



Roles of heat shock factors in gametogenesis and development

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Heat shock factors form a family of transcription factors (four in mammals), which were named according to the first discovery of their activation by heat shock. As a result of the universality and robustness of their response to heat shock, the stress-dependent activation of heat shock factor became a 'paradigm': by binding to conserved DNA sequences (heat shock elements), heat shock factors trigger the expression of genes encoding heat shock proteins that function as molecular chaperones, contributing to establish a cytoprotective state to various proteotoxic stress and in several pathological conditions. Besides their roles in the stress response, heat shock factors perform crucial roles during gametogenesis and development in physiological conditions. First, during these process, in stress conditions, they are either proactive for survival or, conversely, for apoptotic process, allowing elimination or, inversely, protection of certain cell populations in a way that prevents the formation of damaged gametes and secure future reproductive success. Second, heat shock factors display subtle interplay in a tissue- and stage-specific manner, in regulating very specific sets of heat shock genes, but also many other genes encoding growth factors or involved in cytoskeletal dynamics. Third, they act not only by their classical transcription factor activities, but are necessary for the establishment of chromatin structure and, likely, genome stability. Finally, in contrast to the heat shock gene paradigm, heat shock elements bound by heat shock factors in developmental process turn out to be extremely dispersed in the genome, which is susceptible to lead to the future definition of 'developmental heat shock element'.

Introduction

Scientists working on the heat shock response (HSR) have focused on developmental processes because of the remarkably unusual characteristics of heat shock protein (Hsp) expression in pre-implantation embryos and gametogenesis. A strikingly elevated expression of

Hsps is displayed by embryos [1–3], during gametogenesis [4–11], and in stem cell and differentiation models [12–16], and was shown to be stage-specific and tissue-dependent. Moreover, early embryos and stem cell models, as well as male germ cells, exhibited impaired

Abbreviations

Bfsp, lens-specific beaded filament structural protein; FGF, fibroblast growth factor; GVBD, germinal vesicle breakdown; HSF, heat shock factor; Hsp, heat shock protein; HSR, heat shock response; LIF, leukemia inhibitory factor; MI, Metaphase I; MII, Metaphase II; PGC, primordial germ cell; PHL, pleckstrin-homology like; SP1, (GC-box-binding) specific protein 1; Tdag51, T-cell death associated gene 51; VZ, ventricular zone; ZGA, zygotic genome activation.

abilities to mount a classical HSR [1,2,4,17–21]. In parallel, spermatogenesis and pre-implantation embryos showed extreme sensitivity to heat stress [1,22–24].

This led to the first hypothesis that Hsps were required for their chaperone function in developmental pathways, which are believed to be very demanding in terms of protein homeostasis. Correlatively, heat shock factors (HSFs), which also display developmental regulation in expression and activity, were believed to be responsible for the high developmental expression levels of Hsps in nonstress conditions and to constitute a molecular basis of this atypical HSR. We shall overview these hypotheses and emphasize novel aspects in the role of HSFs in development, which brought this field far beyond the first expectations. This review will focus mainly on mammals, in which four HSFs have so far been extensively described. The description of the molecular strategy of the *Hsf* knockout models has been reviewed previously [25]. We will also emphasize the crosstalk existing between developmental programmes and stress responses.

Role of HSF1 and HSF2 in oogenesis and pre-implantation development

Role of HSF1 in meiotic oogenesis and pre-implantation development

The first indication of a role for HSFs in oogenesis was suggested by studies in *Drosophila* [26], which demonstrated that the unique *Drosophila* HSF is essential for oogenesis and implied that its role in oogenesis is mediated not only by the regulation of *Hsp* genes. This gave a new orientation to the field, suggesting that HSF performs a developmental role, which is at least partially unrelated to its stress-responsive function.

Mouse HSF1 is a maternal factor essential for the reproductive success of pre-implantation embryos [27] (Fig. 1). Maternal-effect mutations affect genes that encode RNAs or proteins – transcribed or synthesized in the oocyte, and stored throughout oogenesis – which sustain early embryonic development [28,29]. HSF1 is highly expressed in nonfertilized ovulated oocytes arrested at Metaphase II (MII) and in pre-implantation embryos [30–32]. *Hsf1* inactivation

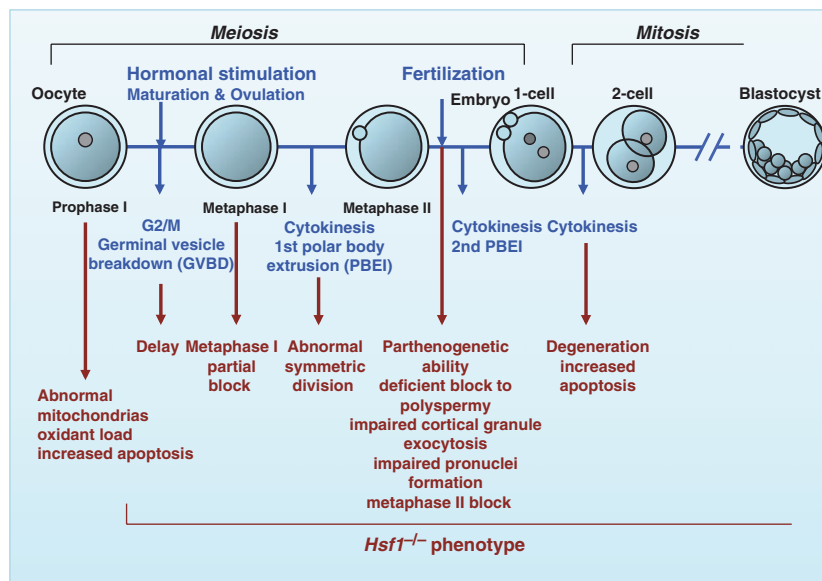


Fig. 1. Multiple effects of the deficiency in maternal HSF1 on oogenesis and pre-implantation development. Oocytes are blocked in prophase I, which occurs in female mice during embryogenesis until puberty. Upon stimulation with physiological concentrations of hormones during the oestrus cycle, a few oocytes in each oestrus cycle will resume meiosis, a hallmark of which is GVBD corresponding to the disappearance of the nucleus (grey circle), until pausing at MII after extrusion of the first polar body. Fertilization then triggers meiotic progression, extrusion of the second polar body and pronucleus formation. HSF1 deficiency results in a series of defects: oocytes, already before GVBD, display abnormal mitochondria and a high oxidant load. These oocytes show delay in GVBD, partial block in MI and abnormal symmetrical division. The ovulated oocytes are prone to parthenogenesis and fertilization is often accompanied by polyspermy and deficient cortical granule exocytosis. The formation of pronuclei is impaired and the ovulated oocytes are frequently arrested in MII. The remaining one-cell stage embryos cannot progress to the two-cell stage but undergo degeneration and apoptosis. The accumulation of these serial partial defects leads to total infertility.

(*Hsf1^{tm1Ijb}*) has multiple effects on oocyte meiosis, through the direct regulation of Hsp90 α expression [33]. During the development of female embryos, oogonia enter meiosis at embryonic day (E)13.5 (i.e. day 13.5 postcoitum) and oocytes remain blocked at prophase I until the completion of their growth. *Hsf1^{-/-}* oocytes show several deviations from this process. First, germinal vesicle breakdown (GVBD)-which signs meiosis resumption upon physiological hormonal stimulation during the oestrus cycle- is delayed. Second, *Hsf1^{-/-}* oocytes also undergo a partial block in Metaphase I (MI). Hsp90 α is the major Hsp expressed by fully grown oocytes and markedly down-regulated by the absence of HSF1 [33]. The authors used an elegant approach to circumvent technical difficulties linked to such scarce material, by treating oocytes with a specific inhibitor of Hsp90, 17-allylamino-17-demethoxygeldanamycin (17AAG). They demonstrated that these defects in meiotic progression are largely caused by the lack of Hsp90 α , in the absence of HSF1. HSF1 directly regulates the transcription of Hsp90 α , and the lack of Hsp90 α leads to the degradation of kinase CDK1, an Hsp90 client protein that controls GVBD. Third, *Hsf1^{-/-}* MII oocytes also display abnormal symmetric division, as a result of the defective migration of the spindle during cytokinesis. In this case, the depletion of Hsp90 α in the absence of HSF1 affects the mitogen-activated protein kinase pathway. This study describes the role of HSF1 as a maternal factor via the strong regulation of expression of a major Hsp and shows how a reproductive defect can originate from multiple impairments in meiotic progression. Other Hsps, whose expression is altered in *Hsf1^{-/-}* oocytes, might also contribute to this complex phenotype [33].

Postovulation development is compromised in *Hsf1^{-/-}* (*Hsf1^{tm1Ijb/tm1Ijb}*) oocytes, with a large increase in the number of eggs presenting only a maternal pronucleus, a sign of impairment in MII arrest, which leads to spontaneous (parthenogenetic) activation. This is associated with supernumerary sperm heads (polyspermy), which seem to be caused by reduced efficiency in cortical granule exocytosis. In line with these findings, the vast majority of *Hsf1^{-/-}* embryos fails to develop to the two-cell stage and thus degenerates. These defects originate in oogenesis, as demonstrated by the fact that pre-ovulated *Hsf1^{-/-}* oocytes display ultrastructural abnormalities (Golgi apparatus, cortical actin cytoskeleton, cytoplasmic aggregates), as well as mitochondrial dysfunction, in conjunction with markedly increased production of reactive oxygen species [27,34]. In line with findings in the heart and kidney [35,36], and together with the down-regulation of many HSPs in

oocytes [33], the deficiency in HSF1 provokes an oxidative stress to which oocytes are particularly sensitive [37]. The redox balance is therefore profoundly affected in mutant oocytes in an HSF1-dependent pathway.

HSF1, zygotic genome activation and chromatin status

It was first hypothesized that mouse HSF1 could be involved in zygotic genome activation (ZGA). In mice, specifically, ZGA occurs at two phases [38]: the first occurs at the late one-cell stage, only involves a restricted number of genes and is characterized by the elevated transcription of *Hsp70.1* (*Hspa1b*) and *Hsp70.3* (*Hspa1a*) genes [33,39–41]; and the second takes place at the two-cell stage and involves regulated global genome activation. The first studies seemed to indicate that heat shock elements (HSEs) were essential for zygotic activation of the *Hsp70* gene [32,42]; however, this was also found to be dependent on GC-box-binding factor (SP1) and GAGA factors [43,44]. Accordingly, *Hsp70* gene transcription during ZGA was not abolished by HSF1 deficiency [27], suggesting that, although HSF1 might contribute to ZGA, it is not essential for the elevated transcription of *Hsp70.1* and *Hsp70.3*, characteristic of ZGA.

Transcription in one-cell embryos is peculiar because the zygotic genome undergoes massive chromatin remodelling [45–49]. During ZGA, the majority of transcription seems to occur in the male pronucleus, which displays higher levels of hyperacetylated histones and of DNA demethylation. Hsp70.1 could, however, have a specific chromatin status. In somatic cells, in contrast to the majority of genes, *Hsp70.1*, as well as *c-Myc*, remains uncompact and accessible because of a process called bookmarking. *Hsp70.1* bookmarking is mediated by HSF2, which interacts with protein phosphatase 2A and inhibits condensin [50–52]. The occupancy of the *Hsp70.1* promoter by HSF1, HSF2 and SP1 in mature spermatozoa [53], together with RNA polymerase II [54], may persist through compaction and fertilization. This was most unexpected because the high level of compaction in sperm chromatin is believed to exclude the majority of transcription factors. Such occupancy could maintain *Hsp70.1* in a transcription-competent state during the first phase of ZGA.

HSF1, HSF2 and the HSR in pre-implantation embryos: possible interplay?

Pre-implantation embryos display an atypical HSR, possibly because of a still-unravelling regulation and interplay between HSF1 and HSF2. Although HSF1 is

stored in the oocyte, heat-inducibility disappears in fully grown oocytes, shortly before meiosis resumes. One-cell stage embryos respond to heat shock by inducing *Hsp70.1*, but at a slow, atypical rate and only a modest increase in *Hsp70.1* is found. This may be linked to the high constitutive levels of *Hsp70*, which are already present at these stages, and which could reduce HSF1 activity. The ability to elicit a normal HSR is acquired progressively during the pre-implantation period where the rapid, strong and transient induction of endogenous *Hsp70* or of an *Hsp70-luciferase* transgene, characteristic of a classical HSR, seem to be established at the blastocyst stage [1]. One- and two-cell embryos are able to respond to osmotic shock, but only *Hsp70.1* (and no other *Hsp* genes) is activated [41]. However, it remains to be determined whether the increase in *Hsp70.1* is HSF1-dependent. In particular, a region containing SP1 (GC-boxes) and HSF-binding sites is known to activate *osp94*, an *hsp110* family member, upon osmotic stress. Such a regulation could operate on *Hsp70.1*, because SP1 is present in cleavage-stage embryos [55] and *Hsp70.1* contains SP1-binding sites. It was first hypothesized that this restriction in eliciting a complete and rapid HSR could be a result of the unusual, strictly nuclear, localization of HSF1 observed in *in vitro* isolated one-cell embryos, suggestive of an atypical mode of activation at this stage [1]. However, HSF1 is cytoplasmic in oocytes in ovarian follicles and in mid-one-cell embryos fixed within Fallopian tubes, indicative of classical HSF1 regulation [33,41]. The nuclear localization of HSF1 in the isolated one-cell embryos might be caused by subtle osmolarity changes [41]. In contrast, the four-cell stage is constitutively devoid of HSF1 and HSE-binding activity [30,31] and cannot respond to heat or osmotic shock [1,30–32,41]. The sharp lowering of HSF1 is believed to be linked to the massive degradation of maternal material that occurs after the two-cell stage [56].

While HSF1 is a maternal factor, *Hsf2* transcripts cannot be detected in oocytes. HSF2 seems to be present at very low levels in the fertilized egg and starts to be synthesized by the zygotic genome at the two-cell stage [1,32]. Expression of HSF2 then shows a progressive increase and is high in blastocysts, in conjunction with the increase in DNA-binding activity that occurs from the four-cell stage to the blastocyst stage [30–32]. The subcellular localization of HSF2 is still controversial: while it is both cytoplasmic and nuclear in the blastocyst [32], its subcellular localization at the one- and two-cell stages is still unclear [1,41]. Nevertheless, the parallel between the increased expression and activity of HSF2 and the progressive ability to mount a

normal HSR is striking and might reveal interplay between HSF1 and HSF2 in early embryos. More precisely, it addresses the question of the role of HSF2 in rendering the ability of the embryo to respond to heat in a HSF1-dependent manner. The influence of HSF2 on the stress response mediated by HSF1 has already been reported in various somatic cell lines [57–61].

Role of HSF2 in oogenesis and pre-implantation development

HSF2 deficiency was reported, by two independent knockout models, to cause a reduction in female fertility (*Hsf2^{tm1Mmr}* and *Hsf2^{tm1Miv}*) (Table 1) [62,63]. This hypofertility phenotype is complex and encompasses multiple defects. The litter size of *Hsf2^{-/-}* female mice is reduced, irrespective of the paternal or embryonic genotype, suggesting that the defect originates in oogenesis. *Hsf2^{tm1Mmr/tm1Mmr}* female mice produce reduced numbers of ovulated oocytes, and 70% of fertilized oocytes appear to be abnormal and unable to proceed to the two-cell stage. Hormonal stimulation of young pubescent female mice restores normal ovulation rates (indicating that in young female mice, ovulation defects are not refractory to hormonal stimulation), but most of the fertilized oocytes are not able to proceed to the two-cell stage. Ovaries are depleted in follicles at all stages and display haemorrhagic cysts, stigmata often reported for the knockout phenotype of meiotic genes, as is the case for *Msh5*, for example [64]. The fact that HSF2 is expressed in primordial germ cells (PGCs) and prophase I oocytes in the embryo (V. M., unpublished data) makes it possible that part of this phenotype could be caused by meiotic defects. Older *Hsf2^{tm1Mmr/tm1Mmr}* female mice develop secondary hormone-related problems, showing very high levels of luteinizing hormone receptor mRNAs. This is probably a consequence of the early hormone-independent ovarian defects, which might have a long-term impact on the hypothalamo–pituitary–ovary axis [62]. Alternatively, it remains to be investigated whether HSF2 could be expressed in granulosa cells and contribute to this ovarian phenotype.

In addition to these pre-implantation defects, increased embryonic lethality is apparent before E9.5 in the *Hsf2^{tm1Mmr}* knockout model [62]. This effect is even stronger in the *Hsf2^{tm1Miv}* model but seems to be of broader occurrence between E7.5 and birth [63]. This would be compatible with aneuploidy and consistent with meiotic defects. HSF1 controls spindle formation and migration during oogenesis, and HSF2 has been shown to modulate microtubule dynamics in brain development (see below). HSF2 deficiency could

Table 1. *Hsf* knockout and overexpression mouse models.

Category	Allele Symbol Gene; allele name; author	Allelic composition (Genetic background)	Observed phenotypes in mouse	
			Developmental and reproductive defects	References
Transgenic (random insertion under the beta-actin promoter)	Tg ^{(ACTB-HSF1)¹Anak} Heat shock factor 1; transgene insertion 1, A. NAKAI	(C57BL/6 × DBA/2)	Reproductive defects: abnormal testis morphology, male meiosis arrest, late pachytene spermatocyte death, male infertility Protection against heat-induced spermatogonia death	69,86,87
Transgenic (random insertion under hst70 promoter)	Heat shock factor 1; transgene insertion 1, P./W. WYDLAK	FVB/N	Reproductive defects: reduced testis size, male meiosis arrest, massive degeneration of the seminiferous epithelium, spermatocyte death, absence of spermatids and spermatozoa, male infertility	85,89,91
Targeted (knockout)	Hsf1 ^{tm1ljb} Heat shock factor 1; targeted mutation 1, I.J. BENJAMIN	129S6/SvEvTac	Reproductive defects: maternal effect mutation, oocyte meiosis defects, oocyte and early embryo ultrastructural defects, polyspermy, pre-implantation development arrest, female infertility, no male infertility observed Reproductive defect in stress conditions: lack of genotoxic proliferation block in spermatogonia, and of genotoxic-induced-cell death decision in meiotic I spermatocytes Developmental defects: abnormal extraembryonic structures (chorioallantoic placenta), partial lethality at E14 and growth retardation	27,33,34,66,83,92
Targeted (knockout)	Hsf1 ^{tm1Miv} Heat shock factor 1; targeted mutation 1, N.F. MIVECHI	129S2/SvPas	Reproductive defects: normal spermatogenesis, no male infertility Complete spermatogenesis disruption in <i>Hsf1/Hsf2</i> double KO Developmental defects: growth defects in <i>Hsf1/Hsf2</i> double KO	72,84
Targeted (knockout)	Hsf1 ^{tm1Anak} Heat shock factor 1; targeted mutation 1, A. NAKAI	(C57BL/6 × CBA × ICR)	Development/maintenance defect : atrophy of olfactory epithelium, proliferation defect, apoptosis Dual reproductive effects in stress conditions: lack of protection against heat-induced spermatogonia death, reduced heat-induced spermatocyte death Dual eye development effects: compensatory effects of HSF4 loss in epithelial lens cells, exacerbated effects of HSF4 loss in lens fiber cells	86,106,110,149
Targeted (knockout)	Hsf2 ^{tm1ljb} Heat shock factor 2; targeted mutation 1, I.J. BENJAMIN	either: [involves: (129S6/SvEvTac × 129X1/SvJ)] or involves: (129S6/SvEvTac × C57BL/6)]	No phenotype observed	65

Table 1. (Continued)

Category	Allele Symbol Gene; allele name	Allelic composition (Genetic background)	Observed phenotypes in mouse	
			Developmental and reproductive defects	References
Targeted (reporter)	Hsf2 ^{tm1Miv} Heat shock factor 2; targeted mutation 1, N.F. MIVECHI	involves: (129S2/ SvPas × 129X1/ SvJ × C57BL/6)	Reproductive/endocrine/exocrine defects: female hypofertility, abnormal ovaries (weight, morphology and number of gametes), reduced testis size, partial arrest of male meiosis, reduced sperm count, light male hypofertility Complete spermatogenesis disruption in <i>Hsf1/Hsf2</i> double KO Developmental defects: embryonic prenatal lethality, growth defects in <i>Hsf1/Hsf2</i> double KO Nervous system developmental defects: enlarged ventricles, intracerebral hemorrhage	63,72
Targeted (reporter)	Hsf2 ^{tm1Mmr} Heat shock factor 2; targeted mutation 1, M. MORANGE, V. MEZGER	involves: (129S2/ SvPas × C57BL/6)	Reproductive/endocrine/exocrine defects: ovulation and and preimplantation defects, abnormal ovaries (weight, morphology and number of gametes), secondary hormonal pathway defects, female hypofertility, reduced testis size, defective synapsis, late pachytene spermatocyte apoptosis, partial arrest of male meiosis, reduced sperm count, no gross impact on male fertility Developmental: embryonic prenatal lethality Developmental nervous system defects: enlarged ventricles, smaller hippocampus and thinner cortex, neuronal migration defects	62,123
Targeted (knockout)	Hsf4 ^{tm1Anak} Heat shock transcription factor 4; targeted mutation 1, A. NAKAI	(C57BL/6 × CBA)F1	Eye developmental defects: abnormal lens capsule and epithelium morphology, hydropic eye lens fibers, cataracts Development/maintenance defect: compensation for the lack of HSF1 in the maintenance of the olfactory epithelium	101,106,149
Targeted (reporter)	Hsf4 ^{tm1Miv} Heat shock transcription factor 4; targeted mutation 1, N.F. MIVECHI	129S2/SvPas	Developmental/morphology defects: abnormal lens fiber cell terminal differentiation, cataracts, microphthalmia	102
Targeted (knockout)	Hsf4 ^{tm1Xyk} Heat shock transcription factor 4; targeted mutation 1, X. KONG	(129X1/SvJ × 129S1/ Sv)F1-Kitl+	Developmental/morphology defects: abnormal lens fibers, cataracts, microphthalmia	105,153

impair proper spindle formation in the first meiotic division and even in the mitotic oogonia stages, which could lead to abnormal chromosomal segregation and aneuploidy. Moreover, HSF2 is involved in the correct pairing of sister chromatids in male meiosis, and the lack of HSF2 in the prophase oocyte could lead to similar defects.

This pleiotropic phenotype is highly dependent on the genetic background. In our hands the penetrance of the *Hsf2*^{tm1Mmr} phenotype is markedly higher on the C57Bl/6N background compared with the C57Bl/6J background. A third *Hsf2* inactivation model (*Hsf2*^{tm1Ijb}) (Table 1) did not exhibit any fertility problems [65]. Although one cannot exclude that

these discrepancies rely on the peculiarities of each inactivation strategy, the differences in genetic background are a more plausible and interesting explanation, which paves the way for the search of modifier genes that would enhance or diminish the impact of HSF2 deficiency.

Pending questions for the roles of HSF1 and HSF2 in oogenesis and in early embryos

The role of HSF2 in oogenesis and in pre-implantation development supports a need for more detailed investigations. Wang *et al.* [63] performed microarray analyses on whole embryos at E8.5 and E10.5 and identified transcripts whose expression profile varies in the absence of HSF2. However, no molecular mechanism has been unravelled to explain these complex fertility defects. Such studies have been hampered by the fact that HSF2 expression seemed to be restricted to PGCs and the ovaries of the female embryo in which the oocytes were in prophase I ([62]; our unpublished results).

The molecular basis underlying the tight regulation of expression of *Hsf1* and *Hsf2* from PGCs to the blastocyst stage is still totally unknown. This regulation is, however, important in respect of possible HSF1/HSF2 interplay. HSF2 is barely detectable in oocytes in the adult ovary; but this remains to be confirmed and would benefit from further mechanistic investigations. HSF2 could either directly interplay with HSF1, if it is expressed in the oocyte, or indirectly influence oogenesis if expressed in ovarian cells (such as granulosa cells) other than oocytes.

HSF1 plays a role not only during the pre-implantation period, but also in postimplantation development. Although HSF1 is present in the nucleus of trophoblastic cells in all layers of the chorioallantoic placenta, HSF1 deficiency specifically results in spongiotrophoblast defects, a layer of cells of embryonic origin. These placental defects could not be attributed to changes in the expression pattern of major Hsps and claim for further investigations for the search of molecular actors [66]. No placental defects were identified in the *Hsf2* KO models, which could have explained embryonic lethality [62].

Roles of HSF1 and HSF2 in spermatogenesis

Role of HSF2 in normal spermatogenesis

HSF2 displays a remarkable stage-specific expression profile during the cycle of the seminiferous epithelium

in rodents [67,68], whereas HSF1 levels are relatively constant during normal testis development and HSF4 is not detected [68,69] (Fig. 2). This led to investigations of the role of HSF2 in normal spermatogenesis. HSF2 is located in the nuclei of early pachytene spermatocytes (stages I–IV) and in the nuclei of round spermatids (Stages V–VII) in the rat [68], consistent with previous findings in the mouse [67]. A very interesting, but yet unexplained, localization has been found in the cytoplasmic bridges that connect germ cells deriving from the same spermatogonia [68]. These two studies, however, showed discrepancies: one study [67] reported that HSF2 was able to constitutively bind HSE in an *ex vivo* electrophoretic mobility shift assay, but no such activity was found in the other study ([68], our unpublished data).

Hsf2 knockout phenotypes

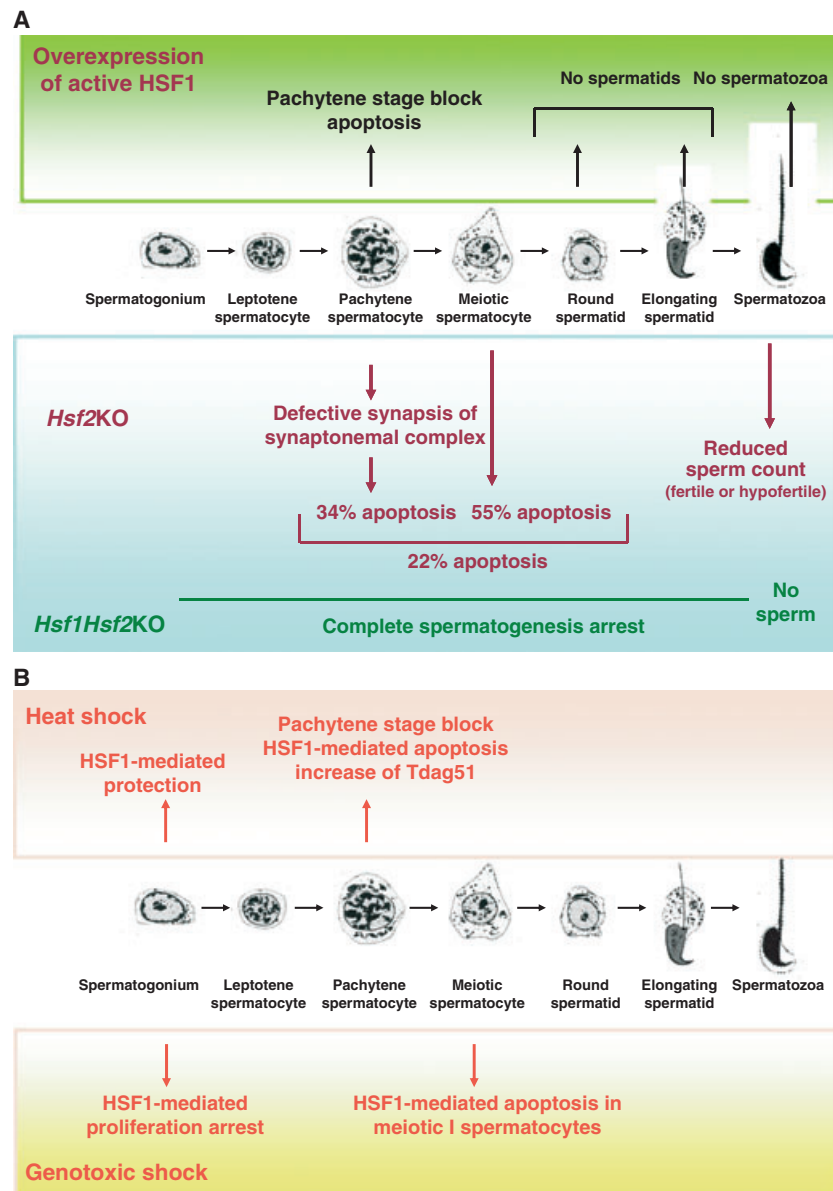
HSF2 deficiency results in reduced testis size, as well as reduced sperm count and vacuolization of seminiferous tubules, both of which are linked to the absence of differentiating spermatocytes and spermatids. Accordingly, late pachytene spermatocytes are eliminated through a stage-dependent apoptotic process (*Hsf2^{tm1Mnr}* [62] and *Hsf2^{tm1Miv}* [63]) (Fig. 2; Table 1). One explanation for this programmed death could be the elevated frequency of synaptonemal complex abnormalities in *Hsf2^{-/-}* spermatocytes. The synaptonemal complex, which forms a proteic axis pairing chromosomes during the pachytene stage, shows defective synapsis indicated by the formation of loop-like structures or the appearance of separated centromeres, susceptible for activating the pachytene checkpoint, which triggers the elimination of defective germ cells by apoptosis [70,71]. The third *Hsf2* knockout model did not report any spermatogenesis defects (*Hsf2^{tm1Ijb}*, [65]) in line with the lack of female fertility and brain phenotypes, which, again, might be a result of the knockout strategy or genetic background effects (Table 1).

Nevertheless, even though *Hsf2* gene inactivation leads to marked defects, it does not cause complete arrest in spermatogenesis, indicating putative compensatory mechanisms for the lack of HSF2. In line with this hypothesis, double disruption of *Hsf1* and *Hsf2* is associated with sterility and complete arrest of spermatogenesis [72].

Elucidation of HSF2 function in spermatogenesis

Attempts were made in the earliest studies to identify target genes for HSF2 in the adult testis, but they were hampered by difficulties in discriminating between cell

Fig. 2. (A) Role of HSF in spermatogenesis under normal conditions. Upper panel. Over-expression of a constitutively active form of HSF1. Lower panel. *Hsf* inactivation studies. Defective synapsis observed in pachytene spermatocytes leads to increased apoptosis in *Hsf2*^{tm1Mmr/tm1Mmr} late pachytene and meiotic spermatocytes (representing 34% and 55% of the total apoptotic cells, respectively [62]; similar phenotype in *Hsf2*^{tm1Miv/tm1Miv} [63]). The third *Hsf2* knockout model did not report any spermatogenesis defects (*Hsf2*^{tm1Ijb}, [65]). Double *Hsf1*^{tm1Miv/tm1Miv}/*Hsf2*^{tm1Miv/tm1Miv} inactivation leads to complete arrest in spermatogenesis and sterility. (B) Dual role of HSF1 towards stress during spermatogenesis. The role of HSF1 in mediating survival of spermatogonia in response to heat shock (upper panel), but selective pachytene-death was shown using *Hsf1*^{tm1Anak/tm1Anak} mice. The role of HSF1 in mediating proliferation block in spermatogonia and cell-death decision in meiotic I spermatocytes was demonstrated, comparing wild-type versus *Hsf1*^{tm1Ijb/tm1Ijb} mice exposed to genotoxic stress (lower panel).



loss caused by apoptosis and the down-regulation of gene expression. One of the most attractive candidates was the testis-specific member of the *Hsp70* family, *HspA2* (formerly *Hsp70.2* in mice and *Hsp70t* in rat), which is essential for spermatogenesis, but was found not to be a target of HSF2 [62,63,65,73]. Recently, a CHIP-on-chip approach, covering around 26,000 promoters of 1.5 kbp in the mouse genome, led to the identification of 546 putative target promoters for HSF2 in wild-type adult testis. Six were validated as being specifically bound by HSF2 in testis: *spermatogenesis associated glutamate (E)-rich protein 4a* (*Speer4a*); *Hspa8* (formerly *Hsc70*); *ferritin mitochondrial* (*Ftmt*); *spermiogenesis specific transcript on the*

Y (*Ssty2*); *Scyp3 like Y-linked* (*Sly*); and *Scyp3 like X-linked* (*Slx*) [73]. Interestingly, the very conserved HSEs of the *Hsp25* gene, which are bound by HSF1 and HSF2 in heat-shocked mouse embryonic fibroblasts [60], are not bound by HSF2 in testis. This interesting result highlights the importance of elucidating the mechanism discriminating various HSEs for HSF2 recruitment in development.

This latter study [73] underlines possible roles of HSF2 in the organization of chromatin and of the genome structure. First, HSF2 binding to its target genes correlates with the acetylation of histones H3 and H4, a frequent mark of transcriptional activity, suggesting that HSF2 may target histone modifications

and influence the accessibility of its target genes. Such targeting has been demonstrated in a stress-dependent manner in the case of HSF1 [74]. Conversely, the binding of HSF2 to its target genes might be favoured by H3 and H4 acetylation. Second, L1 transposable elements (subfamilies 1 and 29 from the large retrotransposon family 'Long Interspread Nuclear Elements') were found to be occupied by HSF2 in the ChIP-chip screen. L1 are transcribed and inserted into the host genome via a copy-and-paste mechanism, which occurs mainly in germ and embryonic cells. This suggests that HSF2 could regulate L1 retrotransposition and consequently would have a global effect on the genome structure and transcriptional activity [75]. Third, studies on the clustering of the HSF2 binding location revealed striking accumulation of HSF2 targets (34) on the Y chromosome. The Y chromosome contains multicopy gene families from diverse origins in the genome that were duplicated and have evolved to perform male-specific roles ([76,77] and references therein). These HSF2 target genes include *Ssty2*, *Sly* and *Similar to Ssty2*, which exist as multicopies throughout the MSYq region (male-specific Y-chromosome long arm), which mostly contains heterochromatin and repetitive sequences. HSF2 occupancy was also found in the X chromosome on numerous copies of the promoter of *Slx*, which share substantial homology with *Sly*. HSF2 occupancy covers 42 Mbp in the MSYq region and 8 Mbp on the X chromosome. HSF2 expression coincides with the abundance of *Ssty2*, *Sly* and *Slx* transcripts in round spermatids (a stage of profound chromatin remodelling), and HSF2 is a transcriptional regulator of *Ssty2*, *Sly* and *Slx*, because the loss of HSF2 results in down-regulation of the levels of *Ssty2* and *Sly* mRNA species, but in the up-regulation of *Slx* mRNA. Recently, *Sly* was demonstrated to post-meiotically repress sex chromosomes [78]. *Sly* deficiency partially mimics MSYq deletions in mice ([79] and references therein), leading to reduced repressive marks and severe impairment of sperm differentiation [78]. Through its effect on *Sly*, HSF2 deficiency might therefore be responsible for the loss of epigenetic marks.

The presence of a Cor1 domain in *Sly* and *Slx* proteins, which presumably helps binding to chromatin, and the high occurrence of head sperm abnormalities related to some MSYq deletions [77,79–81], are suggestive of chromatin remodelling impairment during early sperm head condensation, which includes histone replacement. The impact of HSF2 as a transcriptional modulator of *Sly* and *Slx* in this process was assessed by the elevated frequency of flattened sperm heads. Accumulation of the transition protein TPN2 and reduced levels of protamines 1 and 2 was an evident,

although indirect, effect, because neither genes are HSF2 targets [73]. Thus, DNA integrity is compromised, as shown by DNA fragmentation. The massive occupancy of MSYq by HSF2 is probably crucial for maintaining chromatin structure and sperm quality. In the human population, deletions in MSYq are the most genetic common cause of oligo- or azoospermia. Whether HSF2 defects may be a basis of human male infertility remains an open question.

Functional clustering analyses of HSF2 target genes revealed that the highest ranked biological process are reproduction, followed by gametogenesis. Interestingly, many olfactory receptors were identified as HSF2 target genes, suggesting that HSF2 might play a role in sperm–egg interactions by controlling chemotaxis [73,82]. In addition, the *Neuromedin B receptor* (from the bombesin-like peptide receptor subfamily which have a diverse spectrum of biological activities and have been implicated as autocrine growth factors) and the sex-determination protein homologue, *Femb1*, belong to the list of genes whose expression is altered in the double-knockout *Hsf1^{tm1Miv}/Hsf2^{tm1Miv}* [72]. Interestingly, inducible *Hsp* genes were not found, only the cognate constitutive member (*Hspa8*). The expression of a testis-specific cognate gene *Hsc70t* (*Hspa11*) was found to be modified in double-knockout *Hsf1^{tm1Miv}/Hsf2^{tm1Miv}* testes [72]. Surprisingly, TPN1 was found to be lowered in *Hsf2^{tm1Miv}* and in *Hsf1^{tm1Miv}/Hsf2^{tm1Miv}* knockout testes [72].

Note that the molecular basis of incorrect pairing of sister chromatids and of the lack of integrity of the synaptonemal complex in *Hsf2^{-/-}* spermatocytes is a pending question [62].

Role of HSF1 in the quality control of sperm in stress conditions

Investigation of the role of HSF1 in the quality control of sperm in stress conditions revealed a dual facet. Indeed, whereas it is protective in somatic cells [83,84], HSF1 plays a crucial role in the cell-death decision in male germ cells.

HSF1-induced cell death at the late pachytene stage

This unexpected role played by HSF1 was unravelled in transgenic mice over-expressing a form of HSF1 that was constitutively active for DNA binding [69,85] (Table 1). The most comprehensive study was performed by over-expressing a form of HSF1, which is constitutively active for DNA binding, under the control of the human β -actin promoter [86,87]. HSF1 overexpression resulted in infertility, reduction in testis

size (50%), defective spermatogenesis with block at the pachytene stage, and the general absence of round and elongated spermatids. The authors demonstrated that late pachytene spermatocytes are the target of HSF1-induced cell death (Fig. 2). The similarity between this phenotype and the defects arising in heat-shocked testes in terms of block at the pachytene stage and apoptosis of pachytene spermatocytes suggested that activation of HSF1 would be a major trigger for apoptosis in germ cells. Because, in isolated pachytene spermatocytes, HSF1 is activated at temperatures below the core body temperature (35 °C) [88], the death cascade would therefore be more easily induced in late pachytene spermatocytes than in other germ or somatic cells.

Mechanism of HSF1-induced cell death

Further investigations involving *Hsf1*^{-/-} mice (*Hsf1*^{tm1Anak/tm1Anak}) provided a mechanism for HSF1-dependent heat shock-induced cell death in spermatocytes [86]. Heat shock does not trigger the induction of major heat shock genes in male germ cells. The prominent *Hsp70.2* is even down-regulated. In contrast, heat shock triggers a marked induction of the T-cell death associated gene 51 (*Tdag51*) by direct HSF1 binding of a HSE in the proximal promoter region of the *Tdag51* gene. *Tdag51* is a member of the PHL domain family and its N-terminal region is bound and inhibited by major Hsps. The unique balance of Hsps and *Tdag51* in favour of *Tdag51* in spermatocytes would therefore trigger active HSF1-dependent cell death. Constitutive expression of *Hsp70i* does not protect the seminiferous epithelium against cryptorchidism-induced damage and therefore probably from HSF1-induced death. The fact that the spermatogenic damage provoked by cryptorchidism could not be rescued by *Hsp70i* (*Hsp70.1*) suggests that *Hsp70i* is not sufficient to counteract the induction of *Tdag51* [89, 90]. A marked reduction of *Hsp70.2* precedes apoptosis in spermatocytes that express active HSF1 under the control of the testis-specific *Hst70* promoter, but the effect of HSF1 in this down-regulation seems to be indirect and probably occurs through the misdirection of a transcription factor network [91,92].

Furthermore, studies by Izu and colleagues [86] allowed the discovery of two contrasting roles for HSF1 in male germ cells (Fig. 2). Indeed, HSF1 was found to be protective against heat shock-induced cell death in cells (probably spermatogonia) located in the outermost layer of tubules, in an Hsp-independent mechanism [86]. In contrast, HSF1 is involved in cell death in spermatocytes [86,87]. Once again, this

death-promoting effect occurs without Hsp induction. These two, apparently dual, functions would allow the elimination of damaged spermatocytes in order to prevent passing injured sperm onto the next generation and, conversely, would allow the survival of 'stem' germ cells, maintaining the capability of spermatozoa production if spermatogenesis is allowed to occur under nonstress conditions. Such a model based on cell-specificity was corroborated by Salmand and colleagues [92] who demonstrated that genotoxic stress on another *Hsf1* knockout mouse model (*Hsf1*^{tm1Ijb/tm1Ijb}) causes HSF1-dependent cell death among spermatogonia and meiotic I spermatocytes, highlighting the requirement of HSF1 for proliferation block in mitotic stages and for cell death decision in meiotic stages. Although *Hsf1*^{-/-} spermatogenic cells were more resistant to the reduction of proliferation induced by genotoxic insult, they could not, however, reconstruct spermatogenesis from spermatogonia A, in contrast to *Hsf1*^{+/+} spermatogenic cells (Fig. 2). Interestingly, in rainbow trout, a poikilotherm species, HSF1 activation in germ cells also occurs at lower temperature, and heat shock does not lead to classical Hsp70 accumulation, as in mice, suggesting that the lower set point and lack of typical HSR is not restricted to homeotherm species but might constitute a unique property of germ cells [22].

These studies therefore indicate that HSF1 could have played prominent roles in the maintenance of species during evolution through its differential effects in either protecting against cell death or, conversely, in promoting cell death in stage-specific germ cells in spermatogenesis. It would thus prevent the production of damaged gametes while allowing reconstruction of spermatogenesis.

Pending questions

Interplay of HSF1 and HSF2 in spermatogenesis

No, or only modest, defects in spermatogenesis have been reported in *Hsf1*^{tm1Anak/tm1Anak} [86], *Hsf1*^{tm1Miv/tm1Miv} [72] and *Hsf1*^{tm1Ijb/tm1Ijb} [92] mice (Table 1). However, double-knockout *Hsf1*^{tm1Miv/tm1Miv}/*Hsf2*^{tm1Miv/tm1Miv} leads to male sterility with empty tubules. The examination of spermatogenesis onset in juvenile males shows that germ cells fail to progress beyond the pachytene stage. These data suggest that HSF1 and HSF2 display some redundancy in their functions in spermatogenesis, but incomplete; however. HSF1/HSF2 interplay has been demonstrated in somatic murine and human cell lines [57–61]. Further investigations are currently in progress in Lea Sistonen's laboratory in order to unravel the specific targets of HSF1 in spermatogenesis and to estimate the proportion of com-

mon target promoters between HSF1 and HSF2 and their biological relevance (Lea Sistonen, personal communication).

The identification of HSF2 target genes during spermatogenesis indicated that the vast majority of targets were not heat shock genes. It would be interesting to infer, from these results, whether a 'developmental' HSE could be defined in terms of sequence or localization in the gene body. However, the global approach chosen for the identification of HSF2 target genes in spermatogenesis used first-generation 1.5kbp promoter tiling arrays and it could be difficult to infer new characteristics of HSEs, because they might show greater resemblance to HSEs located in the proximal promoter regions of heat shock genes compared with HSEs identified in other global approaches.

Regulation of HSF2 stage-specific expression in spermatogenesis

Although HSF1 seems, in general, to be constantly expressed during spermatogenesis, HSF2 displays a striking stage-specific pattern [68] (Fig. 2). This raises the question of the molecular mechanism, transcriptional or post-transcriptional, which underlines such a specific profile.

More HSFs in the male germ line?

A heat shock-like factor, sharing partial homology with classical HSFs was discovered that is encoded by the human Y chromosome. However, there are currently no data available to confirm that this *HSFY* gene could play a role in spermatogenesis [93,94].

Role of HSF1 and HSF4 in sensory placode development

Sensory placodes arise from the thickening of cranial ectoderm during formation of the peripheral nervous system and include lens, nasal epithelium, inner ear and the presumptive cranial ganglia. Precursors that contribute to the different placodes are first intermingled and part of a preplacodal domain, and segregate later. In particular, lens and olfactory placodes whose formation is influenced by HSFs form from a common territory [95,96].

HSF4 in lens development

Congenital cataracts account for 10% of cases of childhood blindness, half of which have a genetic cause. Implicated genes can be divided into two

categories: transcription factors ('master gene'-like) that are essential for early stages of lens development and whose mutations prevent the correct formation of lens fibers and are associated with severe phenotypes; and genes that determine or influence lens structure, such as *crystallins* or *lens-specific beaded filament* structural proteins (*Bfsp*).

With an unusual occurrence in the history of HSFs, the role of *HSF4* in lens development was first revealed by mutations in the human *HSF4* gene that were associated with dominant hereditary cataracts [97]. Other *HSF4* mutations have been further identified in familial cases of cataracts. Interestingly, mutations in the DNA-binding domain seem to be associated with dominant cataracts, whereas mutations within (or downstream of) the oligomerization domain correlate with recessive cataracts [98–100]. Strikingly, also, only missense mutations are found in autosomal-dominant cataracts, whereas missense, nonsense, or frameshift mutations can be associated with recessive cataract mutations. It is therefore possible that *HSF4* mutations associated with dominant cataracts may act by a dominant-negative mechanism. The fact that patients have no other symptoms implies that HSF4 would not be essential in other tissues. Accordingly, HSF4 displays extremely high expression levels in the rodent postnatal lens compared with other tissues, and is the major HSF that is constitutively active for DNA-binding in this tissue [101–103].

Lens is composed of only two cell types: epithelial cells and fiber cells. The fiber cells originate from the half posterior epithelial cells, which start to elongate and differentiate from E13.5. They accumulate in concentric layers and gradually lose their nuclei and organelles [104]. Lens is characterized by dehydration, as well as by an extremely high concentration of proteins that cannot turnover and which represent a proteostasis challenge in order to maintain their integrity and solubility throughout the life span.

The inactivation of the *Hsf4* gene in mice causes cataracts in the early postnatal days [101,102,105]. *Hsf4* mRNAs start to be expressed at E13.5 in the two cell types and continue until at least 6 weeks after birth. Two situations are described, depending on the cell type considered.

HSF4 roles in lens fiber cells

Hsf4^{-/-} fiber cells are swollen, histologically abnormal with nuclei, a vacuole-like cavity and inclusion-like structures, which possibly exist in protein aggregates because they contain α B-crystallin. More than 90% of the lens protein is composed of a variety of crystallins;

in mammals, the crystallins are α A and α B; β B1, β B2, β B3, β A3/A1 and β A4; and γ A, γ B, γ C, γ D, γ E, γ F and γ S. In mice, the γ -crystallins are the major contributors among mature lens proteins. A major reduction in γ -crystallins was observed by different laboratories in $Hsf4^{-/-}$ lenses ($Hsf4^{tm1Anak}$, $Hsf4^{tm1Miv}$ and $Hsf4^{tm1Xyk}$) (Table 1). Fujimoto *et al.* [101] detected markedly reduced expression of $\gamma(A-F)$ -crystallin genes, and Min *et al.* [102] detected markedly reduced expression of the $\gamma(F)$ -crystallin gene only. These discrepancies could arise from differences in the $Hsf4$ targeting constructs in their homologous recombination strategies, or from distinct genetic backgrounds. A more recent study also identified a major decrease in the expression levels of the $\gamma(S)$ -crystallin gene [105]. All these γ -crystallin genes possess HSE, which can be bound by HSF4 in ChIP assays [101,102,105]. Interestingly, crossing $Hsf4^{-/-}$ with heterozygous *rncat* mice carrying a recessive cataract mutation in the $\gamma(S)$ -crystallin gene worsens the cataract in the $Hsf4^{-/-}/rncat^{+/-}$ offspring [105]. The similarities between $Hsf4^{-/-}$ and *Bfsp1/2* knockout fiber cell phenotypes led Shi *et al.* [105] to demonstrate that the genes encoding lens-specific beaded filament proteins 1 and 2 (*Bfsp*), which presumably assemble and connect crystallins, are also direct targets of HSF4 and show disturbed expression in $Hsf4^{-/-}$ lenses. In addition, Hsp27 is not expressed in the $Hsf4^{-/-}$ fiber cells, which might correlate with the formation of protein aggregates. The reduction in calpains Lp82 and 2 might account for the abnormal maturation and maintenance of α A-crystallin [105].

HSF4 roles in lens epithelial cells

$Hsf4^{-/-}$ lens epithelial cells display major over-expression of Hsp60 and Hsp70 and exhibit abnormal morphology in correlation with increased proliferation, premature differentiation and elongation, inhibition of denudation and of loss of organelles [101]. In line with this phenotype, fibroblast growth factor (FGF)-4 and FGF-7, which control the proliferation and differentiation of lens epithelial cells, are up-regulated in these $Hsf4^{-/-}$ lens epithelial cells, and FGF-7 was demonstrated to be a direct target gene of HSF4, which inhibits its expression [101].

HSF1 is required for olfactory neurogenesis

Although HSF1 is not required for the development of the nasal epithelium until 3 weeks after birth, mice lacking HSF1 ($Hsf1^{tm1Anak/tm1Anak}$) display abnormal nasal cavities with atrophy of the olfactory epithelium

from 4 weeks on, a time at which proliferation decreases and apoptosis increases. In wild-type mice, no major changes in the levels of HSF1 could be detected in this developing organ, however, HSF1 acquires constitutive DNA-binding activity in the olfactory epithelium in 4- and 6-week old mice, suggesting that its activity should be regulated at the post-translational level [106]. In the absence of HSF1, the high levels of heat shock proteins that can normally be detected in 6-week-old nasal epithelium are markedly diminished. Growth, differentiation and death of olfactory sensory neurons are under the control of many cytokines, including FGFs and LIF. FGF expression is not affected in $Hsf1^{-/-}$ olfactory epithelium, whereas LIF expression is maintained at high levels, instead of decreasing at 6 weeks. Such a high expression inhibits the maturation of olfactory sensory neurons and leads to a reduction in the thickness of the olfactory epithelium via cell death [107,108]. Among 15 upstream sites that resemble HSE, one region containing eight HSEs is exclusively bound by HSF1 in a stage-dependent manner, namely at 4 weeks, but not at 3 weeks [106].

Interplay of HSF1 and HSF4 in lens epithelial and lens fiber cells and in olfactory neurogenesis

Examination of the double $Hsf1Hsf4$ knockout allowed the unravelling of a remarkable interplay between HSF1 and HSF4 with either cooperative or antagonist effects. In lens fiber cells, the phenotype of $Hsf1^{-/-}Hsf4^{-/-}$ lenses is worsened and the reduction in γ -crystallin levels enhanced, suggesting that both factors cooperate to up-regulate these genes. In contrast, in lens epithelial cells, the loss of HSF1, in addition to HSF4, ameliorates the expression of FGF-1, -4 and -7 and partially rescues the phenotype, compared with $Hsf4^{-/-}$ lens epithelial cells. These results demonstrate that in epithelial cells, HSF1 and HSF4 have opposing effects on FGF expression. Similarly, the study of the double $Hsf1Hsf4$ knockout showed that HSF1 and HSF4 have antagonist effects on the LIF gene [106,109].

Note that because HSF4 is believed to constitutively form trimers, one may ask whether the interplay between HSF1 and HSF4 in the lens might involve heterotrimers similar to those formed in response to stress. Interestingly, trimeric HSF4 starts to increase at stages where HSF1 and HSF2, which are expressed in the fetal stages, are decreased [103,110].

Although gene-inactivation studies have mainly focused on the cooperative or competitive roles of mouse HSF1 and mouse HSF4 in lens development, it is possible that other HSFs – HSF1 or HSF2 – might

have a role in the neuronal part of retinal formation, as a result of their expression patterns [111].

HSF4-binding sites in the genome

Human HSF1 is not able to bind discontinuous HSE [112–114]. In contrast, HSF4 preferentially binds to the discontinuous HSE of $\gamma(C)$ -*crystallin*, whereas HSF1 prefers the continuous HSEs in the promoters of $\gamma(A)$ - and $\gamma(B)$ -*crystallin*. These results suggest that the architecture of HSEs is an important determinant in the regulation of HSF target genes. A genome-wide analysis of HSF4 target genes in the immortalized lens epithelial cell line, LEW2d, allowed the definition of a more flexible consensus HSE for HSF4 [110]. The geography of HSF4-binding regions also reveals new features because, in contrast to the classical heat shock genes, these HSEs are not only found in the promoter regions. Actually, only 5% of HSF4-binding regions map to 10-kb promoter proximal regions, whereas 53% map to the introns and exons, and 40% to the distal regions (> 10 kb) of the genes. A substantial number of regions that were found to be occupied by HSF4 are also bound by HSF1 or HSF2. These factors may compete or cooperate on the same regions, depending on the sequence properties of their binding sites. HSF4 binding was shown to be closely associated with reduced methylation of the histone H3K9, irrespective of the relative location of the HSF4-binding regions and of the transcriptional status of genes located around the HSF4-binding regions. This result is thus suggestive of a structural effect of HSF4 on chromatin. In the absence of HSF4, histone H3K9 methylation is induced and HSF1 binding is reduced, indicating that HSF4 facilitates HSF1 binding via chromatin remodelling. Heat shock genes are not HSF4 targets, but HSF4 regulates a set of nonclassical heat shock genes in response to heat shock in the lens. This semicomprehensive study therefore reveals an intimate link between the regulation of the HSR and developmental programmes.

Perspectives in the role of HSF in sensory placodes

The unravelling of the HSF4 role places this factor among genes that occupy the first group in the hierarchy of genes that control lens development. The development of the otic placode, which does not derive from the same territory, is not affected by the loss of HSF1 in young mice, whereas the integrity of sensory hair cells in response to acoustic insult depends on HSF1 [115,116].

The role of HSFs in the sensory placodes is associated with constitutive DNA-binding activity, which was detected in a band-shift assay of either HSF1 or HSF4. One could imagine that such a high expression of HSFs, accompanied by the appearance of constitutive DNA-binding activity, could be linked to the fact that these developing organs are under a type of environmental stress. The extremely high concentration of proteins in the lens, as well as exposure to odorants (or high oxygen tension) in the olfactory epithelium could create a long-term challenge for cell proteostasis. It would perhaps explain why *Hsps* are target genes of HSF in the developmental process. Similarly, the redox challenge which oocytes have to face could perhaps explain why the role of Hsps seems more pronounced than in other developmental process. One possibility would be that the otic placode is protected to a greater degree from environmental stress (such as oxidant stress) and thus less dependent on HSF1.

Role of HSF2 in brain development

We will mainly focus on HSF2, which was demonstrated to influence mouse brain development.

HSF2 expression, nuclear localization and DNA-binding activity correlates with brain development

HSF2 is highly expressed in the neuroepithelium of a wide variety of vertebrates, including zebrafish (zHSF2), chicken (cHSF2), mouse (mHSF2) and rat (rHSF2) [62,63,117–120]. The high expression levels of cHSF2, mHSF2 and rHSF2 are associated with nuclear localization within the developing neural tube [3,62,121]. In addition, mHSF2 is associated with DNA binding in the developing brain, including cortex, striatum, olfactory bulbs and mesencephalon, before birth [62,122,123]. In mice, HSF2 is expressed at all stages in the proliferative neural progenitors of the ventricular zone. Strikingly, it also starts to be expressed in the late cortical plate, when the most superficial layers of young postmitotic neurons of the future six-layer-cortex are being established [62,123] (Fig. 3). HSF2 DNA-binding activity quickly becomes undetectable in the cerebral hemispheres after birth, in parallel with a marked decline of its expression levels in mouse and rat [62,124]. Rat HSF2 is still nuclear in brains at postnatal day 2, but with no detectable HSE-binding activity and is cytoplasmic at postnatal day 30 [124,125]. The marked exception is cerebellum, whose development occurs after birth and consists of the massive

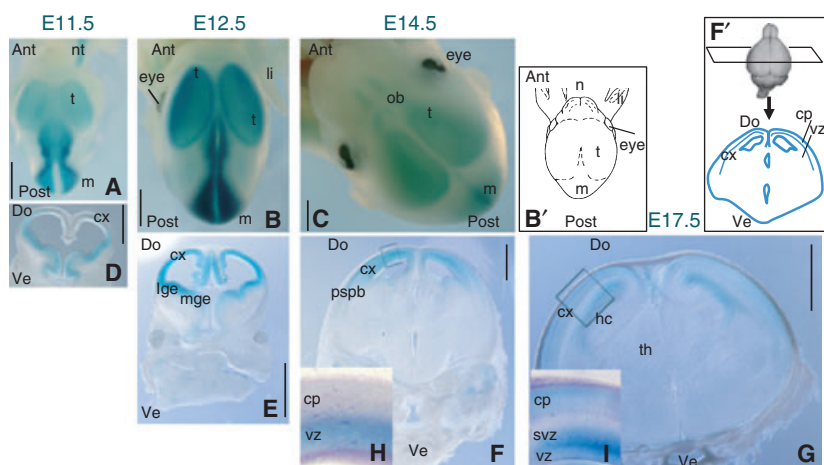


Fig. 3. Dynamic expression of HSF2 during development. *Hsf2-LacZ* expression (in blue) was used as a reporter for *Hsf2* gene transcription in knockout *Hsf2* embryos. (A–C) *In toto* pictures; (B'): dorsal view of the embryonic head schematized for E12.5 in (B) (adapted from [152]). (D–G) Coronal sections, schematized for (E). (H,I) Magnifications of cortical parasagittal sections; (F') dorsal view of the brain with the positioning of a coronal section and scheme of the coronal section at E14.5 in (F). (A) At E11.5, HSF2 is expressed in forebrain, midbrain and in hindbrain except for the midbrain, hindbrain midline. Surprisingly, the HSF2 expression pattern is more ventral than dorsal at this stage (D). (B) From E12.5, the HSF2 expression gradient is visualized along the anterior–posterior axis ([caudal]low to [rostral]high in the forebrain), while HSF2 follows expression along the dorso-ventral axis (E). The HSF2 expression pattern excludes the midline. At this stage, HSF2 is expressed in the whole cortex, in ganglionic eminences and in the dorsal part of the diencephalon (E). (C) HSF2 expression is reduced in the later stages of corticogenesis, and is expressed at E14.5 in the telencephalon dorsal part, in particular in the cortical ventricular zone (F and H). In the midbrain, interestingly, the midline strongly expresses HSF2 in contrast to earlier stages. From E15.5, HSF2 expression is detected mainly in SVZ and only faintly in VZ, and begins in the upper cortical layers (I and K at E17.5). In conclusion, a shift is observed from a more ventral (E11.5) to dorsal (E14.5) expression, with fading at the pallium/subpallium boundary. In addition, HSF2 presents graded expression levels, which follow neurogenesis waves. Scale bars: 1 mm. ant, anterior; cp, cortical plate; cx, cortex; do, dorsal; hc, hippocampus; lge, lateral ganglionic eminence; m, midbrain; mge, median ganglionic eminence; nt, neural tube; n, nose; ob, olfactory bulbs; pspb, pallium subpallium boundary; post, posterior; svz, subventricular zone; t, telencephalon; th, thalamus; ve, ventral; vz, ventricular zone.

migration of cerebellar neurons, which coincides with an increase in rHSF2 levels and HSE-binding activity [124], although no data are currently available to confirm that HSF2 is responsible for this constitutive HSE-binding activity. In older brains, HSF2 levels are low and HSF2 display cytoplasmic and dendritic localization [125]. HSF2 expression and activity profiles are consistent with a major role for HSF2 as a transcription regulator in forebrain and midbrain development, and perhaps also in the cerebellum.

HSF2 expression is mainly regulated at the transcriptional level

Developmental expression of rodent HSF2 seems to be mainly regulated at transcriptional levels [118,122]. Computer-based analysis revealed conserved sites for the binding of transcription factors, including a proximal conserved E-box found to be critical for *Hsf2* promoter activity [126–128]. USF, a major E-box-binding protein in the brain, displays an expression profile compatible with that of HSF2 in various brain regions [128]. The very conserved regulatory region of the

Hsf2 gene also suggests that HSF2 might be expressed in the developing human brain. The unravelling of spatio-temporal HSF transcription requires additional studies. In particular, the very striking mHSF2 expression in the proliferative ventricular zone (VZ) at all stages of the developing mouse cortex and its specific expression in the late postmitotic cortical neurons claim for deeper investigations.

HSF1 displays expression patterns overlapping with HSF2- possible interactions

HSF1 is also expressed in the neuroepithelium and embryonic brain as is HSF2 in zebrafish, chicken, mouse and rat. HSF1 is expressed in similar patterns as HSF2 in zebrafish and chicken embryos [117,120,121]. However, although cHSF1 and cHSF3 display elevated and ubiquitous expression in the developing neural tube as HSF2, only cHSF2 is constitutively nuclear. This suggests that HSF2 could play a more prominent role in CNS development. HSF1 is expressed in the developing mouse brain throughout development (V.M. unpublished results) and rHSF1

was shown to be nuclear in the neuroectoderm of 9.5 cultured embryos [3]. Since HSF1 and HSF2 were shown to interact physically and functionally [57–61] these two factors are susceptible to interact in a spatio-temporal specific manner in the developing mouse cortex and influence development in unstressed conditions. Today, no data are available to estimate the potential impact of HSF1 deficiency on the mouse embryonic brain.

In contrast to HSF2, which rapidly declines from all brain regions except cerebellum, HSF1 protein levels progressively increase in a tissue-specific manner, in the cortex and in the cerebellum of the postnatal brain, between P1 (first day after birth) and P30 where it displays nuclear localization, and then declines but is still detected at significant levels [124,125].

Therefore, the expression of HSF2 at high levels seems to be intimately correlated with neurogenesis and neuronal migration in different parts of the mouse brain. HSF1 and HSF2 might interact in the developing mouse brain in a stage- and tissue-specific region. The levels of HSF1 at P1 are fully sufficient for a robust HSR, which suggests that the increase of HSF1 in later stages reflects a still unknown function in the postnatal differentiated neurons and glial cells. In the postnatal brain, cytoplasmic and dendritic HSF2 might assume a role distinct from its classical role in the regulation of transcription that we will describe below [125].

Regulation of HSF activities during normal brain development

Few data are currently available on the *in vivo* mechanisms regulating HSF ability to bind DNA or to activate transcription. However, *in vitro* and *ex vivo* studies illustrate the importance of posttranslational modifications such as sumoylation and acetylation in these regulations [129].

Alternative splicing or postranslational modifications are likely to add more subtle levels of regulation of HSF2 DNA-binding or transcriptional abilities. The shorter HSF2 β isoform [130,131] is present at higher levels both before and after birth in mouse brains [122,124]. No HSF4 DNA-binding activity was reported in the developing mouse brain so far. However, Hsf4b is expressed in the adult mouse brain, including cerebellum, and in cultured astrocytes together with Dual-Specificity Tyrosine Phosphatase DUSP26, which alters HSF4b DNA-binding abilities. Another level of the regulation of HSF activity in the brain is therefore MAP kinase signaling pathways [132].

Roles of HSFs during brain normal development

Hsf2 gene inactivation studies were performed by three different laboratories. *Hsf2*-disrupted mice are viable and do not display overt morphological abnormalities. While one laboratory did not observe any brain phenotype in adults (*Hsf2*^{tm1Ijb/tm1Ijb}; [65]), embryonic brain defects were reported by the two other groups [62,63]: *Hsf2*^{tm1Miv/tm1Miv} and *Hsf2*^{tm1Mmr/tm1Mmr} adult brains display enlarged ventricles and reduction in hippocampal and striatum size, as well as cortex width in specific areas. Wang *et al.* also reported prominent CNS abnormalities with collapse of ventricular systems and haemorrhages in cerebral regions at early stages [63]. In addition, HSF2 was shown to be involved later in development during the migration phases of the newborn cortical neurons [123].

Cortical neurons are not generated within their final location sites, but are born from the proliferation of neuronal progenitors located in the inner part of the developing cortex, the ventricular zone (VZ), along the cavities in which the cerebrospinal fluid circulates. To reach their final destination, cortical neurons migrate radially towards the outer surface of the developing cortex. During this process, the cortical neurons receive migration inputs [e.g. the Reelin signal, secreted from Cajal–Retzius cells, which are located at the surface of the neocortex (the marginal zone or MZ)]. The cortical neurons benefit from architectural guides provided by radial glia cell fibers, which extend from the VZ to the MZ [133–136]. HSF2 was shown to be involved in multiple aspects of radial neural migration [123]. HSF2 influences the two cell populations that assist radial neuronal migration: it controls the number of radial glia cells and fibers and the number of Cajal–Retzius cells. Thus, the later defect results in disturbances of the Reelin cascade within migrating neurons. Moreover, within the post-mitotic migrating neurons, HSF2 regulates two genes, *p35* and *p39*, encoding *p35* and *p39*, the activators of Cdk5, a kinase essential for migration known to be involved in migration and modulates their expression [123, 137]. As a consequence, Cdk5 activity is markedly reduced in *Hsf2*^{tm1Mmr/tm1Mmr} embryonic cortices. The output of these multiple defects is the mispositioning of cortical neurons of the most superficial layers, in which HSF2 is expressed [123]. HSF2 is therefore able to influence and perhaps maybe couple distinct aspects in the control of radial neuronal migration: it modulates the extent of various cell populations and controls the apparently independent Reelin and Cdk5 signalling pathways, which are believed to operate synergistically for the correct

positioning of cortical neurons. In these processes, given its modest transcriptional abilities, HSF2 is a fine tuner of gene expression, bringing a refined level of coupling and regulation that is needed to establish the most superficial cortical layers, which are also the more evolutionarily recent.

However, the role of HSF2 in cortical development is probably not restricted to this late phase of migration, as it may also regulate the proliferation of neuroepithelial cells and neuronal progenitors of VZ, where it is expressed at high levels (see above). This would be in agreement with defects observed in early central nervous system defects ([63]; V.M., unpublished results). The reduction in the number of radial glia cells is suggestive of such proliferation impairment [123]. The decrease in the Cajal–Retzius cell population could originate from defects in the proliferation of their precursors or from problems of tangential migration by which, from their birthplace, they colonize the whole MZ [138]. Interestingly, many of the genes identified by transcriptome comparison between *Hsf2*^{+/+} and *Hsf2*^{-/-} E10.5 embryos are involved in the control of proliferation [63]. In addition, *Cdk5*, whose activity is regulated by p35 and p39 in postmitotic neurons is also involved in the control of cell cycle exit and differentiation [139], suggesting that the reduction of *Cdk5* activity observed in *Hsf2*^{-/-} neocortices might partially be responsible for deficits in the cell cycle, or survival or differentiation during corticogenesis. Alternatively, HSF2 may participate in the regulation of protein phosphatase 2A [51,140], an M-specific phase molecule, which negatively regulates entry into M phase in *Xenopus* extracts and is also involved in the regulation of microtubule dynamics and centrosome function [141,142].

The search for HSF2 target genes has received great benefit from these gene-inactivation studies, which led to the identification of the first direct HSF2 target gene in development, *p35*. No major modification in basal *Hsp* gene expression during brain development seems to accompany these defects, suggesting that HSF2 might not control their basal expression during normal conditions [62,63]. A genome-wide analysis of the regions bound by HSF2 and of transcriptome should soon allow a broader understanding of the role of HSF2 in brain cortical development.

Hsf2^{tm1Ijb/tm1Ijb} adult mice appear normal, with no overt signs of behavioural problems, which is in agreement with the behavioural studies performed by McMillan *et al.* [65]. Other behavioural tests are currently performed to investigate the impact of incorrect neuronal migration in different parts of the *Hsf2*^{-/-} brains.

A genetic basis for differences in mouse strains in eliciting the HSR has been established as well as for strain differences in heat-sensitivity for the induction of neural tube defects [143]. Therefore, the HSF-dependent brain developmental process can be very sensitive to the genetic background of the strain of mouse. This could provide an explanation for the differences in phenotypes that are observed in *Hsf2* knockout strains [62,63,65] (Table 1).

Although no data are currently available for the role of HSF1 in brain development, HSF1 has been shown to be implicated in the maintenance of the postnatal brain under nonstress conditions [144,145]. *Hsf1* (*Hsf1*^{tm1Ijb}) inactivation results in enlarged ventricles, as in *Hsf2*-null mice, and astrogliosis and neurodegeneration in specific areas. Interestingly, the expression of *Hsp27* and α B crystalline, which protect cells against stress and apoptosis, are deregulated in specific *Hsf1*-null brain regions. The defects observed in the adult brain do not increase with age. These abnormalities must originate either late in gestation (embryonic brains look normal at E18.5 [65]) or before 1 month after birth. The up-regulation of HSF1 levels and nuclear repositioning, which are observed in the first postnatal month, could be linked to the complexification of brain transcriptome at this age spectrum [124]. A high level of ubiquitinated and oxidated proteins, as well as an increased sensitivity to oxidative stress, is also observed [145].

In conclusion, HSF2 acts in brain and neuronal development by fine tuning, and probably coupling, independent signalling pathways and the establishment of distinct cell populations that govern a given process: proliferation, survival, cell fate or migration. In the adult brain, HSF2 is also expressed in niches for neurogenesis (in the anterior SVZ and the hippocampus), which suggest that it might regulate the production of neurons in both the embryonic and the adult brain [62]. This subtle role of HSF2 on murine cortical development might be even more important and critical in species that possess gyrated cortices. Moreover, comparison between knockdown and knockout strategies suggests that murine species display compensatory mechanisms for the loss of other actors in the migration process, such as members of the doublecortin family *Dcx* and *Delc* [146–148].

Conclusions

The emerging landscape of the role of HSFs in development is the regulation of only some Hsps in very specific processes. In addition, HSFs regulate new target genes, which include growth factor genes (FGF,

LIF), genes that are directly or indirectly involved in cytoskeleton dynamics (*Hsp90* and cortical actin in eggs [33,34]; *p35/p39/Cdk5* [123]; β_{IV} tubulin in ciliary-beating activity [149]; and *Bfsp*, lens-specific intermediate filaments [105]). HSFs also direct the establishment of epigenetic marks and might impact genome structure (chromatin condensation state in spermatogenesis, histone methylation or acetylation status [73,110] and retrotransposition [73]) during development. In contrast to *Hsp* genes and in good correlation with the roles of HSFs in chromatin structure, the discovery of new target genes for HSFs during development pointed out that HSEs are located far from promoter regions – within the core gene body or even far upstream or downstream. Although this was, until now, clearly demonstrated for HSF4 only, HSF1 and HSF2 might further reveal similar distal-binding sites in various developmental processes. In addition, the field could evolve towards the definition of ‘developmental’ HSEs whose consensus sequences could be more flexible than the robust HSEs located in heat shock genes [110,123,129]. A new definition of the HSR, which recently culminated with mHSF3 and the large-scale identification of non-*Hsp* targets, foreshadows a new vision of the role of HSF at a crossroads between stress and development [150,151]. HSF3, which regulates the stress-responsive properties of nonheat shock genes, could presumably be involved in development. Whether mHSF3 displays a developmentally regulated expression profile is a pending question. Another feature of this landscape consists of the subtle cooperative or competitive interactions between HSFs. This may occur between homotrimers [109] or through the formation of heterotrimers [61]. An even higher level of complexity, which remains to be explored, is the HSF post-translational modifications that could potentially modulate their developmental abilities [129]. One can wonder whether their roles in normal development make HSFs mediators of stress or, conversely, protectors against stress during development. The effects of HSF1 on different germ cell populations in terms of survival of stress, as demonstrated for spermatogenesis, are suggestive of a dual role, either beneficial or detrimental, that would have been used by evolution to preserve or eliminate certain cell populations (or even individuals) during development. Finally, the basal levels of HSFs, and, even more interestingly, the ratio between different HSFs, which could vary from one individual to another, could contribute to reproductive success versus infertility or to developmental success versus failure in humans.

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