages. Control mice were anaesthetized and left at room temperature; then 24 h later, all the animals were sacrificed. Their right testis and epididymis were collected for immunofluorescence staining and their left testis and epididymis were collected for western blots. This experiment was repeated three times.

### Immunofluorescent staining

The testes and epididymides were fixed in 4 % paraformaldehyde, embedded in paraffin and cut into 5- $\mu$ m sections. Sections were dewaxed, rehydrated and antigen retrieval was accomplished by microwaving in 0.01 M sodium citrate buffer (pH 6.0) for 4 min. After rinsing for 5 min with phosphate buffer saline (PBS), non-specific binding was blocked by incubating the slides in 5 % normal goat serum for 30 min. The sections were then incubated with rabbit anti-HSFY (1:80, ab155418; Abcam) in 5 % NGS at 37 °C for 1.5 h or a normal rabbit IgG control at the same concentration (0.0125 mg/ml, AB-105-C; R&D Systems). For co-staining of HSFY and  $\gamma$ H2AX, Alexa Fluor 488 conjugated mouse anti- $\gamma$ H2AX (1:200, 05-636-AF488; Millipore) was added together with anti-HSFY. After washing in PBS, slides were incubated with Alexa-Fluor 555 conjugated goat anti-rabbit IgG (1:200; Life Technologies) for 1 h. Finally, the sections were mounted using mounting media with DAPI (H1200; Vector Labs). Fluorescence was observed and photographed using a BX53 microscope (Olympus). The rabbit IgG incubated sections were captured in the same conditions and with the same exposure time as those of anti-HSFY incubated section.

### Western blot analysis

Mouse testes and epididymides were homogenized in RIPA buffer. Protein lysates were separated by 12 % SDS-PAGE gel and transferred to polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked in 5 % non-fat milk, subsequently incubated with primary antibodies in blocking solution overnight at 4 °C. After washing in tris-buffer saline with 0.1 % Triton X-100, the membranes were incubated with horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (1:5000, ab6721, Abcam) or HRP conjugated goat anti-mouse IgG (1:5000, ab6789; Abcam) for 1 h at room temperature.



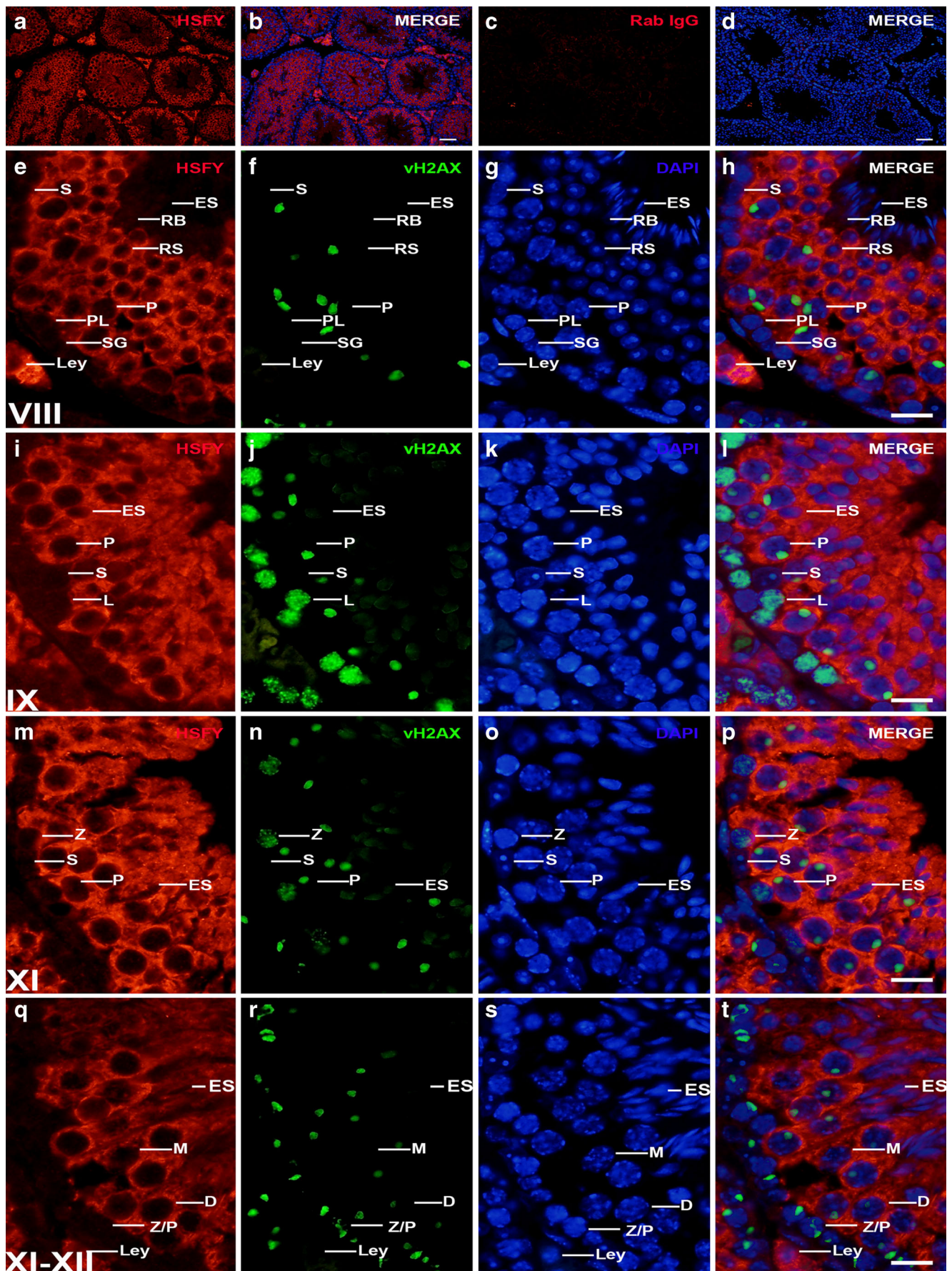
**Fig. 1** Western blot detection of HSFY protein in the testis, caput, corpus, and cauda, and a 43-kDa protein band of HSFY was detected in all four samples.  $\beta$ -actin was used as a loading control

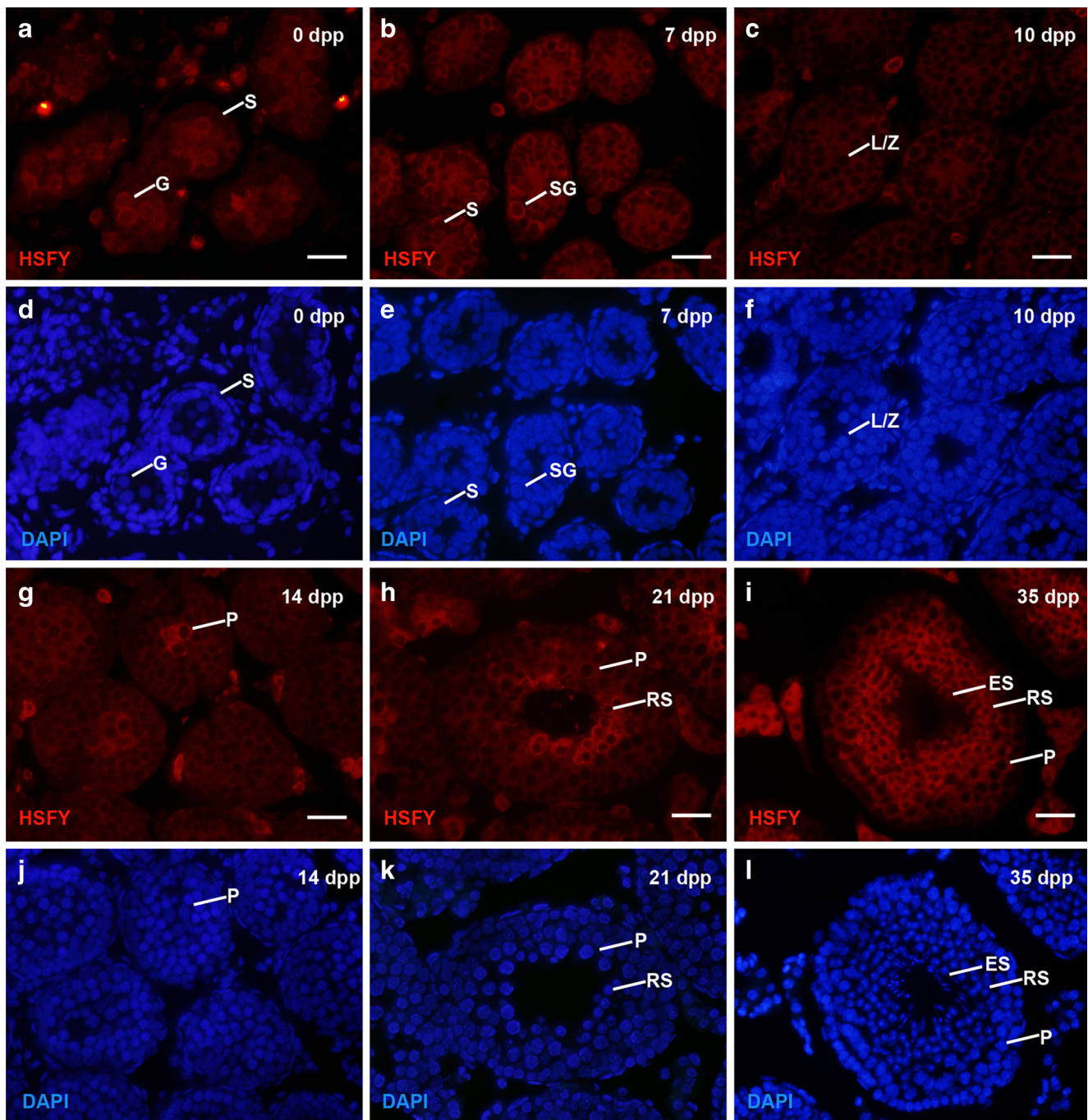
Membranes were incubated with chemiluminescent substrates (Millipore) for detection. The primary antibodies used were as follows: rabbit anti-HSFY (1:2000, ab155418; Abcam), mouse anti-HSP70 (1:2000, ab47455; Abcam) and mouse anti-beta-actin-HRP (1:20000, ab49900; Abcam). Each membrane was stained with all three antibodies one after the other, after being stripped of the previous antibody in stripping solution (25 mM glycine-HCL, 1 % SDS, pH 2.0).

### Results

HSFY was reported to be expressed in the testis but not in other tissues like the colon, prostate and ovary (Tessari et al. 2004). However, the epididymis, an important male reproductive organ, was never tested for HSFY expression. Our results indicate that HSFY, a ~43-kDa protein, is expressed in the testis and in all of the three anatomical regions of epididymis, including the caput, corpus and cauda (Fig. 1). The HSFY protein band in the cauda is much weaker than those in the caput and corpus, indicating a lower expression in the cauda.

**Fig. 2** Localization of HSFY in adult mice testes. Using indirect immunofluorescent staining, HSFY (red) was specifically present in the cytoplasm of germ cells at specific stages. Normal rabbit IgG control is shown in (c, d). Stages of spermatocytes were determined from the  $\gamma$ H2AX staining (green). The central seminiferous tubule in (e, i, m and q) is at the stage of VIII, IX, XI and XI–XII, respectively. SG spermatogonium, PL pre-leptotene spermatocyte, L leptotene spermatocyte, Z zygotene spermatocyte, P pachytene spermatocyte, D diplotene spermatocyte, RS round spermatid, ES elongated spermatid, RB residual body, S Sertoli cell, Ley Leydig cell. Scale bars (b, d) 50  $\mu$ m; (h, l, p and t) 30  $\mu$ m



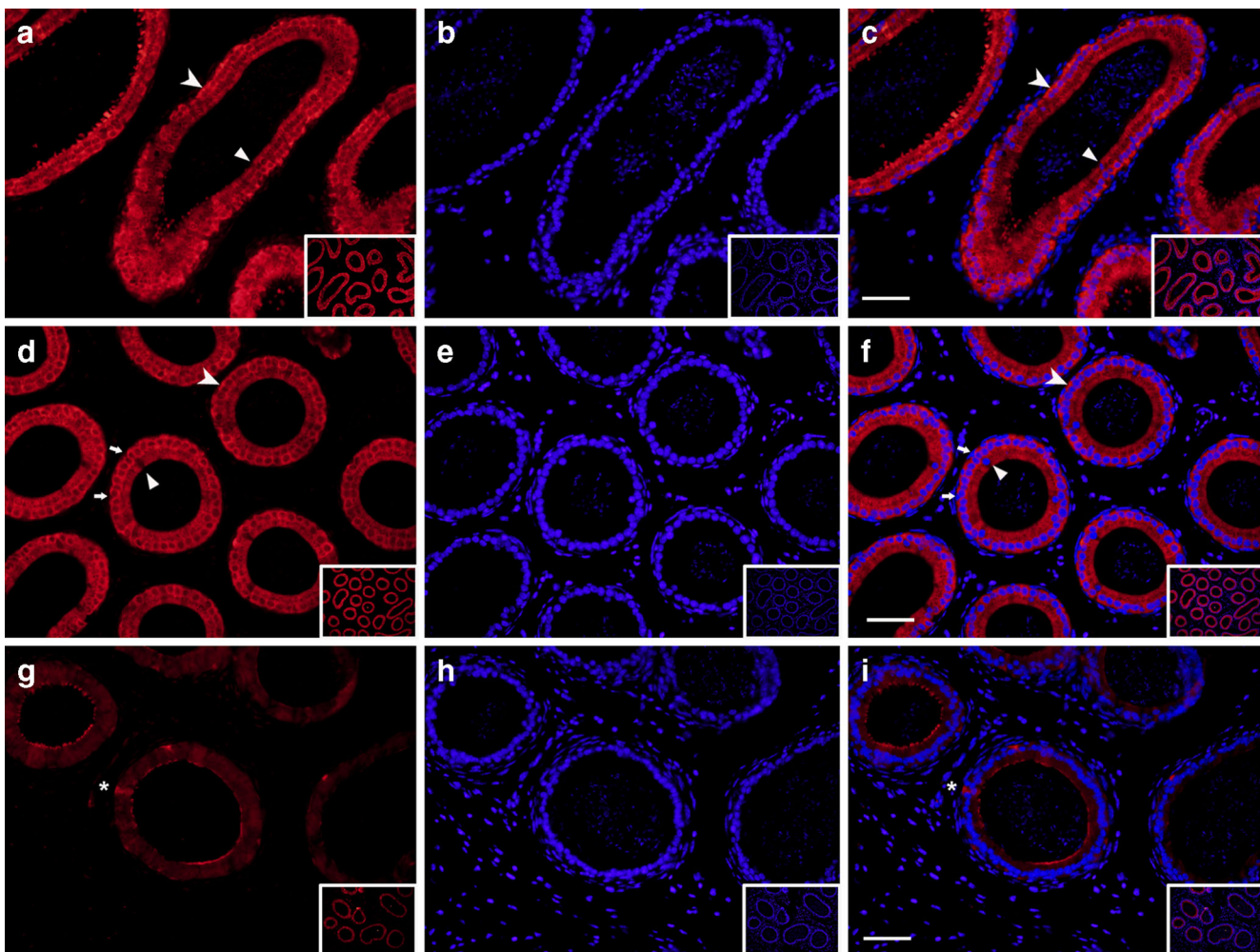


**Fig. 3** HSFY is expressed in mouse testes at various developmental stages. **a, d** Post-parturition day 0 (0 dpp). **b, e** Post-parturition day 7 (7 dpp). **c, f** Post-parturition day 10 (10 dpp). **g, j** Post-parturition day 14 (14 dpp). **h, k** Post-parturition day 21 (21 dpp). **i, l** Post-parturition day 35 (35

dpp). *S* Sertoli cell, *G* gonocyte, *SG* spermatogonium, *L* leptotene spermatocyte, *Z* zygotene spermatocyte, *P* pachytene spermatocyte, *RS* round spermatid, *ES* elongated spermatid. Scale bar 30  $\mu$ m

To investigate the cell type-specific expression of HSFY in mouse testes, we examined HSFY localization using immunofluorescence. HSFY expression was found in the cytoplasm of spermatocytes (Fig. 2), round spermatids (Fig. 2e–h) and elongating spermatids (Fig. 2i–t) but not in spermatogonia (Fig. 2e–h). To further identify the type of spermatocyte that expresses HSFY, we carried out co-immunofluorescence staining of

HSFY and  $\gamma$ H2AX. The results show that HSFY is expressed in pachytene (Fig. 2e–h) and diploleptotene (Fig. 2q–t) spermatocytes but not in pre-leptotene (Fig. 2e–h), leptotene (Fig. 2i–l) or zygotene (Fig. 2m–p) spermatocytes. HSFY protein also exhibited a weak expression level in tubules of stage VIII lumen (Fig. 2e–h). It is tempting to speculate that HSFY weakly localizes to residual bodies (RB) at stage VIII, given that the RB,



**Fig. 4** Localization of HSFY in adult mouse epididymis. Three regions were detected, including caput (a–c), corpus (d–f) and cauda (g–i). HSFY (red) was present mainly in the cytoplasm of principal cells (dovetail

arrowhead) and in some clear cells (asterisk). Basal cells (arrow) and apical cells (triangular arrowhead) did not express HSFY. Scale bar 50  $\mu$ m

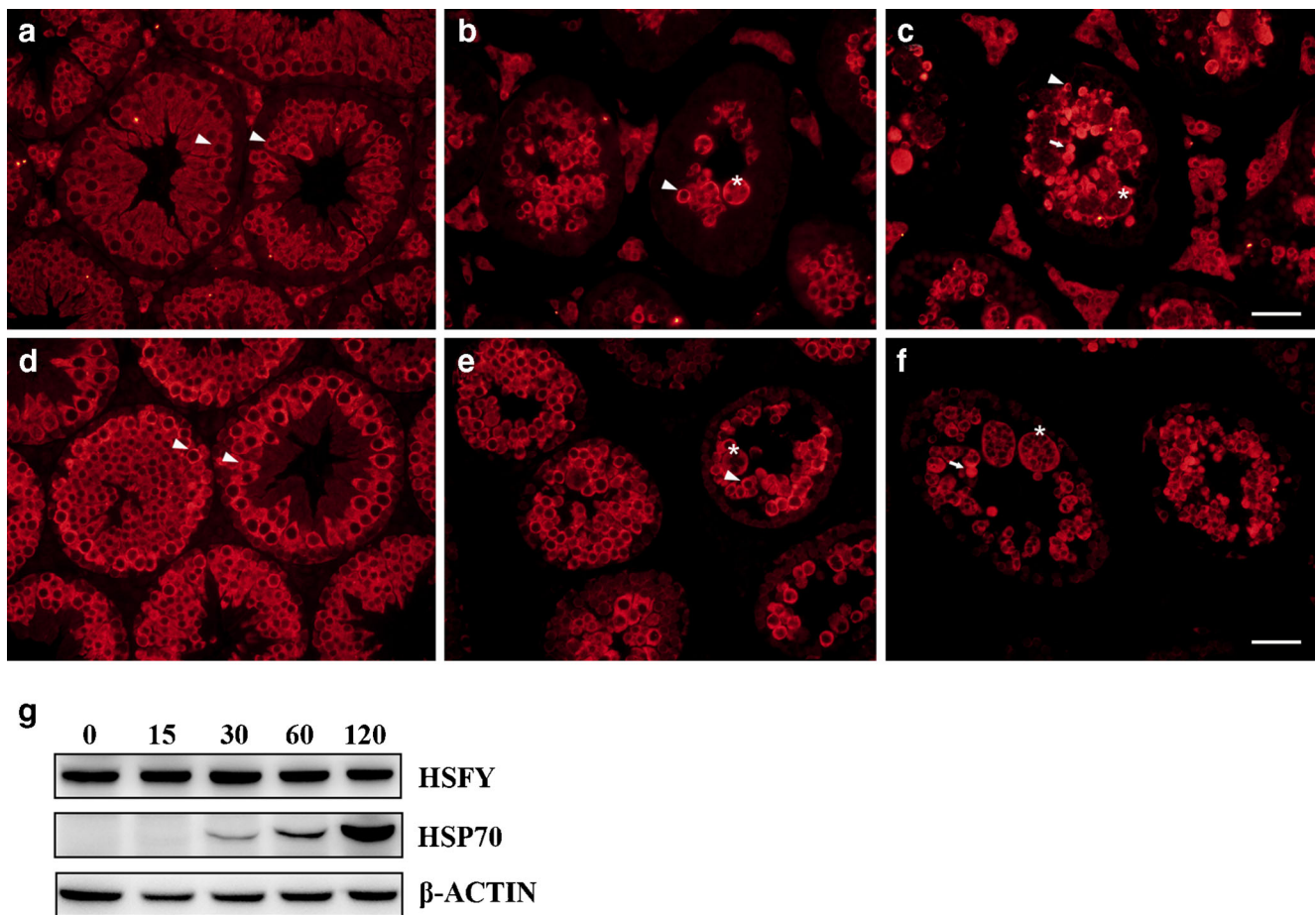
the excessive cytoplasm of spermatids, is only present between stages VII and VIII. Nevertheless, there was no positive staining in epididymal spermatozoa (shown in Fig. 4). Results of the rabbit IgG control at identical concentrations (Fig. 2c, d) showed no obvious fluorescence in the germ cell and weak fluorescence in the cytoplasm of Leydig and Sertoli cells. For this reason, although there was positive HSFY staining in Leydig cells (Fig. 2h, t), we cannot rule out non-specific binding in Leydig cells. In addition, there was no positive staining in Sertoli cells (Fig. 2e–p). In summary, HSFY was stage-specifically expressed in the cytoplasm of germ cells from pachynema to elongating spermatids in adult mice testes.

Next, the expression profile of HSFY protein during mouse maturation was investigated. Tissue sections were prepared from testes ages 0, 7, 10, 14, 21 and 35 days post-parturition (dpp). HSFY was expressed in the cytoplasm of gonocytes in 0 dpp testes and spermatogonia in the 7 dpp testes in a diffused pattern but not in Sertoli cells (Fig. 3a, b). HSFY expression diminished during the differentiation phase of spermatogonia

(Fig. 3c) and reinitiated at the onset of meiosis in pachytene spermatocytes on 14 dpp (Fig. 3g). Furthermore, its expression in round spermatids on 21 dpp (Fig. 3h) and elongated spermatids on 35 dpp (Fig. 3i) confirmed the results in adult testes. Together, our results show that HSFY is a germ cell-specific protein with broad temporal expression dynamics and with restricted expression in specific adult germ cell types.

To explore the expressional pattern of HSFY in the epididymis, we conducted immunofluorescence staining in the cauda, corpus and caput. The result showed that HSFY was predominantly present in the principal cells of the caput and corpus regions (Fig. 4a, d). In the cauda, HSFY expression was very weak in principal cells, whereas it was stronger in a few clear cells (Fig. 4g), validating the western blot result (Fig. 1). It seems that neither basal cells nor apical cells express HSFY (Fig. 4d).

HSFY belongs to the HSF family that has been shown to regulate HSP in heat stress. However, whether HSFY is induced by high temperature like other HSFs is yet unknown.



**Fig. 5** The localization and expression level of HSFY in the mouse testis was not influenced by heat shock. Adult mice scrotums were heated by a warm water bath (43 °C) for different durations. The expression of HSFY (a–c) and germ cell marker MVH (d–f) were detected by

immunofluorescence. **a, d** Control; **b, e** 30 min heat treatment; **c, f** 2 h heat treatment. **g** Western blot analysis of HSP70 and HSFY. HSP70 was used as a heat shock positive control;  $\beta$ -actin was used as a loading control

We employed a single transient scrotal heat stress on mice by immersion in a water bath at 43 °C for different times. Western blot results showed that the expression level of HSFY protein was unchanged in both the testis (Fig. 5g) and epididymis (Fig. 6e). For comparison, the levels of the inducible HSP70 protein increased over time (Figs. 5g; 6e). This indicates that the expressional level of the HSFY protein was not induced by the heat treatment procedures provided in this study.

To examine whether the localization of the HSFY protein was regulated by heat shock, immunofluorescence staining was conducted in the testes and epididymides. Results show that some nuclear and much syncytial staining of HSFY was detected in the seminiferous tubules in the heat shock groups (Fig. 5b, c). To identify whether HSFY's relocalization was specifically induced by heat, or was due to germ cell damage, we immunofluorescence stained for the germ cell marker MVH. MVH had the same expression pattern as HSFY in the heat shock groups (Fig. 5e, f) indicating that the relocalization of HSFY in the nucleus and syncytium was not specific.

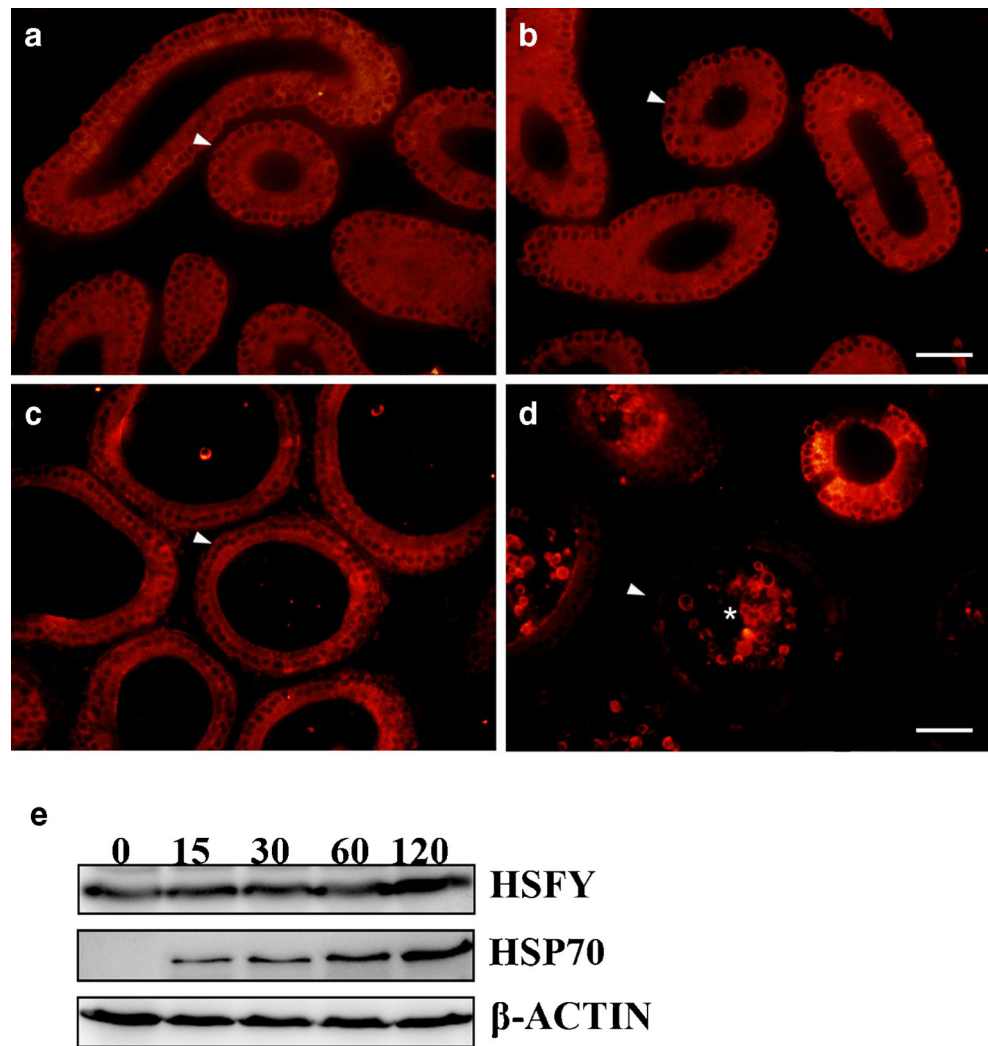
In the caput and corpus, the distributions of HSFY did not obviously change either but some of epitheliums of the corpus showed weaker staining. In addition, many round spermatids were found in the corpus, with strong positive staining.

## Discussion

The human HSFY gene is located in azoospermic factor region b (AZFb) on the Y chromosome (Ferlin et al. 2003) and, therefore, HSFY has been suggested to be a good candidate for an azoospermic factor (Shinka et al. 2004; Vinci et al. 2005). The murine HSFY-like sequence is the mouse orthologue of the HSFY gene (we also referred to it as HSFY in this study), whose HSF-type DBD has 70 % homology to that of human HSFY (Shinka et al. 2004).

HSFY is reported to be testis-specific in human (Tessari et al. 2004), mouse (Kinoshita et al. 2006) and cattle (Hamilton et al. 2011). It is expressed in Sertoli and spermatogenic cells in humans (Shinka et al. 2004) and in round spermatids but not

**Fig. 6** The localization and expression level of HSFY is not influenced by heat shock in the mouse epididymis. Adult mice scrotums were heated by a warm water bath (43 °C) for different durations. The expression of HSFY was detected by immunofluorescence. **a** Control caput; **b** 2-h heat shock caput; **c** control corpus; **d** 2-h heat shock corpus. **e** Western blot analysis of HSP70 and HSFY. HSP70 was used as a heat shock positive control;  $\beta$ -actin was used as a loading control



in Sertoli cells in mice (Kinoshita et al. 2006). In this study, HSFY was detected mainly in spermatocytes and spermatids but not in Sertoli cells, which partially confirms the previous results in mice (Kinoshita et al. 2006). However, due to non-specific staining, it is hard to assert via the techniques used in this study the expression of HSFY in Leydig cells.

Additionally, we report in detail, for the first time, the postnatal expression profile of HSFY in mice and found unexpectedly that it was expressed in the gonocytes at birth and in spermatogonia at 1 week but not in spermatogonia thereafter. This suggests that HSFY potentially plays a role in the proliferation of spermatogonial stem cells and progenitor spermatogonia.

Although HSFY was identified as a testis-specific protein among multiple tissues, the epididymis was not included in those studies. In the present study, we detected the expression of HSFY in the epididymis by western blot and immunofluorescence staining. HSFY heavily stained in the caput and corpus and barely in the cauda. We presume that its different expression patterns are probably related to different functions of HSFY in each segment. Immunofluorescence

staining results revealed that HSFY is dominantly present in principal cells, which are active in the secretion of proteins to and also the endocytosis of proteins from, the epididymal lumen. This indicates that HSFY may be involved in epididymal secretion and endocytosis processes. However, more experimental evidence is needed to prove whether HSFY plays a role in epididymal physiology.

HSFY was postulated to have functions different from those of classical HSFs, such as HSF1 and HSF2 (Shinka et al. 2004), because its presumed DBD shows only 30 % homology to classical DBDs and lacks a structure needed for binding to heat shock elements (HSEs) upstream of the HSP gene (Shinka et al. 2004). Thus, it was not considered to regulate HSPs transcription and be influenced by heat stress. In order to explore whether the expression level of HSFY was regulated by heat stress, we examined its expressional variation after heating the scrotum in a 43 °C water bath for different durations. We detected germ cell syncytium in the testis, apoptosis cells and the induced expression of HSP70 in both the testis and epididymis after heat treatment, which are important features of heat stress (Pei et al.

2012). However, the levels of HSFY remained steady throughout the heat shock treatment in the present study.

Previous studies have shown that post-translational modification of HSFs induced by heat is even more important than their expressional level changes in transcriptional regulation (Xu et al. 2012). For example, HSF1 is a monomeric phosphoprotein that normally interacts with its major repressor, the HSP90 complex. Upon heat shock, HSF1's threonine residue at the 142 site is phosphorylated, which allows it to dissociate from HSP90 and bind HSP genes binding (Soncin et al. 2003). There are also several post-translational modifications on HSFY (Xu et al. 2012), although their functions are currently unknown. Therefore, more functional studies need to be carried out to delineate the precise functions and mechanisms of HSFY in heat shock and spermatogenesis.

In summary, our study revealed the expressional pattern of HSFY in the testis and epididymis and demonstrated that elevated temperatures used in the present study do not result in an expressional change of the HSFY protein.

#### Compliance with ethical standards

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**Conflict of interest** The authors declare that they have no conflict of interest.

**Informed consent** Informed consent was obtained from all individual participants included in the study.

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