Role and regulatory mechanisms of heat shock factor 2

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LIST OF ORIGINAL PUBLICATIONS

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I. Östling P*, Björk J.K*, Roos-Mattjus P, Mezger V, Sistonen L. 2007. Heat shock factor 2 (HSF2) contributes to inducible expression of hsp genes through interplay with HSF1. J. Biol. Chem. 282: 7077-7086.

II. Sandqvist A, Björk J.K, Åkerfelt M, Chitikova Z, Grichine A, Vourc'h C, Jolly C, Salminen T.A, Nymalm Y, Sistonen L. 2009. Heterotrimerization of heat-shock factors 1 and 2 provides a transcriptional switch in response to distinct stimuli. Mol. Biol. Cell. 20: 1340–1347.

III. Björk J.K*, Sandqvist A*, Elsing A.E, Kotaja N, Sistonen L. 2010. miR-18, a member of Oncomir-1, targets heat shock transcription factor 2 in spermatogenesis. Development. 137: 3177-3184.

* Equal contribution

ABBREVIATIONS

AD	activation domain
Ago	argonaute
AIB1	amplified in breast 1
ATP	adenosine-5'-triphosphate
BMPR2	bone morphogenetic protein receptor type II
bp	base pair
BRE	TFIIB recognition element
CBP	CREB-binding protein
CFP	cyan fluorescent protein
CHD	chromo-helicase/ATPase DNA binding
ChIP	chromatin immunoprecipitation
ChIP-chip	chromatin immunoprecipitation on microarray
ChIP-seq	chromatin immunoprecipitation combined with sequencing technology
CTD	C-terminal domain
C-terminus	carboxy terminus of proteins
CTGF	connective tissue growth factor
DBD	DNA-binding domain
DGCR8	DiGeorge syndrome critical region 8
DPE	downstream promoter element
DSIF	DRB sensitivity-inducing factor
eIF	eukaryotic translation initiation factor
EMSA	electrophoretic mobility shift assay
ERα	estrogen receptor alpha
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FRET	fluorescence resonance energy transfer
GR	glucocorticoid receptor
GRS	gene recruitment sequence
GTF	general transcription factor
HCC	hepatocellular carcinoma
hnRNP	heterogeneous nuclear ribonucleoprotein
HR-A/B/C	heptad repeat A/B/C
HSE	heat shock element
HSF	heat shock factor
HSP	heat shock protein
HSR1	heat shock RNA-1
INO80/SWR1	inositol requiring/sick with Rat8 ts
Inr	initiator element
ISWI	imitation switch
MEF	mouse embryonic fibroblast
miRISC	miRNA-induced silencing complex
miRNA	microRNA

mRNA	messenger RNA
MRS	memory recruitment sequence
MSCI	meiotic sex chromosomal inactivation
MSYq	male-specific long arm of the mouse Y chromosome
MTE	motif ten element
NELF	negative elongation factor
N-TEF	negative transcription-elongation factor
NPC	nuclear pore complex
nSB	nuclear stress body
PACT	protein kinase R (PKR) activator
PCK	phosphoenolpyruvate carboxykinase
PI	propidium iodide
PIC	preinitiation complex
P-TEFb	positive transcription-elongation factor-b
PTEN	phosphatase and tensin homolog
PVN	paraventricular nucleus
RD	regulatory domain
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
RNAP II	RNA polymerase II
RT-PCR	reverse transcription polymerase chain reaction
sat III	satellite III
shRNA	small hairpin RNA
siRNA	small interfering RNA
SIRT1	sirtuin 1
SPEER	sperm-associated glutamate-rich
Ssty2	spermiogenesis-specific transcript on the Y 2
SUMO	small ubiquitin-like modifier
SWI/SNF	switching-defective-sucrose non-fermenting
TBP	TATA-box binding protein
TFII	transcription factor II
TGFβ	transforming growth factor beta
T-GIST	transfection of germ cells in intact seminiferous tubules
TRBP	TAR RNA binding protein
Tsp1	thrombospondin-1
TSS	transcriptional start site
UTR	untranslated region
YFP	yellow fluorescent protein

ABSTRACT

Protein homeostasis is essential for cells to prosper and survive. Various forms of stress, such as elevated temperatures, oxidative stress, heavy metals or bacterial infections cause protein damage, which might lead to improper folding and formation of toxic protein aggregates. Protein aggregation is associated with serious pathological conditions such as Alzheimer's and Huntington's disease. The heat shock response is a defense mechanism that protects the cell against protein-damaging stress. Its ancient origin and high conservation among eukaryotes suggest that the response is crucial for survival. The main regulator of the heat shock response is the transcription factor heat shock factor 1 (HSF1), which induces transcription of genes encoding protective molecular chaperones. In vertebrates, a family of four HSFs exists (HSF1-4), with versatile functions not only in coping with acute stress, but also in development, longevity and cancer. Thus, knowledge of the HSFs will aid in our understanding on how cells survive suboptimal circumstances, but will also provide insights into normal physiological processes as well as disease-associated conditions. In this study, the function and regulation of HSF2 have been investigated. Earlier gene inactivation experiments in mice have revealed roles for HSF2 in development, particularly in corticogenesis and spermatogenesis. Here, we demonstrate that HSF2 holds a role also in the heat shock response and influences stress-induced expression of heat shock proteins. Intriguingly, DNA-binding activity of HSF2 upon stress was dependent on the presence of intact HSF1, suggesting functional interplay between HSF1 and HSF2. The underlying mechanism for this phenomenon could be configuration of heterotrimers between the two factors, a possibility that was experimentally verified. By changing the levels of HSF2, the expression of HSF1-HSF2 heterotrimer target genes was altered, implementing HSF2 as a modulator of HSF-mediated transcription. The results further indicate that HSF2 activity is dependent on its concentration, which led us to ask the question of how accurate HSF2 levels are achieved. Using mouse spermatogenesis as a model system, HSF2 was found to be under direct control of miR-18, a miRNA belonging to the miR-17~92 cluster/Oncomir-1 and whose physiological function had remained unclear. Investigations on spermatogenesis are severely hampered by the lack of cell systems that would mimic the complex differentiation processes that constitute male germ cell development. Therefore, to verify that HSF2 is regulated by miR-18 in spermatogenesis, a novel method named T-GIST (Transfection of Germ cells in Intact Seminiferous Tubules) was developed. Employing this method, the functional consequences of miR-18-mediated regulation *in vivo* were demonstrated; inhibition of miR-18 led to increased expression of HSF2 and altered the expression of HSF2 target genes *Ssty2* and *Speer4a*. Consequently, the results link miR-18 to HSF2-mediated processes such as germ cell maturation and quality control and provide miR-18 with a physiological role in gene expression during spermatogenesis.Taken together, this study presents compelling evidence that HSF2 is a transcriptional regulator in the heat shock response and establishes the concept of physical interplay between HSF2 and HSF1 and functional consequences thereof. This is also the first study describing miRNA-mediated regulation of an HSF.

INTRODUCTION

The varieties of different cell types that constitute living organisms as well as the multitude of functions the cell types perform are astounding. This diversity is acquired through the repertoire of proteins that a specific cell expresses. The proteins constitute the workforce of the cell, determining its functions and prevalence. The blueprint for the proteins is contained within the genome, which in humans holds approximately 25 000 genes. Information in the genome is transcribed into RNA molecules and further translated into the corresponding proteins. In this process, the cell is aided by sequence-specific transcription factors that, to certain extent, determine which genes are transcribed and concomitantly, which proteins are synthesized. At any given moment, the correct set of proteins must be expressed for the cell to exert its functions. The cell further needs to be able to respond to changes in the environment through adjusting its gene expression program.

During certain situations, the protein homeostasis in a cell can be disturbed, leading to malfunctional proteins unable to conduct their actions. Elevated temperatures, oxidative stress or infections belong to the inducers of proteotoxic stress. Failure in launching an appropriate response leads to protein aggregation and ultimately to cell death. The heat shock response is a conserved defense mechanism that facilitates cell survival under suboptimal conditions by enhancing expression of heat shock genes. Among them are heat shock proteins (Hsps), which function as molecular chaperones aiding the folding of misfolded proteins and preventing protein aggregation. Transcriptional induction during the heat shock response is regulated by heat shock factors (HSFs), of which there are four in vertebrates (HSF1-4). HSF1 is regarded as the *bona fide* stress-activated member of this family of transcription factors, and binds the promoter of *hsp* genes upon activation. HSF2, on the other hand, has been considered refractory to stress stimuli and instead ascribed functions as a transcriptional regulator in development, particularly spermatogenesis and corticogenesis.

In this thesis, a role for HSF2 in the heat shock response is established. Furthermore, functional interplay between HSF1 and HSF2 as well as a physical interaction in the form of heterotrimerization are demonstrated. The study also presents evidence that the levels of HSF2 determine its function and that in spermatogenesis, the amount, and thus activity, of HSF2 is regulated by a specific microRNA, miR-18. All in all, this study provides novel insight into the function and regulation of HSF2, both in the cellular stress response and in developmental settings.

REVIEW OF THE LITERATURE

1. BASIC PRINCIPLES OF TRANSCRIPTION IN EUKARYOTES

Few molecules have aroused such astonishment as DNA; the bearer of the inherited information across generations in organisms from bacteria to man. In humans, the genetic information is divided on 46 chromosomes, which are further subdivided into genes that hold the code for protein synthesis. All biological processes, such as development, differentiation and growth, depend on accurate expression of genes. The same complete set of genes exists in principally all diploid cells of a given individual. However, different types of cells necessitate expression of specific subsets of genes. Furthermore, gene expression needs to be rapidly adjustable upon changing conditions encountered by the cells. For these reasons, careful regulation of gene expression is a prerequisite. This regulation can be executed on several levels such as transcription, messenger RNA (mRNA) processing, translation or protein stability control. The first step in this process, synthesis of RNA or transcription, is under profound regulation. A multisubunit enzyme, RNA polymerase II (RNAP II), catalyzes the transcription of protein-encoding genes in eukaryotes. Three different classes of regulatory factors facilitate accurate transcription by RNAP II; general transcription factors (GTFs), promoter-specific transcription factors and co-activators (Maston *et al*, 2006; Venters & Pugh, 2009).

1.1. Transcriptional initiation and regulatory elements

Gene expression is dictated by several regulatory elements that influence the positioning of RNAP II and the rate of transcription (Fig. 1). The so called core promoter is located approximately 35 base pairs (bp) upstream or downstream of the transcription start site and contains such regulatory elements. The first core promoter element to be discovered was the TATA box, an AT-rich sequence upstream of the transcription start site. Originally, the TATA box was considered to be required for transcription of most protein-coding genes. However, it has become clear that a TATA box is present only in 10-20% of all mammalian promoters (Gershenzon & Ioshikhes, 2005; Cooper *et al*, 2006; Sandelin *et al*, 2007). Instead, other elements such as the initiator element (Inr), downstream promoter element (DPE), motif ten element (MTE) and TFIIB recognition element (BRE) have been found. None of these elements is universal though, but each is present in only a subset of promoters. Promoters lacking known core elements altogether have also been detected, indicating the existence of more unusual elements, yet to be discovered. The occurrence of multiple core promoter elements provides diversity and complexity in gene regulation. To add to this diversity, most human protein-coding genes can be regulated by several promoter regions, each usually activated in a tissue- or situation-specific manner. Moreover, many genes have several transcription start sites, which either are located in a cluster or dispersed over an area of up to 100 bp (Gershenzon & Ioshikhes, 2005; Juven-Gershon *et al*, 2008).

The core promoter elements interact directly with components of the basal transcription machinery, which is defined as the minimum set of factors necessary to induce transcription executed by RNAP II *in vitro* (Smale & Kadonaga, 2003; Venters & Pugh, 2009). Assembly of a preinitiation complex (PIC; Fig. 2) is typically initiated by the GTF

TFIID binding to the TATA-box, Inr element or other sites at the core promoter. TFIID is a multisubunit complex, including TATA-box binding protein (TBP) and a number of TBP-associated factors (TAFs). Through its binding to the TATA-box, TFIID induces bending of the template DNA, promoting recruitment of other GTFs such as TFIIA, TFIIB, TFIIE, TFIIF, and TFIIH. The functions of the GTFs incorporate promoter melting and directing the RNAP II to the transcription start site (Maston *et al*, 2006; Thomas & Chiang, 2006; Sandelin *et al*, 2007). Although this complex is able to induce transcription from an isolated core promoter *in vitro*, *in vivo*, the action of another multisubunit complex called Mediator is required. This complex consists of roughly 30 proteins and functions as a bridging factor between PIC and sequence-specific transcription factors (Fig. 2) (Malik & Roeder, 2005). Even though the build-up of the transcription machinery frequently is presented as a sequential event in respect to component enrolment, RNAP II is likely recruited as a holoenzyme together with GTFs and components of the Mediator. Furthermore, the composition of the transcriptional machinery is not fixed, and it is plausible that the sequence of the promoter dictates complex composition and assembly (Smale & Kadonaga, 2003; Thomas & Chiang, 2006).

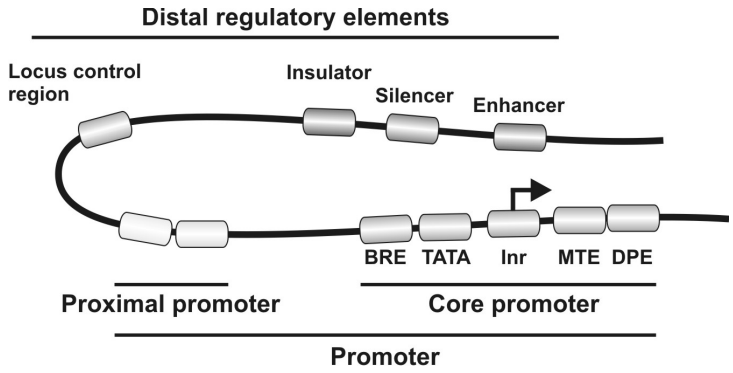

Figure 1. Typical regulatory elements in eukaryotic gene expression. The core promoter region is located close to the transcription start site (arrow), and may contain elements such as the TFIIB recognition element (BRE), TATA box (TATA), initiator element (Inr), motif ten element (MTE) and downstream promoter element (DPE). Binding sites for gene-specific transcription factors are found at the proximal promoter region. Distal regulatory elements include locus control regions, enhancers, silencers and insulators. Modified from (Maston *et al*, 2006; Fuda *et al*, 2009).

In addition to the elements at the core promoter, other regions on DNA contribute to transcriptional control. Such regions are proximal promoter elements and distal regulatory elements (Fig. 1). The proximal promoter elements are located immediately upstream of the core promoter and typically house binding sites for activators (see section 1.2). Distal regulatory elements can, as the name implies, be located far from the transcription start site (up to 1 Mbp), and include enhancers, silencers, insulators, and locus control regions (Maston *et al*, 2006; Narlikar & Ovcharenko, 2009). The existence of various regulatory elements provides combinatorial control, exponentially increasing the number of unique expression patterns.

1.2. Transcriptional activators and repressors

Assembly of PIC on the core promoter is sufficient for low level, or basal transcription. However, transcription is significantly accelerated by promoter-specific transcription factors, also named activators (Fig. 2), which bind to sequence-specific DNA regions typically of small size (6-12 bp). The transcription factors are modular entities, divided into classes based on their DNA-binding domains (DBDs), which confer specificity toward a certain DNA region. Examples of DBD motifs include helix-loop-helix, basic leucine zipper, forkhead and cystein rich zinc finger (Kadonaga, 2004; Maston *et al*, 2006; Georges *et al*, 2009). In addition to the DBD, transcription factors most often contain an activation domain (AD) that is required for their function. Upon activation, by factor-specific stimulatory signals, the AD relays the signal to the general transcription machinery, usually via co-activators which themselves have no intrinsic sequence specificity but function through protein-protein interactions. The activity of the transcription factors affects PIC assembly, initiation, elongation, reinitiation or chromatin modifications (see section 1.4) (Kadonaga, 2004; Maston *et al*, 2006; Weake & Workman, 2010). Another characteristic domain often found in transcription factors is an oligomerization domain and many factors form homo- and/or heterodimers when bound to DNA. The subunit composition can influence the binding specificity as well as regulatory capability of the transcription factor. Furthermore, the sequence of the binding site on DNA can give preference to certain oligomerization partners or influence the structure of a bound transcription factor, thereby affecting its activity (Claessens & Gewirth, 2004; Geserick *et al*, 2005; Georges *et al*, 2009).

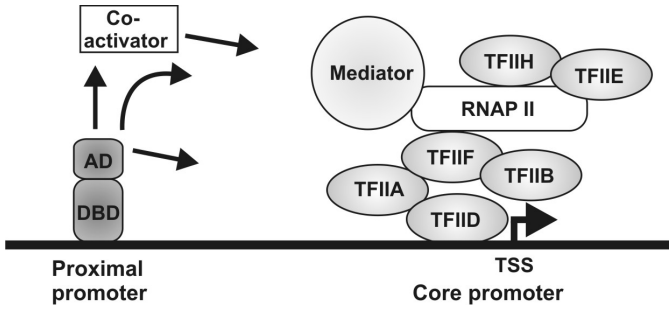

Figure 2. Basic components of the transcription machinery in eukaryotes. General transcription factors including TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH direct RNAP II to the transcription start site (TSS) and constitute, together with RNAP II, the preinitiation complex (PIC). Transcription is enhanced by activators binding to sequence-specific regulatory regions through their DNA-binding domain (DBD). Via the activation domain (AD) activators can directly affect assembly of the PIC, other co-activators or the Mediator complex. Modified from (Maston *et al*, 2006).

A prominent characteristic of transcription factors is their ability to act synergistically, which can arise from identical factors cooperating or collaboration between different factors. The mechanisms underlying the synergy effect are intangible, but one possibility is that the factors create a common interaction surface, which facilitates recruitment of subsequent factors (Georges *et al*, 2009). Besides being positively regulated, the transcription machinery can also be suppressed by transcriptional repressors or co-repressors. The repressors act on several levels, for example by competing with activators for a DNA-binding site, by masking the AD, or by sequestering activators. Furthermore,

many factors have a dual role in that they for instance function as repressors in the absence of an activating signal, but have a positive impact in the company of activators (Courey & Jia, 2001; Thomas & Chiang, 2006).

1.3. RNAP II and the elongation phase of transcription

RNAP II is the key catalytic enzyme in the transcription of protein-encoding genes. Previously, the recruitment of RNAP II to the core promoter was considered the rate limiting step in transcription. However, some genes, such as *Myc* and *Hsp* genes were peculiarly found to be regulated at the level of elongation (Gilmour & Lis, 1985; Bentley & Groudine, 1986). Rougvie and Lis showed that RNAP II was transcriptionally engaged but paused at the uninduced *Drosophila Hsp70* gene after synthesizing 20-50 nucleotides (Rougvie & Lis, 1988). Several studies followed showing similar results, however, it was assumed that pausing of RNAP II merely applied to immediate early genes and a few genes that were poised for rapid activation (Krumm *et al*, 1992; Fivaz *et al*, 2000). This hypothesis was disputed when genome-wide studies, mapping RNAP II along the genes, found that 20-30% of all genes were bound by RNAP II (Guenther *et al*, 2007; Muse *et al*, 2007; Zeitlinger *et al*, 2007; Core & Lis, 2008). Since the polymerase was located both at genes with detectable and undetectable gene expression the studies imply that a post-recruitment step is rate limiting at these genes and point to generality of RNAP II pausing.

RNAP II is being stalled on the genes primarily by two negative transcription-elongation factors (N-TEFs), DSIF and NELF, and the pausing is thought to function as a checkpoint before the polymerase commits to productive elongation. RNAP II can, however, rapidly escape the paused state and the principal executor of the escape from pausing is positive transcription-elongation factor-b (P-TEFb). This factor phosphorylates DSIF and NELF as well as the C-terminal domain (CTD) of RNAP II (Yamada *et al*, 2006; Price, 2008; Weake & Workman, 2010). The long flexible CTD contains multiple repeats of a heptapeptide subjected to phosphorylation and the phosphorylation pattern correlates with the transcriptional activity of RNAP II. Furthermore, phosphorylation of CTD also coordinates recruitment of RNA processing factors in an ordered fashion. These factors travel along with RNAP II and facilitate capping, splicing and polyadenylation of the nascent RNA being produced (Meinhart *et al*, 2005; Saunders *et al*, 2006; Egloff & Murphy, 2008; Venters & Pugh, 2009). In this manner, elongational control is coupled to RNA processing.

1.4. Regulation of transcription at the level of chromatin

If one were to stretch out the DNA that is contained in a single diploid human cell the length of the DNA thread would equal about 2 meters. To fit into the nucleus of cells the DNA has to be tightly packed. In fact, in its densest form, during cell division, the DNA molecule can be packed up to nearly 10 000-fold. The basic compaction of DNA is achieved by histones; two copies each of the histones H2A, H2B, H3 and H4 form a core octamer around which 147 bp of DNA is wrapped. This structure is called a nucleosome. The nucleosomes are spaced along the thread with a stretch of linker DNA between them and stabilized by binding of histone H1. Together with additional proteins the nucleosomes are further compacted into a structure entitled chromatin (Kornberg & Lorch, 1999; Orphanides & Reinberg, 2000; Quina *et al*, 2006). This compaction, albeit indispensable,

forms a formidable structural barrier for the transcription machinery to access the underlying regulatory elements and coding regions. Therefore, modulation of chromatin structure is a crucial step in gene expression and offers an additional level of regulatory control. This modulation is achieved by histone modification, chromatin remodeling, incorporation of histone variants, and histone deportation (Li *et al*, 2007).

Chromatin remodeling complexes utilize ATP hydrolysis to alter the structure, position or composition of nucleosomes thereby shifting the histone-DNA contact. Four families of remodeling complexes have been found; SWI/SNF, INO80/SWR1, ISWI and CHD. Although variations exist between the families, the general mechanism by which the complexes work is similar. The DNA is unwrapped from the histone octamer and a loop is formed, which results in nucleosomes sliding along DNA or complete nucleosome eviction. This consequently influences the accessibility of DNA for components of the transcriptional machinery. Since the chromatin remodeling complexes lack ability to target specific regions on DNA, sequence-specific regulators likely recruit the complexes to the promoter regions. Some of these regulators in turn are directed by another type of regulation, namely histone modification (Li *et al*, 2007; Venters & Pugh, 2009).

Histones are subjected to post-translational modifications, especially on their tail consisting of 20-40 amino acids protruding from the core nucleosome. These modifications include methylation, acetylation, ubiquitination, ADP-ribosylation, sumoylation and phosphorylation (Li *et al*, 2007; Taverna *et al*, 2007). It has been suggested that the various modifications and combinations thereof create a so called histone code that can be read by regulatory proteins to affect the transcriptional state of that region (Strahl & Allis, 2000). Besides providing docking sites for regulators, the histone modifications change the structure of chromatin by altering electrostatic or internucleosomal contacts. This is exemplified by acetylation of lysine residues on histones, which leads to neutralization of the positive charge and thereby loosening the interaction between the nucleosome and DNA. It should, however, be noted that acetylation also works by generating a binding site for proteins containing an acetyl-lysine binding bromodomain. The decondensed regions usually correspond to actively transcribed sequences and are termed euchromatin. By contrast, inactive regions are often highly condensed and are referred to as heterochromatin. Typical post-translational modifications in inactive regions are methylation of histone H3 on lysine residues 9 and 27 (H3K9me and H3K27me, respectively) and low levels of acetylation (Li *et al*, 2007; Taverna *et al*, 2007; Venters & Pugh, 2009). With the appearance on refined techniques such as chromatin immunoprecipitation followed by DNA microarrays (ChIP-chip) or sequencing analysis (ChIP-seq), enabling survey on a genome-wide scale, various patterns of modifications or nucleosome positioning with functional consequences have been uncovered. In extension, this postulates that the chromatin signature of a particular region can be used to predict the transcriptional status of that region. It can thus be concluded that chromatin not merely harbors the blueprint of the molecules produced, but also information on when and where these molecules are made (Hon *et al*, 2009; Venters & Pugh, 2009).

1.5. Genome organization as a means to regulate transcription

In recent years, it has become evident that the activity of genes often correlates with their localization within the nucleus, offering yet another layer of transcriptional control. The genome is spatially organized within the nucleus and the compartmentalization enables the

existence of different nuclear microenvironments. The chromosomes are non-randomly distributed, and even the positioning of genes is a non-arbitrary and dynamic process (Hubner & Spector, 2010). In the interphase nucleus, the chromosomes inhabit specific territories. Within the territories, protein-coding genes are mainly located towards the edges while non-coding regions occupy a more interior position (Kurz *et al*, 1996). With regard to the whole nucleus, peripheral localization has, in general, been associated with a repressive state given that mostly gene-poor chromosome regions are found in the nuclear periphery (Akhtar & Gasser, 2007; Hubner & Spector, 2010). The nuclear lamina binds to chromatin and in a genome-wide search for lamin B-interacting genes in fruit fly it was found that associated genes were silent and lacked histone marks for active transcription (Pickersgill *et al*, 2006). It has also been shown that experimentally induced repositioning of genes to the vicinity of the nuclear lamina promotes silencing in mammalian cells (Finlan *et al*, 2008; Reddy *et al*, 2008), providing a possible explanation for the repressive environment at the nuclear periphery. Likewise, for numerous developmentally regulated genes in various organisms, movement toward the nuclear interior seems to be a common phenomenon upon activation. Examples include the *β-globin* locus during mouse erythroid differentiation, the *IgH* and *Igκ* loci during lymphocyte differentiation, and the *Mash1* locus upon neuronal differentiation of mouse embryonic stem cells (Hubner & Spector, 2010; Egecioglu & Brickner, 2011). On the contrary, a genome-wide analysis in yeast has revealed that several actively transcribed genes are associated with the nuclear pore (Casolari *et al*, 2004). Furthermore, the *Gal* and *Ino1* gene loci translocate to the nuclear membrane upon transcriptional activation (Brickner & Walter, 2004; Casolari *et al*, 2004). The nuclear pore complex (NPC) then binds the promoter of the genes, which indicates involvement in the gene activation process (Schmid *et al*, 2006). Also in fruit fly and mammalian cells, certain genes are associated with the NPC and located to the nuclear periphery when active (Kurshakova *et al*, 2007; Brown *et al*, 2008). It is thus feasible that the nuclear periphery holds specific subregions for active and repressed gene expression.

An intriguing question is how movement of genes, associated with changes in the transcriptional status, is accomplished. One possibility is that the spatial localization of a gene is encoded in the DNA itself. Indeed, it has been shown that a specific enhancer suppressed silencing of a transgene by preventing its localization to centromeric heterochromatin (Francastel *et al*, 1999). Similarly, an insulator sequence affected gene expression by co-localizing distinct loci to insulator bodies at the nuclear periphery (Gerasimova *et al*, 2000). In *S. cerevisiae, cis*-acting Gene Recruitment Sequences (GRSs) have been discovered in the promoter of the *Ino1* gene (Ahmed *et al*, 2010). These sequences function as DNA zip codes that promote localization of loci to the nuclear periphery. The GRS elements confer physical interaction with the NPC, which is important for full activation of the gene. Interestingly, when searching for GRS-containing sequences genome-wide, enrichment for genes interacting with NPC as well as genes induced by protein folding stress was found (Ahmed *et al*, 2010).

Several yeast genes, including *Ino1*, which localize to the nuclear periphery when active, remain at the periphery after they are repressed, and the localization remains through multiple cell divisions (Brickner *et al*, 2007; Kundu *et al*, 2007; Brickner, 2009). The peripheral localization thereby represents an epigenetic form of transcriptional memory, and seems to prime the genes for reactivation (Egecioglu & Brickner, 2011). Although multiple mechanisms for peripheral localization coupled to acquired transcriptional memory have been reported (Kundu *et al*, 2007; Zacharioudakis *et al*, 2007; Tan-Wong *et al*, 2009), for the gene *Ino1*, the mechanism involves another DNA zip code. The zip code

is termed Memory Recruitment Sequence (MRS) and promotes association with NPC and an altered chromatin state (Light *et al*, 2010).

Co-localization of the transcription machinery and target genes is often thought of as being achieved by diffusion of the transcription machinery components through the nucleoplasm. It has, however, been suggested that instead of recruiting components needed for transcription, genes could themselves loop out into specific preassembled territories called transcription factories (Iborra *et al*, 1996; Sutherland & Bickmore, 2009). This hypothesis is based on findings of transcriptionally active units clustered in nuclear foci together with engaged RNAP II, which possibly is attached to a immobile substructure (Carter *et al*, 2008; Sutherland & Bickmore, 2009). Individual factories have been shown to hold genes separated by long distances (over 40 Mbp) or genes located on different chromosomes, and their existence implies co-regulation of gene expression (Osborne *et al*, 2004; Sutherland & Bickmore, 2009). Whether the transcription factories merely constitute foci for accumulation of RNAP II on transcribing genes or possess functional significant roles in regulating transcription and genome organization is at this point a matter of debate.

2. microRNAs AS REGULATORS OF GENE EXPRESSION

An important layer of control in eukaryotic gene expression is sited at the post-transcriptional level and comprises mRNA metabolism (Mata *et al*, 2005). Although only about 1.5% of the mammalian genome is protein-coding, recent data suggest that most of the DNA is transcribed into RNA (Pheasant & Mattick, 2007; Clark *et al*, 2011; Mattick, 2011). The functions of the numerous RNA species produced are, however, largely unknown. Interesting to note is that the coding part of the DNA is highly similar in size and function between organisms as diverse as humans and nematodes, but that the extent of non-coding DNA increases with organism complexity (Clark *et al*, 2011). It can thus be assumed that there are exciting times yet to come in the field of non-coding RNA research. One known group of small non-coding RNA molecules is involved in eukaryotic RNA interference (RNAi) pathways. The small RNAs belonging to this group have varying characteristics but are all regulators of gene expression and genomes, albeit at various levels. Based on the mechanisms of their biogenesis and on the type on proteins they are associated with, these RNA molecules can be divided into three classes; microRNAs (miRNAs), small interfering RNAs (siRNAs) and Piwi-interacting RNAs (piRNAs). The siRNAs are further subdivided into exogenous-siRNAs (exo-siRNAs) and endogenously produced-siRNAs (endo-siRNAs) depending on their origin, while the piRNAs are classified into two subgroups that differ in sequence characteristics, genomic origin, temporal expression, binding partners, and function (Kim *et al*, 2009; Li & Liu, 2011; Meikar *et al*, 2011). miRNAs constitute perhaps the best characterized group, and serve as powerful regulators of gene expression at the post-transcriptional level. The first miRNA, lin-4, was discovered in 1993 and found to regulate developmental timing in the nematode *C. elegans* (Lee *et al*, 1993; Wightman *et al*, 1993). Yet, it took almost a decade before miRNAs were recognized in mammalian species (Lagos-Quintana *et al*, 2001; Lau *et al*, 2001). By now, it is estimated that the human genome contains over 1000 miRNA genes (Berezikov *et al*, 2005) and that the miRNAs control the activity of a majority of the protein-coding genes (Friedman *et al*, 2009). Intense research during the past few years has

revealed that miRNAs are involved in almost all aspects of cellular life such as differentiation, metabolism, development, proliferation, apoptosis, and tumorigenesis.

2.1. miRNA biogenesis

Mature miRNAs are single-stranded RNA molecules of approximately 22 nucleotides in length that target mRNAs. The miRNAs are derived from their own genes and the transcription is mediated by RNAP II, although a minor part of the miRNAs reportedly are transcribed by RNAP III (Borchert *et al*, 2006; Kim *et al*, 2009). The primary transcripts, called pri-miRNAs, vary in length, but can be up to several kilobases long. The pri-miRNAs contain a stem-loop structure that by the action of the nuclear Microprocessor complex is cleaved off from the rest of the transcript (Fig. 3). The Microprocessor complex is composed of the RNase III endonuclease Drosha and its co-factor, which in human cells is DiGeorge syndrome critical region 8 (DGCR8) (Lee *et al*, 2003; Han *et al*, 2004). In addition, several auxiliary factors such as heterogeneous nuclear ribonucleoproteins (hnRNPs), p68, FUS and DEAD-box helicases associate with the Microprocessor complex, albeit with largely unknown functions (Kim *et al*, 2009; Suzuki & Miyazono, 2011). The hairpin-like structure thus formed is about 70 nucleotides long and called pre-miRNA. The pre-miRNA is recognized by Exportin-5, which together with Ran-GTP mediates its translocation from the nucleus (Yi *et al*, 2003). In the cytoplasm, another RNase III protein, Dicer, assisted by the dsRNA-binding proteins TRBP or PACT, cleaves the hairpin-like structure. This results in an imperfectly double-stranded RNA molecule with protruding 2-nucleotide 3' ends (Hutvagner *et al*, 2001; Ketting *et al*, 2001). Together with argonaute (Ago) proteins the dsRNA molecule forms the miRNA-induced silencing complex (miRISC), also referred to as micro-ribonucleoproteins (miRNPs). In humans, four Ago proteins exist (Ago1-4), although significantly different functions between them have not been found as is the case for example in *D. melanogaster* or *C. elegans*, where structural attributes in the pre-miRNA determine the choice of Ago (Pillai, 2005; Kim *et al*, 2009; Suzuki & Miyazono, 2011). Upon assembly of miRISC the miRNA duplex is denatured and, generally, the strand with the thermodynamically less-stable 5' terminus remains associated with the complex while the other strand is degraded (Khvorova *et al*, 2003). The mature miRNA can then guide miRISC to target mRNAs.

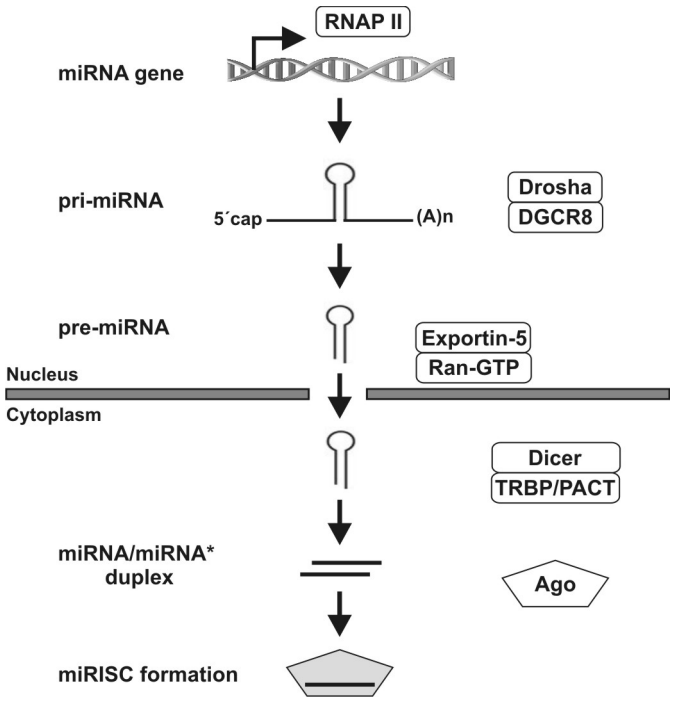

Figure 3. Canonical miRNA biogenesis. miRNAs are transcribed by the action of RNAP II. The transcript, pri-miRNA, folds to form a stem-loop structure, which is processed into pre-miRNA by Drosha and DGCR8. Exportin-5 recognizes the 2-nucleotide 3'overhang of the pre-miRNA and together with Ran-GTP mediates translocation to the cytoplasm. The pre-miRNA is cleaved by Dicer and associated factors TRBP or PACT to form a miRNA duplex. Together with Ago, one strand of the duplex assembles into the RISC, while the other strand, (miRNA*) usually is degraded. Modified from (Kim *et al*, 2009; Suzuki & Miyazono, 2011).

Notably, some miRNA loci are located within protein-encoding regions and the majority of them are found in the intronic parts. Their biogenesis differs from the pathway described above in that they are transcribed as part of the pre-mRNA and processed by Drosha before splicing of the host intron (Kim & Kim, 2007; Kim *et al*, 2009). In addition, a small part of the miRNAs is produced via non-canonical pathways independently of the action of Drosha. These are instead generated by the spliceosome through splicing and debranching of hairpin introns, termed mirtrons (Okamura *et al*, 2007; Ruby *et al*, 2007; Suzuki & Miyazono, 2011). Production of miRNAs bypassing cleavage by Dicer has also been reported (Cheloufi *et al*, 2010; Cifuentes *et al*, 2010).

2.2. miRNA mechanisms of action

miRNA exerts its action in metazoans by Watson-Crick base pairing with the target mRNA, usually in the 3′-untranslated region (UTR). For target recognition, perfect base pairing of the so called seed region, which constitutes nucleotides 2-8 of the miRNA 5′-end, is crucial. Binding at this region then nucleates base pairing of the remaining miRNA and its target, although this interaction seems to be of secondary importance and allows for extensive non-complementarity (Bartel, 2009). Typically, binding of a miRNA leads to target gene silencing via two mechanisms; translational repression or mRNA destabilization (Fig. 4) (Filipowicz *et al*, 2008).

The exact details of how miRNAs induce translational repression are still unclear, but it can probably be carried out via several mechanisms. At the initiation step of mRNA translation miRISC is thought to either interfere with the cap-recognition stage of the eukaryotic translation initiation factors (eIFs) or with the association of the ribosomal 60S subunit (Humphreys *et al*, 2005; Pillai *et al*, 2005; Chendrimada *et al*, 2007). During post-initiation steps the complex seems to be able to slow down the elongation phase, but also to render ribosomes prone to prematurely terminate translation (Maroney *et al*, 2006; Nottrott *et al*, 2006; Petersen *et al*, 2006). Additionally, it is proposed that the miRNA can induce proteolysis of the nascent polypeptide as it exits the ribosome. This hypothesis stems from the observation that repressed mRNAs can be engaged with translationally competent polysomes suggesting that protein synthesis occurs from these mRNAs. So far, no protease performing this task has been identified (Nottrott *et al*, 2006; Filipowicz *et al*, 2008).

For part of the miRNA targets, repression of gene expression is associated with reduced mRNA levels. This is mediated via miRISC-induced shortening of the mRNA poly(A) tail, which leads to mRNA destabilization and degradation by progressive 3'-5' decay. Deadenylation can also provoke decapping followed by 5'-3' degradation of the mRNA (Bagga *et al*, 2005; Behm-Ansmant *et al*, 2006; Wu *et al*, 2006; Filipowicz *et al*, 2008). The decay is thought to occur in P-bodies, which are cytoplasmic structures enriched in components necessary for mRNA turnover as well as for miRNA-mediated gene silencing (Kulkarni *et al*, 2010). mRNA molecules repressed at the translational initiation stage are also found in P-bodies where they are either stored or degraded (Filipowicz *et al*, 2008). Interestingly, Bhattacharyya and colleagues found that the miRNA miR-122 together with its target mRNA CAT-1 localizes to P-bodies when CAT-1 translation is repressed. However, upon amino acid starvation CAT-1 mRNA is relocated from the P-bodies, thus showing that miRNA-induced mRNA storage and repression can be reversed (Bhattacharyya *et al*, 2006).

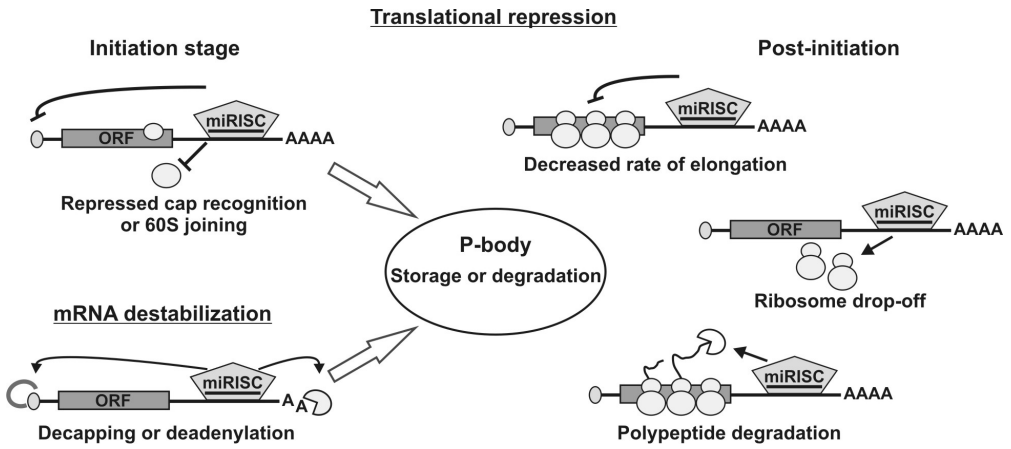

Figure 4. Principal mechanisms of miRNA-mediated post-transcriptional gene repression. miRISC binding to the 3'UTR of target genes leads to translational repression at the initiation stage by hindering cap recognition of the eIFs or the ribosomal 60S subunit from joining. At the post-initiation stage translational repression is achieved via three possible mechanisms: miRISC-mediated decrease in the rate of elongation, ribosomal drop-off, or degradation of the nascent polypeptide. Alternatively, miRISC induces mRNA destabilization, which is achieved via deadenylation, sometimes followed by decapping. mRNAs repressed at the translational initiation stage or via deadenylation are located to P-bodies, where they are stored or degraded. ORF; open reading frame. Modified from (Cannell *et al*, 2008; Filipowicz *et al*, 2008).

In metazoans the miRNAs pair imperfectly with their targets to induce gene silencing by the mechanisms described above. A few reports have nonetheless shown that, occasionally, miRNAs pair with their targets with perfect or near perfect complementarity. This triggers endonucleolytic cleavage of the mRNA by a similar RNAi-like process that prevails in plants (Mansfield *et al*, 2004; Yekta *et al*, 2004; Davis *et al*, 2005; Du & Zamore, 2005). Additionally, it has been suggested that miRNAs hold roles in gene silencing at the transcriptional level, specifically by inducing heterochromatin formation through base pairing with the target gene promoter (Kim *et al*, 2008; Moazed, 2009).

2.3. Biological functions of miRNAs

Perhaps due to their short length and seemingly broad latitude for target base-pairing, a single miRNA can bind numerous mRNA species and, likewise, a single mRNA can be targeted by several different miRNAs. Potentially, this enables build-up of complex regulatory networks (Rajewsky, 2006; Bartel, 2009; Friedman *et al*, 2009). Moreover, regulation via miRNAs is estimated to be involved in most cellular events, and reportedly, miRNAs are particularly often associated with dynamic and developmental processes such as embryogenesis and stem cell proliferation (Stark *et al*, 2005; Rosa & Brivanlou, 2009; Inui *et al*, 2010). Many miRNAs are expressed in a tissue- and cell-specific manner, and as such confer accuracy to gene expression programs at hand, which in turn generates tissue or cell identity (Stark *et al*, 2005; Landgraf *et al*, 2007). In mouse development for example, each embryonic stage is associated with a characteristic miRNA expression profile, and already in the zygote maternally derived miRNAs are present (Mineno *et al*, 2006; Tang *et al*, 2007; Yang *et al*, 2008; Viswanathan *et al*, 2009). Knocking out *Dicer,* and hence bulk miRNA production, is furthermore detrimental for mouse zygote development and no viable embryos can be detected after embryonic day 7.5 in *Dicer*-null mice (Bernstein *et al*, 2003). It has also been shown that a lack of Dicer in maturing oocytes leads to defects at the meiotic stage and to female infertility (Murchison *et al*, 2007; Tang *et al*, 2007).

2.3.1. miRNAs in spermatogenesis

Functional Dicer is needed for male germ cell development, and mice in which *Dicer* is deleted specifically in the testis are infertile. Consequently, this indicates roles for miRNAs in spermatogenesis (Hayashi *et al*, 2008; Maatouk *et al*, 2008; Papaioannou *et al*, 2009; Papaioannou & Nef, 2010). Indeed, several miRNAs are differently expressed as the mouse prepubertal testis differentiates to the adult testis (Yu *et al*, 2005). Moreover, components of the miRNA pathway such as Dicer, Drosha, and the Ago proteins have all been detected in spermatocytes, spermatids and Sertoli cells (Gonzalez-Gonzalez *et al*, 2008). For more details on germ cell types see Section 4.3.1. Interestingly, a study by Kotaja and colleagues showed that miRNA machinery components along with miRNAs are located in the chromatoid body of male germ cells. The chromatoid body resembles the P-body of somatic cells and might function as centers for miRNA-mediated gene regulation during post-meiotic germ cell differentiation (Kotaja *et al*, 2006). Another feature specific for spermatogenic cells is the abundant piRNAs. The piRNAs are longer than miRNAs (between 24-32 nucleotides) and differ from them in that they interact with another class of Ago proteins, namely members of the Piwi subfamily (Kim *et al*, 2009; Papaioannou & Nef, 2010; Meikar *et al*, 2011). In addition, piRNA biogenesis is independent of Dicer

(Vagin *et al*, 2006). The functions of piRNAs are still enigmatic, although some piRNAs are involved in transposon silencing through *de novo* DNA methylation (Aravin *et al*, 2007; Carmell *et al*, 2007; Kuramochi-Miyagawa *et al*, 2008). Since destruction of piRNA biogenesis, via genetic ablation of Piwis, leads to infertility in mice, the piRNAs are likely important regulators of spermatogenesis (Deng & Lin, 2002; Kuramochi-Miyagawa *et al*, 2004; Meikar *et al*, 2011).

2.3.2. miRNAs in disease

With myriad roles in the normal cellular functions, it is not surprising that aberrant miRNA expression or performance is associated with diseases. These conditions range from metabolic syndromes or organ system malfunctioning to cancer of various forms. At the time of writing, over 160 human diseases linked to miRNAs have been reported in the miR2Disease database (Jiang *et al*, 2009). One example is the autoimmune disease multiple sclerosis (MS), characterized by chronic inflammation of myelin sheaths in the central nervous system. Deviant miRNA expression has been detected in several cell types from diseased patients compared to healthy controls (Dai & Ahmed, 2011). One miRNA that was found upregulated in MS patients is miR-326. This miRNA contributes to the pathogenesis by enhancing the differentiation of damaging Th17 cells through targeting Ets-1, a negative regulator of Th17 differentiation (Du *et al*, 2009). In addition, miR-326, together with miR-34a and miR-155, targets CD47, thereby releasing macrophages from inhibitory control, which causes increased phagocytosis of myelin (Junker *et al*, 2009).

In cancer, pathways related to proliferation, differentiation or apoptosis are often dysregulated, and by interfering with these processes, miRNAs can act both as tumor suppressors and as oncogenes (Ventura & Jacks, 2009). One of the first links to cancer was established by the discovery that a genomic region containing two miRNAs was frequently deleted in human chronic lymphocytic leukemia (Calin *et al*, 2002). Several subsequent studies have demonstrated that both loss and amplification of genomic loci containing miRNAs are important hallmarks in various tumors (Calin & Croce, 2006). When comparing cancer tissues with normal tissue counterparts aberrant miRNA levels have been observed, and interestingly, an overall downregulation is detected in many tumors (Lu *et al*, 2005; Ventura & Jacks, 2009). Explicit expression profiles of miRNAs have been connected to various cancer forms, which potentially could be useful in diagnostic purposes for example as a means to distinguish cancer subtypes or reveal the tissue of origin of a metastatic tumor (Ventura & Jacks, 2009). Thus, with the increase in miRNA knowledge, new avenues for diagnostic, prognostic, and therapeutic applications can be envisioned.

2.4. The miR-17~92 cluster

Around half of the mammalian miRNAs are transcribed as clusters with the pri-miRNA molecule containing several hairpin-like structures (Lee *et al*, 2002; Kim *et al*, 2009). These structures are subsequently processed to form individual miRNAs. One such polycistron is the miR-17~92 cluster that constitutes six miRNAs; miR-17, -18a, -19a, -20a, -19b-1, -92a-1. The miRNAs are grouped within an 800 bp region on the human chromosome 13 (chromosome 14 in mouse). The organization of the cluster as well as the sequences of the mature miRNAs are highly conserved in vertebrates, indicating that

coordinated regulation and functions might be vital (Mendell, 2008). Furthermore, gene duplications during early vertebrate evolution have given rise to two paralog clusters, miR-106a~363 and miR-106b~25 (Fig. 5) (Tanzer & Stadler, 2004). The functions of these two paralogs are, however, largely unclear since mice lacking either of the cluster show no apparent phenotype (Ventura & Jacks, 2009).

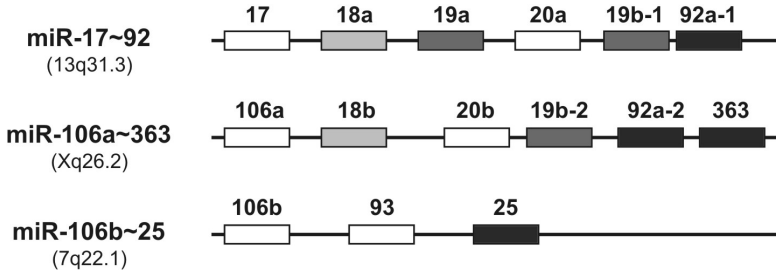

Figure 5. Organization of the human miR-17~92 cluster and its paralogs. The color code designates miRNAs belonging to the same family, which is based on identical seed sequences. The genomic localization is given in brackets. Modified from (Mendell, 2008).

2.4.1. The miR-17~92 cluster in cancer

The miR-17~92 cluster has attracted much attention due to its associations with cancer pathogenesis and was the first miRNA cluster to be identified with oncogenic potential. Mirroring this, the cluster has been named Oncomir-1. Indeed, the chromosomal locus harboring the cluster is frequently amplified in lymphomas and solid tumors and overexpression of the miR-17~92 cluster has been detected in human cancers including breast, lung, colon, prostate, stomach and pancreas, as well as in hematopoietic malignancies (Ota *et al*, 2004; Volinia *et al*, 2006; Mendell, 2008). A causal link between the cluster and tumorigenesis was first provided by the observation that overexpression of the cluster in a mouse model of B-cell lymphomas dramatically accelerates tumorigenesis in cooperation with c-Myc (He *et al*, 2005). Concurrently, the miR-17~92 cluster was shown to be a direct transcriptional target of c-Myc, suggesting that the cluster contributes to its oncogenic potential (O'Donnell *et al*, 2005). Furthermore, among the first identified targets for the miR-17~92 cluster were members of the E2F family of transcription factors, which are regulators of the cell cycle and apoptosis (Table 1) (O'Donnell *et al*, 2005; Sylvestre *et al*, 2007; Woods *et al*, 2007). Since c-Myc via the miR-17~92 cluster can limit the translation of E2F proteins, but also has the ability to activate E2F1 transcription, delicate control over proliferative decisions is at hand. Moreover, E2F1 and E2F3 can directly induce transcription of the miR-17~92 cluster, which establishes a negative feedback loop that promotes cell cycle progression following a proliferative signal (O'Donnell *et al*, 2005; Coller *et al*, 2007; Sylvestre *et al*, 2007; Woods *et al*, 2007; Mendell, 2008). Another mechanism by which the miR-17~92 cluster is thought to affect cell division is via targeting of the cyclin-dependent kinase inhibitor CDKN1a/p21, a negative regulator of the G_1-S checkpoint. It has been shown that TGFβ-induced cell cycle arrest as well as arrest induced by DNA damage, both mediated by p21, can be overridden by cells expressing miR-17~92 or miR-106b~25 cluster members at high levels (Ivanovska *et al*, 2008; Petrocca *et al*, 2008).

In addition to being transactivated by c-Myc, E2F1/3 and also STAT3 (O'Donnell *et al*, 2005; Sylvestre *et al*, 2007; Woods *et al*, 2007; Brock *et al*, 2009), all of which are commonly activated in cancer, the miR-17~92 cluster is repressed by the tumor suppressor p53 under hypoxia (Yan *et al*, 2009). This is mediated via direct binding of p53 to the promoter of miR-17~92, thereby competing for the TATA-box with TBP. Overexpression of the cluster was found to inhibit hypoxia-induced apoptosis and expression of the cluster correlates well with p53 status in colorectal carcinomas. Collectively, this indicates that p53-mediated repression of miR-17~92 might contribute to the tumor suppressive functions of p53 (Yan *et al*, 2009).

The contribution of the individual members of the miR-17~92 cluster in malignant transformation has largely remained unknown. Recently, however, two groups attempted to tackle this question, and both took advantage of a mouse model of c-Myc-driven B-cell lymphomas (Mu *et al*, 2009; Olive *et al*, 2009; van Haaften & Agami, 2010). One of the studies showed that overexpression of the entire cluster enhanced oncogenesis in the mouse model used. Dissecting the function of the individual components, miR-19a and miR-19b-1 were found both necessary and sufficient for lymphomagenesis and identified as the key oncogenic constituents of the cluster (Olive *et al*, 2009). The other study took an opposite approach and crossed the c-Myc mice with mice carrying a conditional *miR-17~92* knockout allele and found that deletion of the cluster resulted in slowed c-Myc-induced oncogenesis. Furthermore, also they identified miR-19a and miR-19b-1 as the most potent oncogenic components since reintroduction of miR-19a/b-1 largely rescued tumorigenicity. However, reintroduction of the entire cluster showed a more potent effect, indicating that also other members than miR-19 contribute to tumorigenicity (Mu *et al*, 2009). To identify the mechanism by which miR-19 mediates its oncogenic potential, both groups investigated putative targets for miR-19. PTEN, a tumor suppressor that negatively regulates the oncogenic PI3K/AKT signaling pathway was, among others, found to be a target of miR-19. Interestingly, suppression of PTEN could alone explain most of the oncogenic effects seen in the mouse models used (Mu *et al*, 2009; Olive *et al*, 2009; van Haaften & Agami, 2010). Worth mentioning is, however, that these results reflect the situation in c-Myc-induced lymphomas of mouse and that the contribution of the miR-17~92 cluster members in other types of tumors and organisms remains to be shown. It is also interesting to note that although indications of a role for the miR-17~92 cluster in promoting tumorigenesis are convincing, a few studies suggest that loss-of-function of these miRNAs might be advantageous for certain cancers (Mendell, 2008). For example, a study designed to determine genome-wide miRNA copy number abnormalities in cancer revealed that the miR-17~92 cluster was deleted in approximately 20% of ovarian cancers, breast cancers, and melanomas (Zhang *et al*, 2006). In agreement, introduction of miR-17 into a breast cancer cell line reduced the cells' ability to proliferate, which was largely an effect of downregulation of AIB1 (amplified in breast cancer 1) by miR-17 (Hossain *et al*, 2006).

2.4.2. The miR-17~92 cluster in development

While most of the research conducted on the miR-17~92 cluster has dealt with its oncogenic effects, a few studies have provided insight into what normal physiological functions the members of the cluster might hold (Mendell, 2008). Ventura and co-workers generated mice lacking the miR-17~92 locus and found that embryos of these mice were drastically smaller in size than their wild-type littermates and died within minutes after birth. The knockout mice exhibited lung hypoplasia and an incompletely closed

interventricular septum in the heart, which together probably account for the early death of the embryos (Ventura *et al*, 2008). In agreement with the observed phenotype, another study reported high expression of the miR-17~92 cluster in embryonic lung and that transgenic overexpression of the cluster increases proliferation and inhibits differentiation of lung epithelial cells (Lu *et al*, 2007). Members of the miR-17~92 cluster can also downregulate the protein levels of bone morphogenetic protein receptor type II (BMPR2), essential for differentiation and proliferation of endothelial and smooth muscle cells, which possibly explains the decreased expression of BMPR2 seen in development of pulmonary hypertension (Brock *et al*, 2009). Still, the precise mechanisms for the involvement of the cluster in normal heart and lung development are unclear.

Due to the reported association of the miR-17~92 cluster in B-cell lymphomas (He *et al*, 2005), Ventura and colleagues investigated a function for the cluster in normal B-cell development using the miR-17~92 knockout mice. Indeed, lack of the miRNA cluster resulted in defects at the pro-B to pre-B transition in both embryonic and adult hematopoiesis (Ventura *et al*, 2008). Furthermore, a marked increase in apoptosis was detected, which curiously was specific for developing B-cells. Absence of the cluster increased the level of Bcl2l11/Bim, a proapoptotic protein with ability to antagonize proteins like Bcl2. The miR-17~92 cluster therefore probably functions as a survival factor by downregulating Bim, in particular since this protein repeatedly has been shown to be a direct target of several cluster members (Koralov *et al*, 2008; Petrocca *et al*, 2008; Ventura *et al*, 2008; Xiao *et al*, 2008). This is in agreement with the reduced expression of Bim detected in a mouse model overexpressing the miR-17~92 cluster in lymphocyte progenitor cells. Moreover, these mice develop lymphoproliferative disease, autoimmunity and die prematurely (Xiao *et al*, 2008). Thus, it is likely that the regulation of Bim by the miR-17~92 cluster is involved in both the tumor-promoting effect of the miR-17~92 cluster as well as its physiological function in normal B-cell development. Furthermore, the miR-17~92 cluster has also been suggested to be a regulator of apoptosis in normal spermatogenesis and the cluster transcript is detected in human testis (Novotny *et al*, 2007). Complementary expression of E2F1 and pri-miR-17 during male germ cell maturation has led to the assumption that translation of E2F1 is inhibited by miR-17. Since the expression of pri-miR-17 is most prominent in pachytene spermatocytes, the role of the miRNA cluster might be to prevent apoptosis during meiotic recombination (Novotny *et al*, 2007).

2.4.3. miR-18

Two isoforms of miR-18 exist; miR-18a and miR-18b, located in the miR-17~92 and miR-106a~363 cluster, respectively (Fig. 5). The mature sequences of the two human miRNAs differ from each other with only one nucleotide, which is situated close to the 3′-end thereby leaving the seed sequences identical. Ectopic expression of members of the miR-106a~363 cluster has indicated that miRNAs are functional and might possess roles similar to the miR-17~92 cluster (Landais *et al*, 2007). However, since endogenous expression of the miR-106a-363 cluster members has proven undetectable or extremely low in normal tissues and cells, the miR-106a-363 cluster has been suggested to represent a pseudogene (Mendell, 2008). In this thesis, the term miR-18 therefore refers to the miR-18a isoform.

Although many miRNAs are incorporated into clusters, differential expression among members of clusters has been detected, which is also the case for the miR-17~92 cluster members (Hayashita *et al*, 2005; Hossain *et al*, 2006; Landais *et al*, 2007; Liu *et al*, 2009;

Jevnaker *et al*, 2011). This shows that control mechanisms for individual miRNA biogenesis exist. In fact, a mechanism for specific processing of miR-18 has been reported (Guil & Caceres, 2007; Michlewski *et al*, 2008). The generation of miR-18 is mediated via attachment of the multifunctional RNA-binding protein hnRNP A1 to the loop of pri-miR-18. The association facilitates relaxation at the stem, likely creating a more favorable cleavage site for Drosha, which in turn enhances selective processing of pri-miR-18 from the miR-17~92 cluster. In agreement, depletion of hnRNP A1 led to reduced levels of miR-18 while other members of the miR-17~92 cluster were unaffected (Guil & Caceres, 2007; Michlewski *et al*, 2008).

Extensive analyses on miR-18 expression or its relation to the abundance of the miR-17~92 cluster transcript have largely been lacking, let alone analyses on its function. Nevertheless, temporally regulated expression of miR-18 in development has recently become evident given that the levels vary during mouse embryogenesis and markedly decrease in several postnatal tissues (Mineno *et al*, 2006; Jevnaker *et al*, 2011). Likewise, miR-18 exhibits decreasing amounts during brain development, which has been detected in rodent, monkey and porcine miRNA expression profiling studies (Miska *et al*, 2004; Podolska *et al*, 2011). In the brain, a function for miR-18 as a regulator of the response to hormone exposure is conceivable since two research groups have reported that miR-18 can downregulate the protein level of glucocorticoid receptor (GR) in cultured neuronal cells (Uchida *et al*, 2008; Vreugdenhil *et al*, 2009). Overexpression of miR-18 also affected GR-mediated events such as impaired activation of a GR-dependent gene (Vreugdenhil *et al*, 2009). Some discrepancies about the relevance of these findings exist since, using luciferase reporter constructs bearing the 3′UTR of GR, one of the studies did not detect direct binding between miR-18 and GR whereas the other study did. After examining expression levels in rats, the latter study (Uchida *et al*, 2008) went on to propose a physiological function for miR-18 in stress-related disorders: glucocorticoids are involved in a variety of physiological processes such as neuronal development, immunity and adaptation to stress and the responsiveness to the glucocorticoids is, among other factors, dependent on the amount of GR protein. Upon stress, adrenal glucocorticoids are produced and released under the control of the hypothalamic-pituitary-adrenal axis and dysregulation of glucocorticoid signaling is associated with vulnerability to a number of psychiatric diseases (de Kloet *et al*, 2005; Seckl & Holmes, 2007). Fischer 344 rats are a strain that is stress-hyperresponsive and that consistently exhibits exaggerated acute-stress-induced release of corticosterone in relation to other strains such as the Sprague–Dawley (SD) strain (Dhabhar *et al*, 1995; Dhabhar *et al*, 1997). In the brains of Fischer 344 rats, Uchida and colleagues found high levels of miR-18 and low levels of GR protein in the paraventricular nucleus (PVN), a region important for the plasticity of the stress response. Concurrently, SD rats showed low levels of miR-18 and high levels of GR protein in the PVN. Together, these observations led to the proposal that miR-18-mediated regulation of glucocorticoid signaling in the brain may underlie susceptibility to stress (Uchida *et al*, 2008).

In cancer, miR-18 seems to regulate another hormone receptor, *i.e.*, estrogen receptor alpha (ERα). Two research groups have recently reported that overexpression of miR-18 in cell culture leads to reduced levels of ERα and that this is mediated via direct binding between the two components (Leivonen *et al*, 2009; Liu *et al*, 2009). In accordance, miR-18 overexpression was found to repress ERα-responsive genes. Indications of an *in vivo* function were provided by the observation that miR-18 shows higher expression in ERα-negative compared to ERα-positive breast cancer tumors (Leivonen *et al*, 2009). Similarly, increased levels of miR-18a in female hepatocellular carcinoma (HCC) tissues correlate

with reduced ERα expression (Liu *et al*, 2009). This suggests that miR-18 is an important regulatory component in the progression of cancers influenced by ERα. Further underscoring miR-18 involvement in carcinogenesis is the finding that miR-18 affects proliferative activity; whereas an increase in miR-18 expression stimulated proliferation of hepatoma cell lines, proliferation was repressed in an estrogen-responsive breast cancer cell line (Leivonen *et al*, 2009; Liu *et al*, 2009). This is in line with the notion that activation of the estrogen signaling pathway acts in a cancer-promoting manner in several estrogen-responsive tissues such as the breast, whereas it, via unknown mechanism, protects against HCC (Vesselinovitch *et al*, 1980; Pike & Spicer, 2000; Yu *et al*, 2003; Liu *et al*, 2009).

A non-cell-autonomous function for miR-18 in cancer development also exists given that miR-18 stimulates tumor angiogenesis. The oncogene c-Myc in known to induce tumor neovascularization (Meyer & Penn, 2008), and using a mouse model of colon cancer Dews and colleagues showed that this capacity is, at least in part, derived from upregulation of the miR-17~92 cluster (Dews *et al*, 2006). Although several members of the miRNA cluster potentially contribute to the tumor vascularization phenotype, demonstrating cooperation between miRNAs derived from a common transcript, miR-18 seems to be the key factor in this process (Dews *et al*, 2006; Suarez *et al*, 2008; Dews *et al*, 2010). Specifically, miR-18 targets the antiangiogenic proteins thrombospondin-1 (Tsp1) and connective tissue growth factor (CTGF). Furthermore, expression profiles from human cancer cell lines showed that high levels of the miR-17~92 cluster transcript negatively correlate with multiple TGFβ-induced antiangiogenic factors. However, the majority of them turned out not to be direct targets of any member of the miR-17~92 cluster. Instead, miR-18 was found to directly target components of the TGFβ-pathway itself such as Smad4 and, together with miR-17 and miR-20a, type II TGFβ receptor. A regulatory network can thus be envisioned in which c-Myc activates the miR-17~92 cluster, whose members, in particular miR-18, downregulate antiangiogenic factors either directly or via attenuation of the TGFβ signaling pathway, hence leading to angiogenesis and tumor growth (Dews *et al*, 2006; Dews *et al*, 2010).

Table 1. Examples of targets of the miR-17~92 cluster members, including all of the proposed miR-18 targets. The majority of the targets are associated with carcinogenesis. See text for details.

miR-17~92 member	**Target**	**Suggested role in**
miR-17, miR-20	E2F1, E2F2, E2F3	Cell cycle, apoptosis
miR-17, miR-20	p21	Cell cycle
miR-17, miR-19, miR-20	PTEN	Oncogenic signaling
miR-17	AIB1	Proliferation of breast cancer cells
miR-17, miR-20	BMPR2	Pulmonary hypertension
miR-17, miR-20, miR-92	Bim	Apoptosis, B-cell development
miR-18	GR	Susceptibility to stress
miR-18	ERα	Cancer progression, proliferation
miR-18	Tsp1	Tumor angiogenesis
miR-18, miR-19	CTGF	Tumor angiogenesis
miR-18	Smad4	Tumor angiogenesis
miR-17, miR-18, miR-20	type II TGFβ receptor	Tumor angiogenesis

3. HEAT SHOCK FACTORS AS TRANSCRIPTIONAL REGULATORS IN CELLULAR STRESS

3.1. The heat shock response

The research field of the heat shock response was initially founded in 1962 when Ferruccio Ritossa observed an unforeseen puffing pattern in the chromosomes of the polytene salivary glands of *Drosophila* larvae exposed to elevated temperatures (Ritossa, 1962). The chromosome puffs were known to indicate RNA synthesis and it was later found that the rapid occurrence upon heat stress was due to robust activation of genes encoding heat shock proteins (Hsps) (Tissieres *et al*, 1974; Lewis *et al*, 1975). Today, it is well known that the heat shock response is a highly conserved phenomenon, shared by organisms from prokaryotes to eukaryotes and from yeast to man (Lindquist, 1986). The preservation across evolution suggests an ancient function vital for cell survival. Indeed, activation of the response protects the cell from the deleterious consequences of protein-damaging insults. Despite its name, the heat shock response is induced also by other environmental stress stimuli such as exposure to heavy metals or oxidative stress, under pathophysiological states such as fever or infections, and under various protein conformational diseases. The response can also be activated under non-stress conditions such as in development and differentiation (Fig. 6) (Morimoto, 1998; Morimoto, 2008).

3.2. Heat shock proteins

For a cell to properly thrive it is dependent on a myriad of functional proteins. The proteins need to be folded correctly and hold an accurate three-dimensional conformation that enables proper functions and interactions. Although the blueprint for correct protein folding is found in the amino acid sequence of the protein itself (Anfinsen *et al*, 1961; Dobson & Karplus, 1999), the folding process is highly facilitated by Hsps. The Hsps are constitutively expressed during cell growth and development and function as molecular chaperones with roles in protein quality control and in promoting folding of nascent polypeptides in the crowded milieu of the cytosol. In addition, when a cell is subjected to stress, such as heat, its proteins are often malformed and hydrophobic residues are exposed. Under these circumstances, the expression of *Hsps* is strongly increased, facilitating protein degradation and prevention of protein aggregation, which enhances cell survival (Lindquist & Craig, 1988; Hartl & Hayer-Hartl, 2002).

Due to the robust stress-inducibility of the Hsps, the heat shock response has extensively been used as a model system to study gene regulation and organization. In fact, the *Hsp* genes of the fruit fly were among the first eukaryotic genes to be cloned (Lindquist, 1986). The various Hsps are divided into families according to their molecular size; the Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and the small Hsp families. All of the Hsps function by binding to exposed hydrophobic surfaces of protein substrates. The best known family is the Hsp70, whose members facilitate *de novo* protein folding through binding and releasing of the substrate in an ATP-dependent manner. Also the Hsp60 family promotes nascent protein folding. However, the mechanism of action differs in that the members of this family form a large cylindrical compartment in which the substrate polypeptide is captured and thereby protected from other non-native proteins while being folded (Hartl & Hayer-Hartl, 2002; Richter *et al*, 2010). The rest of the families do not have the ability to facilitate

de novo protein folding, but can instead possess functions as co-chaperones, such as Hsp40 that endorses efficiency of Hsp70. Other functions include the ability to unfold protein aggregates and regulate different aspects of cell signaling (Hartl & Hayer-Hartl, 2002; Richter *et al*, 2010).

Improper protein folding and protein aggregate formation are notably involved in numerous diseases. The occurrence of protein aggregates is a characteristic feature of neurodegenerative diseases such as Huntington's and Parkinson's disease. These conditions show aggregation of distinct proteins; in Huntington's disease variants of the protein huntingtin aggregate, while Parkinson's disease is accompanied by misfolding of α-synclein leading to death of dopaminergic neurons (Cohen & Dillin, 2008; Arawaka *et al*, 2010). Through their ability to repress protein aggregation in general, the Hsps carry a therapeutic potential towards these diseases and others. In fact, transgenic animal models of neurodegenerative diseases have shown that induction or overexpression of Hsps can reduce neuronal degeneration (Arawaka *et al*, 2010). There is, however, another side of the coin which is that Hsps also confer cancer cells with stress-resilience. Moreover, a prominent feature of the Hsps is their function at key regulatory points of both cell growth and apoptosis. Hence, it is not surprising that atypical expression of Hsps has been found in most forms of malignant tumors, and that for example high expression is associated with poor prognosis and resistance to therapy in breast and gastric cancer (Jolly & Morimoto, 2000; Whitesell & Lindquist, 2005). In view of the protective roles of Hsps and their involvement in numerous physiological and pathophysiological processes, their expression needs to be subjected to strict regulation.

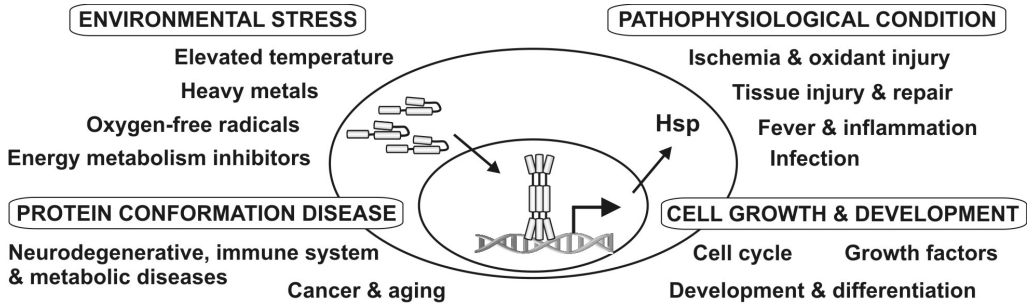

Figure 6. The heat shock response is activated by various forms of environmental stress, in different pathophysiological states, during protein conformational diseases as well as under certain non-stress conditions. Upon activation, HSFs bind DNA as a trimer and induce expression of Hsps. Modified from (Morimoto, 2008).

3.3. The family of HSFs

When a cell is exposed to stress such as heat shock, a hallmark is a general repression of transcriptional activity (Goodrich & Kugel, 2010). In mammals, the repression is, at least in part, mediated by non-coding RNAs: Alu RNA in humans and B2 RNA in mice. These RNAs directly bind RNAP II, thus keeping it from interacting properly with target gene promoters (Espinoza *et al*, 2007; Mariner *et al*, 2008; Goodrich & Kugel, 2010). The decrease in bulk transcription upon stress has been reported in organisms ranging from the fruit fly to humans. However, in stark contrast, production of protective Hsps is rapidly induced following cellular stress. This induction is facilitated by heat shock factors (HSFs),

which are known as the main transcriptional regulators of the heat shock response in eukaryotes (Lindquist, 1986; Fujimoto & Nakai, 2010).

The initial discovery of a factor regulating the expression of Hsps stems from protein-DNA interaction studies in *Drosophila* (Wu, 1984). Preceding this, the cloning of heat-inducible *Hsp* genes had led to the identification of a promoter element, responsible for gene activation (Pelham, 1982). The promoter region was found upstream of *Hsp* genes and was named heat shock element (HSE). The finding of the HSE enabled purification and characterization of an HSE-interacting protein from fruit fly, yeast and human cells (Wu, 1995). In 1988, the gene corresponding to the HSE-binding protein in *S. cerevisiae* was cloned (Sorger & Pelham, 1988; Wiederrecht *et al*, 1988). Soon thereafter, the *Hsf* gene was cloned from fruit fly, plants and mammals (Wu, 1995).

In invertebrates, such as yeasts, nematodes and insects, a single HSF has been found, whereas mammals possess an HSF family consisting of four members: HSF1-4 (Fig. 7) (Lindquist, 1986; Pirkkala *et al*, 2001; Fujimoto & Nakai, 2010). HSF1 is considered the archetype of the HSFs and is the mammalian counterpart of the single HSF found in invertebrates. In agreement, *Hsf1*-deficient fibroblasts and mice are unable to induce expression of Hsps upon thermal insults, revealing that no other HSF can replace its function in the heat shock response (McMillan *et al*, 1998; Xiao *et al*, 1999). Instead, the functions of HSF2 and HSF4 have long been thought to involve development and differentiation-related processes. HSF4 is crucial for the maintenance of sensory organs such as the lens and the olfactory epithelium (Nakai, 2009). The first evidence for a developmental function of HSF4 was provided by population genetic studies where mutations of the *Hsf4* gene were found to be associated with autosomal dominant lamellar and Marner cataract occurring in certain Chinese and Danish families (Bu *et al*, 2002). Three research groups subsequently demonstrated that *Hsf4*$^{-/-}$ mice develop cataract at early postnatal days (Fujimoto *et al*, 2004; Min *et al*, 2004; Shi *et al*, 2009). Lately, HSF4 was attributed a role in the heat shock response as it induces a set of non-classical heat shock genes upon thermal insult (Fujimoto *et al*, 2008). Likewise, the most recently found member of the HSF family, the murine HSF3, responds to heat stress given that it translocates to the nucleus and has the potential to activate the non-classical heat shock genes PDZK3 and PROM2 (Fujimoto *et al*, 2010). In humans, only a pseudogene of *Hsf3* has been observed. The diversity of the mammalian HSF family is increased by distinct HSF isoforms, and alternative splicing appears to be a common feature among the family members (Pirkkala *et al*, 2001; Fujimoto *et al*, 2010). The discoveries of two additional family members: HSFX and HSFY, located on the X and Y chromosomes, respectively, further broaden the picture (Tessari *et al*, 2004; Bhowmick *et al*, 2006). Yet, these novel members remain to be thoroughly characterized.

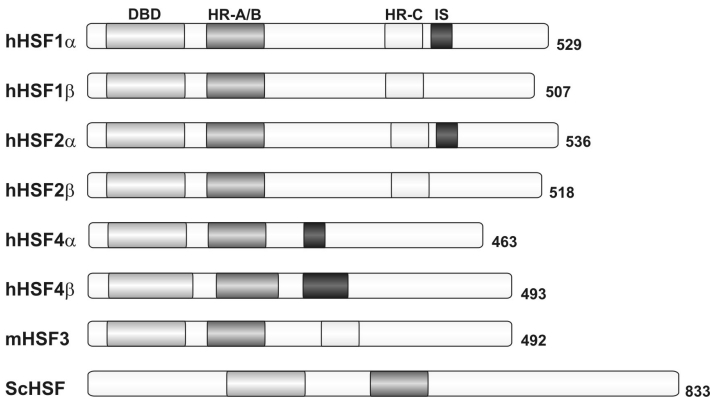

Figure 7. The HSF family members. A conserved DNA-binding domain (DBD) and an oligomerization domain (HR-A/B) are found in the HSFs. In addition, the mammalian HSFs, except HSF4, have a C-terminal domain (HR-C) that inhibits trimerization. HSF1, HSF2 and HSF4 exist in two alternatively spliced isoforms. Apart from HSF3, which only has been found in mouse, human HSFs are described in the figure. Yeast HSF (ScHSF) is drawn as a comparison. The numbers designate amino acids. h, human; IS, isoform-specific region; m, mouse; Sc, *S. cerevisiae*. Modified from (Pirkkala *et al*, 2001; Fujimoto & Nakai, 2010).

3.3.1. Functional domains of HSFs

Similar to most transcription factors, the members of the HSF family are modular proteins composed of functional domains (Fig. 7). The two most conserved domains are the amino-terminal helix-turn-helix DBD and the adjacent oligomerization domain composed of hydrophobic heptad repeats (HR-A/B). Additionally, member-specific domains exist such as a central regulatory domain (RD), a second heptad repeat domain (HR-C), and a transcriptional AD at the carboxy (C-) terminus.

The DNA-binding domain

Among the distinct HSFs, the most prominent common feature is the DBD, and its presence designates membership to the HSF family. The DBD of the HSFs is composed of a winged helix-turn-helix motif (Wu, 1995), which was elucidated through X-ray crystallography of the DBD of *K. lactis* and nuclear magnetic resonance (NMR) solution structures of *K. lactis* and *D. melanogaster* (Damberger *et al*, 1994; Harrison *et al*, 1994; Vuister *et al*, 1994; Damberger *et al*, 1995). Overall, the DBD takes on a globular shape with a core of three α-helices packed against a small four-stranded, anti-parallel β-sheet. The third α-helix constitutes the DNA-recognition element and binds in the major groove of the DNA double helix. Between β-strands 3 and 4, and protruding from the globular structure, is a flexible wing or loop (Wu, 1995). The loop does not contact DNA, as is the case in other winged helix-turn-helix motifs, but rather mediates protein-protein interactions such as between adjacent HSF molecules in a trimer or even between adjacent trimers bound to DNA (Littlefield & Nelson, 1999). The loop is furthermore thought to dictate DNA-binding characteristics since swapping of the mouse HSF1 and HSF2 loops exchanges their respective DNA-binding profiles, as determined by DNase I footprinting (Ahn *et al*, 2001).

Trimerization and interaction with the heat shock element

Upon activation, the HSFs assemble as trimers (Wu, 1995). This notion has been brought about by a number of studies beginning with Perisic and co-workers who performed chemical cross-linking experiments, and Sorger and Nelson who analyzed the number of heteromeric complexes formed when allowing HSF polypeptides of different length to randomly associate (Perisic *et al*, 1989; Sorger & Nelson, 1989). The confirming studies that followed utilized techniques such as electrophoretic mobility shift assay (EMSA), gel filtration and density gradient centrifugation (Baler *et al*, 1993; Rabindran *et al*, 1993; Sarge *et al*, 1993; Westwood & Wu, 1993; Sistonen *et al*, 1994).

The trimerization process is mediated by the oligomerization domain composed of the hydrophobic heptad repeats HR-A/B that through interactions of the hydrophobic residues form a coiled-coil. Although unusual for helical coiled-coil structures, which typically form dimers, these form a triple-stranded configuration (Sorger & Nelson, 1989; Wu, 1995). The trimerization is repressed by another more C-terminal heptad repeat; HR-C, the deletion of which renders HSF1 constitutively trimeric (Rabindran *et al*, 1993; Zuo *et al*, 1994). In the inactive state, HSF1 exists as a monomer, and it is assumed that the HR-C folds back to interact with the HR-A/B domain, thereby preventing oligomerization (Wu, 1995). Accordingly, yeast HSF and mammalian HSF4, both lacking the HR-C, are constitutively trimeric (Chen *et al*, 1993; Nakai *et al*, 1997). In addition, mutations in the linker region that connects the DBD and the HR-A/B have been shown to generate constitutively trimeric HSF1, suggesting that this region also might be involved in modulating oligomerization (Liu & Thiele, 1999).

There is a close link between trimerization and DNA-binding ability, and the trimerization process is essential in order to achieve high-affinity binding to DNA (Wu, 1995). The HSEs, present in HSF target promoters, are composed of an array of inverted repeats of the pentamer nGAAn. Each DBD recognizes one nGAAn, and thus, an HSE typically contains three pentameric repeats (Amin *et al*, 1988; Xiao & Lis, 1988; Wu, 1995; Sakurai & Enoki, 2010). Analyzing sequences bound by HSF1, Trinklein and colleagues found that guanine is the most conserved nucleotide in the nGAAn pentamer, an observation that also has been reported by Xiao and Lis (Xiao & Lis, 1988; Trinklein *et al*, 2004b). Furthermore, Trinklein and colleagues found that in a pair of inverted repeats, a TTC triplet 5' to a GAA triplet is separated by a pyrimidine-purine dinucleotide, whereas the two nucleotides separating a GAA triplet 5' of a TTC triplet is unconstrained (Trinklein *et al*, 2004b). Many target promoters enclose more than three 5 bp repeats and it has been shown that HSF trimers bind to DNA in a cooperative manner, where binding of one HSF trimer facilitates binding of the next trimer. Binding of multiple HSFs also affects the dissociation rate from DNA, which is significantly lower from an HSE containing six or more 5 bp repeats than from an HSE composed of three 5 bp repeats (Xiao *et al*, 1991).

The Hsp70 gene as a model for stress-induced transcription

With an extraordinary rapid transcriptional induction upon heat stress, the *Hsp70* gene has long served as a model system for inducible gene expression in eukaryotes. The mammalian *Hsp70* promoter holds two HSEs, one proximal containing five inverted repeats and one distal containing six repeats (Abravaya *et al*, 1991a). Thus, simultaneous binding of up to four HSF trimers is theoretically feasible, albeit not all can display perfect high-affinity binding. Other regulatory elements on the *Hsp70* promoter include a TATA-

box and two binding sites for the transcription factors Sp1 and CTF (CCAAT-box binding transcription factor), respectively (Wu *et al*, 1986; Morgan *et al*, 1987; Abravaya *et al*, 1991a).

The uninduced *Hsp70* promoter is primed for quick activation by harboring a transcriptionally engaged, but stalled RNAP II near the transcription initiation site (Rougvie & Lis, 1988; Brown *et al*, 1996; Fuda *et al*, 2009). It has been suggested that the dormant state of the RNAP II is sustained by the presence of nucleosomes since the stalling of RNAP II *in vitro* is enhanced by nucleosome formation (Brown *et al*, 1996). Upon heat shock, HSF1 rapidly translocates to the *Hsp70* promoter where it interacts with BRG1, an ATPase subunit of the chromatin remodeling complex SWI/SNF, and stimulates the release of RNAP II (Sullivan *et al*, 2001; Corey *et al*, 2003). Furthermore, both absence of HSF1 or mutations in the BRG1-binding region of HSF1 prevent nucleosomal displacement and *Hsp70* gene expression, demonstrating that HSF1 provides a signal for chromatin rearrangement through direct interaction with the SWI/SNF complex (Corey *et al*, 2003). However, HSF1 also stimulates SWI/SNF action by inducing acetylation of histone H4 on the *Hsp70* promoter upon heat shock (Thomson *et al*, 2004). In addition to nucleosomal remodeling, the release of the paused RNAP II is mediated by P-TEFb. Upon heat shock, P-TEFb localizes to the *Hsp70* promoter in an HSF1-dependent manner and phosphorylates Ser2 residues on the CTD of RNAP II, which switches RNAP II into the elongating mode (Lis *et al*, 2000; Ni *et al*, 2004; Weake & Workman, 2010). Another component recruited to the *Hsp70* promoter by HSF1 is the Mediator co-activator complex. This complex acts by conveying activating signals from transcription factors to the basal transcription machinery. The complex is recruited by a direct interaction with HSF1 upon heat shock and in *Drosophila* this occurs between the AD of HSF and the Mediator subunit dTRAP80 (Park *et al*, 2001). In addition, HSF1 has been reported to interact with TBP and the GTF TFIIB *in vitro*, suggesting that HSF1 directly affects also the PIC (Mason & Lis, 1997; Yuan & Gurley, 2000).

3.3.2. HSF1

Activation and regulation of HSF1

By far the best characterized member of the HSF family is HSF1. In eukaryotes, HSF1 is constitutively expressed in most tissues and cell types (Fiorenza *et al*, 1995), but under normal growth conditions it is kept inactive through a number of intra- and intermolecular interactions, various post-translational modifications and subcellular localization (Morimoto, 1998; Anckar & Sistonen, 2011). In the inactive state, HSF1 prevails as a monomer, and can be detected both in the cytoplasm and in the nucleus, as it is constantly being shuttled between the two compartments (Sarge *et al*, 1993; Mercier *et al*, 1999; Vujanac *et al*, 2005). In response to various environmental and physiological stress stimuli, HSF1 is rapidly activated in a multistep fashion, involving a monomer-to-trimer conversion, nuclear accumulation, increased phosphorylation, and acquisition of DNA-binding as well as transactivation capacity (Fig. 8) (Morimoto, 1998).

Early discoveries of interactions between HSF1 and Hsps led to a proposal of a negative feedback loop, where excess Hsps keep HSF1 inactive (DiDomenico *et al*, 1982; Abravaya *et al*, 1992; Baler *et al*, 1992; Ali *et al*, 1998; Morimoto, 1998; Zou *et al*, 1998). In response to proteotoxic stress, the Hsps are sequestered to denatured proteins and HSF1 is

released from the chaperone complexes to induce transcription of heat shock genes. Once the pool of Hsps is saturated, the chaperones are again able to bind HSF1 and negatively regulate its function (Morimoto, 1998; Voellmy, 2004). Interestingly, HSF1 associates with different Hsp complexes in different phases of the activation cycle. In its monomeric state HSF1 is bound to Hsp90, while stress leads to dissociation of this affiliation and to HSF1 trimerization (Ali *et al*, 1998; Zou *et al*, 1998). Trimeric HSF1 in turn can be kept inactive via binding to the multichaperone complex Hsp90-p23-FKBP52 (Ali *et al*, 1998; Bharadwaj *et al*, 1999; Guo *et al*, 2001). Yet another complex, Hsp70 and its co-chaperone Hsp40, is involved in inhibiting the transactivation capacity of HSF1, without affecting its DNA binding (Shi *et al*, 1998).

Post-translational modifications of HSF1

A hallmark of HSF1 in response to stress is an immense increase in its phosphorylation (Sorger & Pelham, 1988). Using mass spectrometry, at least 12 serine residues were found to be phosphorylated upon heat stress, and most of the sites reside in the RD positioned between the HR-A/B and HR-C domains of HSF1 (Guettouche *et al*, 2005). Intriguingly, under basal conditions, an intact RD is necessary for repressing the AD that encompasses the 150 most C-terminal residues of HSF1 (Green *et al*, 1995; Newton *et al*, 1996). Thus, stress-induced phosphorylation of key serines within the RD might work as a trigger that relieves the inhibition of the AD and enables transactivation capacity of HSF1. Unexpectedly, a comprehensive mutagenesis analysis of the phosphorylation sites only revealed one residue, serine 326, whose phosphorylation enhanced the transcriptional competence of HSF1 (Guettouche *et al*, 2005). In addition, stress-dependent phosphorylation of serine 230 has been found to promote HSF1 activation (Holmberg *et al*, 2001). However, HSF1 is a phosphoprotein also under normal conditions and at least serines 230, 303, 307 and 363 are constitutively phosphorylated. Of these serine residues, phosphorylation of the three last ones seems to repress the transactivation capacity of HSF1 (Chu *et al*, 1996; Knauf *et al*, 1996; Kline & Morimoto, 1997; Holmberg *et al*, 2001; Holmberg *et al*, 2002; Wang *et al*, 2004; Batista-Nascimento *et al*, 2011). Interestingly, serine 303 is linked to another post-translational modification as phosphorylation of this site is a prerequisite for sumoylation on the adjacent lysine 298 (Hietakangas *et al*, 2003). Although the stress-dependent sumoylation event initially was thought to enhance HSF1 DNA-binding activity (Hong *et al*, 2001), later anlayses showed that sumoylation hampers the transactivation capacity of HSF1 (Hietakangas *et al*, 2006). More specifically, in cells subjected to mild heat shock phosphorylation-dependent sumoylation of HSF1 is sustained whereas progressive HSF1 desumoylation correlates with increasing temperatures (Hietakangas *et al*, 2006), indicating that sumoylation restrains HSF1 activity under moderate stress conditions. Noteworthy, data on phosphorylation-dependent sumoylation of HSF1 led to the identification of an extended motif combining the SUMO consensus site with an adjacent proline-directed phosphorylation site, ψKxExxSP (where ψ is a hydrophobic amino acid, K is the SUMO-accepting lysine and X is any amino acid). The motif was named PDSM (phosphorylation-dependent sumoylation motif) and is recurrently found in proteins related to transcriptional regulation (Hietakangas *et al*, 2006; Anckar & Sistonen, 2011).

Upon heat stress, HSF1 rapidly binds the target promoter *Hsp70*, but the binding is transient and reaches a peak at approximately 30 minutes after a moderate heat shock, followed by a slow decrease in the level of binding (Abravaya *et al*, 1991a; Abravaya *et al*,

1991b; Kline & Morimoto, 1997). This indicates that also the attenuation phase of the activation cycle of HSF1 is strictly regulated and involves both ceased DNA-binding and ceased transcriptional activity. The attenuation mechanism cannot be explained merely by the chaperone-mediated negative feedback loop, since increased levels of Hsps, although leading to inhibition of transcription, do not cause a release of HSF1 from its target promoter (Rabindran *et al*, 1994; Shi *et al*, 1998). Instead, it has been demonstrated that HSF1 is subjected to stress-induced acetylation, which negatively affects the DNA-binding activity (Fig. 8). Intriguingly, HSF1 deacetylation is regulated by sirtuin 1 (SIRT1), resulting in prolonged binding of HSF1 to its target promoter (Westerheide *et al*, 2009).

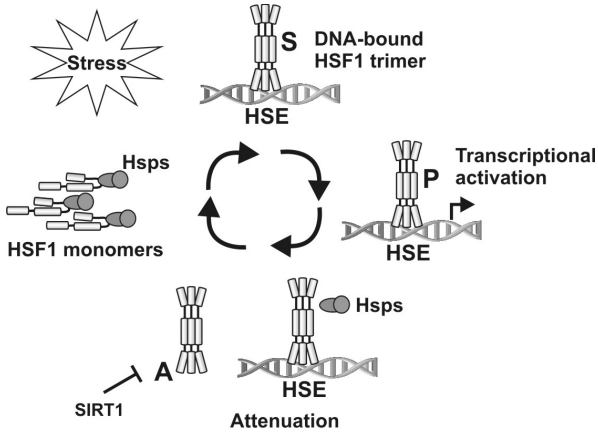

Figure 8. The activation cycle of HSF1. In its inactive state, HSF1 is monomeric and negatively regulated by interactions with Hsps. Upon stress, HSF1 is converted to a DNA-bound trimer and sumoylated. The stress-induced hyperphosphorylation correlates with target gene induction. Attenuation of the transactivation capacity of HSF1 is mediated through negative feedback from Hsps and the DNA-binding activity is inhibited by acetylation. SIRT1 regulates the attenuation phase by preventing HSF1 acetylation. A, acetylation; P, phosphorylation; S, sumoylation. Modified from (Björk & Sistonen, 2010).

In search of the stress sensing mechanism of the heat shock response

As previously stated, activation of HSF1 is a rapid process and HSF1 is found on the *Hsp* promoters within minutes following heat stress (Abravaya *et al*, 1991a; Abravaya *et al*, 1991b; Kline & Morimoto, 1997). However, how the cell senses stress and conveys this information to HSF1 has remained elusive. HSF1 is activated by diverse stimuli, ranging from environmental stress and pathophysiological conditions to certain developmental settings, but a common denominator is the occurrence of misfolded or aggregated proteins (Morimoto, 1998). Thus, a long-standing hypothesis is that a disturbance in the protein homeostasis, leading to an augmented need for Hsps, is responsible for the activation. In line with this theory, denatured but not native proteins injected into *Xenopus* oocytes are capable of activating HSF1 (Ananthan *et al*, 1986). On the other hand, kinetic studies on *Drosophila* HSF favors a model where exposure to stress leads to direct activation of HSF (Boehm *et al*, 2003). Furthermore, both *Drosophila* HSF and mammalian HSF1 have been shown to display intrinsic stress-sensing capacity as recombinant HSFs undergo trimerization and DNA binding in response to various stress stimuli such as heat shock, low pH, H_2O_2 and increased calcium levels *in vitro* (Mosser *et al*, 1990; Goodson & Sarge, 1995; Larson *et al*, 1995; Farkas *et al*, 1998; Zhong *et al*, 1998). In addition, Ahn and

Thiele found that the capability of mammalian HSF1 to directly sense heat and oxidative stress, stemmed from two conserved cysteine residues, cysteines 35 and 105, situated in the DBD. The redox-dependent activation required formation of disulfide bonds to induce trimerization and target gene expression. In agreement, HSF1 was rendered refractory to stress upon mutation of the cysteine residues (Ahn & Thiele, 2003).

Intriguingly, the threshold temperature for HSF1 activation seems to be dependent on the host cell type or organism: when human HSF1 is expressed in *Drosophila* cells, the threshold temperature of HSF1 activation is decreased to the temperature that induces activation in *Drosophila* (Clos *et al*, 1993). This observation points to additional regulatory mechanisms. One such mechanism is represented by an RNA molecule named heat shock RNA-1 (HSR1), proposed to function as a thermosensor (Shamovsky *et al*, 2006). According to the suggested model, heat induces a conformational change in HSR1, which, together with the translation elongation factor eEF1A, facilitates trimerization and activation of HSF1. In accordance, physiological concentrations of purified HSR1 and eEF1A were capable of activating HSF1 in *in vitro* experiments (Shamovsky *et al*, 2006). This model demonstrates similarities between the mammalian heat shock response and the bacterial as activation of the bacterial heat shock sigma factor σ^{32} includes a heat-induced conformational change in the σ^{32} RNA molecule (Morita *et al*, 1999). Another feasible stress sensory mechanism in the mammalian heat shock response involves cellular membranes. Stress-induce perturbations such as altered composition of lipids and proteins affect membrane fluidity and leads to activation of *Hsp* genes (Soti *et al*, 2005). Furthermore, impact of membrane microdomain reorganization has been suggested as the membrane fluidizer benzyl alcohol, which changes the microdomain structure in a similar way as heat stress, was shown to induce DNA-binding and transcriptional activity of HSF1 (Nagy *et al*, 2007). The precise signaling pathway originating from the membrane is yet to be elucidated.

Although HSF1 activation follows the same principles upon various forms of stress, there are stimulus-dependent variations, arguing against a single universal signal pathway that activates HSF1. As an example, HSF in *S. cerevisiae* is differently phosphorylated when exposed to either heat or oxidative stress (Liu & Thiele, 1996). Other studies have demonstrated that induction of the HSF target gene *CUP1* occurs through distinct mechanisms upon activation by heat shock or glucose starvation. Specifically, CUP1 expression following glucose starvation is dependent on HSF-phosphorylation by the Snf1 kinase, while heat shock-induced expression is Snf1-independent (Tamai *et al*, 1994; Liu & Thiele, 1996; Hahn & Thiele, 2004).

3.3.3. HSF2

At the time of the discovery of HSF1, another HSF family member, HSF2, was cloned from human and mouse cells (Sarge *et al*, 1991; Schuetz *et al*, 1991). When comparing the amino acid sequences, HSF2 shows only about 35% overall identity with HSF1, however, the DBD and the oligomerization domains are highly conserved between the factors (Pirkkala *et al*, 2001). As HSF1, also HSF2 binds HSEs, but the two factors seem to display certain binding site preferences concerning the architecture of the HSE. Using DNase I footprinting analyses it became evident that HSF1 protects all five nGAAn repeats in the proximal HSE of the *Hsp70* promoter, whereas HSF2 protects a smaller area equivalent in size to the footprint of a single trimer (Sistonen *et al*, 1992; Kroeger *et al*,

1993). Likewise, in a screen to identify new binding sites of HSF1 and HSF2 using a library of random oligonucleotides, HSF1 bound long arrays of HSE units, typically four to five, while HSF2 preferred shorter arrays of two to three pentameric units (Kroeger & Morimoto, 1994). The dissimilar affinities between HSF1 and HSF2 are thought to originate from differences in the potential for cooperative DNA-binding of adjacent trimers (Xiao *et al*, 1991; Kroeger & Morimoto, 1994). In agreement, Yamamoto and colleagues showed that an HSE composed of four continuous nGAAn repeats binds two trimers of HSF1 but only a single HSF2 trimer (Yamamoto *et al*, 2009). The difference in the cooperative ability has been pinpointed to the flexible loop in the DBD of the HSFs. If the loops of HSF1 and HSF2 are switched between the proteins, HSF2 gains cooperative DNA-binding ability and ability for stress-responsive trimerization (Ahn *et al*, 2001). The specificity of HSF1 and HSF2 indicates selectivity in activating target genes based on the arrangement of the HSEs, and implies that the two HSFs have specialized and unique functions. Accordingly, human HSF1 and HSF2 expressed in yeast disrupted of the *Hsf* gene showed preferential transcriptional activation when comparing two distinct yeast heat shock-responsive genes (Liu *et al*, 1997). Similarly, expression of HSF1, HSF2 or HSF4 in yeast cells leads to differentially regulated target genes in correlation with the type of HSE present in the genes (Yamamoto *et al*, 2009).

HSF2 exists in two different isoforms, α and β, generated by alternative splicing of exon 11. This renders HSF2-α with an 18 amino acid long isoform-specific sequence situated in the vicinity of the HR-C domain (Fig. 7) (Fiorenza *et al*, 1995; Goodson *et al*, 1995). Although the functional differences between the isoforms are somewhat unclear, HSF2-α is thought to be a more potent transcriptional activator, as shown in reporter gene assays (Goodson *et al*, 1995; He *et al*, 2003). Also, overexpression of HSF2-β leads to inhibition of hemin-induced erythroid differentiation and Hsp expression in K562 erythroleukemia cells, while overexpression of HSF2-α enhances the induced response (Leppä *et al*, 1997). Presumably because of the functional differences, expression of the isoforms is spatially regulated. In adult mice HSF2-α dominates in the testis and in the germ cells pachytene spermatocytes and round spermatids, whereas HSF2-β is more abundant in the brain and heart (Goodson *et al*, 1995). The abundance of the isoforms is also regulated temporally as the dominance switches from HSF2-β to HSF2-α during the postnatal development of mouse testis (Goodson *et al*, 1995). It can thus be assumed that the ratio between the isoforms is important for the functional outcome of HSF2 expression. However, the precise mechanisms regulating different expression levels are still to be elucidated.

Regulation of HSF2

In stark contrast to HSF1, HSF2 does not seem to be regulated by phosphorylation. However, an indication of HSF2 being subjected to other post-translational modifications came from a yeast two-hybrid screen where HSF2 was found to interact with the SUMO-conjugating enzyme Ubc9 (Goodson *et al*, 2001). Indeed, HSF2 is primarily sumoylated on lysine 82, located in the flexible loop within the DBD that confers paralog-specific DNA-binding (Ahn *et al*, 2001; Goodson *et al*, 2001; Anckar *et al*, 2006). Initially, sumoylation was thought to positively alter the DNA-binding ability of HSF2 (Goodson *et al*, 2001; Hilgarth *et al*, 2004). Subsequent studies have on the contrary reported that the modification rather hinders the DNA-binding activity, possibly through sterical interference (Anckar *et al*, 2006; Tateishi *et al*, 2009). Interestingly, although a similar loop structure with an accessible SUMO consensus site exists in both HSF1 and HSF2, only the

loop of HSF2 is sumoylated. This specificity was found to be determined by amino acids in the vicinity of the sumoylation site, which present the SUMO consensus motif to Ubc9 by affecting the flexibility of the loop (Anckar *et al*, 2006).

HSF2 is a rather unstable protein and, as investigated in both K562 and HEK293 cells, it has a half-life of approximately 1-2 hours (Mathew *et al*, 1998; Ahlskog *et al*, 2010). Ubiquitination-mediated degradation has been suggested to control its abundance, in particular since treatment with proteasome inhibitors leads to accumulation of HSF2 protein as well as acquired DNA-binding activity (Kawazoe *et al*, 1998; Mathew *et al*, 1998; Pirkkala *et al*, 2000). Cullin3, a subunit of a Cullin-RING E3 ubiquitin ligase, was recently reported to interact with HSF2, which could direct HSF2 to ubiquitin/proteasome-mediated degradation (Xing *et al*, 2010). Another study showed that HSF2 is subjected to stress-induced ubiquitination and subsequent degradation, mediated by the ubiquitin E3 ligase anaphase-promoting complex/cyclosome (APC/C). Specifically, direct interactions were found between HSF2 and the APC/C co-activators Cdc20 and Cdh1, as well as the subunit Cdc27 (Ahlskog *et al*, 2010).

Under normal growth conditions, HSF2 primarily exists as a dimer, while the active DNA-binding form is trimeric (Sistonen *et al*, 1994; Mathew *et al*, 1998). Two ADs are located in the C-terminus of HSF2, but these domains have significantly less transcriptional capacity compared to the AD of HSF1. Presumably, the ADs are under negative regulation by adjacent regions, although no mechanism for the regulation has been detected (Yoshima *et al*, 1998a; Zhu & Mivechi, 1999). Interestingly, a GAL4-HSF2 fusion protein containing the ADs and regulatory regions is not activated by hemin or heat shock (Zhu & Mivechi, 1999). Knowledge on how the transcriptional activity of HSF2 is induced is thus fragmental. However, HSF2 activation could be connected to its level of expression, an issue that will be further discussed in the Results and Discussion section of this thesis.

Is there a role for HSF2 in the heat shock response?

Initial observations following the cloning of HSF2 found the transcription factor refractory to stress (Sarge *et al*, 1991; Sistonen *et al*, 1992). Whereas HSF1 produced in an *in vitro* translation system bound DNA in response to heat shock, HSF2 DNA-binding was not induced upon stress (Sarge *et al*, 1991). Also in K562 cells subjected to heat shock HSF1 was the primary DNA-binding factor as elucidated by EMSA and antibody supershifts (Sistonen *et al*, 1992). Instead, HSF2 bound DNA as well as stimulated expression of Hsp70 upon hemin-induced erythroid differentiation (Sistonen *et al*, 1992; Sistonen *et al*, 1994). In addition, HSF2 was reported to constitutively bind DNA in early embryogenesis, embryonal carcinoma cells, in the developing heart and during spermatogenesis (Mezger *et al*, 1994; Murphy *et al*, 1994; Sarge *et al*, 1994; Eriksson *et al*, 2000). Collectively, these early studies led to the conclusion that HSF2 might not be involved in the heat shock response, but rather functions in development- and differentiation-related processes.

However, whether HSF2 has a function in the heat shock response has since been much debated. Sheldon and Kingston noticed that the localization of HSF2 is altered upon heat shock as HSF2 translocated to punctuate structures in the nucleus of heat-treated HeLa cells (Sheldon & Kingston, 1993). The structures were later identified as nuclear stress bodies (nSBs) (Alastalo *et al*, 2003; Biamonti, 2004) and are described below. In a ChIP-based study on heat shock gene promoter occupancy, both HSF1 and HSF2 were found to

bind numerous promoters upon heat shock or hemin treatment, indicating a role for HSF2 in stress-induced gene expression (Trinklein *et al*, 2004a). In agreement, elevated expression of HSF2 has been reported to potentiate HSF1-mediated transcription of the *Hsp70* promoter in a luciferase reporter system after exposure to heat stress (He *et al*, 2003). In yeast, human HSF2 was able to compensate for the lack of endogenous HSF and induce target gene transcription upon heat shock (Liu *et al*, 1997). HSF2 has also been assigned a role in gene bookmarking as HSF2 inactivates the condensin complex on the *Hsp70* promoter in mitotic cells thereby preventing compaction of the site. Inhibiting bookmarking by RNAi-mediated silencing of HSF2 decreased the induction of Hsp70 and survival of stressed cells (Xing *et al*, 2005). On the other hand, arguing against a role for HSF2 in the heat shock response is the finding that the amount of *Hsp* transcripts was unaltered when comparing wild-type mouse embryonic fibroblasts (MEFs) and MEFs deficient in *Hsf2* after heat stress exposure (McMillan *et al*, 2002). Using cells derived from mice with another genetic background, Paslaru and co-workers found that lack of HSF2 lowered the threshold temperature for Hsp70 expression, indicating that HSF2 could even hold a negative role in the heat shock response (Paslaru *et al*, 2003). Taken together, the contradicting results on the role of HSF2 in the heat shock response warrant further investigations and will be discussed in the Results and Discussion section herein.

3.4. Nuclear stress bodies

A hallmark for activation of the heat shock response is the formation of nSBs, which are subnuclear organelles clearly distinct from other nuclear granules. The nSBs vary in size and are between 0.3 and 3 μm in diameter, and peculiarly, they are unique for human and primate cells (Sandqvist & Sistonen, 2004; Biamonti & Vourc'h, 2010). Initially, the nSBs were identified as sites of HSF1 accumulation and already within 30 seconds of heat shock HSF1 can be detected in the bodies (Sarge *et al*, 1993; Jolly *et al*, 1999). The localization is reversible and HSF1 disappears from the nSBs during attenuation and recovery from stress (Cotto *et al*, 1997). A decade after the discovery of HSF1 in nSBs (Sarge *et al*, 1993), HSF2 was interestingly found to co-localize with HSF1 and even influence its accumulation in the nSBs (Alastalo *et al*, 2003). Apart from HSFs, a number of RNA-processing factors are also found in the nSBs, although the kinetics for their translocation is somewhat delayed in comparison to that of the HSFs (Weighardt *et al*, 1999; Denegri *et al*, 2001). The factors include the hnRNPs HAP (hnRNP A1-associated protein), hnRNPM, and Sam68 (Scr-activated during mitosis). In addition, certain members of the serine/arginine-rich family of splicing factors such as SRp30c, SF2/ASF and 9G8 are recruited to the nSBs (Weighardt *et al*, 1999; Denegri *et al*, 2001; Metz *et al*, 2004).

Upon repeated heat shocks, Jolly and co-workers noticed that the nSBs formed on the same nuclear locations (Jolly *et al*, 1999). This finding, together with the observation that the number of nSBs roughly correlates with cell ploidy, led to the assumption that nSBs are formed on specific chromosomal loci. However, opposite to as first envisioned, the localization did not correlate with *Hsp* gene loci (Cotto *et al*, 1997; Jolly *et al*, 1997). Instead, two research groups simultaneously identified the pericentromeric band q12 on human chromosome 9 as the primary site for nSB formation (Denegri *et al*, 2002; Jolly *et al*, 2002). This region is composed of long tandem arrays of satellite III sequences (*sat III*). Interestingly, although the 9q12 locus has been considered constitutively heterochromatic, the detection of histone acetylation and RNAP II in the nSBs suggested otherwise (Jolly *et al*, 2004; Rizzi *et al*, 2004). Indeed, stress induces transcription of the *sat III* sequences,

and this is mediated via direct interaction between HSF1 and the *sat III* DNA. The produced transcripts are non-coding and heterogeneous in size (Jolly *et al*, 2004; Rizzi *et al*, 2004). Based on the prevailing data on nSBs, a model for their dynamic formation and disassembly has been put forward (Biamonti, 2004). Accordingly, transcriptionally active HSF1 binds the heterochromatic *sat III* locus following stress, recruits histone acetyltransferase CREB binding protein (CBP) and initiates chromatin remodeling. RNAP II is recruited to the site and transcription of the *sat III* sequences commences. Through direct interaction with the newly formed *sat III* transcripts RNA-binding proteins are then recruited (Chiodi *et al*, 2004; Jolly *et al*, 2004; Metz *et al*, 2004; Rizzi *et al*, 2004). These ribonucleoprotein complexes form perichromatin granules, which cluster and correspond to the mature nSBs (Chiodi *et al*, 2000). During attenuation of the stress response HSF1 dissociates from the nSBs, perhaps as a consequence of increased levels of Hsp70 (Cotto *et al*, 1997; Alastalo *et al*, 2003). CBP and RNAP II also leave the nSBs, followed by disassembly of the perichromatin granules as the RNA-binding proteins are dispersed in the nucleoplasm. In contrast, the *sat III* transcripts stay bound to the locus for an extended time period (Jolly *et al*, 2004; Rizzi *et al*, 2004). Eventually, the *sat III* transcripts are degraded, and reinstatement of the original chromatin state occurs (Biamonti, 2004; Rizzi *et al*, 2004).

The function of the nSBs and the *sat III* transcripts has remained an enigma, although many plausible roles have been proposed. The massive accumulation of HSFs and other factors involved in transcription suggests that nSBs are storage sites for these factors and thereby have a role in transcriptional control (Jolly & Lakhotia, 2006; Biamonti & Vourc'h, 2010). A similar hypothesis is that the function lays in the control of alternative splicing events. This is based on the finding that the *sat III* transcripts contain binding motifs for RNA-processing factors and provide scaffolds for their docking and recruitment to the nSBs. The trapping of factors to the nSBs is a selective process where only certain factors are sequestered (Denegri *et al*, 2001; Chiodi *et al*, 2004; Metz *et al*, 2004). Since splicing decisions are made based on the ratio between different splicing factors available, the nSBs could influence the kind of splicing events that take place following stress (Denegri *et al*, 2001; Biamonti, 2004; Biamonti & Vourc'h, 2010). Moreover, several proposed roles for the *sat III* transcripts include control of the chromatin state and nuclear organization (Jolly & Lakhotia, 2006; Biamonti & Vourc'h, 2010). For example, since the *sat III* transcripts stay associated with the genomic locus from which they derive they could be involved in protecting this region following stress. Alternatively, the transcripts may be involved in the re-establishment of heterochromatin on the 9q12 locus in a manner analogous to that of the *Xist* transcript that confers X chromosome inactivation (Heard, 2004). Another way the *sat III* transcripts could be involved in heterochromatin assembly is through incorporation into the RNAi system (Biamonti, 2004). According to the hypothesis, this could be comparable to the process in *S. pombe* where small double-stranded RNAs, generated from longer transcripts, direct the RITS (RNA-induced transcriptional silencing) complex to complementary DNA sequences and facilitate heterochromatin formation (Verdel *et al*, 2004; Eymery *et al*, 2009a). Hypothetically, *sat III* transcription could also activate gene expression. Because pericentromeric heterochromatin can have a repressive effect on genes located in *cis* or *trans* (Fisher & Merkenschlager, 2002), the activation of the 9q12 region following stress could influence the activity of genes in its vicinity through positioning effects (Eymery *et al*, 2009a). A recent study analyzing transcriptomic data did, however, not find an effect on transcription of neighboring genes upon activation of the *sat III* locus

(Eymery *et al*, 2010). Whatever functions the nSBs might hold, their HSF1-dependent formation points to a broader role for the HSFs than previously envisioned.

Concurrently with the finding of human chromosome 9 as the primary site for nSB formation, two additional chromosomes, chromosome 12 and 15, were reported to harbor nSBs upon stress (Denegri *et al*, 2002). Recently, the presence of so called secondary nSBs was observed on 12 additional chromosomes (Eymery *et al*, 2010). The secondary nSBs form on pericentromeric regions and contain *sat II* or *sat III* repeated sequences. Furthermore, the regions correspond to HSF1-binding sites and hold active transcription. Thus, it seems that upon heat shock, HSF1 induces genome-wide trancriptional activation (Eymery *et al*, 2010). This is in line with another study by Eymery and colleagues demonstrating global HSF1-dependent transcription from pericentromeric sequences of heat-shocked HeLa cells (Eymery *et al*, 2009b). Interestingly, transcription of pericentromeric regions is also detected in human testis and anomalous expression is apparent in certain cancers (Eymery *et al*, 2009b).

4. ROLES OF HSF1 AND HSF2 BEYOND THE HEAT SHOCK RESPONSE

In yeast, HSF is essential for viability and deletion of the gene renders the cells unable to grow also under normal conditions (Sorger & Pelham, 1988; Wiederrecht *et al*, 1988; Gallo *et al*, 1993). Apart from regulating stress-responsive genes to protect the cell during proteotoxic insults, the mammalian HSFs are involved in a multitude of processes including murine gametogenesis, corticogenesis, maintenance of sensory organs, cancer and aging (Xiao *et al*, 1999; Christians *et al*, 2000; Kallio *et al*, 2002; McMillan *et al*, 2002; Hsu *et al*, 2003; Wang *et al*, 2003; Fujimoto *et al*, 2004; Min *et al*, 2004; Morley & Morimoto, 2004). Correspondingly, HSF target genes under non-stressful conditions represent a vast variety, ranging from chemokines and cytokines to sex-chromosomal multicopy genes in the testis and fibroblast growth factors in the lens (Fig. 9) (Åkerfelt *et al*, 2007; Abane & Mezger, 2010).

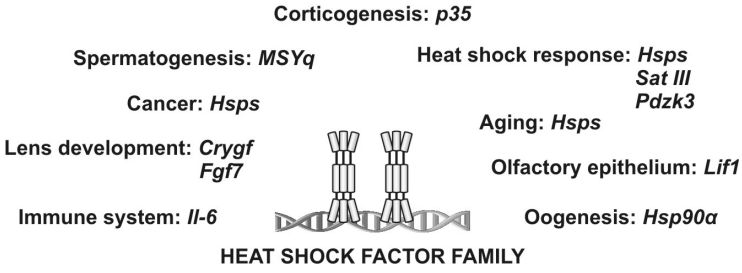

Figure 9. The mammalian HSFs are involved in a wide range of biological processes. Direct target genes identified *in vivo* are shown. *Crygf*, crystalline γF; *Fgf7*, fibroblast growth factor 7; *Il-6*, interleukin-6; *Lif1*, leukemia inhibitory factor; *MSYq*, male-specific long arm of the mouse Y chromosome; *Pdzk3*, PDZ domain-containing 3, *Sat III*, satellite III repeats. Modified from (Åkerfelt *et al*, 2010a).

4.1. HSF1 as a developmental factor

Indications of HSFs being developmental factors came from deletion experiments of the *Drosophila Hsf,* which resulted in defective oogenesis and early larval development

(Jedlicka *et al*, 1997). The developmental defects were not imposed by improper Hsp regulation, suggesting that additional HSF target genes exist. Several genome-wide analyses have proven that this is indeed the case. In yeast, HSF was found to bind a wide range of target genes, implicated in functions such as protein folding and degradation, energy metabolism, cell signaling, transcription and vesicular transport (Hahn *et al*, 2004). Using mammalian cells deficient in *Hsf1* or with downregulated levels of HSF1, numerous target genes were found also under non-stressful conditions (Trinklein *et al*, 2004b; Page *et al*, 2006). Via gene ontology analysis, the HSF1-regulated target genes were connected to processes including RNA splicing, protein folding, ubiquitination, and cell survival (Page *et al*, 2006).

Mice lacking HSF1 can survive to adulthood, although high prenatal lethality is indefeasible. This is likely a result of placental defects, specifically in a layer of cells with embryonic origin. Other developmental defects in the *Hsf1* knockout mice include growth retardation and female infertility (Xiao *et al*, 1999). HSF1 is a maternal factor since fertilized oocytes from *Hsf1*$^{-/-}$ females are unable to develop even when transplanted into wild-type females (Christians *et al*, 2000). In developing oocytes, HSF1 regulates the expression of Hsp90α and is needed for meiotic maturation (Metchat *et al*, 2009). Post-ovulated oocytes with disrupted *Hsf1* show mitochondrial damage and are sensitive to oxidative stress, leading to reduced survival (Bierkamp *et al*, 2010). In spermatogenesis, lack of HSF1 causes modest defects under normal conditions (Izu *et al*, 2004; Wang *et al*, 2004; Salmand *et al*, 2008; Abane & Mezger, 2010; Åkerfelt *et al*, 2010b). During thermal stress, however, HSF1 seems to hold a dual role as it protects early germ cells, but induces apoptosis of pachytene spermatocytes (Izu *et al*, 2004). This could indicate a delicate quality control mechanism, where HSF1 secures the stem germ cell population while hindering damaged sperm to instigate abnormal development in the following generation. Also genotoxic stress induces cell type-specific apoptosis in an HSF1-dependent manner (Salmand *et al*, 2008). A role for HSF1 in cell-death decisions is further demonstrated by the finding that overexpression of constitutively active HSF1 causes death of pachythene spermatocytes and inhibits spermatogenesis (Nakai *et al*, 2000; Widlak *et al*, 2003).

A requirement for HSF1 is evident also in maintenance of sensory organs. In lens epithelial cells HSF1 directly binds the growth factor *Fgf7* promoter and activates its transcription, whereas in the olfactory epithelium HSF1 negatively regulates the expression of the cytokine LIF1 (Fujimoto *et al*, 2004; Takaki *et al*, 2006). In addition, HSF1 plays a role in the maintenance of the postnatal brain. Mice deficient in HSF1 show enlarged ventricles, astrogliosis and neurodegeneration as well as accumulation of ubiquitinated proteins under non-stress conditions (Santos & Saraiva, 2004; Homma *et al*, 2007). Interestingly, accompanied with impaired neurogenesis, *Hsf1*$^{-/-}$ mice show altered affective behavior such as increased aggression and depression but reduced anxiety (Zhu *et al*, 2008; Uchida *et al*, 2011). By reintroducing HSF1 into the hippocampus of neonatal mice, Uchida and colleagues were able to reverse the deviant anxiety- and depression-like behaviors (Uchida *et al*, 2011).

4.2. HSF1 in aging and cancer

HSF1 protects against polyglutamine diseases and in the nematode *C. elegans* deletion of *Hsf1* increases protein aggregation. The lack of HSF1 is accompanied by a substantial reduction in the lifespan of the organism, while conversely, increased levels of HSF1

promote longevity (Hsu *et al*, 2003; Morley & Morimoto, 2004; Fujimoto *et al*, 2005; Cohen *et al*, 2006; Cohen *et al*, 2010). In aging organisms, a tendency to decreased protein homeostasis and impaired heat shock response is apparent (Kregel, 2002; Morimoto, 2008). Recent cell-based aging experiments propose that an age-related decline in HSF1 activity and the heat shock response is connected to progressive loss of the expression and activity of SIRT1, a deacetylase that keeps HSF1 in a DNA-binding state (Westerheide *et al*, 2009). While the beneficial effects of HSF1 are unambiguous for the organism under both normal and stressful conditions, HSF1 provides also cancer cells with a survival advantage. Intrinsic stress is a feature shared by most cancer cells and, perhaps consequently, high levels of HSF1 are found in several types of human cancers (Tang *et al*, 2005; Khaleque *et al*, 2008; Whitesell & Lindquist, 2009). In accordance, mice lacking HSF1 are less prone to develop tumors induced by targeted mutations, and human cancer cell lines are to a higher degree dependent on HSF1 to proliferate and survive than non-transformed cells. The ability to support malignant transformation stems from the role of HSF1 as an orchestrator of a range of cellular functions such as glucose metabolism, protein synthesis and proliferation (Dai *et al*, 2007).

4.3. HSF2 in developmental processes

Whereas HSF1 is evenly expressed in most tissues, the level of HSF2 varies both temporally and spatially (Fiorenza *et al*, 1995; Rallu *et al*, 1997; Abane & Mezger, 2010), indicating specific roles in development. During murine embryogenesis, expression of HSF2 increases progressively throughout the whole embryo, until the second half of gestation when the expression becomes restricted to the developing brain. The increase in HSF2 levels is mirrored by enhanced DNA-binding activity (Rallu *et al*, 1997). Constitutive DNA-binding has also been detected in embryonic carcinoma cells and stem cells. However, high DNA-binding activity was not found to correlate with altered expression of Hsps, suggesting that HSF2 regulates other target genes in development (Mezger *et al*, 1994; Murphy *et al*, 1994; Rallu *et al*, 1997). The generation of *Hsf2* knockout mice by several laboratories confirmed that Hsp expression in development is not affected by HSF2 (Kallio *et al*, 2002; McMillan *et al*, 2002; Wang *et al*, 2003). Instead, while one of these studies did not find any marked developmental abnormalities (McMillan *et al*, 2002), the two other studies revealed aberrant corticogenesis and gametogenesis of the *Hsf2*$^{-/-}$ mice. In females, disruption of *Hsf2* results in reduced fertility, probably due to meiotic defects that are perceptible as a reduction in the number of ovarian follicles and production of abnormal eggs. Increased prenatal lethality of the embryos is also observed (Kallio *et al*, 2002; Wang *et al*, 2003). In the surviving embryos, brain defects are evident, and the adult knockout mice show enlarged ventricles and reduced size of hippocampus, striatum and cortex, as well as a collapse of the ventricular systems (Kallio *et al*, 2002; Wang *et al*, 2003; Chang *et al*, 2006). An intriguing finding was that *Hsf2*$^{-/-}$ mice display mispositioning of neurons during cortex formation (Chang *et al*, 2006). Newborn cortical neurons migrate to the outermost layers guided by radial glial cells and the positioning signal Reelin, which is secreted by Cajal-Retzius cells. In *Hsf2* knockout mice, a reduction in the number of both glial and Cajal-Retzius cells is observed as well as a disturbance in the Reelin signaling pathway. Moreover, decreased expression of p35, an activator of the kinase Cdk5, crucial for radial migration, is apparent in the *Hsf2*$^{-/-}$ cortex. Indeed, *in vivo* ChIP experiments revealed *p35* as the first direct HSF2 target gene in development. In agreement, reduced activity of Cdk5 was detected in the cortex of *Hsf2* null mice (Chang *et*

al, 2006). The results establish that HSF2 affects neuronal migration during the development of the cortex through influencing, and perhaps coupling, multiple signaling pathways.

4.3.1. HSF2 in spermatogenesis

Basic principles of spermatogenesis

Spermatogenesis is the process by which male diploid stem cells, spermatogonia, go through a complex series of events leading to production of mature haploid germ cells, spermatozoa. The process is highly organized both in a spatial and temporal manner and takes place within the seminiferous tubules, which are tightly coiled inside the testis. Stem cells adjacent to the tubule wall divide and as the development progresses the cells proceed toward the innermost part or the lumen. From there the immature germ cells are transported to epididymis where the cells reach their final maturation. The whole process takes approximately 35 days in the mouse, and can be divided into three distinct phases; the mitotic phase, the meiotic phase, and spermiogenesis (Oakberg, 1956; Clermont, 1972). During the first phase spermatogonial stem cells proliferate and maintain their number by self-renewal. The spermatogonia then form into primary spermatocytes. At this stage the spermatocytes replicate their DNA and are called preleptotene spermatocytes. These develop, in succession, into leptotene, zygotene, pachytene and diplotene spermatocytes, during which two meiotic divisions take place finally giving rise to haploid round spermatids. Four spermatids thus form from one primary spermatocyte and, due to random inclusion of the maternal and paternal chromosomes, all spermatids contain unique genetic material. Chromosomal crossover that takes place during meiosis I, when the chromosomes align and synapse, also ensures genetic variance (Clermont, 1972; Russell *et al*, 1990; Sassone-Corsi, 2002). Via cytoplasmic bridges the developing germ cells remain in contact with each other during each division. It has been suggested that the function of the cytoplasmic bridges is to compensate for the non-identical distribution of the chromosomes between the cells, since material, such as gene products and the mRNA containing chromatoid body, can be shared (Braun *et al*, 1989; Ventelä *et al*, 2003). The last phase, spermiogenesis, does not include cell divisions but massive morphological and biochemical changes as the elongated spermatids are formed and further develop into mature spermatozoa. The changes include formation of the acrosome, elongation of the tail and packing of the genetic material into the spermatid head. During this process, somatic histones are replaced by transition proteins that are DNA packing proteins unique to male germ cells. The transition proteins are later replaced by protamines, which further induce DNA compaction. Concurrently, the cell loses part of its cytoplasm to gain a smaller size (Russell *et al*, 1990; Sassone-Corsi, 2002).

The various cell types in the seminiferous tubules are not arranged at random but form cellular associations of specific subsets of cells. When observing the mouse seminiferous tubules in cross section, principally one such association or stage is seen, and at a given stage spermatids of a specific developmental state are always found with the same type of spermatocytes and spermatogonia. The stages follow each other in a fixed sequence and the seminiferous epithelium progresses in a cyclic manner. Thus, this feature is often termed "the seminiferous epithelial cycle" (Fig. 10). In mouse, twelve stages can be identified during one cycle (Oakberg, 1956; Clermont, 1972). The development of germ cells can also be described as a wave-like feature because the stages are orderly distributed along the

seminiferous tubules. When viewed under a dissection microscope the seminiferous tubule has a distinct light absorption pattern originating from defined stages. This transillumination pattern enables isolation of specific stages of the epithelial cycle (Kotaja *et al*, 2004).

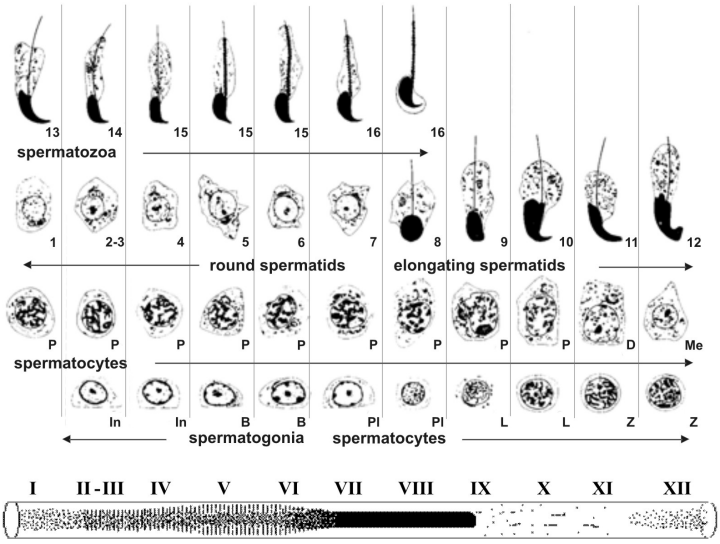

Figure 10. Schematic presentation of the twelve stages that constitute the seminiferous epithelial cycle in mouse. The development of germ cells proceeds from lower left corner to the right, moves up one row and starts again from the left until the cycle is completed. Each stage, depicted by Roman numerals, is defined by the specific set of cells present. The transillumination pattern of the tubule in each stage is shown at the bottom. In, intermediate spermatogonia; B, type B spermatogonia; PL, preleptotene spermatocyte; L, leptotene spermatocyte; Z, zygotene spermatocyte; P, pachytene spermatocyte; D, diplotene spermatocyte; Me, meiotic division; 1–16; 16 steps of spermatid differentiation. Modified from (Russell *et al*, 1990; Kotaja *et al*, 2004).

In addition to the germinal cells, the seminiferous tubules contain somatic Sertoli cells. These cells function as supporting cells that nourish the germ cells and transport them from the base of the seminiferous tubule to the inner lumen. Sertoli cells also structure the blood-testis barrier by forming tight junctions with each other (Mruk & Cheng, 2004). Another type of somatic cells is Leydig cells, located adjacent to the seminiferous tubules. The Leydig cells produce androgens such as testosterone, which is decisive to maintain germ cell development. Both endocrine and paracrine hormonal influences are crucial for normal spermatogenesis, in particular since these, directly or indirectly, affect the activity of transcription factors. The highly complex process of spermatogenesis is exceedingly dependent on a large number of genes that need to be expressed at the right time at the right place. Transcriptional control is hence crucial. However, chromatin compaction occurring during spermiogenesis is incompatible with transcription (Braun, 1998; Eddy, 1998). In addition, the X and Y chromosomes are transcriptionally silenced already in spermatocytes seeing as they are condensed into heterochromatin and secluded into a subnuclear compartment called a sex body. Due to a lack of homology the sex chromosomes suffer incomplete synapsis during meiosis, and it is thought that the sex body masks the incomplete synapsis and allows the cell to escape the meiotic surveillance system. The silencing process is called meiotic sex chromosomal inactivation (MSCI) and the repressed

state is maintained throughout round spermatid development (Turner, 2007). Thus, to retain appropriate gene expression, despite compromised control over transcription, rigorous regulation is executed at the post-transcriptional and translational levels. In fact, transcripts needed for later stages of spermatogenesis are commonly generated in advance and stored until their translation (Braun, 1998; Kleene, 2003). One mechanism to accomplish post-transcriptional control in the testis, that lately has emerged, is via the action of miRNAs. Their importance in spermatogenesis is demonstrated by observations that mice with a germ cell-specific conditional knockout of *Dicer*, required for miRNA biogenesis, show abnormal spermatids, spermatogenic arrest and are infertile (Hayashi *et al*, 2008; Maatouk *et al*, 2008; Papaioannou & Nef, 2010; Meikar *et al*, 2011). Furthermore, disruption of *Dicer* exclusively in Sertoli cells also causes infertility in mice (Papaioannou *et al*, 2009).

Hsf2 knockout phenotype in the testis

Comparing different tissues of adult mice, HSF2 is most abundantly found in the testis (Sarge *et al*, 1994; Fiorenza *et al*, 1995). Specifically, HSF2 is expressed in a developmental, stage-, and cell type-dependent manner, with highest expression in spermatocytes and round spermatids of the mouse and rat (Sarge *et al*, 1994; Alastalo *et al*, 1998). Furthermore, as detected in the murine testis, HSF2 exists in a constitutively active DNA-bound form (Sarge *et al*, 1994), suggesting that HSF2 holds a role as a regulator of gene expression during germ cell maturation. In agreement, an abnormal phenotype is seen in spermatogenesis of *Hsf2* knockout male mice, including reduced size of the testis and severe disruption and vacuolarization of the seminiferous tubules. Increased apoptosis particularly at the late pachytene stage is also apparent. The amount of mature sperm is consequently reduced and the sperm more frequently display an anomalous head shape than the wild-type counterpart (Kallio *et al*, 2002; Wang *et al*, 2003; Åkerfelt *et al*, 2008). Moreover, the synaptonemal complex, which forms between pairs of homologous chromosomes in pachytene spermatocytes, is malformed and shows abnormal loop-like structures, indicating disorganized synapsis (Kallio *et al*, 2002). This defect could conceivably activate the pachytene checkpoint, which induces elimination of malfunctioning germ cells, and thereby account for apoptosis seen in *Hsf2* null spermatocytes.

Notwithstanding the defects of the *Hsf2* knockout mice, spermatogenesis is not completely impaired, given that the males remain fertile (Kallio *et al*, 2002; Wang *et al*, 2003). However, disruption of both *Hsf1* and *Hsf2* results in a more pronounced phenotype associated with arrest in spermatogenesis and male sterility (Wang *et al*, 2004). The testis of the double knockout animals, which is drastically reduced in size, displays seemingly normal somatic Leydig and Sertoli cells, but a complete absence of spermatozoa. This is likely caused by failure of the germ cells to progress beyond the pachytene stage. Large vacuolar structures and multinucleated giant cells in the seminiferous tubules are other characteristics of the double knockout mice (Wang *et al*, 2004).

HSF2 as a regulator of gene expression in spermatogenesis

The Y chromosome is essential for male germ cell development, but contains only a small number of genes. The chromosome can be divided into a male-specific region and a short pseudoautosomal region, needed for chromosome pairing (Ellis & Affara, 2006). The male-specific region on the mouse Y chromosome long arm (MSYq) harbors numerous copies of

a few gene families, which are critical for chromatin packing and sperm differentiation. Interestingly, the phenotype of mice carrying 2/3 deletions of the MSYq resembles the phenotype of the *Hsf2*$^{-/-}$ mice in regard to the abnormal sperm head shape (Toure *et al*, 2004; Ellis *et al*, 2005; Ward & Burgoyne, 2006; Åkerfelt *et al*, 2008). The distorted shape is thought to reflect improper chromatin organization in the nucleus (Toure *et al*, 2005). In agreement, the *Hsf2* knockout mice display frequent DNA fragmentation of the sperm. Atypical levels of chromatin packing proteins, such as transition proteins and protamines, are also observed. The aberrant expression levels are likely an indirect effect since binding of HSF2 to these genes has not been detected. However, by performing ChIP-chip analysis on whole testis, more than 500 target genes of HSF2 were found. Strikingly, HSF2 target genes show an accumulation on the Y chromosome and specifically the multicopy genes of the MSYq are occupied by HSF2. When comparing wild-type and *Hsf2*$^{-/-}$ testis, the expression of the multicopy genes *Sly* and *Ssty2* is clearly reduced in the absence of HSF2. In addition, *Slx*, a *Sly* paralogue residing on the X chromosome, shows increased expression in *Hsf2*$^{-/-}$ testis (Åkerfelt *et al*, 2008). In a recent study, HSF1 was shown to regulate the same sex chromosomal multicopy genes as HSF2, and furthermore, abnormal sperm head shape was also detected in the *Hsf1* knockout mice (Åkerfelt *et al*, 2010b). Given that the sex chromosomes are transcriptionally silenced during meiosis by MSCI, and remain repressed throughout round spermatid development (Turner, 2007), it is interesting to note that the multicopy genes are expressed mainly in round spermatids (Toure *et al*, 2005; Mueller *et al*, 2008). Intriguingly, detailed analysis of HSF1 localization during spermatogenesis revealed that HSF1, as the first known transcription factor, accumulates in the sex body of meiotic cells and within the sex chromocenter of post-meiotic cells (Åkerfelt *et al*, 2010b). These results together suggest that HSF1, concomitant with HSF2, enables the sex chromosomal multicopy genes to escape repression. Furthermore, the above described studies show that, in addition to the unique functions of HSF1 and HSF2 in the testis, both HSFs are required for chromatin remodeling and sperm differentiation during spermatogenesis (Åkerfelt *et al*, 2008; Åkerfelt *et al*, 2010b). Noteworthy is that deletions in the Y chromosome is the most common genetic cause for spermatogenetic failure in humans (Krausz, 2005). Whether HSF malfunction similarly is involved in male infertility remains an intriguing question.

In addition to the high occurrence of HSF2 target genes on the Y chromosome in the mouse testis, the ChIP-chip screen revealed putative HSF2 promoter binding on all autosomal chromosomes. Among the target genes, HSF2 binding has been verified for *Speer4a*, *Hsc70* and *Ftmt* by standard *in vivo* ChIP assays (Åkerfelt *et al*, 2008). Speer4a belongs to the recently found SPErm-associated glutamate (E)-Rich protein (SPEER) family, the members of which are expressed in germ cells during the spermatocyte-spermatid transition. The proteins possess motifs suggesting interactions with cytoskeletal components and a function as nuclear matrix proteins involved in reorganization of the haploid germ cell nucleus (Spiess *et al*, 2003). Ftmt, in turn, belongs to the iron storage protein family ferritin, and has been implicated to play a role in protecting mitochondria from iron-dependent oxidative damage. It is expressed in a wide range of tissues but is highly abundant in the testis, where it is found in both interstitial and germinal cells (Santambrogio *et al*, 2007; Arosio & Levi, 2010). Binding of HSF2 to the above mentioned autosomal genes, in addition to the sex chromosomal multicopy genes, occurs in the testis and is not detected in other tissues such as brain, muscle or kidney (Åkerfelt *et al*, 2008). This indicates that the promoter sequence *per se* is not sufficient for HSF2 binding and that HSF2 activity in regulated in a tissue-specific manner. How this regulation is achieved warrants further investigations.

OUTLINE OF THE STUDY

The overall objective of this thesis was to elucidate a putative stress-responsive role for HSF2 as well as the regulatory mechanisms behind HSF2 function and activity. Prior to the work described herein, HSF1 and HSF2 were considered functionally distinct: while HSF1 held an undisputed role in the heat shock response, HSF2 was primarily associated with developmental processes and the determinants for its activity were as yet enigmatic. However, results emerging from our laboratory had shown that HSF1 and HSF2 co-localize in nSBs upon heat stress. This led us to investigate a possible involvement of HSF2 in the heat shock response, with emphasis on the DNA-binding activity and contribution to inducible *Hsp* transcription in response to stress. The results obtained, showing dependency on HSF1 for HSF2 to bind the *Hsp70* promoter, together with an earlier observation that HSF1 and HSF2 interact, urged us to explore a physical interaction between the two factors. From these analyses in turn, it became evident that the activity of HSF2 might be determined by its level of expression. To uncover by what means HSF2 is controlled, we hypothesized that miRNAs are involved in this process and thus examined the option using mouse spermatogenesis as a model system. In addition, during the course of the project we noticed a lack of methods for *in vivo* studies in spermatogenesis. We therefore sought to develop a technique enabling treatment of germ cells residing in intact seminiferous tubules.

In short, the aims of my thesis were:

- To establish whether HSF2 holds a role in the heat shock response.
- To characterize HSF2 interplay with HSF1 and the consequences thereof.
- To identify the mechanism regulating expression and activity of HSF2.

EXPERIMENTAL PROCEDURES

1. MICE (II-III)

Hsf2$^{-/-}$ and wild-type mice, used in study II, were obtained by matings of heterozygous mice described earlier (Kallio *et al*, 2002) and were maintained in the C57BL/6N background. Male C57BL/6N mice were used in study III. The pathogen-free mice were housed under controlled environmental conditions and fed with complete pellet chow and allowed tap water. The mice were sacrificed by CO_2 asphyxiation and handled in accordance with the institutional animal care policies of the Åbo Akademi University (Turku, Finland; Central Animal Laboratory of the University of Turku, permission no. 061002). Adult (60-80 days old) mice were used for isolation of testes.

2. CELL CULTURE AND EXPERIMENTAL TREATMENTS (I-III)

K562 erythroleukemia cells were cultured in RPMI 1640 medium, while HeLa, HEK293T, GC-1 spg (spermatogonia), ST15A, and MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich). The media were supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, penicillin, and streptomycin. K562, HeLa, HEK293T and MCF-7 are all human cells, while GC-1 spg are derived from mouse and ST15A from rat. MEFs from *Hsf1*$^{-/-}$ and wild-type mice (McMillan *et al*, 1998) were cultured in DMEM supplemented with 10% FCS, 10 mM non-essential amino acids, 0.96 µl β-mercaptoethanol/100 ml, 2 mM L-glutamine and antibiotics. MEFs from *Hsf2*$^{-/-}$ and wild-type mice (Kallio *et al*, 2002) were cultured in DMEM containing 10% FCS, 1.2 mM sodium pyruvate, 2 mM L-glutamine, and antibiotics. All cell lines were maintained at 37ºC in a humified 5% CO_2 atmosphere. Heat shock treatments were performed in a 42°C (K562, HeLa, and HEK293T) or 43°C (MEFs) water bath. Hemin (Fluka) was used at a final concentration of 40 µM for the indicated time periods and the proteasome inhibitor MG132 (Peptide Institute Inc.) was used at a concentration of 10 µM for 5 h.

3. PLASMID CONSTRUCTION (I-III)

Expression vectors encoding mouse HSF2-α and HSF2-ß with C-terminal Myc tags were constructed by PCR and cloned into the EcoRI site in the pcDNA3.1(-) MycHis B vector (Invitrogen) in frame with the MycHis tag (Alastalo *et al*, 2003). The mouse HSF1-cyan fluorescent protein (CFP), containing amino acids 1-226, was cloned into the HindIII and XhoI sites of pcDNA4 (Invitrogen). The human HSF2-yellow fluorescent protein (YFP), containing amino acids 1-214, was constructed by PCR and cloned into the BamHI and XhoI sites of pEYFP-N1 (Clontech). The tandem CFP-YFP construct was described earlier (Kim *et al*, 2002) and was generously provided by Richard I. Morimoto (Northwestern University, Evanston, IL, USA). Vectors with N-terminal GST-tagged human HSF1 and HSF2 proteins for bacterial expression were constructed by PCR and cloned into the XhoI and EcoRI sites of pGEX-4T-2 and EcoRI and BamHI sites of

pGEX-2TK (Amersham Pharmacia Biotech), respectively. For generation of a Luciferase reporter construct containing part of the 3'UTR of HSF2 a 258-nucleotide long fraction of the human HSF2 3'UTR was amplified using the primers: 5'-CATCCACTAGTTCCCCAGGAAGTGGACTTTAC-3' and 5'-CATCCAAGCTTGGAGAAAAATGGCCATTTGAATCC-3'. The PCR fragment was digested with SpeI and HindIII and cloned into the pMIR-REPORT vector (Ambion). A construct containing the miR-18 binding site mutations was made by Quick Change site-directed mutagenesis (Stratagene) in two sequential reactions. For the mutated sequence, see study III, Fig. 3D. For plasmids used in RNAi, see below; section 5. All constructs were verified by sequencing.

4. TRANSFECTION (I-III)

Transient transfections were performed by electroporation on K562, HeLa, and HEK293T cells. 6 x 10^6 cells were resuspended in 0.4 ml OptiMEM (Invitrogen) and placed in a 0.4-cm gap cuvette (BTX). Plasmid DNA (a total of 30 μg) was incubated with the cells for 5 min at room temperature. Cells were subjected to a single electric pulse (220-250 V, 975 μF) using BioRad Gene Pulser electroporator and thereafter incubated for 15 min at room temperature and transferred to 10 ml media/transfection. Cells were incubated at 37ºC for at least 40 h prior to further treatments. MEF *Hsf2*$^{-/-}$ cells were plated to a confluence of 80-90% in 6-well plates one day before transfection using Lipofectamine 2000 (Invitrogen). Briefly, the Lipofectamin reagent and plasmid DNA were diluted in OptiMEM and incubated for 5 min at room temperature before mixing and further incubation for at least 20 min to enable complex formation. The mixture was added to the cells and the cells incubated at 37 ºC. Six hours later the transfection solution was replaced with fresh DMEM and the cells again incubated awaiting further treatments. MCF-7 cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. In short, 2.5 x 10^5 cells were plated the day before transfection in a 12-well tissue culture dish. 1.6 μg DNA was used for transfection. The pD40-His/V5-c-Myc plasmid was described earlier (Yeh *et al*, 2004) and was a kind gift from Rosalie Sears (Oregon Health & Science University, Portland, OR, USA). pEGFP-C1 (Clontech) was used to control for transfection efficacy and unspecific effects. HEK293T and GC-1 spg cells were transfected with the indicated amounts of miRIDIAN miRNA mimics (Dharmacon) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions.

5. RNA INTERFERENCE (RNAi) (I-II)

The pSUPER vector (Oligoengine) was used for generating specific RNA sequences that are processed to functional small hairpin RNAs (shRNAs) when the vector is transfected into cells (Brummelkamp *et al*, 2002). A double-stranded 64-nucleotide oligonucleotide containing the unique 19-nucleotide sequence from the target transcript both in sense and antisense orientation, separated by a 9-nucleotide spacer sequence (TTCAAGAGA), was ligated into the pSUPER vector at BglII and HindIII restriction sites. Two constructs for downregulation of HSF1 (HSF1 RNAi I and II) were generated producing the double-

stranded oligonucleotides (GCT CAT TCA GTT CCT GAT C and GTA CTT CAA GCA CAA CAA C, respectively). Two constructs for downregulation of HSF2 (HSF2 RNAi I; CAG GCG AGT ACA ACA GCA T, and HSF2 RNAi II; CTA TTG AGT CCA GGC TTT C) were similarly generated. The sequences did not have any significant homology to the other HSFs or any other known gene using BLAST (Altschul *et al*, 1990). The scrambled sequence (GCG CGC TTT GTA GGA TTC G) was used as a control and did not correspond to any known gene in the data bases. For generation of RNAi-resistant HSF1 and HSF2 constructs the expression vectors pcDNA3.1/myc-His(-)A and pcDNA4/TO/myc-HisA (Invitrogen) encoding hHSF1 and hHSF2α, respectively, were used. Silent mutations in the shRNA target sequences (underlined nucleotides) were introduced using the QuikChange site-directed mutagenesis kit (Stratagene) with the following primer: for HSF1 mutI: C GTC AAC AAG CTC ATT CAA TTT CTG ATC TCA CTG GTG CAG, for HSF1 mutII: G CTG CCC AAG TAC TTC AAA CAT AAC AAC ATG GCC AGC TTC, for HSF2 mutI: GTT GAA CAG GCG AGC ACG ACA GCA TCA TCA GAA G, and for HSF2 mutII: CAG GAA ACT ATT GAG TCC CGG CTC TCT GAA TTA AAA AGT GAG. The HSF1 R71A construct (Inouye *et al*, 2003) was mutated with the primer CAT GGC CAG CTT CGT GGC GCA GCT CAA CAT GTA TGG C and thereafter made RNAi-resistant as above. The pSUPER HSF1 and HSF2 shRNA vectors and the RNAi-resistant constructs were transfected into K562 or HEK293T cells by electroporation. Cells were incubated 72 h prior to further treatments. A stable cell line for downregulation of HSF1 was generated by transfecting HeLa cells with the pSUPER HSF1 RNAi I plasmid, and single clones were established after selection with neomycin. A stable cell line with the scrambled plasmid was similarly generated. For transient downregulation of HSF2 (in study II), small interfering RNA (siRNA) against HSF2 or AllStars negative control siRNA was transfected using HiPerFect Transfection reagent (all from Qiagen).

6. WESTERN BLOT ANALYSIS (I-III)

Soluble cell extracts were prepared as described previously (Mosser *et al*, 1988) or lysed in Laemmli sample buffer. The lysates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) followed by transfer to nitrocellulose membrane (Protran nitrocellulose, Schleicher and Schuell). Proteins were detected with the following antibodies: anti-HSF1 (Sarge *et al*, 1993; Holmberg *et al*, 2000), anti-HSF2 (Sarge *et al*, 1993; Östling *et al*, 2007), anti-Hsc70 (SPA-815, StressGen), anti-Hsp70 (SPA-810, StressGen) anti-V5 (MCA1360GA, AbD Serotec), and anti-β-actin (A4700, Sigma-Aldrich). Secondary antibodies were horseradish peroxidase-conjugated (Promega and GE Healthcare). The blots were developed with an enhanced chemiluminescence method (ECL kit, GE Healthcare).

7. BIOTIN-MEDIATED OLIGONUCLEOTIDE PULLDOWN ASSAY (I)

The oligonucleotide assay was performed as previously described (Anckar *et al*, 2006), with minor changes. The double-stranded HSE contained the sequence 5'-biotin-AACGAGAATCTTCGAGAATGGCT-3' and the scrambled control oligonucleotide

5'-biotin-AACGACGGTCGCTCCGCCTGGCT-3' (Oligomer). Buffer C extracts (Mosser *et al*, 1988) containing 300-400 µg protein were incubated with 0.5 µM annealed oligonucleotide in binding buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 10% glycerol). Salmon sperm DNA was added (0.5 µg/µl), and proteins were allowed to bind the oligonucleotide for 15 min at room temperature and 30 min at 4°C. The samples were precleared and the remaining DNA was precipitated with 15 µl of a 50% slurry of UltraLink streptavidin gel (Pierce) for 1 h at 4°C. Bound fractions were washed twice with binding buffer and three times with binding buffer containing 0.2% Triton X-100. DNA-bound proteins were eluted with denaturing buffer followed by SDS-PAGE and Western blotting.

8. CHROMATIN IMMUNOPRECIPITATION (ChIP) (I-II)

The ChIP protocol was modified from (Takahashi *et al*, 2000). K562 and MEF cells were cross-linked with a final concentration of 1% formaldehyde followed by quenching with a final concentration of 125 mM glycine. Samples were lysed in buffer (1% SDS, 10 mM EDTA, 50 mM Tris, complete protease inhibitor cocktail (Roche Applied Science), and the chromatin fragmented to an approximate size of 500 bp by sonication with Bioruptor (Diagenode). Immunoprecipitation was performed after preclearing with 50% slurry of protein G-coated Sepharose beads containing bovine serum albumin (100 µg/ml, Amersham Biosciences) at 4°C overnight. The following antibodies were used: HSF1 (SPA-901, Stressgen); HSF2 (clone 3E2, NeoMarkers, (Sarge *et al*, 1993), and rabbit polyclonal antibodies specific to mouse HSF2 produced in the Sistonen laboratory (SFI57 and SFI58)); acetylated histone H4 antibody (Upstate Biotechnology); and normal rabbit serum (Jackson Immuno Research Laboratories). Washing of immunocomplexes was performed three times in wash buffer 1 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, pH 8.0, 150 mM NaCl, 20 mM Tris-HCl, pH 8.0), twice in wash buffer 2 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, pH 8.0, 500 mM NaCl, 20 mM Tris-HCl, pH 8.0), and three times in wash buffer 3 (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 10% glycerol). The samples were incubated overnight at 65°C to reverse the cross-links. DNA was purified with phenol:chloroform, and PCR analysis was performed on 10% of each ChIP sample using puRe Taq Ready-to-go PCR beads (Amersham Biosciences). For primer sequences see Table 2. The etidiumbromide-agarose gel was scanned with FluorImager 595 (GE Healthcare) or Alpha Imager (Alpha Innotech). The input lanes represent 1% of the material used in the immunoprecipitation.

Table 2. Primers used in ChIP experiments. h, human; m, mouse; F, forward primer; R, reverse primer.

Gene	ChIP primer sequence
m*Hsp70.1*	F: 5'-CAC CAG CAC GTT CCC CA-3' R: 5'-CGC CCT GCG CCT TTA AG-3'
m*Pck*	F: 5'-GAG TGA CAC CTC ACA GCT GTG G-3' R: 5'-GGC AGG CCT TTG GAT CAT AGC C-3' (Cissell *et al*, 2003)
h*Hsp70.1*	F: 5'-CCA TGG AGA CCA ACA CCC T-3' R: 5'-CCC TGG GCT TTT ATA AGT CG-3'
h*β-actin*	F: 5'-AAC TCT CCC TCC TCC TCT TCC TC-3' R: 5'-GAG CCA TAA AAG GCA ACT TTC GG-3'
h*Sat III*	F: 5'-AAT GAA CCC GAT GCA AT-3' R: 5'-CCA TTC TTG TTG AAT CCA TT-3'(Valgardsdottir *et al*, 2005)

9. GENERATION OF RECOMBINANT PROTEINS (II)

Recombinant proteins of human HSF1 and HSF2 were produced with the above mentioned (Section 3) GST-tagged human HSF1 and HSF2 constructs in *E. coli* BL-21 Codon Plus cells (Stratagene). The BL-21 cells were grown at 37ºC to an OD_{600} of 0.8 and expression of HSF proteins was induced by addition of isopropyl-b-D-thiogalactopyranoside to 1 mM for 3 h. Cells were harvested and resuspended in ice cold lysis buffer (20 mM trietanolamin, 10 mM Tris pH 8.0, 60 mM NaCl, 2 mM dithiotreitol, 1 mM EDTA, 0.05% Triton X-100, 4 mM benzamidin, and 0.2 µg/ml leupeptin, pepstatin and aprotonin). Lysozyme was added to a final concentration of 1 mg/ml for 30 min. Incubation with 2.5 mM DNase I disrupted the DNA, and the crude lysate was centrifuged 10 000 rpm for 30 min. The soluble extract was incubated with glutathione Sepharose 4 B affinity resin for 2 h, washed, and the GST-moiety cleaved with thrombin. Coomassie blue staining of SDS-PAGE gels was used to determine the purity of the HSF proteins.

10. ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA) (I-II)

EMSA analysis was performed as previously described (Mosser *et al*, 1988). Briefly, whole cell extracts were prepared (12 µg protein) and incubated with a ^{32}P-labeled oligonucleotide corresponding to the proximal HSE of the human *Hsp70.1* promoter. Alternatively, recombinant HSF1 or HSF2 protein was added as indicated and incubated with the ^{32}P-labeled oligonucleotide probe pHuR98 (Moyzis *et al*, 1987). The protein-DNA complexes were analyzed on a native 4% polyacrylamide gel. After the gel was dried, the signal was detected by autoradiography.

11. IMMUNOPRECIPITATION (IP) (II)

For co-immunoprecipitation, testes were isolated from *Hsf2*$^{-/-}$ and wild-type mice (Kallio *et al*, 2002) and lysed in 2 ml of lysis buffer (25 mM HEPES, 100 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 20 mM ß-glycerophosphate, 20 mM *para*-nitrophenyl phosphate, 100

μM ortovanadate, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiotreitol, 1x complete mini protease inhibitor cocktail (Roche Applied Science)) supplemented with 20 mM *N*-ethylmaleimide, followed by centrifugation for 25 min at 15,000 g at 4°C. After protein extraction, 200-500 μg total cell protein was preincubated with slurry of protein-G/Sepharose (GE Healthcare) in TEG buffer (20 mM Tris-HCl pH 7.5, 1 mM EDTA, 10% glycerol) containing 150 mM NaCl and 0.1% Triton X-100 for 30 min at 4°C followed by a brief centrifugation. The precleared cellular lysate was incubated with anti-HSF1 (NeoMarkers), anti-HSF2 (NeoMarkers), or anti-FLAG M2 (Sigma-Aldrich) antibodies at 4°C for 1 h under rotation, after which 40 μl of a 50% slurry of protein-G/Sepharose was added to the reaction mixture and incubated for 1 h at 4°C under rotation. After centrifugation, the Sepharose beads were washed with supplemented TEG buffer. The immunoprecipitated proteins were run on 8% SDS-PAGE and analyzed by Western blotting.

12. IMMUNOFLUORESCENCE (II-III)

For immunofluorescence analysis, HeLa cells cultured on coverslips were washed with phosphate-buffered saline (PBS), fixed with -20°C methanol for 6 min or with 3% paraformaldehyde in PBS for 15 min. Following three washes with PBS-0.5% Tween 20, the cells were incubated in blocking solution (1% bovine serum albumin in PBS-0.5% Tween 20) for 1 h. Rabbit anti-HSF1 (Holmberg *et al*, 2000), rat anti-HSF1 (NeoMarkers), rabbit anti-HSF2 (Sarge *et al*, 1993), or rat anti-HSF2 (NeoMarkers) antibodies were diluted in blocking solution and added for 1 h. After washes secondary antibodies, anti-rabbit Alexa 488 and anti-rat Alexa 568 (Invitrogen), were incubated for 1 h. Coverslips were mounted and DNA was visualized using Vectashield mounting medium with 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). The cells were analyzed with an LSM510-Meta scanning confocal microscope (Carl Zeiss) equipped with the SP2 (version 3.2) software. The images were acquired using a Plan-Apochromat 63x/1.4 oil differential interference contrast objective and processed using Adobe Photoshop (Adobe Systems) and CorelDRAW software (Corel Corporation).

For immunofluorescence analysis of squash preparations, slides were postfixed for 10 min in 4% paraformaldehyde (PFA), washed in PBS and permeabilized in 0.2% Triton X-100 for 5 min. After additional washes, slides were blocked in 10% FCS in B1 solution (0.1 M Tris pH 7.5, 0.15 M NaCl) for 1 h at room temperature. For detection of HSF2 a polyclonal rabbit anti-HSF2 antibody (4506, produced in the Sistonen laboratory) was used overnight at 4°C. The secondary antibody, anti-rabbit Alexa 546 (Molecular Probes), was incubated for 1 h. Nuclei were visualized using Vectashield mounting medium with DAPI (Vector Laboratories). Cryosections for protein detection were prepared as for *in situ* hybridization. Following sectioning, the slides were allowed to air-dry and thereafter blocked and stained for HSF2 as above. The cryosections and squash preparations were analyzed with a LSM510-Meta scanning confocal microscope (Carl Zeiss) equipped with the SP2 (version 3.2) software. The images were acquired using a Plan-Apochromat 40x/1.4 Oil objective or a Plan-Neofluar 20x/0.5 objective.

13. TWO-PHOTON FLUORESCENCE LIFETIME IMAGING MICROSCOPY (FLIM) (II)

FLIM on HeLa cells was performed with an inverted two-photon laser scanning microscope Axiovert 200M (LSM510 NLO META, Carl Zeiss). Measurements were performed using a 63x/1.4 oil immersion plan-apochromat objective. The cells were grown on LabTek chambered coverslips and prior to the experiments the medium was replaced with buffered culture medium without phenol-red. During the experiment, the cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 by using an on-stage incubator (PeCon). The fluorescence decays were measured by the time-correlated single photon counting technique. Fluorescence decays were fitted using a biexponential model and the corresponding mean decay time in each pixel was color coded to obtain FLIM images (SPCImage software, Becker & Hickl). Fluorescence resonance energy transfer (FRET) was identified by the shorter lifetime of donor (CFP) in the presence of acceptor (τ_{DA}) as compared with that in the control donor-only cells (τ_D). The FLIM/FRET efficiency was calculated as:
$E_{\mathrm{FILM/FRET}} = 1 - \tau_{DA} / \tau_D$.
Additional acceptor photobleaching experiments were carried out on the same cell and completed with FLIM measurements to confirm FRET. At least five cells were measured for each experimental condition in three independent experiments.

14. FLUORESCENCE-ACTIVATED CELL SORTING-BASED FLUORESCENCE RESONANCE ENERGY TRANSFER (FACS-BASED FRET) (II)

An LSR II flow cytometer (Becton Dickinson) was used for FRET analysis. HeLa cells (10^6 cells/ml) were excited using the 405 nm violet laser. The filters utilized for detecting the CFP and YFP signal were 480/40BP and 520/50BP, respectively. The 405 nm laser does not excite YFP and the background signal to YFP detector was very weak. The analysis was performed using CFP *vs.* YFP dot plots, and $3x10^5$ cells were counted.

15. STRUCTURAL MODELING (II)

The structural model of the human HSF heterotrimer consisting of two HSF1 molecules (amino acids 16-205) and one HSF2 molecule (amino acids 8-194) was done in three steps. First, a template of the DBD of six *Kluyveromyces lactis* HSF monomers bound to a 32 bp DNA was generated using SYBYL 7.3 (Tripos Inc.). This was done by aligning three dimers of the crystal structure of *K. lactis* HSF bound to DNA next to each other as previously suggested (Littlefield & Nelson, 1999). Second, the HR-A domains of HSF1 and HSF2 were aligned against the *Escherichia coli* Lpp-56 X-ray structure (Shu *et al*, 1999), while the HR-B domains of HSF1 and HSF2 were aligned against the mH38-P1 GCN4 Leucine Zipper X-ray structure (Shu *et al*, 1999). This resulted in the template structure for the HR-A/B trimerization domain. The alignments were done according to the characteristic heptad repeat sequence $(abcdefg)_n$ seen in coiled coil structures. Third, by using the X-ray structure of human GABPα protein the DBD and HR-A/B domains were

linked to generate the final template used for modeling the heterotrimer consisting of the two domains (Batchelor *et al*, 1998). In the resulting model of the heterodimer, HSF2 makes both head-to-head and tail-to-tail contacts with HSF1. For sequence alignments, MALIGN and MALFORM (Johnson & Overington, 1993) were used within the BODIL visualization and modeling package (Lehtonen *et al*, 2004). Ten models were generated with the Modeler program (Sali & Blundell, 1993), and the model with the lowest objective function was selected. Sequence alignments (Study II, Suppl. Fig. 3) were generated with the program ALSCRIPT (Barton, 1993). The structural models (Study II, Figs. 2A, B) were created with the PYMOL Molecular Graphics System (DeLano Scientific).

16. SEMIQUANTITATIVE REVERSE TRANSCRIPTION (RT)-PCR AND QUANTITATIVE REAL-TIME RT-PCR (I-III)

For quantitative real-time RT-PCR, RNA was isolated using the RNeasy kit (Qiagen). For each sample, 1 µg of RNA was treated with RQ1 DNase (Promega) and reverse transcribed using Moloney Murine Leukemia Virus RNase H(-) (Promega). For analysis of mRNA in mouse GC-1 spg cells, reverse transcription was done using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). ABsolute QPCR ROX Mix (Thermo Scientific) was used, and the PCR was performed with ABI Prism 7700 or 7900HT (Applied Biosystems). Relative quantities of the target gene were normalized against *Gapdh* or *β-actin*, and fold inductions were determined. The results were analyzed with SDS 2.3 and RQ manager software (Applied Biosystems). For mRNA analysis of transfected and sorted pachytene spermatocytes, RNA was isolated using the RNeasy Micro Kit (Qiagen). Reverse transcription was performed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) and, instead of oligo-dT primers or random hexamers, the reverse primers were used as gene-specific RT primers, which was due to the low yield of transfected cells. Primers and probes for h*HSF2*, m*HSF2*, m*Hsp40*, m*Hsp110*, m*Ssty2*, and m*Speer4a* were designed using Universal Probe Library Assay Design Center (Roche Applied Biosciences) and the fluorescent probes were obtained from the Universal Probe Library (Roche Applied Biosciences). The murine *Hsp25.1*, *Hsp70.1* and *Gapdh* probes, as well as the h*Hsp70.1* probe were from CyberGene, while h*Gapdh* and m*β-actin* probes were from MedProbe and Oligomer, respectively. For primer and probe sequences, see Table 3. Differences between *Hsps* expression in *Hsf2*$^{-/-}$ MEFs and MEFs in which HSF2 was reintroduced were examined for statistical significance with Student's *t* test (Study I, Fig 6).

For analysis of s*at III* expression, RNA was isolated with the RNeasy kit (Qiagen). Contaminating genomic DNA was removed with two DNase I treatments according to the RNeasy protocol (Qiagen). Of each sample, 1 µg of RNA was subjected to reverse transcription using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). For quantitative real-time RT-PCR, ABsolute sybrgreen mix (Advanced Biotechnologies Ltd.) and the ABI Prism 5700 and 7900HT (Applied Biosystems) were used. Primers for *sat III* and *Gapdh* were described previously (Shumaker *et al*, 2006). For semiquantitative RT-PCR, ABsolute QPCR ROX mix (Advanced Biotechnologies Ltd.) was used and the PCR was run 40 cycles. The same *sat III* primers as for ChIP were used. Relative RNA quantities were normalized to *Gapdh*.

Table 3. Primers and probes used in RT-PCR experiments. Forward primer (F), reverse primer (R), probe (P), dark quencher dye (Q).

Gene	**Primer and probe sequences**
Quantitative real-time RT-PCR:	
m*Hsp25.1*	F: 5'-CACTGGCAAGCACGAAGAAAG-3' R: 5'-GCGTGTATTTCCGGGTGAAG-3' P: 5'-FAM ACCGAGAGATGTAGCCATGTTCGTCCTG TAMRA-3'
m*Hsp40*	F: 5'-ACCGCTATGGAGAGGAAGG-3' R: 5'-GAGGTACCATTAGCACCACCA-3' P: 5'-FAM GGAGGAAG Q-3'
m*Hsp70.1*	F: 5'-AGGTGCTGGACAAGTGCCAG-3' R: 5'-AACTCCTCCTTGTCGGCCA-3' P: 5'-FAM CATCTCCTGGCTGGACTCCAACACG TAMRA-3'
m*Hsp110*	F: 5'-ACGCTCAATGCAGACGAAG-3' R: 5'-CCGGAGAAAGAATTGCACAC-3' P: 5'-FAM CAGAGGCT Q-3'
m*HSF2*	F: 5'-GGTGTCAGAACTAAGAGCAAAGC-3' R: 5'-CCAATGTAACAATAAACTGGACAATC-3' P: 5'-FAM CCCAGCAG Q-3'
m*Gapdh*	F: 5'-TGCACCACCAACTGCTTAG-3' R: 5'-GGATGCAGGGATGATGTTC-3' P: 5'-FAM CAGAAGACTGTGGATGGCCCCTC TAMRA-3'
m*β-actin*	F: 5'-TGGCTCCTAGCACCATGAAGA-3' R: 5'-GTGGACAGTGAGGCCAGGAT-3' P: 5'-FAM CAAGATCATTGCTCCTCCTGAGCGCA TAMRA-3'
m*Speer4a*	F: 5'-CAAGCAGGAGTTCAAGAAGGAGCT-3' R: 5'-GCTGCAATATCGCCAACTTT-3' P: 5'-FAM TGGAGGAG Q-3'
m*Ssty2*	F: 5'-CAAGAAGAAGAGTAGGAGGAAGCA-3' R: 5'-GAGAAATTCTGCAGCCAACA-3' P: 5'-FAM GGCCCTGG Q-3'
h*Hsp70.1*	F: 5'-GCCGAGAAGGACGAGTTTGA-3' R: 5'-CCTGGTACAGTCCGCTGATGA-3' P: 5'-FAM TTACACACCTGCTCCAGCTCCTTCCTCTT TAMRA-3'
h*HSF2*	F: 5'-GGAGGAAACCCACACTAACG-3' R: 5'-ATCGTTGCTCATCCAAGACC-3' P: 5'-FAM GGAGCCAG Q-3'
h*Gapdh*	F: 5'-GTTCGACAGTCAGCCGCATC-3' R: 5'-GGAATTTGCCATGGGTGGA-3' P: 5'-FAM ACCAGGCGCCCAATACGACCAA TAMRA-3'
h*Gapdh*	F: 5'-ACCCACTCCTCCACCTTTGA-3' R: 5'-CTGTTGCTGTAGCCAAATTCGT-3' (Shumaker *et al*, 2006)
h*Sat III*	F: 5'-AATGGAATGCAATGGAATGG-3' R: 5'-CCTGTACTCGGGTTGATTCC-3' (Shumaker *et al*, 2006)
Semiquantitative RT-PCR:	
h*Sat III*	F: 5'-AATGAACCCGATGCAAT-3' R: 5'-CCATTCTTGTTGAATCCATT-3'(Valgardsdottir *et al*, 2005)
h*Gapdh*	F: 5'-ACCCACTCCTCCACCTTTGA-3' R: 5'-TTGCTGTAGCCAAATTCGTTGT-3'

17. miRNA QUANTITATIVE REAL-TIME RT-PCR (III)

The expression levels of miR-18 were determined using TaqMan miRNA Assays (Assay ID: 2422, 1094, 1232, Applied Biosystems), performed according to the manufacturer's instructions. For analysis of human MCF-7 cells, RNA was isolated using mirPremier microRNA Isolation Kit (Sigma-Aldrich) and 15 ng of RNA was used in the RT reaction. The small nucleolar RNA RNU44 was used as an endogenous control. For analyses of miR-18 expression in mouse, total RNA derived from pooled tissues from Swiss Webster mice (7800, Ambion) were used as starting material. The small RNA Sno202 was utilized for normalization. All PCR reactions were performed in triplicates with ABI Prism 7900HT (Applied Biosystems) and analyzed as above.

18. LUCIFERASE ASSAY (III)

ST15A cells (10^5 cells) were plated in a 24-well plate the day before transfection. 180 ng of the Luciferase reporter constructs and the internal control pRL-SV40 (Clontech) together with miRIDIAN miRNA mimics (Dharmacon) or a negative control were incubated with 1 µl of Lipofectamine 2000 (Invitrogen) and transfected into the cells. After 5 h the transfection solution was replaced by fresh DMEM. 24 h after transfection cell lysates were obtained and assayed using the Dual-Luciferase Reporter Assay System (Promega) and Luminoskan (Labsystems). The firefly luciferase activity was normalized to that of Renilla luciferase. MCF-7 cells were transfected with the luciferase reporter constructs and the internal control pMIR-REPORT Beta-gal (Ambion), together with pD40-His/V5-c-Myc (Yeh *et al*, 2004) or pcDNA3.1 (Invitrogen). 48 h after the transfection cell lysates were analyzed for firefly luciferase activity as described above. The luciferase activity was normalized to beta-galactosidase expression measured with Multiskan Ascent (Thermo Scientific).

19. TRANSFECTION OF GERM CELLS IN INTACT SEMINIFEROUS TUBULES (T-GIST) (III)

Mouse testes were isolated and decapsulated in Dulbecco's Modified Eagle's Medium:Ham's F12 Nutrient Mixture (DMEM:Ham's F12, D8437, Sigma-Aldrich). Stage IX of the seminiferous epithelial cycle was identified based on the light absorption pattern and segments were cut as previously described (Kotaja *et al*, 2004). The isolated stages were placed in a 96-well dish with 50 µl medium and transfected with FITC-labeled miRCURY LNA knockdown oligonucleotides (Exiqon) at a final concentration of 200 nM. A non-targeting scrambled control or a knockdown oligonucleotide specific for miR-18 was diluted in 30 µl Opti-MEM I (Gibco) together with 1.3 µl Lipofectamine RNAiMAX. The mixture was incubated for 20 min and added to the wells. Following incubation at 34°C in 5% CO_2 for 5 h, DMEM:Ham's F12 supplemented with antibiotics was added and the tubules were further cultivated for 35 h. Transfected germ cells were subsequently identified and analyzed with squash preparation or FACS, see below. For an illustrated description, see study III, Supplementary Fig. S3.

20. SQUASH PREPARATIONS (III)

Squash preparations were performed as previously described (Kotaja *et al*, 2004). Transfected seminiferous tubule segments were transferred onto a Superfrost Plus slide (Thermo Scientific) and a coverslip was placed over the tubule. Excess fluid was removed with a filter paper which allowed the cells to float out from the tubule forming a monolayer. The slide was frozen in liquid nitrogen, the coverslip was removed, and the slide was fixed in 96% ethanol for 3 min and air-dried for 4 h. Immunofluorescence was subsequently performed, see above.

21. FLUORESCENCE-ACTIVATED CELL SORTING (FACS) OF TRANSFECTED GERM CELLS (III)

Seminiferous tubules were transfected as described above (T-GIST). Following cultivation, cells were released from the tubule segments using scissors, pelleted by centrifugation at 600 g for 5 min, and resuspended in PBS. For fixation, ice cold ethanol was added to a final concentration of 70% in 150 µl, and samples were stored at -20°C for at least 24 h. The cells were centrifuged at 600 g for 5 min and stained with Propidium Iodide (PI) to visualize the DNA content in FACS. PI was used at 40 µg/ml diluted in PBS with 0.05% Tween 20. Transfected pachythene spermatocytes were collected based on the DNA content (4N) and the signal from the transfected FITC-tagged inhibitors with a FACSVantage SE cell sorter (Becton Dickinson).

22. *IN SITU* HYBRIDIZATION (III)

In situ hybridization was done essentially as described earlier (Obernosterer *et al*, 2007). Mouse testes were dissected and prefixed in 4% fresh PFA for 2 h, incubated overnight in 0.3 M sucrose, embedded in Tissue Tek OCT (Sakura) and stored at -80°C. 10 µm thick cryosections were cut with a cryostat (Leica) and collected on Superfrost Plus slides (Thermo Scientific). The slides were dried, postfixed in PFA, acetylated, treated with proteinase K and pre-hybridized in room temperature. Digoxigenin-labeled LNA scrambled probe and miR-18 antisense probe (15 nM, Exiqon) were hybridized to the slides at 51°C overnight. All steps thus far were kept strictly RNase free. Following washes and blocking, anti-digoxigenin antibody (Roche Applied Science) was added (diluted 1:2000). Alkaline phosphatase color reaction was performed with Fast Red Substrate (Dako). Slides were mounted and DNA visualized using Vectashield mounting medium with DAPI (Vector Laboratories). A LSM510-Meta scanning confocal microscope (Carl Zeiss) equipped with the SP2 (version 3.2) software was used for analysis. The sequences of the miR-18 probe and the scrambled probe were 5'-CTATCTGCACTAGATGCACCTTA-3' and 5'-GTGTAACACGTCTATACGCCCA-3', respectively.

RESULTS AND DISCUSSION

1. HSF2 AS A TRANSCRIPTIONAL REGULATOR IN THE HEAT SHOCK RESPONSE (I)

Right after the discovery of mammalian HSF2, hemin-induced differentiation of K562 erythroleukemia cells was found to activate HSF2 as it bound to the *Hsp70* promoter (Sistonen *et al*, 1992; Sistonen *et al*, 1994). Subsequent studies reported that HSF2 possesses DNA-binding activity in mouse embryonic carcinoma cells, blastocysts and the testis (Mezger *et al*, 1994; Murphy *et al*, 1994; Sarge *et al*, 1994). More lately, roles for HSF2 in processes such as corticogenesis and spermatogenesis have been established (Abane & Mezger, 2010). Thus, HSF2 has from early on been considered to be a developmental factor, whereas its role in the heat shock response has remained largely unresolved.

1.1. Both HSF1 and HSF2 bind the *Hsp70* promoter

To determine whether HSF2 participates in the heat shock response we investigated the binding of HSF1 and HSF2 to the stress-inducible human *Hsp70* promoter by performing ChIP assays. Since HSF1 and HSF2 have been shown to acquire DNA-binding activity upon heat stress and hemin treatment, respectively (Sistonen *et al*, 1992; Sarge *et al*, 1993; Sistonen *et al*, 1994; Alastalo *et al*, 2003), we included both of these conditions in the experiments. In contrast to heat shock that represents a rapidly induced stress response, hemin mediates erythroid differentiation and induces prominent *Hsp* transcription at a much slower rate (Theodorakis *et al*, 1989; Pirkkala *et al*, 2001). Interestingly, we detected binding to the *Hsp70* promoter by both HSF1 and HSF2 during heat shock as well as hemin treatment (I, Figs. 1B, C). These results are partly in agreement and contradictory to previous reports. Several earlier studies concluded, based on antibody supershift EMSAs, that HSF1 is the main DNA-binding factor during heat shock and HSF2 during hemin-induced differentiation (Sistonen *et al*, 1992; Sarge *et al*, 1993; Sistonen *et al*, 1994; Alastalo *et al*, 2003). It is possible that weak binding of a factor could have been overlooked, since the sensitivity of the supershift assay is questionable (Pirkkala & Sistonen, 1999). Using an ORIGEN-based assay, in which HSF binding was quantified based on equal affinity for electrochemiluminescent secondary antibodies to monoclonal HSF1 and HSF2 antibodies, Mathew and colleagues detected binding of both HSF1 and HSF2 to an HSE oligonucleotide upon hemin treatment (Mathew *et al*, 2001). Less clear cut results were reported concerning the binding after heat shock: increased binding of only HSF1 was detected in mouse 3T3 cells subjected to heat treatment, whereas HSF2 accounted for about 20% of the overall HSF signal in heat-shocked K562 cells. Subsequently, Trinklein and co-workers assayed HSF binding to numerous heat shock genes using ChIP, and detected HSF1 and HSF2 on several target promoters upon heat shock or hemin-induced differentiation of K562 cells. It was, however, concluded, that HSF1 bound its targets with higher affinity during heat shock than during hemin and that HSF2 exhibited strong binding only following hemin treatment (Trinklein *et al*, 2004a).

1.2. Recruitment of HSF2 to the *Hsp70* promoter is HSF1-dependent

The presence of both HSF1 and HSF2 on the *Hsp70* promoter (I, Fig. 1) could designate interplay between the factors on DNA. To investigate this possibility we utilized loss-of-function studies enabled by the advent of RNAi technology. Plasmids were generated that produce shRNAs targeted against *Hsf1* or *Hsf2*, in addition to a control plasmid encoding a non-targeting scrambled shRNA. When transiently transfected into K562 cells, the shRNAs downregulated the protein levels of HSF1 and HSF2, respectively, while the scrambled control did not cause any observable effect (I, Fig. 2A). We thereafter performed EMSA on cells transfected as above and treated with heat shock, hemin or left untreated. As expected, HSE-binding activity was detected after both heat shock and hemin treatment in cells expressing the control shRNAs (I, Fig. 2B, lanes 2, 3). On the contrary, downregulation of HSF1 abolished HSE-binding activity in heat-shocked and hemin-treated cells (I, Fig. 2B, lanes 5, 6). When HSF2 was downregulated, the HSE-binding activity after heat shock was unaffected, whereas the binding activity in hemin-treated cells was reduced (I, Fig. 2B, lanes 8, 9). These results are in accordance with previous studies stating that HSF1 is the main DNA-binding factor during heat shock. Interestingly, however, DNA-binding activity after hemin treatment includes not only HSF2 but also HSF1.

In order to ensure the accuracy of the results, we generated an additional set of shRNA producing plasmids targeted against *Hsf1* or *Hsf2*, respectively. The two *Hsf1* targeting constructs showed identical effects when various biological consequences of the downregulation, such as DNA-binding activities and Hsp70 protein induction, were examined. The same applied to the two *Hsf2* targeting constructs (unpublished results). This suggests that the obtained results were due to specific decrease in HSF1 or HSF2 expression, respectively, and not due to downregulation of other unspecific proteins. In addition, RNAi-resistant HSF1 or HSF2 constructs were made by inserting silent mutations in the shRNA target sequences. When co-transfected with the shRNA constructs the effects on DNA-binding activity and Hsp70 protein induction seen when HSF1 or HSF2 were downregulated were reversed (unpublished results).

The results obtained (I, Figs. 1, 2) point to interplay between HSF1 and HSF2 when bound to DNA, but they also show that HSF1 could be the steering factor in the process. Therefore, we sought to examine this aspect more closely. The DNA-contacting arginine 71 of HSF1 was mutated to alanine (R71A). This renders HSF1 unable to bind HSEs albeit still competent to trimerize (Inouye *et al*, 2003), and is thus presumably capable of interacting with HSF2. The construct was furthermore made RNAi-resistant by nucleotide substitutions in the sequence otherwise targeted by the shRNAs. This enabled us to minimize the effect of endogenous HSF1, via RNAi, simultaneously with HSF1 R71A overexpression. Transfection of RNAi-resistant wild-type HSF1 or HSF1 R71A restored the protein levels in cells where endogenous HSF1 was downregulated (I, Fig. 2C). When assaying for HSE-binding activity, overexpression of wild-type HSF1 reinstated HSE complex formation that was abolished by HSF1 depletion, as expected (I, Fig. 2D, lanes 5, 6, 8, 9). Overexpression of HSF1 R71A did not restore HSE-binding activity after heat shock or hemin treatment (I, Fig. 2D, lanes 11, 12). This shows that the mere presence of HSF1 is not enough for HSF2 HSE-binding upon heat shock or hemin treatment, and that HSF2 requires HSF1 that is capable of binding DNA. Surprisingly, this is the case not only in heat-treated but also in hemin-treated cells, a situation in which HSF2 has been thought to be the main HSE-binding factor. Noteworthy is that HSF2 protein levels were decreased in the absence of HSF1 following heat shock (I, Fig. 2C), an issue that will be discussed in

section 3.1. Moreover, in hemin-treated cells, HSE complex formation was also abolished, despite ample amounts of HSF2 in the lysate (I, Fig. 2C).

To further decipher the contribution of HSF1 and HSF2 to the HSE-binding complex and whether HSF1 indeed steers HSF2 participation, biotin-mediated oligonucleotide pulldown assays using MEFs derived from *Hsf1*$^{-/-}$ mice (McMillan *et al*, 1998) were performed. This way, we were able to overcome the poor sensitivity of EMSA in determining the specific composition of the HSF-HSE complexes (Pirkkala & Sistonen, 1999) as well as possible errors posed by the RNAi technique in not providing absolute protein knockdown. In heat-shocked wild-type MEFs both HSF1 and HSF2 bound the HSE-containing oligonucleotide (I, Fig. 3B, left panel). In accordance with the results obtained with EMSA (I, Fig. 2), binding of HSF2 was abolished in MEFs lacking HSF1 (I, Fig. 3B, left panel). To investigate whether HSE-binding by HSF2 requires the presence of HSF1 that is able to bind DNA also in this experimental set up, we transfected *Hsf1*$^{-/-}$ MEFs with wild-type HSF1 or HSF1 R71A. While wild-type HSF1 restored HSF2 HSE-binding, HSF1 R71A did not (I, Fig. 3B, right panel), suggesting that HSF1 DNA-binding activity is a prerequisite for HSF2 to take part in heat-induced complex formation.

To explore the situation *in vivo* we performed ChIP assays on the endogenous murine *Hsp70* promoter. In wild-type MEFs, binding of both HSF1 and HSF2 could be detected on the promoter following heat shock, which is in line with our results obtained using EMSA and biotin-mediated oligonucleotide pulldown assays. Moreover, when HSF1 was absent, binding of HSF2 was eradicated (I, Fig. 3C). When the same experiment was performed in MEFs derived from *Hsf2*$^{-/-}$ mice (Kallio *et al*, 2002), binding of HSF1 to the *Hsp70* promoter was intact (I, Fig. 3D). These results confirm HSF1-dependency of HSF2 to be able to bind the *Hsp70* promoter following heat stress. The results were not caused by the antibody used, since the same effects were obtained using several different HSF2 antibodies (unpublished results).

Our results reveal an unexplored dimension in the interplay between HSF1 and HSF2. DNA-binding of both factors upon stress was detected in human K562 cells as well as in two different lines of MEFs. Using both RNAi knockdown as well as cells with genetically ablated *Hsf1*, a dependency on HSF1 for HSF2 DNA-binding was discovered. Together, these results suggest a novel co-occupancy of HSF1 and HSF2 when binding to the *Hsp* promoter under stressful conditions. In addition, the finding that the R71A HSF1 mutant was not able to rescue stress-induced HSF-HSE binding shows that the mere presence of HSF1 is not sufficient to render HSF2 stress-responsive in terms of binding DNA. The requirement of DNA-binding competent HSF1 indicates that HSF1 might bring HSF2 to DNA, perhaps as a heterocomplex. Although our ChIP analyses show that both HSF1 and HSF2 bind the *Hsp* promoter following stress, we cannot determine whether heterocomplexes between the HSFs are formed. This is due to that the promoter of the *Hsp70* gene examined contains two HSEs located approximately 100 bp apart from each other. The sonication step in the ChIP protocol used produces DNA fragments of roughly 500 bp in size and thus a signal for HSF binding could originate from either or both HSEs, perhaps with HSF1 and HSF2 binding to separate HSEs. In a study by Loison and colleagues this obstacle was overcome by investigating the binding of HSF1 and HSF2 to the *clusterin* promoter, which contains only one HSE (Loison *et al*, 2006). Furthermore, the *clusterin* HSE is composed of three nGAAn pentamers, which suggests that only one HSF trimer binds. Upon proteotoxic insults caused by the proteasome inhibitor MG132 or by incorporation of the amino acid analog azetidin, binding of both HSF1 and HSF2 was

detected on the *clusterin* promoter and hence heterotrimer formation was suggested. However, no conclusive data concerning configuration of a heterocomplex can be obtained from the study by Loison and co-workers, since simultaneous occupancy of HSF1 and HSF2 on the same promoter fragment was not discerned. To elucidate co-occupancy sequential-ChIP (Geisberg & Struhl, 2004) would provide a suitable alternative for future studies.

1.3. HSF2 modulates the inducible expression of *Hsp*s

The occupancy of both HSF1 and HSF2 on the *Hsp70* promoter under stressful conditions should likely be reflected in transcriptional activation, an aspect we consequently investigated. Because binding of both HSF1 and HSF2 was detected on the *Hsp70* promoter after hemin treatment, we first analyzed the contribution of the HSFs in hemin-induced K562 cells by downregulating either factor. In comparison to the expression in cells transiently transfected with a scrambled control, downregulation of HSF1 or HSF2 markedly reduced *Hsp70* mRNA and protein levels, as measured with real-time RT-PCR and Western blotting, respectively (I, Figs. 4A, B). The decrease was particularly prominent after HSF1 knockdown. These results show that both HSFs act as transcriptional activators of *Hsp70* during hemin-induced differentiation, but suggest that HSF1 is the major factor in this endeavor. This suggestion is in line with Yoshima and colleagues who, using reporter assays, concluded that HSF1 mediates hemin-induced *Hsp70* expression (Yoshima *et al*, 1998b). However, despite detected HSE-binding, Yoshima *et al.* did not find involvement of HSF2 in transcriptional activation upon hemin treatment. This is in contrast to an earlier report showing that overexpression of HSF2-α enhances hemin-induced *Hsp* expression (Leppä *et al*, 1997) as well as to our results demonstrating that HSF2 contributes to hemin-induced *Hsp70* expression. A reason for the contradictory results might be methodological differences. Whereas Yoshima and co-workers used reporter assays with GAL4 fusion constructs, we scrutinized the situation using loss-of-function techniques *in vivo*. Putative interplay between HSF1 and HSF2 was not considered in the Yoshima study and the HSF1 and HSF2 GAL4 fusion constructs were used separately in the experiments. Given the dependence of HSF2 on HSF1 for DNA-binding (I, Figs. 2, 3), it is undoubtedly conceivable that a reliance on HSF1 prevails for HSF2 to act as a transcriptional regulator.

Because both HSF1 and HSF2 bound the *Hsp70* promoter during exposure to heat treatments (I, Figs. 1, 3), we wanted to decipher the contribution of the HSFs on *Hsp* expression also after thermal stress. The importance of HSF1 for induction of *Hsp*s during heat shock is evident from numerous studies (McMillan *et al*, 1998; Xiao *et al*, 1999; Zhang *et al*, 2002), which enabled us to concentrate on the potential impact of HSF2, a matter that has remained unclear. To this end, wild-type and *Hsf2*$^{-/-}$ MEFs were subjected to the following forms of stress: 1 h heat shock, 0.5 h heat shock followed by a 3 h recovery period or treatment with the proteasome inhibitor MG132 for 5 h. Measuring *Hsp70* expression using real-time RT-PCR revealed that HSF2 influenced the expression since less *Hsp70* mRNA was detected in cells lacking HSF2 in comparison to wild-type cells exposed to any of the three stress treatments (I, Fig. 5A). This demonstrates that HSF2 participates in transcriptional regulation of *Hsp70* after different forms of stressful conditions. To extend our study we analyzed the expression of *Hsp25*, *Hsp40* and *Hsp110* in cells exposed to the same treatments as above. The results revealed that lack of HSF2 slightly affects the expression of *Hsp25* after a 1 h heat shock, but no effect was detected in

cells allowed to recover from heat shock (I, Fig. 5B). Upon MG132 treatment, induced expression was detected in the absence of HSF2, inferring that HSF2 could function as a repressor. The *Hsp* genes have different kinetics of activation and since no marked induction in *Hsp40* and *Hsp110* could be detected after a 1 h heat shock alone (unpublished data), only the results following recovery from heat shock and MG132 treatments are shown. Interestingly, the expression of *Hsp40* and *Hsp110* increased in the absence of HSF2 in the recovery period after heat shock as well as after MG132 treatment (I, Fig. 5B), again demonstrating an inhibitory role for HSF2. To ensure that the effects seen on *Hsp* expression were a result of HSF2 absence, we re-introduced HSF2 into *Hsf2*$^{-/-}$ MEFs using transient transfections. Following a selection of stressful conditions, exogenous HSF2 was indeed able to restore *Hsp* expression (I, Fig. 6), which clearly demonstrates that HSF2 participates in induction of *Hsp*s during various forms of proteotoxic stress.

The obtained results are surprising given that previous Northern blot analyses showed no change in *Hsp* expression between heat-shocked wild-type and *Hsf2*$^{-/-}$ MEFs (McMillan *et al*, 2002). The same study further reported that induction of *Hsp70* after treatment with the proteasome inhibitors MG132 or lactacystine was unaffected by the absence of HSF2. There are several putative reasons for the deviating results. Our study was conducted with immortalized MEFs, whereas McMillan and colleagues used primary MEFs. The MEFs were furthermore derived from mice with different genetic backgrounds and dissimilar strategies have been employed to achieve *Hsf2* knockout (Kallio *et al*, 2002; McMillan *et al*, 2002). Moreover, different methods were utilized to detect mRNA levels, *i.e.* Northern blotting and real-time RT-PCR, and of these, the latter is generally regarded as more sensitive. On the other hand, our results are in agreement with He and co-workers who suggested that elevated expression of HSF2-α potentiates HSF1-mediated transcription of the *Hsp70* promoter upon heat shock, as measured with a luciferase reporter system (He *et al*, 2003). A role for HSF2 in Hsp regulation has also been proposed by Xing and colleagues, given that they found reduced Hsp70 induction and survival of stressed cells in which HSF2 was downregulated using RNAi. The underlying mechanism for this phenomenon was suggested to be involvement of HSF2 in preventing compaction of the *Hsp70* gene in mitotic cells, so called gene bookmarking (Xing *et al*, 2005).

Our study expands beyond previous ones since HSF2 DNA-binding was assessed *in vivo*, on endogenous *Hsp70* promoters and under several proteotoxic stress conditions. The results state that although HSF1 is the main factor in the heat shock response, HSF2 can be ascribed a modulatory role in *Hsp* induction. Furthermore, HSF2 was found to possess both an activating and an inhibiting role in gene expression (I, Figs. 5, 6). While the HSFs generally are thought of as transcriptional activators, several reports on HSFs functioning as repressors exist. When comparing genome-wide heat-induced binding of yeast HSF with HSF target gene expression profiles, Hahn and colleagues found that though the majority of the target genes were upregulated, transcription of a small number of genes was either unchanged or decreased after heat shock. This indicates a transcriptionally neutral or repressive function of HSF (Hahn *et al*, 2004). In agreement, a negative impact on transcription of certain genes has been reported for mammalian HSF1 (Cahill *et al*, 1996; Singh *et al*, 2000; Xie *et al*, 2002; Xie *et al*, 2003; Khaleque *et al*, 2008). In line with our results, a subsequent study detected decreased expression of the HSF2 target genes *Sly* and *Ssty2* but increased expression of *Slx* in the testis of *Hsf2*$^{-/-}$ mice, as compared to expression in the wild-type counterpart (Åkerfelt *et al*, 2008). Importantly, our study demonstrates that the consequence of HSF2 activity in the heat shock response is gene-specific since diverging effects were observed on different *Hsp* promoters. The dual role of HSF2 in

regulating *Hsp* genes, both positively and negatively, might establish that different chaperones are expressed to a gene-specific level in a synchronous manner, thereby forming a precise chaperone population to manage the protein damage arisen. Moreover, the nature of HSF2 function was found to be variable also depending on the type of proteotoxic stress. The results thus allow for a scenario in which HSF2 ensures the composition of the chaperone network in a stress-specific manner. In line with the idea of stress-specific functions of HSF2, a recent study suggested that the requirement for HSF2 is dependent also on the severity of thermal stress and showed that HSF2 facilitates cell survival especially under sustained mild heat shock conditions (Shinkawa *et al*, 2011).

2. HETEROTRIMERIZATION BETWEEN HSF1 AND HSF2 (II)

Study I shows that HSF2 participates in the heat shock response and functions in alliance with HSF1 during stressful situations. The results, however, raised intriguing questions such as by which mechanism HSF2 is recruited to stress-inducible promoters, how HSF2 is activated, and how the functional relationship between HSF1 and HSF2 is executed. Further investigations to address these questions were thus prompted.

2.1. Translocation of HSF2 to the nSBs is HSF1-dependent

During stress, HSF1 and HSF2 co-localize in nSBs (Alastalo *et al*, 2003), a finding that points to the existence of interplay between the factors. Based on this, we chose to use nSBs as a model system in the forthcoming study. Previously, it was shown that HSF1 binds *sat III* DNA in nSBs (Jolly *et al*, 2002), and to investigate whether also HSF2 binds the *sat III* sequences, we performed ChIP in K562 cells. Upon heat treatment, binding of HSF1 and HSF2 was observed (II, Fig. 1A), indicating that both factors could occupy the same *sat III* DNA fragment. Study I showed dependency on HSF1 for HSF2 to bind the *Hsp70* promoter, and we therefore hypothesized that HSF1 similarly could affect the localization of HSF2 to nSBs. To this end, we generated a HeLa cell line stably downregulating HSF1 by an shRNA producing plasmid (II, Fig. 1C) and followed the localization of HSF1 and HSF2 after heat treatment. Knockdown of HSF1 abrogated the translocation of both HSF1 and HSF2 to the nSBs (II, Fig. 1B, Supplementary Fig. 2A), which indicates that HSF2's stress-responsiveness and ability to bind DNA require the presence of HSF1, and is in line with study I.

2.2. Molecular modeling of heterotrimerization between HSF1 and HSF2

Trimerization of HSFs highly increases the affinity for DNA, and it has generally been assumed that the HSFs form homotrimers (Xiao *et al*, 1991; Wu, 1995). However, a feasible explanation for the dependency of HSF2 on HSF1 for both stress-induced translocation and DNA-binding activity could be physical interaction between the factors, perhaps in the form of heterotrimers. This is a reasonable scenario considering the highly homologous oligomerization domains between HSF1 and HSF2 (Pirkkala *et al*, 2001). Indeed, interaction between the HSFs has previously been detected and based on analyses of deletion mutants, the association is mediated via the HR-A/B domains (Alastalo *et al*, 2003; He *et al*, 2003). To elucidate the possibility of heterotrimer formation between HSF1

and HSF2 we began by aligning the HR-A/B domains of HSF1 and HSF2 and illustrated the heptad repeats (abcdefg) as a helical wheel (II, Supplementary Figs. 3A, B). Amino acids involved in trimerization were found conserved, especially within the midsection of the HR-A/B domains. The heptad repeats showed the characteristics typical of a trimeric supercoil, which are hydrophobic residues at positions a and d, polar residues at positions b, c, and f, and charged residues at positions e and g (Mason & Arndt, 2004). To demonstrate how two HSF1 HR-A/B helices and one HSF2 HR-A/B could form a left-handed coiled coil, a structural model was made (II, Fig. 2A). As apparent from the model, all buried polar residues in the coiled coil structure are conserved (II, Fig. 2A). For heterooligomerization, buried polar residues have been suggested to play a role in structural specificity and partner verification. In addition, surface salt bridges aid in trimerization of coiled coils (Kammerer *et al*, 2005) and such interactions could be formed between positions e and g of the HSFs (II, Supplementary Fig. 3B).

Based on the crystal structure of *K. lactis* HSF in complex with DNA (Littlefield & Nelson, 1999), a model of a human HSF1-HSF2 heterotrimer bound to DNA was generated (II, Fig. 2B). The heterotrimer is composed of the DBD and the HR-A/B domains of HSF1 and HSF2 and for comparison a corresponding HSF1 homotrimer is shown. Indications of HSF1 and HSF2 interacting could theoretically stem from associations between HSF1 and HSF2 homotrimers or other form of complexes, and not from heterotrimer formation as we hypothesized. This possibility must be considered given that higher order complexes of HSFs, such as hexamers, under certain circumstances have been detected (Sorger & Nelson, 1989; Clos *et al*, 1990). Furthermore, the association previously observed between the HR-A/B domains of HSF1 and HSF2 (Alastalo *et al*, 2003; He *et al*, 2003) has remained uncharacterized and could tentatively be a result of interacting homotrimers. Thus, we wanted to know whether interactions can occur between neighboring HSF trimers bound to DNA. The two HSF complexes in our model are bound to a 32 bp DNA double-stranded helix composed of inverted repeats of the nGAAn sequence. The model reveals that the distance between the coiled coils is approximately 40 Å (II, Fig. 2B), a gap large enough to exclude non-covalent interactions which normally occur within proximities of less than 4 Å (Laberge, 1998). Also electrostatic interactions are unlikely to transpire over a distance of 40 Å (Creighton, 1993). Therefore, if HSF1 and HSF2 interact on DNA, the interaction should be mediated through heterotrimers and not via adjacent trimers.

2.3. Heterotrimers of DNA-bound HSF1 and HSF2

After demonstrating that there is no steric hindrance for HSF1-HSF2 heterotrimerization and that interaction between adjacent DNA-bound trimers is unlikely, we sought to examine if the HSFs do interact on DNA in nSBs. For this purpose, we used different FRET-based techniques. CFP and YFP were fused to HSF1 and HSF2 constructs, respectively. The HSF constructs were deleted of their C-terminus (HSF1 contained amino acids 1-226 and HSF2 1-214), leaving the critical domains for the experimental set-up intact, *i.e.* the DBD and the HR-A/B domains (II, Fig. 2C). The constructs spontaneously localized to nSBs (II, Supplementary Fig. 4A; Jolly *et al*, 2002), a feature which circumvented the use of heat treatments. As a means to measure interaction we combined FRET with FACS (FACS-based FRET), and verified the applicability of the method (II, Supplementary Fig. 4B, dot plot 1-4). To examine FRET between HSF1 and HSF2, HeLa cells were transfected with HSF1-CFP alone or together with HSF2-YFP. After exciting

CFP, only co-expression of HSF1-CFP and HSF2-YFP generated a FRET signal (II, Supplementary Fig. 4B, dot plot 5, 6), implying that HSF1 and HSF2 interact.

To confirm that interaction between HSF1 and HSF2 takes place on DNA we applied FLIM-FRET in the nSBs. HeLa cells were transfected with the above mentioned HSF1-CFP and HSF2-YFP constructs and the fluorescence lifetime of HSF1-CFP was measured. An increase in the lifetime of the donor (HSF1-CFP) was detected in cells where the acceptor (HSF2-YFP) was photobleached as compared to unbleached cells (II, Fig. 2D). Since energy transfer from the donor molecule to the acceptor molecule affects fluorescence lifetime, the detected difference indicates that FRET occurred. The possibility of FRET taking place between adjacent HSF1 and HSF2 homotrimers cannot be fully excluded. However, the efficiency of FRET would be expected to be drastically lower than within a trimer. In the experiments, the mean FRET efficiency was 10%. When compared to the 14% efficiency of an HSF1-CFP/HSF1-YFP pair, likely forming homotrimers, this can be considered a high value, indicative of heterotrimers (II, Fig. 2E). All in all, these results show that FRET occurs between HSF1 and HSF2 in nSBs, demonstrating that HSF1 and HSF2 interact when bound to DNA. In addition to the evidence presented in our study, data from Loison and colleagues support the notion of HSF heterotrimers (Loison *et al*, 2006). Binding of both HSF1 and HSF2 was demonstrated on the *clusterin* promoter and using gel filtration of DNA-protein complexes, the two factors were found in the same fraction, which in mass corresponded to that bound by HSF1 homotrimers. Although no definitive evidence was provided in the study, the finding that the *clusterin* promoter matches the binding site for only one HSF trimer makes binding of a heterotrimer likely. Taking all evidence together, we propose that HSF1 and HSF2 interact on DNA as a heterotrimeric complex.

2.4. Heterotrimerization as a regulatory step in HSF activity

To explore the impact of heterotrimerization between HSF1 and HSF2 in transcriptional regulation, we abrogated heterotrimer formation by transient downregulation of HSF1 in HEK293T cells (II, Fig. 1E). This eradicated stress-induced *sat III* transcription as measured by real-time RT-PCR and semiquantitative RT-PCR (II, Fig. 1D, Supplementary Fig. 2B), an effect that could be rescued by overexpressing an RNAi-resistant HSF1 mutant (unpublished data). The result is in line with the dominant role for HSF1 in the nSBs (II, Fig. 1B) and with previous studies showing that HSF1 initiates chromatin remodeling and recruits RNAP II to the 9q12 locus following stress (Jolly *et al*, 2004; Rizzi *et al*, 2004). The function of HSF2 was similarly examined and following downregulation of HSF2 in HEK293T cells (II, Fig. 3B), an increase in stress-induced expression of *sat III* transcripts was detected (II, Fig. 3A). In this situation, the disruption of heterotrimers likely enabled HSF1 homotrimers to bind the *sat III* locus, in particular since HSF2 downregulation did not affect translocation of HSF1 to nSBs upon stress (II, Fig. 3C). Intriguingly, HSF2 lacking the DBD has been shown to prevent accumulation of HSF1 into nSBs during heat stress (Alastalo *et al*, 2003). A putative explanation is that HSF1 in this case can interact with HSF2, forming heterotrimers, but due to the compromised HSF2, the complex is unable to bind the 9q12 locus. When HSF2 is absent (II, Figs. 3A, B) or lacking its HR-A/B domain, which disrupts the interaction with HSF1 (Alastalo *et al*, 2003), HSF1 forms only homotrimers that have intact ability to bind DNA.

Disruption of heterotrimer formation revealed that heterotrimers function in regulating the strength of the stress-induced transcriptional response. HSF1 and HSF2 have different transactivation potentials, with HSF2 being the weaker activator (Yoshima *et al*, 1998b). Thus, heterocomplexes might have lower activation potential than HSF1 homotrimers, which would account for the positive effect on *sat III* transcription in the absence of HSF2 (II, Fig. 3A). This is also in line with the increase in *Hsp40* and *Hsp110* following heat stress when intact *Hsf2* is lacking (I, Fig. 5B). The situation is, however, more complex, since heterotrimers have a positive effect on heat-induced *Hsp70* and *Hsp25* transcription, as judged by the decrease in their expression when the *hsf2* gene is disrupted (I, Figs. 5A, B). One explanation could be differences in the composition of HSEs in the target gene promoters. HSF1 and HSF2 homotrimers are known to prefer different HSE sequences (Kroeger *et al*, 1993; Kroeger & Morimoto, 1994; Yamamoto *et al*, 2009), and the formation of heterotrimers likely brings yet other preferences. Minute differences in the promoter sequences might evoke distinct regulation of HSF target genes, accounting for the opposing effects of *hsf2* ablation seen in the expression of individual Hsps (I, Figs. 5A, B). Influence of other factors binding to specific promoters, with or without interacting with the HSFs, should, however, not be excluded.

Another means to achieve variation in binding specificity and regulatory activity could be by altering the composition of the heterocomplexes. Formation of distinct heterocomplexes, and combinations thereof, might provide an efficient way of integrating the functions of the HSFs in response to the plethora of different stimuli. Furthermore, by forming heterocomplexes with different compositions, a gradient of transactivation could be accomplished. Our model of a DNA-binding heterotrimer is composed of two HSF1 molecules and one HSF2 molecule (II, Fig. 2B). However, based on the existing data, the complex possesses no specified stoichiometry and trimers with two HSF2 molecules are equally likely. A way to affect the composition of the heterotrimers would be to change the amount of available HSF molecules. As a matter of fact, in contrast to the stable levels of HSF1, the levels of HSF2 are known to vary between cell types and tissues and fluctuate during certain processes (Fiorenza *et al*, 1995; Rallu *et al*, 1997; Alastalo *et al*, 1998). Therefore, we investigated how increased expression of HSF2 might influence the function of the heterotrimers. Interestingly, as examined in both HeLa and HEK293T cells, elevated expression of HSF2 caused not only HSF2 but also HSF1 to spontaneously localize to nSBs (II, Fig. 4D, Supplementary Fig. 5D), indicating that HSF2 concentration regulates DNA-binding activity of heterotrimers. This conclusion is supported by the observation that HSF1 and HSF2 remained dispersed in the nucleoplasm of cells moderately overexpressing HSF2 (II, Fig. 5E). Elevated levels of HSF2 (II, Fig. 4C, Supplementary Fig. 5C) furthermore resulted in a marked increase in *sat III* transcription (II, Fig. 4A, Supplementary Fig. 5A). The elevated transcriptional activity was induced in the absence of heat stress, suggesting that the mere concentration of HSF2 is enough to mediate heterotrimer activity in the nSBs. To extend the study to classical HSF target genes, we measured the expression of *Hsp70*, and found that it spontaneously increased in HeLa cells when the HSF2 concentration was elevated (II, Fig. 4B). In HEK293T cells, no similar induction could be detected (II, Supplementary Fig. 5B), a result that probably is due to the constitutive HSF activity in these cells and the consequently high levels of Hsps present already in untreated cells (Phillips *et al*, 1991). Altogether, these results suggest that HSF2 is regulated by its concentration, which influences the activity of HSF1 and the formation of DNA-bound heterotrimers.

3. HSF2 INTERACTS WITH HSF1 IN STRESS AND DEVELOPMENT (I, II)

3.1. HSF2 acts in concert with HSF1 during the heat shock response

The results presented in studies I and II expand the prevailing view of HSF2 and stipulate a role in the heat shock response, a function that might have been overseen due to the absolute requirement of HSF1. The finding that HSF1 and HSF2 are able to form heterotrimers sheds new light on how HSF2 contributes to stress-induced transcriptional activation. HSF2 seems to be dependent on HSF1 to respond to heat stress and gains DNA-binding activity in a complex with intact HSF1. Therefore, our results explain how HSF2 binds DNA during heat stress without contradicting the notion that HSF2 lacks intrinsic stress-responsiveness (Sarge *et al*, 1991; Sistonen *et al*, 1994; Ahn *et al*, 2001).

Another feature demonstrating a link between HSF2 and the heat shock response, detected during the course of studies I and II, was a slight decrease in the protein levels of HSF2 following heat shock (I, Fig. 2C; II, Figs. 1C, 3B). Subsequently, our laboratory reported that HSF2 interacts with the ubiquitin E3 ligase APC/C, which mediates HSF2 degradation during the acute phase of the heat shock response (Ahlskog *et al*, 2010). Interestingly, as noted by us and others (Rossi *et al*, 2006), the decrease in HSF2 protein levels following heat shock is markedly accentuated when HSF1 is downregulated (I, Fig. 2C; II Fig. 1C). The effect is not due to unspecific downregulation by the RNAi construct since similar results were obtained using different HSF1 targeting constructs (II, Supplementary Fig. 6; unpublished results). This suggests that HSF1 has a stabilizing influence on HSF2. As the two HSFs form heterotrimers upon heat stress (II), it is possible that complex formation protects HSF2 from being degraded. In fact, introduction of an RNAi-resistant HSF1 mutant unable to bind HSEs (HSF1 R71A) does not rescue the levels of HSF2 following heat shock to the same extent as RNAi-resistant wild-type HSF1 does (I, Fig. 2C, compare lanes 5, 8, 11).

On the other hand, decreasing the protein levels of HSF2 might be an efficient way to influence heterocomplex composition, and thereby activity, during heat stress (for a model see Fig. 13). Recently, we proposed that it is in fact the promoter-bound pool of HSF2 that is subjected to heat-induced degradation (Ahlskog *et al*, 2010). This suggestion is based on our finding that the APC/C co-activator Cdc20 as well as the proteasome subunit α2 are recruited to the HSF2 target promoter *Hsp70* during heat shock. Furthermore, the diverging kinetics of HSF1 and HSF2 with regard to *Hsp70* promoter occupancy during a heat shock time course (Ahlskog *et al*, 2010), implies that the trimeric complexes binding the promoter change their composition during progression of the stress response. Pointing in the same direction is the observation that disruption of *Hsf2* has a different outcome on *Hsp25* induction depending on when following a heat shock the expression is measured (I, Fig. 5B, compare HS with HS+R). In effect, this stipulates that HSF2 function could be restricted to, or be more critical during, a specific phase of the heat shock response, most likely in a promoter-specific manner.

Protein homeostasis is crucial for the well-being of organisms, but can be disturbed by external stress stimuli as well as during normal growth or development. Particularly age-related pathologies and protein misfolding diseases are circumstances in which the protein network is damaged and for which therapeutic interventions are warranted. To restore protein homeostasis, the protective functions of Hsps are evident. Since HSF1 has been

considered the major regulator of the heat shock response and is implicated in both the control of lifespan and progression of neurodegenerative diseases, HSF1 has constituted an attractive target for pharmacological regulation (Westerheide & Morimoto, 2005; Powers *et al*, 2009). In fact, small molecule regulators, such as the HSF1 activators HSF1A and the natural plant compound celastrol, have been identified and show potential in drug discovery (Westerheide *et al*, 2004; Neef *et al*, 2010). Our results demonstrating that; 1) HSF2 is a transcriptional regulator of Hsps during various forms of stress, 2) HSF2 heterotrimerizes with HSF1, and 3) the two factors can mutually regulate each other's activity, strongly suggest that also HSF2 should be taken into consideration when developing therapeutic strategies aimed at achieving protein homeostasis. Furthermore, approaches to activate HSF1 might rebound given that elevated levels of HSF1 have proven detrimental in both germ cells and neuronal cells (Nakai *et al*, 2000; Dirks *et al*, 2010). In addition, both HSF1 and high levels of Hsps are reportedly beneficial in tumor malignancy (Jolly & Morimoto, 2000; Whitesell & Lindquist, 2005; Dai *et al*, 2007). Therefore, in regard to the modulatory role of HSF2 on Hsp expression (I), targeting HSF2 might be a more subtle way of controlling Hsps and shifting the balance back towards protein homeostasis without providing cells with the amount of Hsps that could offer an upper hand in carcinogenesis. The therapeutic potential of HSF2 was reinforced by a recent publication demonstrating that HSF2 protects the cell against thermal stress within the physiological febrile range and suppresses accumulation of misfolded proteins (Shinkawa *et al*, 2011). Moreover, loss of HSF2 was found to accelerate disease progression and shorten the lifespan of Huntington's disease model mice (Shinkawa *et al*, 2011).

3.2. Putative HSF1/HSF2 interplay in development and consequences thereof

As noted by us (II, Fig. 4), HSF2 gains DNA-binding ability following increased concentration. This could reflect the situation in development since a correlation between high HSF2 levels and DNA-binding activity has been reported, for example in embryogenesis (Murphy *et al*, 1994; Rallu *et al*, 1997; Min *et al*, 2000). Since we found that increased HSF2 levels also activate HSF1 and induce transcription of target genes (II, Fig. 4), probably through heterotrimerization, it is possible that a similar interaction occurs in certain developmental processes. In agreement, upon hemin-induced differentiation of K562 cells along the erythroid pathway, both HSF1 and HSF2 bind the *Hsp70* promoter (I, Figs. 1, 2), implicative of interplay during development. To test this hypothesis, we investigated the situation in the mouse testis, because HSF1 and HSF2 are both involved in spermatogenesis (Sarge *et al*, 1994; Fiorenza *et al*, 1995; Alastalo *et al*, 1998; Nakai *et al*, 2000; Kallio *et al*, 2002; Wang *et al*, 2003; Izu *et al*, 2004; Wang *et al*, 2004; Salmand *et al*, 2008; Åkerfelt *et al*, 2008; Åkerfelt *et al*, 2010b). Using co-immunoprecipitation on mouse whole testis, physical interaction between HSF1 and HSF2 was detected (II, Fig. 4F). This implies that HSF1 and HSF2 form heterotrimers during spermatogenesis and that heterotrimerization could act as a regulatory mechanism of transcription also beyond the heat stress response (for a model see Fig. 13).

Several phenotypical analyses indicate that HSF1 and HSF2 have both unique and overlapping functions in male germ cell development (Abane & Mezger, 2010; Åkerfelt *et al*, 2010a). As an example, mature spermatozoa from *Hsf1*$^{-/-}$ mice display comparable head shape morphology to spermatozoa lacking intact *Hsf2* (Åkerfelt *et al*, 2008; Åkerfelt *et al*, 2010b). Interestingly, simultaneous disruption of both *Hsf1* and *Hsf2* potentiates the phenotype of *Hsf2*$^{-/-}$ mice, and leads to a lack of mature spermatozoa and male infertility

(Wang *et al*, 2004), indicating similar and synergistic functions of the HSFs. In ChIP-chip analyses performed on whole testis, both HSF1 and HSF2 were found to bind numerous target genes, and of the approximately 700 putative targets found for HSF1, around 15% were shared with HSF2 (Åkerfelt *et al*, 2008; Åkerfelt *et al*, 2010b). Thus, binding of HSF1 and HSF2 in heterotrimeric complexes during spermatogenesis could be the norm on part of the HSF-regulated promoters, in line with the detected interaction between the HSFs (II, Fig. 4F), and might at least to some extent explain the severe phenotype of the *Hsf1/Hsf2* double knockout mice.

Heterotrimerization as a regulatory mechanism of transcription might also be envisioned to take place in other physiological settings. Both HSF1 and HSF2 are involved during development of the brain and in maintaining homeostasis of the central nervous system (Abane & Mezger, 2010). Analogously to the situation in the testis, *Hsf1/Hsf2* knockout mice exhibit an exacerbated phenotype compared with mice disrupted of only *Hsf1* that suffer from developmental defects particularly in myelination. Intriguingly, also disruption of *Hsf1/Hsf4* leads to more severe defects than displayed by *Hsf1*$^{-/-}$ mice (Homma *et al*, 2007). Although no formal proof has been presented, it is tempting to speculate that heterotrimerization could occur also between other members of the HSF family than HSF1 and HSF2. Conceivably in agreement with an incorporation of HSF4 in heterotrimers, a genome-wide DNA-binding analysis in mouse lens revealed that a substantial number of the regions bound by HSF4 are co-occupied by HSF1 and/or HSF2 (Fujimoto *et al*, 2008). Further studies are warranted to clarify the existence of heterocomplexes harboring HSF4, in particular since HSF4 exists in a constitutively trimeric form with high HSE-binding affinity.

On a different note, Fig. 4 (II) revealed that increased concentration of HSF2 induces formation of nSBs harboring HSF2 and HSF1 as well as expression of *sat III* transcripts. As already stated above, HSF2 shows high levels of expression accompanied with DNA-binding activity in certain developmental programs, which could indicate that *sat III* transcripts are induced and play a role in various physiological settings. Indeed, although nSBs have only been detected upon exposure to different forms of stress, *sat III* expression has been reported to occur in the human testis and in embryonic cells (Jehan *et al*, 2007; Eymery *et al*, 2009b; Faulkner *et al*, 2009). While the studies did not link the *sat III* expression and HSFs, these cells likely house active HSF2, as earlier reported (Mezger *et al*, 1994; Murphy *et al*, 1994; Sarge *et al*, 1994; Rallu *et al*, 1997; Min *et al*, 2000). It is thus tempting to speculate that HSF2-induced heterotrimers are responsible for the *sat III* transcription observed also beyond the heat shock response. Intriguingly, anomalous transcription of pericentromeric regions, harboring various *sat* sequences, is apparent in both somatic and male germ cell cancers (Eymery *et al*, 2009b), a finding that warrants further investigations pertaining to HSF involvement.

4. REGULATION OF HSF2 BY miR-18-MEDIATED REPRESSION (III)

Assuming HSF2 activity is dependent on its concentration, as suggested in study II, the abundance of HSF2 should be under strict spatiotemporal control. A swift way to adjust gene expression, bypassing transcriptional regulation, is via miRNA-mediated repression. We hypothesized that HSF2 concentration could be managed by miRNAs. In fact, additional features point to HSF2 being a miRNA target, such as its varying levels and the general propensity of miRNAs to target transcription factors (Shalgi *et al*, 2007; Davis & Hata, 2009).

Furthermore, miRNAs are prone to function as rheostats in dynamic and developmental processes (Stark *et al*, 2005; Inui *et al*, 2010), which harmonizes with the profile of HSF2.

To initiate the study, we used the target prediction programs TargetScan, miRanda, PicTar and miRBase (Lewis *et al*, 2003; Krek *et al*, 2005; Betel *et al*, 2008; Griffiths-Jones *et al*, 2008) and searched for miRNAs that potentially could bind HSF2. By comparing the results of the predictions, taking aspects into account such as frequency of the miRNAs to turn up in the programs and conservation of the miRNA-target pair, we chose to further investigate miR-18a, miR-18b, miR-182, miR-185, miR-464, miR-494 and miR-495. When these miRNAs were transfected into cells in the form of miRNA mimics, only miR-18a and miR-18b showed an effect on the protein level of HSF2 (unpublished results), and we therefore focused on them. In HEK293T cells, a clear decrease in the protein amount of HSF2 was detected after transfection of either miR-18a or miR-18b as compared to transfection of a negative control or two unrelated miRNAs (III, Fig. 2A). The same result was obtained in human HeLa and rat ST15A cells (unpublished results), proving that the effect was not restricted to the cell type used. miR-18a and miR-18b are derived from paralogous miRNA clusters; miR-17~92 and miR-106a~363, respectively, and differ in sequence with only one nucleotide in humans and two in mice (Tanzer & Stadler, 2004; Mendell, 2008). Because expression of the miR-106a~363 cluster is rarely detected, and mice lacking the cluster show no obvious phenotype (Ventura *et al*, 2008), we continued the study with miR-18a only, hereafter termed miR-18. To strengthen the obtained results, mouse GC-1 spg cells were transfected with increasing amounts of miR-18 and a concentration-dependent decline in the protein level of HSF2 was apparent (III, Fig. 2B). Interestingly, miR-18 also affected the mRNA levels of *Hsf2*, in a concentration-dependent manner (III, Fig. 2C), suggesting that miR-18 is able to operate through destabilizing *Hsf2* mRNA.

4.1. Identification of the miR-18 binding site on *Hsf2*

Alignment of human *Hsf2* and miR-18 revealed a putative target site for miR-18 at position 112-134 of the *Hsf2* 3'UTR (III, Fig. 3A, upper panel). The site is composed of an exact match in the seed region constituting nucleotides 2-7 and at position 8, making it a so called 7mer-m8 site (Grimson et al., 2007). The site is conserved in the *Hsf2* 3'UTR of several species (III, Fig. 3A, lower panel), which increases the likelihood of it constituting a target site since conservation of DNA sequences often is coupled to functionality. To investigate if *Hsf2* is a direct target of miR-18, we generated a reporter construct containing a 258 nucleotide stretch of the 3'UTR of *Hsf2* downstream of the luciferase gene (III, Fig. 3B). ST15A cells were transfected with the reporter construct together with miR-18, the unrelated miR-494 or a negative control. Only transfection of miR-18 inhibited expression of the reporter as measured by luciferase assays (III, Fig. 3C). To more closely pinpoint the site of interaction, we mutated the reporter construct by substituting seven nucleotides in the putative binding region of miR-18, and as expected, miR-18 no longer affected the activity of the reporter construct (Fig. 3D). These results confirm that miR-18 directly targets *Hsf2* and identify the site of interaction in the 3'UTR of *Hsf2*.

4.2. Endogenous miR-18 targets *Hsf2*

We next sought to elucidate whether the regulation between miR-18 and HSF2 occurs in more physiological settings, *i.e.* without the use of exogenous miRNAs. miR-18 is

transcribed as a polycistron together with members of the miR-17~92 cluster, and it has been shown that expression of the cluster is regulated by c-Myc, which directly binds the promoter region of the miR-17~92 locus (O'Donnell *et al*, 2005; Dews *et al*, 2006). In MCF-7 cells we thus overexpressed c-Myc in order to achieve activation of endogenous miR-18 (III, Fig. 4A). As detected by real-time RT-PCR, an increase in the level of miR-18 was apparent in cells overexpressing c-Myc as compared to control transfected cells (III, Fig. 4C). When monitoring the protein level of HSF2, a decrease in HSF2 was evident in cells where c-Myc, and consequently miR-18, was up-regulated (III, Figs. 4A, B). The decrease in HSF2 levels reached its nadir at 48 h after transfection of c-Myc, (as compared to 24 and 72 h, unpublished data), while the increase in c-Myc peaked at 24 h, suggesting that c-Myc is up-stream of the effect on HSF2. Furthermore, a decrease was also detected in the mRNA level of *Hsf2* (III, Fig. 4D). To demonstrate that the effect on HSF2 is mediated via miR-18, the reporter construct bearing the 3'UTR of *Hsf2* was utilized. Co-transfection with c-Myc led to a clear decrease in luciferase activity, while the reporter construct mutated in the miR-18 binding site was not affected (III, Fig. 4E). This data establishes HSF2 as a novel target of miR-18 and demonstrates that endogenous miR-18 affects HSF2 expression.

4.3. Mutually exclusive expression of HSF2 and miR-18 in spermatogenesis

The above described data evidently show that miR-18 can regulate HSF2. However, whether the regulation takes place on the organismal level remained unrequited. HSF2 is involved in mammalian spermatogenesis and mouse deficient in *Hsf2* display profound defects such as reduced size of the testis and lower number of mature spermatozoa (Kallio *et al*, 2002; Wang *et al*, 2003). The apparent variations in the amount of HSF2 in spermatogenic cells and during different stages of the seminiferous epithelial cycle (Sarge *et al*, 1994; Alastalo *et al*, 1998), indicate that HSF2 is regulated in a stage-specific manner during male germ cell development. To assess whether this regulation could be achieved via miR-18, we investigated miR-18 expression in various mouse tissues, and found particularly high levels in testis as well as in thymus and midterm embryos (III, Fig. 1A). The high level in testis was detected also by Northern blotting (unpublished data). *In situ* hybridization on cross-sections of mouse testis revealed a cell- and stage-specific expression pattern of miR-18 during spermatogenesis (III, Figs. 1B, C).

To elucidate the physiological link between miR-18 and HSF2 we followed their expression in detail by examining cryosections showing the twelve developmental stages that constitute the epithelial cycle (III, Fig. 5B) (Kotaja *et al*, 2004). Each stage comprises a specific subset of germ cells in different phases of differentiation and the precise stage of the epithelial cycle was determined on the basis of DAPI staining. The criteria used in the staging analysis were; 1) presence of specific cell types and combinations of different cell types; 2) organization of the cell types such as association of early stage germ cells with the basal lamina, presence and position of the elongating spermatid bundles and position of the elongating spermatids released from the bundles; 3) the size of late spermatocytes, which increases dramatically as the cells proceed towards meiotic division. HSF2 and miR-18 were detected using immunostaining and *in situ* hybridization, respectively, and since these assays were performed on consecutive sections, comparisons of the expression of miR-18 and HSF2 in the same cells could be made. Interestingly, like miR-18, also HSF2 showed a cell- and stage-specific expression pattern, which remarkably was complementary to that of miR-18 (III, Fig. 5A, Supplementary Fig. S1). HSF2 was highly expressed in spermatogonia, decreased as cells developed into spermatocytes and reappeared in

spermatids where it stayed expressed through the elongation phase. miR-18 on the other hand displayed low expression in spermatogonia and spermatids but high expression in spermatocytes (III, Fig. 5A, Supplementary Fig. S1). The inverse correlation between miR-18 and HSF2 expression patterns, schematically presented in Fig. 5B (III) and Fig. 11, strongly suggests that miR-18 regulates HSF2 in male germ cell development.

Our results on HSF2 expression are in line with previous studies in which high expression of HSF2 was detected in spermatids of mouse and rat (Sarge *et al*, 1994; Alastalo *et al*, 1998). However, in these earlier studies, HSF2 was observed in large quantities also in pachytene spermatocytes, whereas we detected relatively low levels, in particular compared with the high expression seen in spermatids. The discrepancy might arise from variations in HSF2 expression between the animals used since one of the studies was conducted on rat while the two others used mice of different strains. It is also possible that the dissimilarity is due to different antibodies or techniques employed. Concerning miR-18, and in agreement with our findings, the miR-17~92 cluster transcript has earlier been detected in the testis (Novotny *et al*, 2007). Moreover, analogously to miR-18 and HSF2, expression of the cluster member pri-miR-17, showing highest abundance in pachytene spermatocytes, correlates inversely with that of the E2F1 protein, a known target of the cluster (O'Donnell *et al*, 2005; El-Darwish *et al*, 2006; Novotny *et al*, 2007). Another intriguing aspect is that concomitantly with the detection of the miR-17~92 cluster in testis, c-Myc mRNA was also detected, albeit its expression in specific cell types was not specified (Novotny *et al*, 2007). It would be interesting to compare the expression of c-Myc, miR-18 and HSF2 in detail in order to elucidate whether HSF2 could be a downstream target of c-Myc-mediated regulation in spermatogenesis. Noteworthy is, however, that apart from c-Myc, several other transcription factors are thought to regulate expression of the miR-17~92 cluster (O'Donnell *et al*, 2005; Sylvestre *et al*, 2007; Woods *et al*, 2007; Brock *et al*, 2009; Yan *et al*, 2009). Furthermore, mechanisms yet to be clearly defined probably exist for steering selective generation of the individual cluster members since they are expressed to variable degrees in several tissues (Guil & Caceres, 2007; Jevnaker *et al*, 2011).

4.4. miR-18 inhibition in spermatocytes affects HSF2 levels and target genes

In order to provide compelling evidence that miR-18 regulates HSF2 in spermatogenesis, we set out to alter the activity of miR-18 *in vivo*. Spermatogenesis constitutes a complex differentiation program where germ cell types are strictly organized in the seminiferous epithelium and depend on interactions with nurturing cells and intricate regulatory programs such as sophisticated hormonal signaling (Russell *et al*, 1990; Sassone-Corsi, 2002; Wang *et al*, 2009). For these reasons, cell lines that are able to mimic the differentiation steps of spermatogenesis are lacking. To provide a system in which spermatogenic cells could be manipulated and would survive cell culture conditions we developed a novel method that was named T-GIST (III, Supplementary Fig. S3). By this method, seminiferous tubules are isolated from adult mice and specific stages of the epithelial cycle are identified. This is based on the transilluminating pattern of the tubules, which correlates with specific stages (Toppari & Parvinen, 1985; Kotaja *et al*, 2004). The stages are dissected using scissors and placed in growth media. Here the germ cells can be treated, for example by liposome-mediated transfection. Due to the fact that the cells reside in their natural environment, *i.e.* inside the tubules, survival is highly facilitated and the cells can be sustained in culture for an extended period of time. Subsequently, the effect of the treatment can be detected using several methods, such as squash preparations, enabling

e.g. immunostaining and *in situ* hybridization, or cell sorting, providing pools of specific cells that can be further analyzed (III, Supplementary Fig. S3).

For our purpose, we chose to isolate stage IX due to several reasons. First, HSF2 and miR-18 co-localize in pachytene spermatocytes, and HSF2 is expressed at a relatively low level making it a likely miR-18 target in this stage. Secondly, since release of mature spermatids takes place in the preceding stage (Russell *et al*, 1990), stage IX contains one less layer of cells, which probably facilitates the liposome particles used in subsequent transfections to reach germ cells embedded in the seminiferous epithelium. Next, miRNA inhibitors tagged with FITC for detection purposes were transfected into the cells. After incubation, squash preparations were made by squeezing out the cells thus forming a monolayer (Toppari & Parvinen, 1985; Kotaja *et al*, 2004). Immunostainings of HSF2 revealed that in pachytene spermatocytes, where miR-18 was inhibited, the HSF2 levels increased in comparison to untransfected cells of the same type (III, Fig. 6A, upper panel). No change in the levels of HSF2 could be detected in cells transfected with a non-specific scrambled inhibitor (III, Fig. 6A, lower panel). This data demonstrates that miR-18 downregulates HSF2 in spermatogenesis.

If desired, a more quantitative method to demonstrate the effect on the HSF2 levels following miRNA inhibition using T-GIST would be to employ FACS with antibody-based protein detection. This procedure would allow distinction of transfected and untransfected cells based on the FITC marker as well as distinction of the germ cell types based on their size and DNA content. The latter distinction is achievable given that the DNA content of germ cells varies so that spermatogonia, spermatocytes and spermatids contain 2C, 4C and 1C, respectively (Toppari *et al*, 1985). The intensity of HSF2 staining, correlating with the amount of HSF2 protein, in specific cell populations could thereby be measured. Moreover, another method to visualize the effect on HSF2 protein levels, albeit with compromised accuracy in quantification, would be to perform Western blotting following transfections. However, the current set-up of T-GIST does not allow for such analyses due to the small amount of positively transfected cells of a specific cell type. A convenient method that provides quantitative results and requires a relatively small amount of cells is real-time RT-PCR. Thus, following transfections performed as described above, we conducted FACS by which transfected spermatocytes were collected, isolated RNA, and measured the mRNA levels of *Hsf2*. In cells where miR-18 was inhibited, a modest increase (8%) in the amount of *Hsf2* was detected in comparison to the amount in cells transfected with the unspecific scrambled inhibitor (unpublished data). However, miRNAs can repress translation or promote mRNA degradation of its targets, and the mechanism of action can differ in a cell type-specific manner (Mishima *et al*, 2006; Filipowicz *et al*, 2008). Thus, mRNA measurements may not be an appropriate approach to demonstrate the effect of miRNA-mediated regulation at all times. Therefore, although a clear increase was detected in the protein amount of HSF2 in spermatocytes (III, Fig. 6A), the corresponding mRNA levels are not necessarily increased to the same extent.

Finally, the consequence of miR-18 steered regulation of HSF2 was delineated. We chose to examine *Speer4a* and the multicopy gene *Ssty2* (spermiogenesis-specific transcript on the Y 2), since the promoters of these genes are bound by HSF2 during spermatogenesis (Åkerfelt *et al*, 2008). Seminiferous tubules were transfected as described above to inhibit the function of miR-18. Transfected spermatocytes, corresponding to the cell type in which miR-18-dependent increase in HSF2 had been detected (III, Fig. 6A), were collected and their mRNA analyzed. Interestingly, in cells where miR-18 was inhibited a clear reduction in the expression of *Speer4a* and *Ssty2* was evident (III, Fig. 6B). Using target prediction programs, the

possibility that miR-18 would target *Speer4a* and *Ssty2* directly was ruled out. Taken together, the results propose that correct regulation of HSF2, via miR-18, is critical for accurate transcriptional regulation during male germ cell development (for a model see Fig. 11).

4.5. Aspects on miR-18-mediated regulation of HSF2 in spermatogenesis and beyond

The miR-17~92 cluster has mainly been associated with cancer, and although studies on mice lacking the cluster have implicated roles in development, little has been reported concerning its physiological functions (Mendell, 2008; Ventura *et al*, 2008; Ventura & Jacks, 2009). In particular, both roles and targets of the cluster member miR-18 were in essence unidentified when work for this thesis was initiated. Here, we demonstrate a novel function for miR-18, as a regulator of HSF2 in mouse spermatogenesis. The miR-18-mediated regulation influences HSF2 target genes *Speer4a* and *Ssty2*, however, the functional consequences can at this point only be speculated upon. Speer4a belongs to a family of testis-specific proteins that based on expression and sequence homology with other proteins might hold roles in reorganization of the post-meiotic nucleus (Spiess *et al*, 2003). This is in consonance with the role of HSF2 in chromatin organization and proper germ cell maturation (Kallio *et al*, 2002; Wang *et al*, 2003; Åkerfelt *et al*, 2008). Likewise, Ssty2 is thought to regulate chromatin remodeling in post-meiotic cells (Toure *et al*, 2004). It would thus be exciting to investigate whether the action of miR-18 affects chromatin compaction and maturation processes and thereby influences correct morphology and function of the spermatozoa. For these kinds of endeavors, *in vivo* electroporation would be a vital method since it provides means to elucidate putative effects in the context of intact spermatogenesis in testis. Furthermore, because HSF2 targets a large number of gene promoters as analyzed in whole testis (Åkerfelt *et al*, 2008), it is likely that miR-18-mediated regulation affects a considerably broader scale of genes than demonstrated in this study, an aspect that could be explored using this method (Fig. 11).

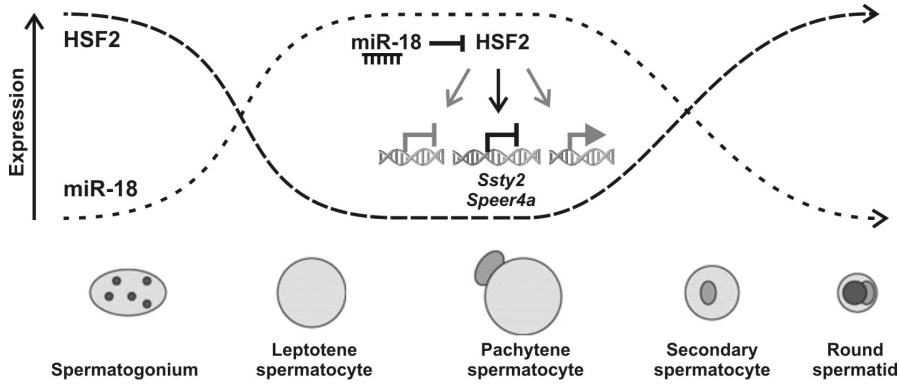

Figure 11. Schematic presentation (based on III, Fig. 5) of the inverted expression profiles displayed by HSF2 and miR-18 (dashed lines) during mouse spermatogenesis. In spermatocytes, where miR-18 is abundantly expressed, miR-18 targets *Hsf2*, which affects the expression of HSF2 target genes *Ssty2* and *Speer4a*. Since HSF2 binds a significant number of promoters during spermatogenesis, miR-18-mediated regulation could impinge, perhaps both positively and negatively, on transcription in a broader range (depicted with grey arrows).

In spermatogenesis, HSF2 function is to some extent coupled to HSF1, since for example part of the target genes of HSF1 and HSF2 are shared (Wang *et al*, 2004; Åkerfelt *et al*, 2010b). A physical interaction between the factors in testis was also observed (II, Fig. 4F), indicating presence of heterocomplexes. It is tempting to speculate that miR-18 therefore could influence HSF1. The levels of HSF1 are, however, not affected when miR-18 is overexpressed in cell culture (Fig. 12). Instead, since increased levels of HSF2 activate HSF1 as detected in nSBs (II, Figs. 4D, E), one could envision that by regulating the amount of HSF2, miR-18 indirectly affects the activity of HSF1.

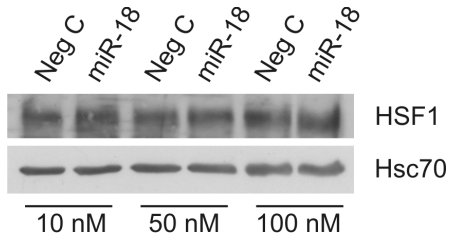

Figure 12. The protein levels of HSF1 are unaffected by miR-18 overexpression. GC-1 spg cells were transfected with increasing amounts of miR-18 or a negative scrambled control (Neg C, miRIDIAN miRNA mimics). HSF1 expression was analyzed 48 h later by Western blotting. Hsc70 was used as a loading control. (Björk & Sandqvist, unpublished results.)

In addition to HSF2, another HSF family member that conceivably could be a direct target of miRNA-mediated regulation is HSF4. In common with prime candidates for miRNA regulation, HSF4 is a transcription factor involved in development and displays varying expression levels. A case in point is rodent lens in which HSF4 is present already during fetal development, shows a peak in its abundance in the postnatal period and then declines. Furthermore, maximal expression levels correlate with the emergence of HSE/HSF4 complexes on target gene promoters (Fujimoto *et al*, 2004; Somasundaram & Bhat, 2004; Fujimoto *et al*, 2008). Considering that HSF4 possesses major HSE-binding activity, is constitutively in a DNA-bound trimeric form, binds numerous regions genome-wide, and affects the methylation status of histone H3K9 in its binding regions (Fujimoto *et al*, 2004; Somasundaram & Bhat, 2004; Fujimoto *et al*, 2008), careful regulation can be assumed a prerequisite. In fact, our preliminary data indicates that a specific miRNA, miR-491, could constitute a regulator of HSF4, since transfection of miR-491 into mouse C2C12 cells reduced the protein levels of HSF4 as assessed by Western blotting (unpublished data). Additional experimentation is, however, required to verify the finding.

Another intriguing aspect, to view the issue from a different angle, is whether HSFs could constitute regulators of miRNA expression. Although knowledge is scarce on the mechanisms steering miRNA generation, mapping of miRNA promoters using nucleosome positioning and ChIP-chip analyses have revealed that the promoter structure of miRNA genes, in regards to histone modifications and promoter elements, is indistinguishable to mRNA promoters (Ozsolak *et al*, 2008; Corcoran *et al*, 2009). Indeed, there are numerous recent findings of transcription factors, such as c-Myc and p53, that bind to the promoter region of miRNA genes (O'Donnell *et al*, 2005; Dews *et al*, 2006; Raver-Shapira *et al*, 2007; Davis & Hata, 2009; Wang *et al*, 2010). Autoregulation has also been reported, in which the transcription factor regulating expression of a miRNA is targeted by the miRNA itself. Depending on whether the regulation is positive or negative, direct or indirect,

complex regulatory networks are constructed, enabling tight control of miRNA and transcription factor levels (Davis & Hata, 2009; Inui *et al*, 2010). To elucidate whether HSF2 could regulate the expression of miR-18, we performed a series of experiments in which the levels of HSF2 were altered. This was achieved either by overexpressing HSF2 or downregulating it using RNAi in K562 cells, or by treating the cells with hemin or heat shock, known to increase (Sistonen *et al*, 1992; Sistonen *et al*, 1994) or decrease (I, II; Ahlskog *et al*, 2010) the levels of HSF2, respectively. A putative effect on the levels of miR-18 was measured using real-time RT-PCR, but no significant changes in the amount of miR-18 could be detected across the samples (unpublished data). Nevertheless, it cannot be excluded that HSF2 regulates miR-18 under other circumstances such as in spermatogenesis, since different regulatory mechanisms might be at work in different tissues or cell types. Moreover, considering the function of the HSFs as versatile transcription factors, it is conceivable that one of the HSFs, singlehandedly or in concert with other family members, would regulate expression of other miRNAs, apart from miR-18. A starting point for exploring this aspect could be to utilize bioinformatics to search for HSEs in miRNA promoters. However, the HSEs allow great variation (Trinklein *et al*, 2004b) and a more direct approach could be to perform miRNA-specific ChIP-chip analysis or ChIP-seq to achieve high resolution concerning the exact binding site. To investigate whether HSFs affect transcription, miRNA expression profiles could be determined using microarrays. Presumably, HSF-mediated regulation could take place in specific developmental settings but also in response to stress, perhaps as part of the heat shock response in which radical rearrangements in the cellular activity occur.

5. REGULATION OF HSF2 LEVELS, *e.g.* BY miR-18, DETERMINES ACTIVITY (I, II, III)

In contrast to HSF1, whose activity is induced by external stimuli and regulated through multiple post-translational modifications such as phosphorylation, sumoylation and acetylation (Åkerfelt *et al*, 2010a), the mechanisms regulating HSF2 are less well characterized. Several observations from different studies are, however, indicative of HSF2 activity being steered via its concentration. Firstly, HSF2 levels fluctuate comparing different cells and tissues (Fiorenza *et al*, 1995). Secondly, in the majority of cell types, HSF2 exists in a latent non-DNA binding form. In contrast, in cells where HSF2 is expressed at markedly high levels, such as in mouse germ cells, embryonal carcinoma cells, and mouse blastocysts, HSF2 is constitutively in an active DNA-binding form (Mezger *et al*, 1994; Murphy *et al*, 1994; Sarge *et al*, 1994). Likewise, during embryogenesis, HSF2 exhibits temporal-expression pattern, which coincides with DNA-binding activity (Rallu *et al*, 1997; Min *et al*, 2000). Thirdly, a similar correlation between increased HSF2 levels and acquisition of DNA-binding activity is seen as erythroleukemia cells differentiate following hemin treatment. Simultaneously, transcription of the target gene *Hsp70* is induced (Sistonen *et al*, 1992; Sistonen *et al*, 1994). Fourthly, and in agreement with above, ectopically increased expression of HSF2 potentiates the HSF1-mediated response to stress as measured by reporter assays (He *et al*, 2003). Fifthly, HSF2 is a short-lived protein (Mathew *et al*, 1998; Ahlskog *et al*, 2010) with varying levels, and for example upon heat shock, HSF2 shows a decrease in its protein abundance (I, II). Thus, HSF2 abundance seems to be strictly regulated, indicative of its importance. In line with this, mechanism regulating HSF2 levels have been reported, such as degradation via the ubiquitin-proteasome pathway (Mathew *et al*,

1998; Ahlskog *et al*, 2010), and as concluded in study III, miRNA-mediated repression (III). Sixthly and finally, we show that by increasing the concentration of HSF2, either by ectopic expression (II) or via inhibition of miR-18 (III), HSF2 gains DNA-binding activity, which affects the expression of HSF2 target genes (II, Fig. 4; III, Fig. 6). Taken together, these observations all point to HSF2 being regulated by its concentration. Furthermore, although HSF2 likely forms homotrimers when bound to DNA, in certain cases and at certain promoters, HSF1 and HSF2 heterotrimerize (II). Thus, as transactivation can be modulated via the levels of HSF2, HSF2 concentration could provide a switch in the formation, composition and activity of heterotrimers that integrates both the response to stress and developmental stimuli (Fig. 13).

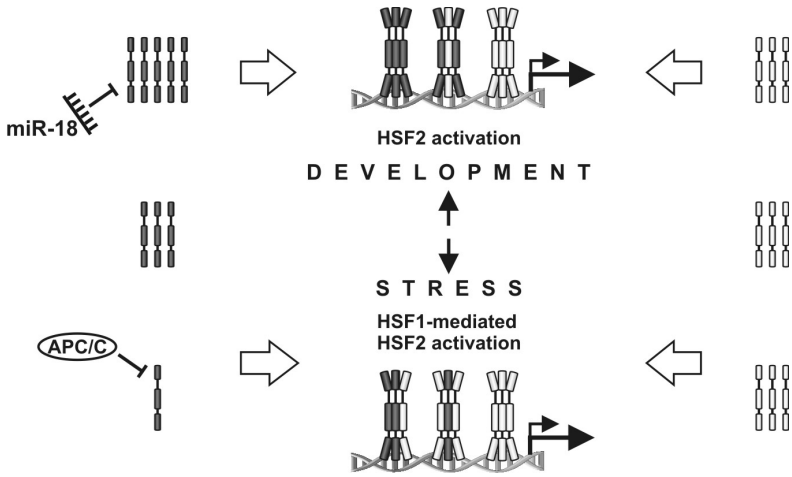

Figure 13. Schematic presentation on how HSF2 levels and activity might affect heterotrimerization between HSF1 and HSF2 in response to stress or during development. Upon stress, HSF1 is activated, which confers DNA-binding ability to HSF2. However, the amount of HSF2 rapidly decreases, which is at least in part due to APC/C-mediated ubiquitination. The remaining levels of HSF2 might affect heterotrimer composition and the transcriptional outcome (depicted by a small and large arrow) in a temporal manner during the stress response. In various developmental settings, HSF2 levels are elevated leading to activation and possible heterocomplex formation with HSF1 on certain promoters. The transcriptional activity is steered via tightly regulated concentrations of HSF2, *e.g.* via miR-18-mediated repression, in a temporal- and tissue-specific manner. HSF2 is depicted in black and HSF1 in white. Note that the inactive monomeric and dimeric forms of HSF1 and HSF2, respectively, are not indicated in the presentation.

CONCLUDING REMARKS

When this work was initiated, HSF2 was predominantly associated with developmental processes and its roles and regulatory mechanisms were largely unidentified. The notion of a putative function in the heat shock response was awakened by observations that HSF2, together with HSF1, localizes to nSBs in heat-stressed cells, but the functional consequences had remained unresolved. We discovered that HSF2 responds to various forms of stress by binding to the endogenous *Hsp70* promoter. Interestingly, the stress-related function depends on HSF1 since HSF2 DNA-binding requires the presence of intact HSF1. Nonetheless, HSF2 was found to affect expression, both positively and negatively, of various Hsps, revealing that HSF2 is a transcriptional regulator of the heat shock response. The novel intertwined actions of HSF2 and HSF1 that were exposed, prompted further studies. To this end, we established that HSF2 heterotrimerizes with HSF1 when bound to DNA in nSBs following heat stress. Also in this scenery, HSF2 is dependent on HSF1 and localization to nSBs is abrogated in the absence of HSF1. Nonetheless, HSF2 is able to incorporate HSF1 into a transcriptionally competent heterotrimer, given that increased levels of HSF2 lead to localization of both HSF2 and HSF1 to nSBs and production of *sat III* transcripts even without stress stimuli. In extension, this suggests that the activity of HSF2 could be regulated by its concentration. In fact, HSF2 levels recurrently vary, both in response to different forms of stress, but also in developmental settings such as during differentiation of male germ cells. We used mouse spermatogenesis as a model system to elucidate how particulate concentrations of HSF2 are achieved, and found that HSF2 is under direct control of miR-18. This finding is the first describing miRNA-mediated repression of a member of the HSF family. Given that inhibition of miR-18-mediated repression impinged on HSF2 target gene expression, the study links miR-18 with HSF2-regulated processes such as germ cell maturation and quality control, and demonstrates that the levels of HSF2 affect HSF2 activity.

In conclusion, this thesis reinforces a place for HSF2 in the heat shock response and provides a breakthrough in our understanding on how HSF2 interacts, both physically and functionally, with HSF1. All organisms depend on the ability to withstand stress provoked as a consequence of normal growth and developmental, by environmental cues or under pathophysiological conditions. Since HSF1 has been considered the primary regulator in the response to proteotoxic stress, HSF1 has been proposed a target in different therapeutic strategies. However, with regard to our results, it would be of outmost importance to also consider HSF2 when developing future therapies. Finally, the results presented herein provide a mechanism for how HSF2 levels, and thereby activity, is regulated; *i.e.* by miR-18, a finding that simultaneously endows miR-18 with a physiological role as a rheostat of gene expression in male germ cell development.

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