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2023

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ISBN 978-952-12-4307-3 (Printed)
ISBN 978-952-12-4308-0 (Digital)
Painosalama, Turku, Finland 2023

Till min familj

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ABSTRACT

Protein-damaging stress frequently occurs during the lifetime of a cell. To survive these insults, cells rely on the action of proteins called transcription factors that adjust the expression of genes, such as chaperones, according to the environmental requirement. Heat shock factors (HSFs) are key transcription factors that modulate gene expression upon proteotoxic insults; however, they are also important during several physiological processes related to development. To regulate transcription, HSFs undergo a complex activation process that involves nuclear accumulation, trimerization, and post-translational modifications (PTMs). However, there are big gaps in our knowledge regarding the importance of PTMs in the activation of HSFs, and it remains to be established if different HSF family members modulate transcription similarly during different stress conditions. Importantly, advances in our understanding of HSFs' pro-survival roles have revealed that their misregulation can provoke pro-tumorigenic gene expression programs, which various cancers rely on to promote survival and invasion.

This thesis comprises three studies that characterize different elements of HSF biology. The first study seeks to determine how phosphorylation in the regulatory domain of HSF1 affects its transcriptional capacity. The focus in the second study is placed on the ability of HSF1 and HSF2 to collaborate in the modulation of transcription, and it aims to map the network of genes and enhancers regulated by both HSF during heat and oxidative stress. The final study differs from the previous two in that it deals with the involvement of HSFs in the progression of cancer. Based on an earlier study showing that some cancer cells downregulate HSF2 to promote invasion, the third study aims primarily to uncover the relevant signaling pathway that suppresses the expression of HSF2. Also, the study seeks to determine why reduced levels of HSF2 are advantageous for certain cancer cells. Together, these studies characterize fundamental aspects of HSFs in stress regulation and explore how suppression of HSF2 may benefit cancer progression.

SAMMANFATTNING (Swedish Abstract)

Proteinskadande stress uppstår ofta under en cells livstid. För att överleva denna skada förlitar sig cellen på proteiner som kallas för transkriptionsfaktorer vilka justerar uttrycket av gener, inklusive chaperoner, i enlighet med miljöbehovet. Värmechockfaktorer (HSFs) är viktiga transkriptionsfaktorer som modulerar genuttryck vid proteinskadande stress, men de är också viktiga under flera fysiologiska processer relaterade till utveckling. För att reglera transkription genomgår HSF:s en komplex aktiveringsprocess som involverar nukleär ackumulering, trimerisering och post-translationella modifieringar (PTMs). Det finns dock stora luckor i vår kunskap om betydelsen av PTMs i aktiveringen av HSFs, och det återstår att fastställa om olika HSF-familjemedlemmar modulerar transkription på liknande sätt under olika stressförhållanden. I samband med framsteg i vår förståelse av HSFs roll i överlevnad har det framstått att deras felaktiga reglering kan stimulera uttrycket av flera gener som stöder utvecklingen av cancer, och olika typer av cancer förlitar sig på dessa nätverk av gener för att understöd deras överlevnad och invasionsförmåga.

Denna avhandling sammanställer tre studier vars syfte är att karakterisera olika aspekter av HSF-biologi. Målet i den första studien är att fastställa hur fosforylering i den regulatoriska domänen av HSF1 påverkar dess transkriptionskapacitet. Fokuset i den andra studien är riktad på HSF1:s och HSF2:s förmåga att samarbeta i regleringen av transkription, och studien strävar till att kartlägga nätverket av gener och förstärkare, som regleras av både HSF under värme och oxidativ stress. Den sista studien skiljer sig från de två föregående i den meningen att den är inriktad på att utreda HSFs involvering i processer relaterat till cancer. På basen av en tidigare studie, som visade att vissa cancerceller minskar på uttrycket av HSF2 för att stimulera invasion, är det huvudsakliga målet i den tredje studien att utreda den relevanta signalräcka som kan styra uttrycket av HSF2. Dessutom strävar den tredje studien att bestämma varför låga nivåer av HSF2 är fördelaktiga för cancerceller. Sammantaget karakteriserar dessa studier grundläggande aspekter av HSFs i stressreglering och utforskar hur ett reducerat uttryck av HSF2 kan gynnar utvecklingen av cancer.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the listed publications and one manuscript, which are referred to by roman numerals (I-III). Additionally, some unpublished results are included in the thesis. These original publications are reproduced which the permission of the copyright holders.

I. Budzyński MA., **Puustinen MC**., Joutsen J., and Sistonen L. (2015). Uncoupling stress-inducible phosphorylation of heat shock factor 1 from its activation. *Molecular and Cellular Biology*. 35: 2530-40. doi: 10.1128/MCB.00816-14.

II. Himanen SV., **Puustinen MC**., Da Silva AJ., Vihervaara A., and Sistonen L. (2022). HSFs drive transcription of distinct genes and enhancers during oxidative stress and heat shock. *Nucleic Acids Research*. 50: 6102-6115. doi:10.1093/nar/gkac493

III. Pessa JC.*, **Puustinen MC**.*, Hästbacka HSE., Da Silva AJ., Pihlström S., Gramolelli S., Joutsen J., and Sistonen L. TGF-beta mediates the loss of HSF2 to activate pro-tumorigenic gene expression programs. Manuscript

*Equal Contribution

PUBLICATIONS NOT INCLUDED IN THE THESIS

de Thonel A., Ahlskog JK.*, Daupin K.*, Dubreuil V., Berthelet J., Chaput C., Pires G., Leonetti C., Abane R., Barris LC., Leray I., Aalto AL., Naceri S., Cordonnier M., Benasolo C., Sanial M., Duchateau A., Vihervaara A., **Puustinen MC**., Miozzo F., Fergelot P., Lebigot É., Verloes A., Gressens P., Lacombe D., Gobbo J., Garrido C., Westerheide SD., David L., Petitjean M., Taboureau O., Rodrigues-Lima F., Passemard S., Sabéran-Djoneidi D., Nguyen L., Lancaster M., Sistonen L., and Mezger V. (2022). CBP-HSF2 structural and functional interplay in Rubinstein-Taybi neurodevelopmental disorder. *Nature Communications*. 13: 7002. doi: 10.1038/s41467-022-34476-2.

Björk JK.*, Åkerfelt M.*, Joutsen J., **Puustinen MC**., Cheng F., Sistonen L., and Nees M. (2016). Heat-shock factor 2 is a suppressor of prostate cancer invasion. *Oncogene*. 35:1770-84. doi: 10.1038/onc.2015.241.

Murillo-Garzón V., Gorroño-Etxebarria I., Åkerfelt M., **Puustinen MC**., Sistonen L., Nees M., Carton J., Waxman J., and Kypta RM. (2018). Frizzled-8 integrates Wnt-11 and transforming growth factor-β signaling in prostate cancer. *Nature Communications*. 9:1747. doi: 10.1038/s41467-018-04042-w.

*Equal Contribution

REVIEWS

Puustinen MC., and Sistonen L. (2020). Molecular mechanisms of heat shock factors in cancer. *Cells*. 9:1202. doi: 10.3390/cells9051202.

AUTHOR CONTRIBUTION

I. The author contributed to the study by performing research, analyzing results, and assisting in writing the manuscript. The author and B.MA performed the following experimental procedures: cell culture and transfections, site-directed mutagenesis, protein analysis using western blotting, immunofluorescence microscopy, mRNA quantification using qRT-PCR, and measuring DNA-binding capacity by EMSA. The author assisted in sample preparation for the ChIP and luciferase assays, which B.MA performed. J.J performed: site-directed mutagenesis and protein analysis using western blotting. B.MA. wrote the first draft of the manuscript together with L.S, which was revised based on the comments from all authors.

II. The author contributed to the study by performing research and assisting in writing the manuscript. The author performed the following experimental procedures: cell culture and sample preparation for ChIP-seq. The ChIP-seq DNA library preparation was performed by the author, and H.SV. D.S.A conducted the experiments requiring immunofluorescence microscopy. H.SV performed all PRO-seq experiments. V.A assisted in the analysis of the PRO-seq data. The ChIP-seq and PRO-seq data analysis was performed by H.SV, who also wrote the first draft of the manuscript together with L.S, which was revised based on the comments from all authors.

III. The author contributed to the study by performing research, analyzing results, and assisting in writing the manuscript. The author and P.JC performed the following experimental procedures: cell culture and transfections, genetic engineering of plasmids, protein analysis using western blotting, mRNA quantification using qRT-PCR, sample preparation for RNA-seq, cell-cell adhesion analysis using ULA-assay, sample preparation for wound-healing assay, cell proliferation analysis using CCK8-assay, sample preparation for luciferase-assay. P.JC also performed wound-healing, luciferase, and vasculogenic mimicry assays. P.S performed protein analysis using western blotting and helped to optimize the luciferase assay. G.S generated stable cell lines. The analysis of the RNA-seq data was performed by H.HSE, and the author aided in the analysis. The author performed the statistical analysis for the functional assays, luciferase assay, and qRT-PCR data. J.J helped with analyzing results and assisting in writing the manuscript. D.S.A performed wound-healing assays and helped with analyzing results. P.JC. wrote the first draft of the manuscript together with L.S, which was revised based on the comments from all authors.

ABBREVIATIONS

Bp	base pair
BRD4	bromodomain-containing protein 4
BRG1	brahma-related gene-1
CDK	cyclin-dependent kinase
ChIP	chromatin immunoprecipitation
DBD	DNA-binding domain
ECM	extracellular matrix
EMT	epithelial-to-mesenchymal transition
ERK	extracellular signal-regulated kinase
eRNA	enhancer RNA
FGF	fibroblast growth factor
GTF	general transcription factor
HAT	histone acetyltransferase
HDAC	histone deacetylase
HIF	hypoxia-inducible factor
HR-A/B/C	heptad repeat A/B/C domain
HSE	heat shock element
HSF	heat shock factor
HSP	heat shock protein
HSR	heat shock response
MET	mesenchymal-to-epithelial transition
MMP	matrix metalloproteinases
mRNA	messenger RNA
ncRNA	non-coding RNA
NELF	negative elongation factor
PIC	pre-initiation complex
Pol I-III	RNA polymerase I-III

PTM	post-translational modification
RD	regulatory domain
ROS	reactive oxygen species
SUMO	small ubiquitin-like modifier protein
SWI/SNF	switch/sucrose-non-fermentable
TAD	transactivation domain
TAF	TBP-associated factor
TBP	TATA-binding protein
TF	transcription factor
TGF	transforming growth factor
TSS	transcription start site
WT	wild-type

INTRODUCTION

Elevated temperatures and oxidative reagents can detrimentally impact cellular macromolecules, including proteins. Luckily, cells have transcriptional programs that safeguard their integrity by inducing the production of proteins that aid in the refolding process of damaged proteins, thereby combating environmental stress conditions. For this, cells rely on both protein complexes that maintain basal transcription and specialized transcription factors that adjust the transcription rate, which includes the stress-responsive heat shock factors (HSFs). HSF1 and HSF2 are the most studied HSFs, and in addition to regulating the transcription of genes encoding proteins, they also modulate transcription at enhancers, which are short non-protein coding gene-regulatory elements in the DNA. The activation mechanisms of HSFs are complex and multifaceted. However, they require nuclear accumulation and trimerization to bind target genes. Additionally, PTMs, including phosphorylation, have been linked to promoting HSF1's activity, but some phosphorylation events can even suppress its transcriptional capacity. Importantly, structural similarities in HSF1 and HSF2 allow them to assemble heterotrimers. Hence, differences in trimer composition affect DNA-binding selectivity and transcriptional induction of genes. However, we lack an understanding of the cooperative nature of these HSFs in various environments.

Using animal models has significantly helped the scientific community assess the function of proteins. In the case of HSFs, it has been uncovered that the proper expression of these transcription factors is important in several processes related to development and health. In the latter example, research has proven that HSFs affect cancer development, as the irregular expression of HSFs impacts several hallmarks of cancer. While HSF1 displays pro-tumorigenic activities and is typically overexpressed in multiple types of cancer, the expression and function of HSF2 in cancer are less established. Importantly, prostate epithelial cancer cells have been demonstrated to downregulate HSF2 to promote invasion. Hence low levels of HSF2 may benefit cancer progression in an unknown manner.

This thesis covers three principal studies that characterize different aspects of HSF1 and HSF2. The first study investigates how the phosphorylation of HSF1 affects the parameters required for its activation. Also, it aims to address if HSF1's transcriptional capacity depends on stress-inducible phosphorylation within its regulatory domain. The second study explores the ability of both HSF1 and HSF2 to regulate transcription. This study uses various genome-wide sequencing techniques to determine if HSF1 and HSF2 operate independently or synergistically to regulate the expression of genes and enhancers during heat and oxidative stress. The third study explores the relevance of HSF2 in processes related to cancer development. Building upon previous data showing that cancer cells sometimes reduce the expression of HSF2 to promote invasion, the third study aims to uncover the pro-tumorigenic signaling pathway that influences the expression of HSF2. Furthermore, this study also aims to determine if changes in the expression of HSF2 impact gene expression and pro-malignant processes in cancer cells. Taken together, this thesis aims to address some of the gaps in our knowledge concerning the fundamental functions of HSF1 and HSF2 in stress regulation, and it also explores the nature of HSF2 in cancer-related processes.

REVIEW OF THE LITERATURE

1 Principles of transcription in eucaryotes

All organisms access genetic information from DNA via RNA molecules generated during transcription. Ribosomes can decode the data in so-called messenger RNA (mRNA) during the translation process to generate an amino acid chain, which is the core component of a protein. However, most RNAs are not translated; hence they are categorized as non-coding RNAs (ncRNAs), and some of these are processed into smaller products while others form large complexes (Panni et al., 2020). A subcategory of the ncRNAs that display an essential role in translation is transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs). Notably, certain ncRNA species, including microRNAs (miRNAs), long non-coding RNAs (lncRNAs), enhancer RNAs (eRNAs), and Xist, can promote or inhibit diverse biological processes (Chow et al., 2005; Panni et al., 2020; Han and Li, 2022). Taken together, the term transcription encompasses the synthesis of a diverse range of RNA species, which through their multifaceted interactions, participate in major processes in the cell.

1.1 Chromosomal DNA is packaged into chromatin

Eucaryotes have large DNA molecules condensed into chromosomes, which efficiently package the genetic material within the nucleus. The compact and complex structure of chromosomes results from DNA interactions with proteins that facilitate the formation of chromatin fibers. Nucleosomes form the basic structural units in chromosomes, and they consist of segments of DNA wrapped around four duplicates of the histone proteins H2A, H2B, H3, and H4 (Morrison and Thakur, 2021). Additionally, these histone octamers are connected to each other via the linker histone H1. The formation of nucleosomes across the DNA promotes additional wrapping of the DNA structure, which forms chromatin fiber that displays a varying degree of compactness (Morrison and Thakur, 2021). Lightly packaged chromatin is called euchromatin, and this structure permits various proteins to contact the DNA and initiate transcription. Tightly packaged chromatin is called heterochromatin, and this compact structure does not generally allow proteins access to DNA; hence heterochromatin is associated with a low rate of transcription (Morrison and Thakur, 2021).

1.2 Histone modifications and chromatin remodelers

The access of proteins to DNA is crucial during transcription, DNA repair, and replication (Morrison and Thakur, 2021). Hence, eucaryotic cells rely on proteins called chromatin remodelers to rearrange the chromatin structure, which can reduce the degree of compactness in chromatin. A reduction in the wrapping of the chromatin fibers generates euchromatin, which supports transcription. However, when transcription needs to be suppressed, some chromatin remodelers stimulate the formation of less transcriptionally active heterochromatin (Morrison and Thakur, 2021). Significantly, the recruitment of chromatin remodelers to chromatin is heavily influenced by PTMs on histones, which include acetylation, methylation, and SUMOylation.

There are families of chromatin remodelers, and each class of these enzymes utilizes ATP for their catalytical function (Flaus et al., 2006). An important chromatin remodeler is called the switch/sucrose-non-fermentable complex (SWI/SNF complex), and it is recruited to

acetylated lysine in histone H3 and H4 (Hassan et al., 2001). Histone acetylation is catalyzed by histone acetyltransferases (HATs), whereas deacetylation is carried out by histone deacetylases (HDACs) (Yang and Seto, 2007). Acetylation can partially neutralize the positive charge in lysine, which is believed to destabilize the nucleosome-DNA interaction. However, removing nucleosomes from protein-coding genes is mainly facilitated by the SWI/SNF complex once it has docked to the acetylated histones (Phelan et al., 2000).

A fundamental histone modification, histone methylation, can either facilitate or impede the access of chromatin remodelers to the DNA, which includes the nucleosome remodeling factor (NURF). NURF requires tri-methylation of histone H3 (H3K4me3) to form a more transcriptionally active chromatin conformation (H. Li et al., 2006). Importantly, in contrast to H3K4me3, transcriptional silencing is encouraged by the tri-methylation of histone H3 at lysine 9 (H3K9me3) (Kumar and Kono, 2020). This modification attracts heterochromatin protein 1 (HP1) to the DNA, inducing the reorganization of the chromatin into heterochromatin (Maison and Almouzni, 2004). Moreover, HP1 binds histones containing small ubiquitin-like modifier proteins (SUMOs) (Shiio and Eisenman, 2003). In addition to HP1, SUMOylation of histone H4 attracts HDAC1, promoting histone deacetylation and heterochromatin formation. Furthermore, SUMOylation of histones promotes transcriptional silencing by recruiting various non-histone proteins to the chromatin, negatively impacting the activity of RNA polymerase II (Ryu and Hochstrasser, 2021).

1.3 Transcription

The fundamental process of transcription, wherein genetic information is transcribed into different types of RNA molecules, is governed by evolutionarily conserved multi-subunit RNA polymerases. The eucaryotic RNA polymerases are numbered one to three (Pol I-III) and transcribe different DNA regions. Pol I only transcribes regions encoding ribosomal RNAs (Russell and Zomerdijk, 2006), whereas Pol II and Pol III transcribe regions encoding numerous ncRNAs, and Pol II is the only one that transcribes protein-coding genes (Khatter et al., 2017; Schier and Taatjes, 2020). Decades of biochemical studies have characterized the mechanisms by which the RNA polymerases operate, which briefly includes cycles in which they form different intermediates that execute the core phases of transcription: transcriptional initiation, elongation, and termination (Mooney and Landick, 1999). Notably, the process for transcription initiation is highly similar between the RNA polymerases, and only the mechanisms of Pol II will be described here.

1.3.1 Formation of the pre-initiation complex

Transcription is usually initiated at a defined position termed the transcription start site (TSS) within the promoter of a gene. The TSS is centered in a region called the core promoter, which consists of short segments of ~50 base pairs (bp) upstream and downstream of the TSS. The core promoter is surrounded by regions called proximal-promoters, and some genetic sequences in the core promoter operate as DNA motifs to which general transcription factors (GTFs) and Pol II can bind (Sainsbury et al., 2015) (Fig. 1). The formation of the pre-initiation complex (PIC), which is the first step in transcription, is initiated once the GTFs that include TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH bind to their recognized motifs in the core promoter (Roeder, 1996). Importantly, the formation of the PIC is often initiated when TFIID, composed of TATA-binding protein (TBP) and several TBP-associated factors

(TAFs), binds to the regions in the core promoter. Next, Pol II and other GTFs are recruited to the core promoter, which forms the PIC (Sainsbury et al., 2015). In cases when TBP is absent from the PIC, TAFs facilitate the formation of the PIC by binding to distinct motifs in the core promoter. These motifs include the initiator motif (Inr), downstream promoter element (DPE), and motif ten element (MTE) (Martinez et al., 1994; Smale and Kadonaga, 2003; Lim et al., 2004).

1.3.2 The elongation phase in transcription

Following the formation of the PIC, the multi-subunit complex TFIIH promotes DNA strand separation in the core promoter, and its kinase domain can phosphorylate a residue in the carboxy-terminal domain (CTD) of Pol II (Rimel and Taatjes, 2018). This phosphorylation event stimulates the activation of Pol II, and once it has transcribed 20-50 bp, it is subjected to a phenomenon called promoter-proximal pausing, which hinders further transcription (Strobl and Eick, 1992; Rasmussen and Lis, 1995; Mayer et al., 2017). This will, in turn, cause the accumulation of transcriptionally engaged Pol II in the proximal-promoter of numerous genes. It has been suggested that promoter-proximal pausing functions as a regulatory step for gene induction. For instance, stimuli such as heat shock, mitogens, and pro-inflammatory signals can promote the release of paused Pol II from numerous genes (Adelman et al., 2009; Mahat et al., 2016a), and by these means, it is possible that certain gene products can more rapidly be synthesized without delays originating from the time-intensive recruitment of Pol II to the core promoter.

Promoter-proximal pausing of Pol II is maintained by negative elongation factor (NELF) and DRB sensitivity-inducing factor (DSIF), and DSIF can function either as a negative or positive elongation factor (Qiu and Gilmour, 2017). The release of the paused Pol II is initiated when positive transcription elongation factor b (P-TEFb) is recruited to the promoter (Lis et al., 2000; Yang et al., 2005). P-TEFb is a multi-protein complex composed of cyclin-dependent kinase 9 (CDK9), cyclin T1, and bromodomain-containing protein 4 (Brd4) (Itzen et al., 2014; Zhou et al., 2022). The bromodomains in Brd4 facilitate the tethering of P-TEFb to acetylated lysines in histone H3 and histone H4, which are often found in euchromatin. The subunits CDK9 and cyclin T1 are essential for P-TEFb since they phosphorylate DSIF, NELF, and the CTD of Pol II (Dey et al., 2003). The phosphorylation of NELF disrupts its interaction with Pol II, while the phosphorylation of DSIF converts it into a positive elongation factor that stimulates the activity of Pol II (Fujinaga et al., 2004; Yamada et al., 2006). The phosphorylation of the second residue in the CTD by P-TEFb is also important for promoting Pol II activity (Marshall et al., 1996; Marshall and Price, 1995) (Fig. 1).

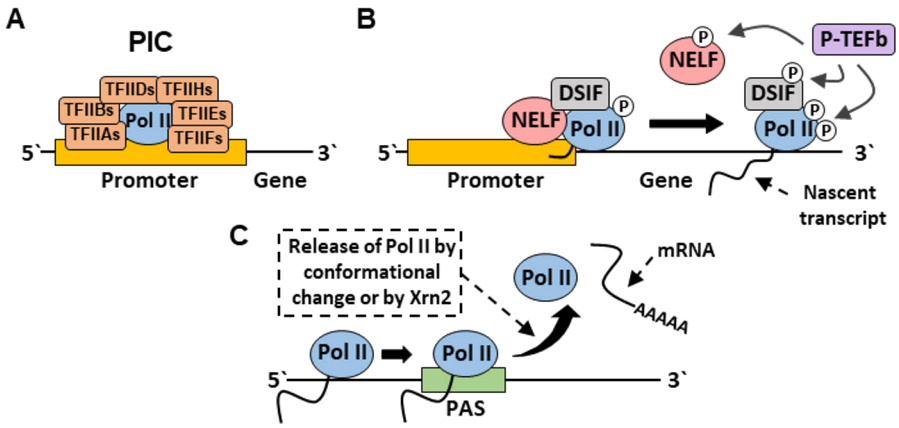

Figure 1. The main phases of Pol II-mediated gene transcription. (A) The initiation phase of transcription begins with forming the pre-initiation complex (PIC) at the promoter. The principal components that form the PIC include Pol II and the GTFs: TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH. (B) Transcriptionally engaged Pol II is paused at several genomic locations by NELF and DSIF. This promoter-proximal pausing is alleviated by P-TEFb, which phosphorylates NELF, DSIF, and the CTD of Pol II. These phosphorylation events promote the release of Pol II; hence it can proceed with transcription. (C) The termination phase of transcription is initiated once transcriptionally engaged Pol II transcribes the PAS-sequence in the 3`end of a protein-coding gene. The termination phase includes cleavage and polyadenylation of the mRNA transcript, as well as the release of Pol II from the DNA, which Xrn2 can partially facilitate. P = phosphorylation.

P-TEFb activation is primarily regulated by a complex called 7SK small nuclear ribonucleoprotein (7SK-snRNP) (Yang et al., 2001). During optimal growth conditions, when basal transcription rates are favored, 7SK-snRNP sequesters the majority of P-TEFb. However, when the cell is subjected to stress, such as DNA damage, the P-TEFb-7SK-snRNP complex becomes disrupted, which releases P-TEFb. Subsequently, an increase in the amount of active P-TEFb can stimulate the transcription of DNA damage-responsive genes (Studniarek et al., 2021; Zhou et al., 2022). In addition to DNA damage, protein-damaging stress, such as elevated temperature, can prompt the release and recruitment of P-TEFb to the chromatin. The recruitment of P-TEFb to the stress-responsive genes is facilitated by HSF1 (Lis et al., 2000). The involvement of P-TEFb in stress-dependent transcription is further discussed in section 2.3.

1.3.3 Termination of transcription

Termination is the final stage of transcription, and it facilitates the release of the newly synthesized nascent transcript from transcriptionally engaged Pol II. The key event that initiates transcription termination is when Pol II reaches a polyadenylation signal (PAS) at the end of the gene. This enables 3`-end processing, including cleavage and polyadenylation of the mRNA transcript (Proudfoot, 2011). Cleavage of the mRNA transcript requires polyadenylation specificity factor (CPSF), cleavage stimulation factor (CstF), and other cleavage factors (Takagaki et al., 1990; McCracken et al., 1997; Zhu et al., 2018; Boreikaite et al., 2022). Next, the enzyme called poly-A polymerase conjugates adenines to the 3`-end

of the cleaved RNA molecule, generating the so-called poly(A)tail, which stabilizes the molecular structure and improves translational efficiency (Edmonds and Abrams, 1960; Passmore and Coller, 2022)

For transcriptional termination to take place, Pol II must cut and release the mRNA transcript. Also, it needs to disengage itself from the DNA. Currently, two potential models describe how Pol II achieves termination. In the first model, the attachment of CPSF and CstF to the mRNA molecule causes a conformational change in Pol II, which reduces the enzyme's processivity and causes the release of the protein complex from DNA (Nag et al., 2007). The second model states that once the mRNA molecule is cleaved, Pol II continues to transcribe DNA downstream of the protein-coding sequences (Eaton and West, 2018). However, the lack of the protective 5`-Cap in the new transcript allows exonuclease Xrn2 to cleave the transcript, resulting in Pol II disconnecting from the DNA (West et al., 2004). Importantly, a recent study by Eaton and colleagues demonstrated that transcriptional termination likely relies on both mechanisms, wherein a cleavage factor, which is involved in the initial cleavage event, subsequently promotes the activation of Xrn2 (Eaton et al., 2020) (Fig. 1).

1.4 The function of enhancers in gene regulation

The eucaryotic genome contains a plethora of non-protein-coding sequences that are called enhancers, which can either promote or suppress transcription (Koch et al., 2011). The first discovery describing the function of eucaryotic enhancers revealed that these genomic elements mediate tissue-type specific regulation of the human immunoglobulin heavy chain gene locus (Banerji et al., 1983). Subsequential studies have revealed that regions encoding enhancers contain transcription factor binding sites, and numerous proteins, including transcription factors, co-regulators, Pol II, and chromatin architectural proteins bind to these regions (Kagey et al., 2010; Liu et al., 2014). This considerable assembly of proteins often displaces nucleosomes, a feature that has been taken advantage of experimentally to map the genomic distribution of enhancers (Dorschner et al., 2004). Importantly, the lack of nucleosomes at an enhancer facilitates intrachromosomal looping, wherein the core promoter of a neighboring gene is brought into close vicinity of the enhancer (Soutourina, 2018). Moreover, the cis-acting mechanism that an enhancer relies on to contact its target gene is not limited by the distance between them, nor does the presence of intersecting genes affect the target specificity (Kvon et al., 2014). The distance between an enhancer and its target gene can typically range from a few kilobases (kb) to tens of megabases (Mb), and substantial modifications in the chromatin landscape are required to facilitate the enhancer-promoter interaction.

The development of several genome-wide sequencing techniques has made it possible to identify enhancers (Heintzman et al., 2007; Vihervaara et al., 2017). Some of the common enhancer markers include the histone variants H3.3 and H2A.Z, as well as mono-methylation on histone H3 lysine 4 (H3K4me1) and acetylation of histone H3 lysine 27 (H3K27ac) (Calo and Wysocka, 2013). Furthermore, studies from the last decade have demonstrated that enhancers are integral elements of the transcriptional networks that regulate inflammation and proteotoxic stress, underscoring their far-reaching impact on gene regulation beyond development (Hah et al., 2015; Vihervaara et al., 2017).

1.4.1 The mediator complex

The formation of enhancer-mediated intrachromosomal loops often requires the mediator complex. This large multi-subunit protein complex, comprised of 30 subunits in humans, is considered to be essential for Pol II-mediated transcription (Soutourina, 2018). Enhancers influence transcription primarily by recruiting the mediator complex to the enhancer-gene interface with the assistance of pre-bound transcription factors at the enhancers (Fig. 2). Moreover, the formation of the enhancer-promoter contact also requires chromatin architectural proteins, including cohesin and the cohesin-loader protein called nipped-B-like protein (NIPBL) (Kagey et al., 2010). Nipbl has been found to co-localize with the mediator complex near enhancers, where it aids in the attachment of cohesin to DNA. During the binding process, cohesin forms a ring-shaped structure around the DNA, thereby creating a loop in the chromatin. Apart from the mediator complex and its associated factors, intrachromosomal looping is also regulated by the CCCTC-binding factor (CTCF) (Y. Li et al., 2020). CTCF achieves this in part by modulating the attachment of cohesin to the chromatin. Moreover, the function of CTCF in cohesin positioning across the chromatin helps to establish stable chromatin domains, which are essential for the 3D chromatin architecture (Lebeau et al., 2022).

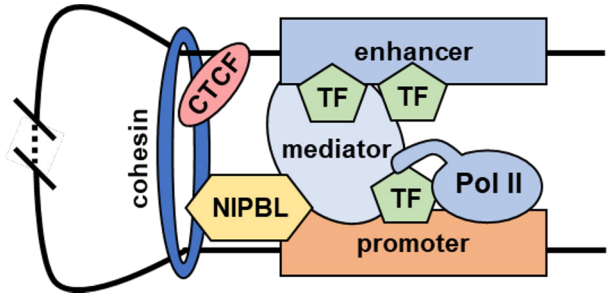

Figure 2. Formation of an intrachromosomal loop between an enhancer and a promoter. Interactions between enhancers and promoters are facilitated by loops in the chromosome, which require the mediator complex. The mediator complex interacts with Pol II and transcription factors, including those in the PIC. Intrachromosomal loops are stabilized by the structural protein cohesin, which forms a ring-like structure. Nipbl is required for the loading of cohesin to the chromatin, and CTCF aids in cohesin positioning across the chromatin.

After the enhancer-promoter connection is established, the mediator can, through intermediate transcriptional regulators, either stabilize PIC assembly or promote the release of Pol II from the proximal-promotor (Takahashi et al., 2011; Soutourina, 2018). The impact of the mediator complex in the PIC assembly is best characterized in yeast, where it cooperates with TFIID to bind chromatin, thereby enabling TFIID to initiate the assembly process (Johnson et al., 2002). Moreover, another important general transcription factor that the mediator can bind is TFIIH. This interaction is facilitated by the mediator complex subunit 11 (MED11), and when complete, TFIIH can phosphorylate the CTD in Pol II through the subunit known as cyclin-dependent kinase 7 (CDK7) (Esnault et al., 2008). This phosphorylation event activates the release of Pol II, thus facilitating further transcription. In addition to CDK7, the mediator has been shown to transiently interact with a mediator-

associated kinase called the CDK8 kinase module (CKM), which may influence transcriptional elongation through stimulating phosphorylation of the CTD in Pol II (Hengartner et al., 1998). Moreover, CKM can promote the phosphorylation of the CTD through its interplay with P-TEFb (Donner et al., 2010), a transcriptional regulator which was discussed in section 1.3.2. Importantly, the effect of CKM on gene expression can also be negative depending on cell type and environment (Galbraith et al., 2013; Chen et al., 2017)

1.4.2 Enhancer RNA

A genome-wide sequencing study has revealed that Pol II transcribes the regions that form enhancers, spanning 50 to 2000 base pairs (Kim et al., 2010). These transcripts are classified as non-coding RNA molecules, and they are referred to as enhancer RNAs (eRNAs) (Fig. 3). Transcription of eRNAs requires the same components used for transcribing mRNA; however, unlike mRNAs, eRNAs are not polyadenylated, and thus they are unstable (Han and Li, 2022). Interestingly, the transcription of eRNAs occurs from both strands in the DNA, yet, an analysis of single cells indicates that only one strand is transcribed at a time (Kouno et al., 2019).

Several studies have explored the implications of enhancer transcription on gene expression, and interestingly, the downregulation of certain eRNAs was found to influence the expression of protein-coding genes near the respective enhancer regions (Wang et al., 2011; Li et al., 2013; Melo et al., 2013). Still, evidence suggest that certain eRNAs may be non-functional and are likely byproducts of the general transcriptional processes. For instance, a study has demonstrated that the mutation of the locus of an enhancer, which maintains the expression of the gene encoding cyclin-dependent kinase inhibitor 1B, caused severe disruption to eRNA synthesis, while the expression of the gene remained unchanged (Paralkar et al., 2016). Still, numerous studies have identified mechanisms through which eRNAs can modulate the transcription of protein-coding genes (Li et al., 2013; Tsai et al., 2018).

The underlying mechanisms that enable eRNAs to influence transcription depend on the mediator complex and chromatin remodelers. In the former mechanism, eRNAs associate with the mediator complex at the enhancer-promoter interface, stabilizing and reinforcing the intrachromosomal loop (Lai et al., 2013; Li et al., 2013). The latter mechanism is more complex, as it involves eRNAs regulating transcription through the interaction of transcriptional co-activators and chromatin remodelers. For instance, eRNAs can promote the acetylation of histones, thus increasing the accessibility of the transcriptional machinery to the chromatin. For this, eRNAs bind to a specific region of the histone acetyltransferase known as the CREB Binding Protein (CBP) (Bose et al., 2017). This interaction enhances the capabilities of CBP to acetylate histone H3, which then draws transcriptional regulators to the enhancer-promoter interface, promoting the formation of the PIC and release of Pol II from the proximal-promoter (Fig.3). Additionally, eRNAs can increase the transcription of genes by directly engaging with transcriptional regulators that govern the release of paused Pol II. This is primarily accomplished by eRNAs binding to and sequestering NELF from promoters, and secondarily eRNAs promote the recruitment of P-TEFb to the vicinity of promoters, thereby increasing the phosphorylation of the CTD in Pol II (Schaukowitch et al., 2014; Zhao et al., 2016) (Fig. 3).

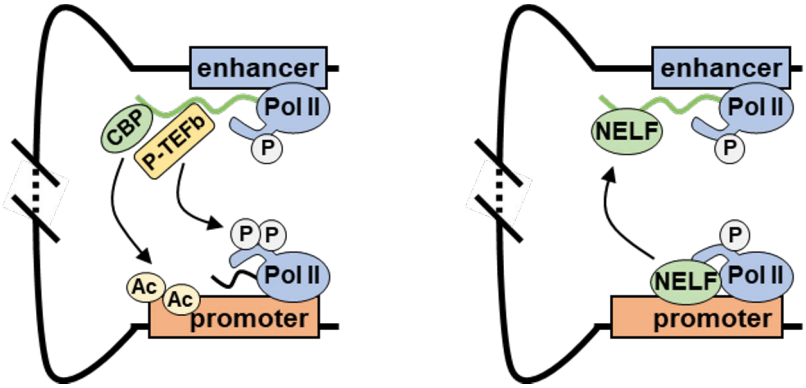

Figure 3. eRNA-mediated regulation of transcription. (A) Pol II transcribes eRNA that attracts CBP and P-TEFb, consequently promoting the acetylation of histones and phosphorylation of the CTD in Pol II. Histone acetylation forms a transcriptionally active chromatin conformation, and the phosphorylation of the CTD promotes the release of paused Pol II. (B) Pol II transcribes eRNA that functions as a decoy, to which NELF binds. Dissociation of NELF from Pol II promotes the release of paused Pol II from the promoter, thus, allowing transcription to proceed. Abbreviations: Ac = acetylation, P = phosphorylation.

2 Cellular stress responses

Life begins with a single cell, which forms the foundation for more complicated multicellular organisms like humans and plants. Apart from the DNA, which encodes all genetic information, cells require several organelles, such as the mitochondria, Golgi apparatus, lysosomes, chloroplasts, and endoplasmic reticulum, to sustain life. Each organelle performs its own distinct set of tasks which require specialized proteins to function correctly. Still, the environment is ever-changing, and cellular homeostasis is often disrupted by protein-damaging stresses such as elevated temperatures, viruses, and harmful compounds. To survive in a stressful environment, cells have developed distinct stress-responsive mechanisms that allow them to cope with and process damaged macromolecules (Fig. 4). It is important to note that while the stress responses enable cells to combat stress, their pro-survival function can be subverted in pathophysiological conditions. For instance, neurodegenerative conditions can disrupt some of the the mechanisms that drive stress responses, whereas diseases such as cancer can hijack the stress-combating mechanisms to ensure survival and disease propagation (Gomez-Pastor et al., 2018; Hughes and Mallucci, 2019; Puustinen and Sistonen, 2020; Brandes and Gray, 2020).

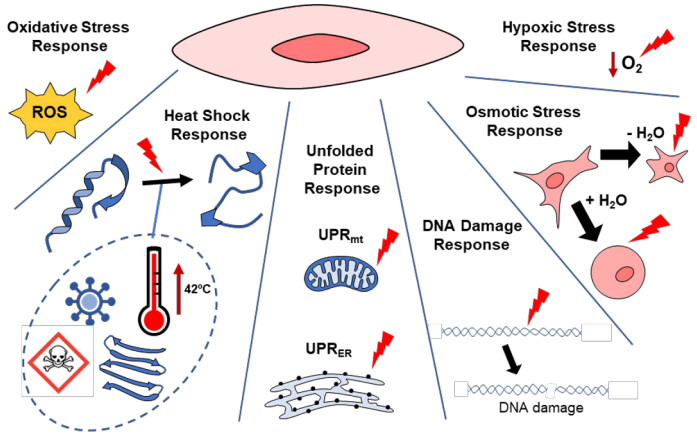

Figure 4. Cellular stress responses. The cell combats various forms of cellular stress by activating specific stress responses. Exposure to reactive oxygen species (ROS) can trigger the oxidative stress response, which induces the expression of proteins that processes oxidized macromolecules and restores the redox state in the cell. The heat shock response is activated upon protein-damaging stress, including elevated temperatures, toxins, protein aggregates, and viruses. This response induces the production of proteins that help other proteins maintain their structure and function. High rates of protein synthesis can disrupt protein folding in the endoplasmic reticulum (ER) or the mitochondria (mt), resulting in the activation of the unfolded protein response (UPR) in the respective organelles. During the UPR, the expression of proteins that aid in protein folding processes increases, whereas the general synthesis rate of other proteins is reduced. Exposure to radiation or reactive chemicals can cause DNA damage. To repair modified nucleotides or breaks in the DNA strands, the cell depends on the DNA damage response, which encompasses various mechanisms that can repair the damaged DNA. The osmotic stress response is triggered when water (H2O) influx or efflux to the cell is abnormal. This response induces the production of water-transport channels and various compounds that can rebalance the osmolarity in the cell. During oxygen deprivation (hypoxia), cells activate the hypoxic stress response to express angiogenic factors that, over time, can restore oxygen levels to their normal levels.

2.1 The oxidative stress response

Reactive oxygen species (ROS) are produced as a natural by-product of the normal metabolism of oxygen during energy conversion in the mitochondria. These reactive compounds include superoxide, which is further processed into other reactive oxygen species (ROS), such as hydrogen peroxide and hydroxyl molecules (Sies et al., 2017). ROS are highly reactive molecules that can cause oxidative stress by damaging macromolecules such as DNA, proteins, and lipids. Oxidative damage to DNA is repaired through the processes of the DNA damage response, particularly the base excision repair pathway (Shafirovich and Geacintov, 2017). In addition to endogenous sources, exogenous sources of oxidative stress can include drugs, pathogens, and heavy metals (Sies et al., 2017). Fortunately, high levels of reactive oxygen species (ROS) activate the oxidative stress response, a process that promotes the synthesis of protective enzymes to neutralize ROS and aid in balancing the proteome and the redox state of the cell.

The oxidative stress response is mainly regulated by a transcription factor called nuclear factor E2-related factor 2 (NRF2). During non-stress conditions, NRF2 is sequestered to the actin or myosin cytoskeleton by Kelch-like ECH-associated protein 1 (Keap1) (Kang et al., 2004). Importantly, Keap1 binds the ubiquitin ligase complex Cullin 3 (Cul3), which ubiquitinates NRF2 and directs it to proteasomal degradation (Kobayashi et al., 2004). Oxidative stress induces the oxidation of cysteine residues in Keap1, resulting in structural modifications that release NRF2 from the NRF2-Keap1-Cul3 complex. Following this, the liberated NRF2 migrates to the nucleus and binds to transcriptional regulators, forming a complex that binds to the promoters of NRF2's target genes, including antioxidant enzymes, thus regulating their transcription (Venugopal and Jaiswal, 1996).

2.2 The heat shock response

Most organisms live and survive within a certain temperature range that does not substantially shift above and below the optimum growth temperature. Hence, temperature is one of the biggest barriers to life, and it was not before the early '60s that two distinct studies uncovered some fundamental aspect of how heat affects organisms. According to Roger Milkman's research, when exposed to heat shock, *Drosophila* pupae cultivated in a slightly elevated temperature displayed a higher survival rate and fewer physiological defects than the control group (Milkman, 1962). Milkman's research also indicated that the temperature adaptation resulted from elevated expression of a protein speculated to be involved in protein denaturation. Later in the same year, Ferruccio Ritossa reported that heat shock induces a unique puffing pattern of the polytene chromosomes in *Drosophila buscki* larvae, which indicates loci-specific transcriptional activation (Ritossa, 1962). This response was termed the heat shock response (HSR), and it took more than a decade to demonstrate that the puffing pattern corresponded to the induction of proteins known today as heat shock proteins (HSPs) (Tissières et al., 1974), which is further discussed in section 2.5. The HSR is conserved across organisms, and numerous forms of protein-damaging stress, including heavy metals, toxins, and oxidating agents, can activate the response (Saydam et al., 2003; Yamamoto et al., 2007)

Further characterization of the heat-responsive genomic regions uncovered similarities in certain sections in the promoter sequences for the *Hsps*. These motifs are regulatory elements called heat shock elements (HSEs), which consist of pentameric nGAAn repeats (Amin et

al., 1988). After identifying the HSEs, research teams conducted *in vitro* purification experiments to isolate and identify the transcription factor suspected of interacting with the HSEs in *Drosophila melanogaster* and *Saccharomyces cerevisiae* (Sorger and Pelham, 1987; Wiederrecht et al., 1987; Wu et al., 1987). The binding protein for the HSEs was termed heat shock factor 1 (HSF1), and subsequent research has uncovered several other HSFs in humans, which will be discussed in section 2.6.

HSF1 is the main transcription factor in the HSF family that modulates the expression of *Hsps* during protein-damaging conditions. Therefore, numerous studies have focused on characterizing the non-exclusive mechanisms by which HSF1 is activated upon exposure to acute stress. Per the classical model termed the chaperone titration model, at optimal growth conditions, HSP70, HSP90, and the TCP1 ring complex (TRiC) sequester monomeric HSF1 (Neef et al., 2014; Le Breton and Mayer, 2016) (Fig. 5B). When protein-damaging stress is severe and unfolded proteins start to accumulate, chaperone operations are shifted to process the damaged proteins, which releases and activates HSF1 (Neef et al., 2014). Once activated, HSF1 forms trimers and is subjected to various PTMs, which will be discussed in section 2.6.2. Next, the trimers are translocated to the nucleus and attach to the promoters of the genes encoding HSPs, subsequently inducing their transcription (Sarge et al., 1993). Once the levels of the HSPs are high, proteins such as HSP70 will promote the attenuation of the HSR (Abravaya et al., 1992). During this process, the trimers are released from the DNA, monomerized, and degraded via the proteasome (Raychaudhuri et al., 2014; Kmiecik et al., 2020; Zhang et al., 2022). Importantly, evidence to support the chaperone titration model includes the findings that pharmacological inhibition or knockdown of HSP70, HSP90, or TRiC leads to the activation of the HSR (Powers and Workman, 2007; Neef et al., 2014; Pesonen et al., 2021).

After HSEs were identified, a study conducted by Larson and colleagues reported that human HSF1 in cytoplasmic cell extracts of non-stressed cells had the capacity to bind to DNA *in vitro* when the extract was heated (Larson et al., 1988). This result indicated that HSF1 could autonomously detect heat and trigger its activation. Subsequently, *in vitro* studies have demonstrated that purified HSF1 monomers, subjected to protein-damaging conditions like heat and oxidative agents, can form trimers and bind to DNA (Goodson and Sarge, 1995; Ahn and Thiele, 2003). It is important to note that the structural analysis of HFS1 monomers has revealed that its thermosensory ability is partly attributed to the heat-induced unfolding of two regions in the protein, which results in the formation of trimers (Hentze et al., 2016). Taken together, these studies indicate that 'intrinsic stress responsiveness' is one feature driving HSF1's activation (Fig. 5A). Another important parameter for trimer formation *in vitro* is the quantity of purified HSF1 monomers. Therefore, high concentrations of monomers can spontaneously trimerize, and intrinsically activated HSF1 trimers cannot regress to the monomeric state without the aid of chaperones like HSP70 and heat shock cognate 71 kDa protein (HSC70) (Hentze et al., 2016; Kmiecik et al., 2020). The concentration-dependent activation of HSF1 also appears *in vivo*. For instance, overexpressing HSF1 in human cells can induce the HSR, and cancer cells with elevated levels of HSF1 also exhibit an active HSR (Sarge et al., 1993; Björk et al., 2018).

The third documented mechanism underlying HSR regulation is specific to *C. elegans*, and it is based on 'neuronal control' (Fig. 5C). These multicellular organisms display unique AFD

thermosensory neurons that monitor temperature and react to heat by inducing the HSR in distally located tissue (Prahlad et al., 2008). This signaling transduction requires AFD serotogenic neurons to release serotonin when activated by the thermosensory neurons, resulting in the induction of the HSR in distal cells (Tatum et al., 2015). Intriguingly, Clark and colleagues discovered that thermosensory neurons assist roundworms in locating the optimal temperature needed to support growth and reproduction, thus enabling them to avert environmental stress or cope with it (Clark et al., 2007). Moreover, *C. elegans* can induce the HSR in response to heavy metal exposure, a type of stress that thermosensory neurons cannot recognize, suggesting that the worm utilizes mechanisms other than neuronal control to regulate the activity of the HSR (Prahlad et al., 2008).

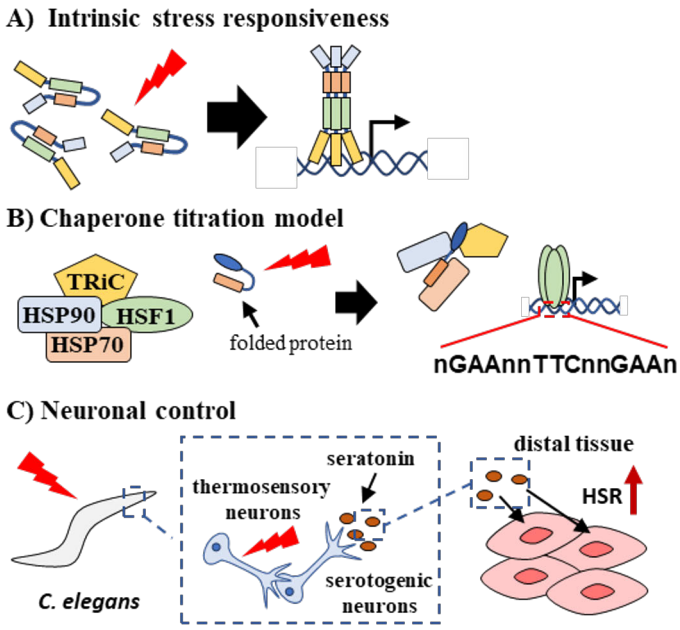

Figure 5. Mechanisms driving the activation of the HSR. (A) Protein-damaging stress causes conformational changes in purified HSF1 that promote its activation, which include trimerization and gain of DNA-binding capacity. This model of activation is termed "intrinsic stress responsiveness". (B) According to the "chaperone titration model", in non-stress environments, HSF1 is sequestered by HSP70, HSP90, and TRiC. However, during protein-damaging stress, proteins are unfolded, which shifts the operation of the HSPs and TRiC to stabilize the damaged proteins, thus causing the release and activation of the HSFs. The activated monomeric HSFs form homo/heterotrimers that bind to HSE sequences (GAAnnTTCnnGAA) located in the promoter region of stress-responsive genes, thereby stimulating transcription. (C) The HSR can be activated in *C. elegans* via "neuronal control". This mechanism relies on thermosensitive neurons that react to heat, and via seratogenic neurons, they indirectly promote the release of serotonin, which subsequentially activates the HSR in distal cells.

2.3 Activation mechanisms of Pol II during the HSR

The protein complex responsible for transcribing DNA into mRNA is RNA polymerase II (Pol II), and its transcription rate is strictly controlled. These rate-limiting controls can trigger

the accumulation of Pol II near the promoter regions of stress-responsive genes, which is thought to hasten their induction during stress. Key steps affecting Pol II activation include the removal of nucleosomes from a gene and releasing Pol II from the proximal-promotor, which was discussed in section 1.3.2.

The gene encoding HSP70 has been used in numerous studies as a model for investigating the activation mechanisms of the HSR, which depends on the release of Pol II from the paused state at the genes encoding HSPs (O'Brien and Lis, 1991; Mahat et al., 2016a). Characteristically the *Hsp70* gene is kept nucleosome-free under control conditions by the actions of HSF1 and replication protein A (RPA) (Fujimoto et al., 2012). The interplay between RPA and HSF1 attracts the chromatin remodeler BRG1 and the histone chaperone FACT, which promotes nucleosome removal and preloading of Pol II to the gene (Fujimoto et al., 2012). Hence, these modifications to the chromatin enable the transcriptional machinery to access the promoter and other regulatory regions of the gene. Importantly, during stress, the distribution of nucleosomes across the chromatin is regulated by poly (ADP-ribose) polymerase 1 (PARP1) in an HSF1-dependent manner. For instance, in *Drosophila melanogaster*, nucleosome depletion in the *Hsp70* gene loci is facilitated by PARP1 during stress, whereas the mammalian PARP1 requires the scaffold protein PARP13 to mediate this process (Fujimoto et al., 2012; Petesch and Lis, 2012).

To induce the expression of *Hsp70*, the removal of nucleosomes must be accompanied by the activation of several transcriptional regulators. During the HSR, HSF1 recruits several regulatory factors to the *Hsp70* promoter, including SWI/SNF, P-TEFb, Shugoshin 2 (SGO2), and activating transcription factor 1 (ATF1). SWI/SNF promotes Pol II's activity by facilitating the remodeling of the chromatin, whereas SGO2 and P-TEFb promote the recruitment and release of Pol II at the gene, respectively (Takii et al., 2019; Lis et al., 2000; Sullivan et al., 2001). HSF1-mediated recruitment of ATF1 to the *Hsp70* promoter enables the binding of the co-activator complexes BRG1 and p300/CBP (Takii et al., 2015). The ATF1-BRG1 complex can establish an active chromatin state during heat shock, whereas the ATF1-p300/CBP complex promotes attenuation by decreasing HSF1's DNA-binding activity during the recovery phase from stress. Adding to this, many other co-activators of HSF1 have been implicated in modulating HSF1 activity during various stress conditions; however, it is unclear to what extent these factors affect either the status of Pol II or the chromatin landscape (Hong et al., 2004; Chen et al., 2014; Srivastava et al., 2021)

2.4 Transcriptional repression during the HSR

Transcriptional repression is an important feature during stress conditions that allow the downregulation of genes involved in metabolism and the cell cycle. This enables cells to prioritize the synthesis of pro-survival factors, such as chaperones, while non-essential cellular functions are transiently shut down. To date, four principal mechanisms contribute to heat stress-induced transcriptional repression. The first mechanism relies on the chromatin remodeler NELF, which is recruited to promoters of repressed genes via the action of mitogen-activated protein kinase 14 (p38α) and ubiquitinated nascent proteins (Aprile-Garcia et al., 2019). Once NELF is bound to the chromatin, it will repress Pol II, thus resulting in paused Pol II at the genes. The second mechanism for transcriptional repression is based on heat shock inducible non-coding Alu RNA molecules (Mariner et al., 2008). High levels of Alu negatively impact Pol II by hindering its binding to the promoter regions of genes. The

third mechanism relies on heat-inducible SUMOylation of chromatin. This global SUMOylation is promiscuously distributed, and it is unknown how this causes gene silencing (Niskanen et al., 2015). However, a general depletion of the SUMO-protein reservoir might contribute to the repression mechanism (Niskanen et al., 2015). Additionally, it has been postulated that global SUMOylation promotes Pol II pausing via Pol II pausing factors and the general transcription factors (Niskanen and Palvimo, 2017). The final mechanism shown to promote transcriptional repression is heat-inducible chromatin rearrangement of topologically associating domains (TADs) (Lyu et al., 2018). TADs are interacting genomic regions, manifesting as chromatin loops that facilitate enhancer-promoter interaction (Kagey et al., 2010). During heat shock, TADs are reorganized in a fashion that disrupts the enhancer-promoter interaction, thus resulting in the downregulation of the gene (Lyu et al., 2018).

2.5 Heat shock proteins

Heat shock proteins (HSPs) are the primary products of the HSR, and their ability to support the refolding of misfolded proteins provides a pivotal pro-survival function to cells. Additionally, during normal physiological conditions, HSPs stabilize many cellular protein structures, including receptors and cytoskeletal structures (Liang and MacRae, 1997; Streicher, 2019). The HSP families are classified based on their molecular weights, and each family contains many members (Hu et al., 2022). Major HSPs involved in stress regulation include HSP90, HSP70, HSP40, and HSP27 (Hu et al., 2022)

The small HSPs, including HSP27, possess chaperone-like activity and bind partially denatured proteins to secure them from further denaturation (Sun and MacRae, 2005). Importantly, small HSPs do not use ATP in their binding activity and cannot refold damaged proteins, which is a task that requires ATP-dependent chaperones such as HSP70 and HSP90 (Haslbeck et al., 2019). To refold damaged proteins back to their native state, HSP70 cooperates with HSP90 in a process termed the HSP70-HSP90 chaperone cascade (Morán Luengo et al., 2019). In this mechanism, HSP70 does the initial folding of the early- and core-segments of the protein, whereas HSP90 binds late-folding intermediates, facilitating the release of the processed protein (Karagöz et al., 2014; Morán Luengo et al., 2018). However, to refold proteins, HSP70 requires the assistance of the co-chaperone HSP40 and nucleotide exchange factors (NEFs) (Bracher and Verghese, 2015; Li et al., 2009). HSP40's interaction with HSP70 stimulates ATP hydrolysis by HSP70, whereas NEFs facilitate the replacement of ADP with ATP in HSP70. Additionally, several co-chaperones modulate the activities of HSP90, and some function as adaptor proteins for recruiting client proteins (Schopf et al., 2017). An important co-chaperone that impacts both HSP70 and HSP90 is the Hsp70-Hsp90 organizing protein (Hop). Hop functions as an adaptor that connects HSP70 and HSP90 and thereby helps in the transfer of proteins that are being folded from HSP70 to HSP90 (Schopf et al., 2017; Lott et al., 2020).

Apart from stress, HSPs have key tasks in directing protein folding in a wide range of cellular housekeeping processes, including folding newly synthesized polypeptides and protein-complex assemblies (Beckmann et al., 1990; Streicher, 2019). For this reason, cells already express high levels of HSPs at normal physiological conditions; however, atypical expression of HSPs is associated with many diseases, such as cancer. Cancer cells typically overexpress HSPs, and these proteins often attenuate apoptosis by inhibiting pro-apoptotic proteins and stabilizing receptors and kinases, which are involved in signaling pathways that facilitate

cancer cell survival (Garrido et al., 2001; Makhnevych and Houry, 2012; Hu et al., 2022). For instance, HSP90 is essential for the maturation, activation, and stability of numerous client proteins that are involved in facilitating tumorigenesis, including mutant-type p53, ERBB2 (HER2), Bcr-Abl, and c-Raf (Nagata et al., 1999; Chiosis et al., 2001; Nimmanapalli et al., 2001).

2.6 The HSF family

Following the discovery of the HSR, several research groups focused on identifying the transcription factors involved in driving the response. HSF1 was the first and only HSF family member discovered in *Drosophila melanogaster*, and it was later found in yeast (Parker and Topol, 1984; Sorger and Pelham, 1987). However, these HSF1 variants are the only ones expressed in invertebrates or yeast. Interestingly, the human HSF family members, HSF1 and HSF2, were discovered by two research groups within the same year (Rabindran et al., 1991; Schuetz et al., 1991). Later, HSF3, HSF4, HSF5, HSFX, and HSFY were identified, of which HSFX and HSFY are sex chromosome-specific HSFs (Nakai et al., 1997; Fujimoto et al., 2010; Gomez-Pastor et al., 2018). Importantly, each HSF encoding gene occupies a different chromosomal locus, yet they are not very homologous, except in some conserved regions.

2.6.1 The molecular structure of HSFs

The fundamental structure of all HSFs is the evolutionarily conserved N-terminal DNA-binding domain (DBD) (Fig. 6). HSFs rely on the DBDs to attach to the binding sites known as heat shock elements (HSEs), which requires HSEs to possess three or more nGAAn sequence inverted repeats (Amin et al., 1988). The DBD has a winged helix-turn-helix motif, through which it recognizes and binds HSEs. Importantly, recently published X-ray crystal structures indicate that the “wing-loop”, termed the “wing domain” within the DBD, mediates important protein-protein interactions between the DBDs in HSF1 and HSF2 hetero/homo-trimers (Feng et al., 2021). Therefore, it is considered that the wing domain provides a synergistic effect amongst DBDs, which improves trimer-binding to the HSE. Adjacent to the DBD is the conserved region called the oligomerization domain, or the HR-A/B domain. This domain contains hydrophobic heptad repeats, which enables HSFs to form either hetero- or homotrimers upon activation (Peteranderl et al., 1999; Feng et al., 2021). Some HSFs contain an HR-C domain, which functions as a repressor motif that inhibits the spontaneous trimerization of HSFs under control conditions (Hentze et al., 2016). The HR-C domain interacts with the HR-A/B domain to achieve repression (Hentze et al., 2016), and since human HSF4 is missing an HR-C domain, it continuously forms active trimers (Nakai et al., 1997). The transactivation domain (TAD), located in the C-terminus of most mammalian HSFs, enables HSFs to modulate the transcription of target genes (Nieto-Sotelo et al., 1990). This domain is rich in hydrophobic and acidic amino acid residues, which generate binding sites for transcription co-regulators, such as proteins in the basal transcription complex (Park et al., 2001; Sullivan et al., 2001). An important domain in HSF1 is called the regulatory domain (RD), situated in the middle of the protein (Green et al., 1995; Newton et al., 1996). Using reporter assays to analyze the activity of HSF1 fusion proteins with different segments of HSF1, Green and colleagues demonstrated that the RD could suppress HSF1 activation at regular growth temperatures (Green et al., 1995). Importantly, this study also uncovered that the RD facilitates stress-induced activation during heat stress. Further characterization of the

regulatory domain revealed that it alone could induce heat stress-mediated activation in a chimeric construct containing VP16's viral transactivation domain, emphasizing the importance of this domain for stress responsiveness (Newton et al., 1996). Interestingly, some segments of the RD are also located in HSF2 and HSF4, but they remain poorly characterized.

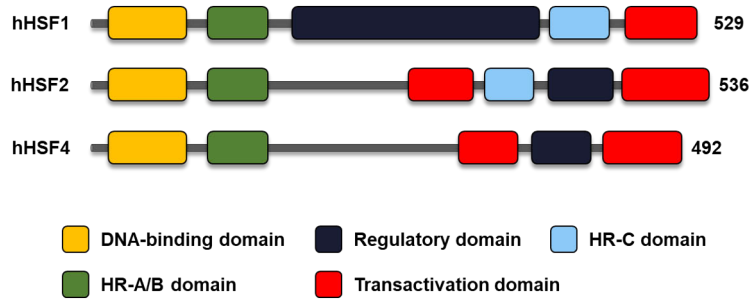

Figure 6. The functional domains of human HSFs. Schematic illustration of the functional domains in human HSFs (hHSF1), (hHSF2), and (hHSF4). The denoted number indicates the total amount of amino acids per HSF. Note: The figure is not drawn to scale.

2.6.2 Post-translational modifications of HSF1 and HSF2

In various stressful environments, HSFs are subject to post-translational modifications (PTMs) that affect their ability to regulate transcription. The foremost characterized PTMs in mammalian HSFs include phosphorylation, acetylation, SUMOylation, and ubiquitination (Anckar and Sistonen, 2011) (Fig. 7). While mass spectrometry has been widely used to identify many PTMs in HSFs, most detection methods still require the overexpression of essential proteins, such as kinases and acetyltransferases, both *in vivo* and *in vitro*. For instance, heat-induced HSF1 SUMOylation was initially identified by overexpressing exogenous SUMO-1 and SUMO-2 in cells, subjecting them to heat stress, followed by immunoprecipitation of HSF1, and protein analysis by western blotting (Hietakangas et al., 2003). Additionally, since SUMOylation requires specific consensus motifs (Rodriguez et al., 2001), the SUMOylation of a few HSF1 mutants was evaluated to determine which lysine had been modified (Hietakangas et al., 2003). The impact of phosphorylation of HSF1's activities has also been assessed using various HSF1 mutants (Knauf et al., 1996; Holmberg et al., 2001; Hietakangas et al., 2006). Furthermore, numerous phosphorylation sites in HSF1 were uncovered using methods such as phosphopeptide mapping and mass spectrometry (Chu et al., 1996; Kline and Morimoto, 1997; Guettouche et al., 2005). Moreover, *in vitro* kinase assays have been used to promote phosphorylation of HSF1 before mass spectrometric analysis, thereby enabling the identification of the kinases responsible for phosphorylation in some cases (Chou et al., 2012; W. Lu et al., 2022).

Since the phosphorylation of HSF1 has been associated with the activation of the HSR (Kline and Morimoto, 1997), it has prompted many research teams to investigate the connection between this post-translational modification and HSF1 function (Budzynski and Sistonen, 2017). Several investigations have uncovered that HSF1 is heavily phosphorylated under acute stress conditions, and, to date, 24 phosphorylation sites have been reported in human

HSF1, whereas two phosphorylation sites have been found in HSF4 (Guettouche et al., 2005; Hietakangas et al., 2006; J. Zhang et al., 2014; W. Lu et al., 2022). While it remains to be established how each phosphorylation event mechanistically affects HSF1, it is known that the conjugation of a phosphate group to an amino acid generates a local negative charge in the protein, and, in some instances, this can trigger a conformational change or generate a docking site for interacting proteins (Ubersax and Ferrell, 2007).

Due to the number of phosphorylation sites in HSF1, with the majority being present in the RD, studies have mainly been directed at characterizing how the phosphorylation of individual sites impacts HSF1. Remarkably, phosphorylation has, in several instances, been shown to either positively or negatively impact HSF1's transcriptional capacity (Anckar and Sistonen, 2011). For instance, the stress-induced phosphorylation of Ser230 by CaMKII increases the induction of the HSR, and the phosphorylation of Ser320 by PKA facilitates the localization of HSF1 to the nucleus, thus promoting its ability to regulate transcription (Holmberg et al., 2001). Additionally, one of the most studied phosphorylation sites linked to HSF1 activation is Ser326, which numerous kinases, including the kinases p38, MEK, Akt, and mTOR, can phosphorylate (Chou et al., 2012; Tang et al., 2015; Dayalan et al., 2016; W. Lu et al., 2022). The exact mechanism of how phosphorylation of Ser326 impacts HSF1 is still unknown, but a recent study suggests that phosphorylation of this site enhances its transcriptional capacity by allowing TFIIB and CDK9, general transcription factor components, to bind to the promoter of certain stress-responsive genes (W. Lu et al., 2022). These transcriptional regulators were discussed in sections 1.3.2.

Phosphorylation can not only enhance but also limit the transcriptional capacity of HSF1. For example, AMPK-mediated phosphorylation of Ser121 reduces the DNA-binding ability of HSF1, thus limiting the induction of HSR (Dai et al., 2015). Similarly, phosphorylation of Ser303 and Ser307 by the kinases GSK-3β and ERK1/2 suppresses HSF1 activity, partly by inducing the nuclear export of HSF1 (Wang et al., 2003). Additionally, once Ser303 and Ser307 are phosphorylated, it enables FBXW7 ubiquitin ligase to attach to and ubiquitinate HSF1, which leads to the degradation of HSF1 (Kourtis et al., 2015). It is important to note that in cancer cells, the deletion of the loci encoding FBXW7 is correlated with an elevated expression of HSF1, suggesting that FBXW7 is a significant regulator of HSF1's expression (Kourtis et al., 2015). Interestingly, a recent study demonstrated, in a knockout mouse model, that the permanent removal of Ser303 and Ser307 increased HSF1's stability, nuclear accumulation, and transcriptional capacity during optimal growth conditions and exposure to stress (Jin et al., 2018b). This study also highlighted that when phosphorylation is absent at Ser303 and Ser307, it significantly reduces the activation threshold for HSF1, thus leading to its activation under mild stress conditions.

The stress-induced phosphorylation of Ser303 in HSF1 is also a prerequisite for the SUMOylation at Lys298 (Hietakangas et al., 2006). It is important to note that this SUMOylation event is predicated on a specific consensus motif in HSF1 termed the phosphorylation-dependent SUMOylation motif (PDSM), which includes Ser303 but not Ser307 (Hietakangas et al., 2006). The SUMOylation of Lys298 reduced the transcriptional capacity of HSF1 upon heat shock (Hietakangas et al., 2003), and it has been demonstrated *in vitro* that this modification does not affect its DNA-binding affinity or the trimer to monomer transition (Kmiecik et al., 2021). Although Lys298 is the only confirmed

SUMOylation site, evidence suggests that other SUMOylation sites may be present in the N-terminal region of HSF1 (Kmiecik et al., 2021). In contrast to HSF1, the SUMOylation of Lys82 and Lys139 in HSF2 has been demonstrated to alter its DNA-binding capacity (Anckar et al., 2006). Lys82 is located within the wing domain of the DBD in HSF2, and SUMOylation of this site can negatively affect its DNA binding activities both *in vitro* and *in silico* (Anckar et al., 2006; Tateishi et al., 2009; Feng et al., 2016). However, one study has also reported that SUMO-1 conjugation to Lys82 can increase the DNA-binding affinity *in vitro* (Goodson et al., 2001).

Another important PTM that affects the function of HSF1 is acetylation, a modification identified in HSF1 using mass spectrometry (Westerheide et al., 2009). This PTM can affect the stability and DNA-binding activity of HSF1. Notably, the acetylation of Lys80 and Lys118, which are situated in the DBD, have been shown to inhibit the DNA-binding activity of HSF1 (Westerheide et al., 2009; Raychaudhuri et al., 2014). Importantly, it is assumed that the acetylation of these residues aid in attenuation during the HSR. Acetylation of Lys208 and Lys298, located in the HR-A/B and RD regions, respectively, enhances the stability of HSF1 (Raychaudhuri et al., 2014). It is important to note that Lys298 can be SUMOylated or acetylated. Therefore, it is likely that acetylation may impede SUMOylation at this site, which would enhance HSF1's transcriptional activity since SUMOylation has been reported to have an inhibitory effect on HSF1's activities (Hietakangas et al., 2006)

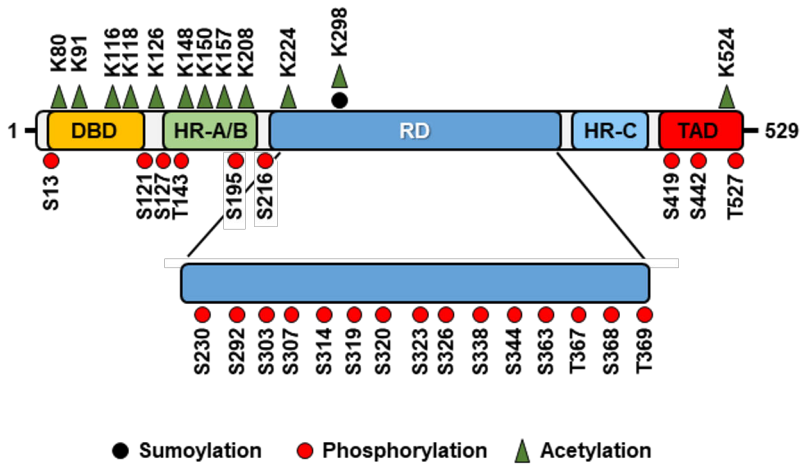

Figure 7. The post-translational modifications of human HSF1. Human HSF1 is phosphorylated at 24 residues, most of which reside in the regulatory domain (RD). HSF1 is acetylated at 12 residues, most of which reside in the DNA-binding domain (DBD) and the oligomerization domain (HR-A/B). Note that lysine 298 (K298) is the only residue known to be either acetylated or SUMOylated. The other modified domains include the oligomerization domain (HR-C) and the transactivation domain (TAD). The first and last amino acids are indicated with a number.

A recent study using mass spectrometry analysis has shown that, like HSF1, HSF2 is acetylated at multiple sites. Eight residues (Lys82, Lys128, Lys135, Lys197, Lys209, Lys210, Lys395, and Lys401) were acetylated under optimal growth conditions by CBP and

EP300 (de Thonel et al., 2022). Moreover, the analysis of single-point mutations revealed that most of the acetylation occurred at Lys128, Lys135, and Lys197 in the HR-A/B region. Importantly, the acetylation of these residues counteracts the proteasomal degradation of HSF2 in both control and stress circumstances (de Thonel et al., 2022). Another PTM that can influence the stability of HSFs is ubiquitination, and particular sites on both HSF2 and HSF4 have been observed to be susceptible to this modification (Ahlskog et al., 2010; Xing et al., 2010; S. Liao et al., 2015). The ubiquitination of HSF2 is partially directed by the ubiquitin E3 ligase anaphase-promoting complex/cyclosome (APC/C), which prompts the degradation of HSF2 upon moderate heat shock (Ahlskog et al., 2010). As a whole, the complex interplay between different PTMs in HSFs provide signatures that likely adjust their activity across multiple biological processes, including stress responsiveness and developmental processes.

2.7 HSFs as developmental factors

After discovering HSF1, numerous investigations were conducted to uncover the biological roles associated with HSFs. One significant study showed that HSF1 is required to maintain cell viability in yeast under ideal growth conditions; hence, it has essential functions apart from stress regulation (Sorger and Pelham, 1988). Moreover, the lack of HSF1 in *Drosophila melanogaster* and *C. elegans* is detrimental to larval development (Jedlicka et al., 1997; J. Li et al., 2016). Importantly, HSF1's essential role during nematode development was connected to its regulation of a selection of genes, which includes some stress-responsive genes. Additionally, HSF1 and a second transcription factor known as EFL-1/DPL-1 had to synergistically modulate this transcriptional network (Li et al., 2016). Therefore, this study highlighted that the cooperation between transcription factors is essential for governing transcriptional programs in more complex organisms. Adding to the complexity, since mammals express multiple HSFs, HSF-null mice were generated to provide suitable genetic models to uncover the physiological processes in which HSFs are engaged. Remarkably, several of these processes involve developmental processes that influence fertility and sensory organs; hence, HSFs are paramount in mammals' non-stress-associated processes (Xiao et al., 1999; Kallio et al., 2002; Uchida et al., 2011; Jin et al., 2018a).

2.7.1 HSF1 and HSF2 in fertility

Spermatogenesis is a strictly regulated developmental process that occurs in the seminiferous tubules of the adult male testes, and during this process, millions of haploid spermatozoa are continuously produced from the spermatogonial diploid cells (Neto et al., 2016). Due to the intricate nature of spermatogenesis, animal models are often required to investigate this process *in vivo*. Thus, it was intriguing when both HSF1- and HSF2-null mice were found to have defects in spermatogenesis and other fertility-related processes, which was not evident from previous *in vitro* experiments (Widlak and Vydra, 2017). For instance, male HSF1-null mice produced ~ 20% less sperm than wild-type males due to deteriorating seminiferous tubules (Salmand et al., 2008). This suggests that HSF1 plays a critical role in the maintenance of the seminiferous tubules and the arrangement of germ cell layers within them. Additionally, the importance of HSF1 in gestation is highlighted by female HSF1-null mice, which have defects in the development of oocytes and the chorioallantoic placenta, the latter of which has been linked to increased prenatal mortality (Xiao et al., 1999; Christians et al., 2000).

HSF2-null mice have also been shown to have impairments in gametogenesis. For example, female HSF2-null mice display abnormal oocyte production, whereas male mice have increased apoptosis of spermatocytes (Kallio et al., 2002). The defective spermatogenesis observed in male mice was partially attributed to abnormal regulation of multicopy Y-chromosomal genes, which HSF2 typically regulates to maintain sperm quality (Åkerfelt et al., 2008). An important factor associated with HSF2's ability to control some of these genes was the microRNA miR-18, which decreased the expression of HSF2 at certain stages of male germ cell development, thus directly adjusting HSF2's ability to modulate transcription (Björk et al., 2010). Similarly to male HSF1-null mice, the reduced sperm count and quality had only a minor impact on fertility in male HSF2-null mice. Therefore, it has been suggested that the similar impacts on spermatogenesis observed when either HSF1 or HSF2 is knocked out are due to their ability to collaborate in regulating transcription. This idea is supported by a genome-wide analysis, revealing that HSF1 and HSF2 bind to the same genomic loci in spermatogenic cells under optimal growth conditions (Korfanty et al., 2014). Moreover, it has been shown that double knockout of both *Hsf1* and *Hsf2* leads to severe defects in spermatogenesis; thus, male mice lacking both HSFs are infertile (Wang et al., 2004). These studies indicate that in an optimal environment, these HSF distinctly and synergistically influence the processes that regulate sperm production and gestation, and both HSF is required for optimal fertility.

2.7.2 HSF2 and HSF4 in brain development and sensory organs

HSF2 is the primary HSF involved in neuronal and brain development. For instance, HSF2-null mice display brain abnormalities, such as enlarged ventricles, small hippocampus, and misarrangement of neurons (Kallio et al., 2002; Chang et al., 2006). These phenotypes are partially explained by research demonstrating the necessity of HSF2 for the accurate expression of p35, p39, and upstream activators of cyclin-dependent kinase 5 (CDK5), which influence neuronal migration (Chang et al., 2006). The significance of HSF2 in facilitating proper neuronal migration is further underscored in a study on fetal alcohol spectrum disorder (FASD), which generally encompasses brain development defects resulting from maternal alcohol consumption (El Fatimy et al., 2014). In this study, an FASD mouse model was used to examine how the consumption of alcohol affected the activities of HSF1 and HSF2 in rodent fetuses. Remarkably, the results showed that alcohol triggered a stress response in the fetal brain, necessitating the activation of both HSF1 and HSF2 to induce the expression of pro-survival genes. Despite the stress response promoting neuronal survival, it also interfered with neuronal migration in the developing fetal cortex. Importantly, HSF2-null mice did not show a similar defect in the cortex in response to alcohol exposure, but other abnormalities were observed in their brains under non-stress conditions. Therefore, HSF2 is important for rodent brain development, and its interplay with HSF1 can help neuronal cells endure stress; yet, this has a detrimental effect on neuronal migration (El Fatimy et al., 2014).

In addition to mice, HSF2 plays an integral role in the development of the human brain. A recent study by de Thonel and colleagues demonstrated that improper regulation of HSF2 is associated with the underlying mechanisms driving the development of Rubinstein-Taybi syndrome (RTS), a rare condition affecting numerous processes, including human neuronal development (de Thonel et al., 2022). This syndrome results from detrimental changes in the genes encoding the acetyltransferases CBP and p300; thus, HSF2 and other substrates under the control of CBP cannot be acetylated (de Thonel et al., 2022). The study revealed that, in

a normal environment, HSF2 levels are regulated by acetylation. However, in RTS cells where HSF2 is not acetylated, its expression decreases, thereby impairing HSF2's ability to effectively regulate the expression of genes that play a vital role in brain development (de Thonel et al., 2022).

Unlike HSF2, which has important roles in brain development, HSF4 is critical for lens and olfactory development (Fujimoto et al., 2004; Takaki et al., 2006). HSF4-null mice typically display lens deterioration that leads to the development of postnatal cataracts and blindness (Fujimoto et al., 2004). The pathophysiological cause of the degeneration of the lens epithelial cells is mainly attributed to abnormal growth and differentiation. Additionally, the lack of HSF4 negatively impacts the gene expression of crystallins, which are major structural proteins required in the lens (Somasundaram and Bhat, 2004; Shi et al., 2009). The impact of HSF4 on cell proliferation was further investigated in a study, revealing that HSF4 can regulate cell proliferation by binding to p53 and stabilizing its expression, consequently leading to cell cycle arrest and decreased proliferation (Huang et al., 2015). Importantly, the relevance of HSF4 in human physiology was recently highlighted by reports showing that numerous mutations in the DBD of HSF4 negatively impact the protein's functionality, resulting in autosomal-dominant hereditary cataracts (Lv et al., 2014; Berry et al., 2018). In addition to lens development, HSF4 has been reported to assist in the normal development of olfactory sensory neurons (Takaki et al., 2006). In this context, both HSF4 and HSF1 precisely modulate the expression of leukemia inhibitory factor (LIF), an essential cytokine required for the normal development of olfactory sensory neurons.

2.8 HSFs in cancer

In the past two decades, numerous studies have demonstrated that various cancer types require high levels of HSPs to survive, grow and metastasize (Puustinen and Sistonen, 2020). Subsequently, many attempts have been made to target HSPs pharmacologically (Wu et al., 2017; Yuno et al., 2018), and various drug trials are still underway; however, to date, no monotherapies using HSP inhibitors have been approved by the FDA. This is mainly attributed to harmful side effects, including activating HSF1, which induces the expression of genes encoding various HSPs (Bagatell et al., 2000; Pesonen et al., 2021), hence, forming a positive feedback loop that is advantageous for cancer cells (Guo et al., 2005). Fortunately, drug developers have started to direct efforts toward targeting HSFs because they modulate the expression of a wide range of genes, including *Hsps* and newly identified cancer-specific genes (Mendillo et al., 2012; Dong et al., 2019; Smith et al., 2022).

The first study to investigate a link between the elevated expression of HSPs in cancer and HSF1 originated in Roy-Burman's laboratory (Hoang et al., 2000). This study presented pathological evidence that HSF1 is overexpressed in cancerous prostate tissue, and the elevated levels stimulated the basal expression of HSP27. Following this discovery, attention was directed to uncovering the role of HSF1 in tumorigenesis. Two pioneering studies exploring the connection between HSF1 and cancer in animals employed distinct mouse models (Dai et al., 2007; Min et al., 2007). In the study by Dai and colleagues, HSF1-null mice showed resistance to cancer caused by either a carcinogen or gene modification, and *in vitro* analysis of different cancer cells showed that the knockdown of HSF1 reduced cell survival and growth (Dai et al., 2007). Another significant finding in this study was that heterozygous HSF1-knockout mice, which contained mutated p53 with gain-of-function

properties, did develop tumors, yet, the tumor types significantly differed from those in the wild-type mouse. In the same year, Min and colleagues published a study examining the relationship between HSF1 and tumorigenesis in HSF1/p53-null mice (Min et al., 2007). This study found that the absence of HSF1 reduced tumor formation and changed the range of tumor types, which is consistent with the results in Dais' study. Importantly, the influence of HSF1 on tumor evolution was partly attributed to its importance in maintaining genomic stability and eliciting a proper immune response in the tumor microenvironment. Moreover, in 2012, a study by Jin and colleagues further highlighted the significance of HSFs in tumor evolution by demonstrating that double-knockout of HSF4 and p53 also reduces tumorigenesis in mice and shifts the formation of tumor types, albeit differently than in HSF1/p53-null mice (Jin et al., 2012). Therefore, these studies collectively indicate that different HSF family members can support tumor formation in a tissue-specific manner.

Further research has revealed that HSF1 is rarely mutated in cancer, yet it is commonly overexpressed in several types of cancer (Santagata et al., 2011; Tsukao et al., 2017; Björk et al., 2018; Cyran and Zhitkovich, 2022a). The elevated levels of HSF1 in tumors have partially been attributed to the amplification of chromosome region 8q24.3, which harbors the genes encoding HSF1 and MYC, the latter of which is a proto-oncogene (Brusselaers et al., 2019; Cyran and Zhitkovich, 2022a). The expression of HSF2 and HSF4 in cancer is not as extensively documented as that for HSF1; however, lower levels of HSF2 have been observed in prostate and breast cancer, while higher levels are observed in lung cancer tissue (Björk et al., 2016; Zhong et al., 2016). Furthermore, only a few studies have documented the levels of HSF4 in cancer, and it appears that colon and lung cancer are the most common types that overexpress HSF4 (Yang et al., 2017; Ma et al., 2020). While HSFs are mainly associated with their role in controlling *Hsp* expression, it is important to recognize that they also influence the expression of other target genes involved in various processes in cancer (Puustinen and Sistonen, 2020).

2.8.1 HSF1-associated transcriptional networks in cancer

It is believed that HSF1's activity is finely tuned to the level of cellular stress. Therefore, in moderately stressed cells, the induction of *Hsps* is modest, whereas, during acute stress, the induction of *Hsps* is markedly higher. However, cancer cells alter the expression and activity of HSF1 to maximize the production of HSPs, thereby ensuring the adequate levels of chaperones needed for maintaining a balanced proteome (Y. Liao et al., 2015; Kourtis et al., 2018). Additionally, certain types of cancers utilize HSFs to induce the expression of genes other than *Hsps* to promote growth and invasion (Mendillo et al., 2012; Smith et al., 2022).

Thanks to advances in genome-wide sequencing techniques, it has been possible to identify HSF-mediated transcriptional programs distinct from the HSR. For instance, the paradigm-shifting study by Mendillo and colleagues demonstrated that HSF1 regulates cancer-specific transcription programs that promote cell proliferation, metabolism and suppress cell adhesion (Mendillo et al., 2012). This HSF1-dependent transcriptional program termed the HSF1 cancer signature (HSF1-CaSig), predominantly contains uniquely regulated genes that differ from those in the HSR. Furthermore, the pathophysiological importance of the HSF1-CaSig is highlighted by two key findings. First, several genes belonging to the signature, including HSF1, are located in chromosome 8q, typically amplified in numerous cancer types (C. Zhang et al., 2017; Brusselaers et al., 2019). Second, breast cancer patients with a prominent

HSF1-CaSig demonstrate poorer survival than those with low expression (Mendillo et al., 2012).

Another impactful study that mapped the network of genes regulated by HSF1 revealed that cancer-associated fibroblasts (CAFs) residing in the stroma are dependent on HSF1 to trigger transcriptional reprogramming that plays a critical role in maintaining a tumor microenvironment that enhances survival and proliferation of cancer cells (Scherz-Shouval et al., 2014). The stroma-associated transcriptional program is distinct from that of HSF1-CaSig, and the CAF-mediated survival mechanism relies on cytokine signaling to promote cancer cell growth. Moreover, in recent years, similar HSF1-dependent stromal-associated transcriptional programs have been identified in gastric, pancreatic, and colorectal cancers (Levi-Galibov et al., 2020; Grunberg et al., 2021; Shaashua et al., 2022).

In addition to studies investigating the transcriptional networks of HSF1, several independent studies have explored the post-translational mechanisms that control its activity in cancer. For instance, one study revealed that the transcriptional regulator IER5 and protein phosphatase 2 (PP2A) regulates the activity of HSF1 in breast cancer cells (Asano et al., 2016). This is achieved by IER5, PP2A, and HSF1 forming a complex in which PP2A subsequently dephosphorylates HSF1 at certain sites that represses HSF1's transcriptional capacity. This hypo-phosphorylated form of HSF1 can induce the transcription of pro-survival genes, including *Hsps*, increasing anchorage-independent cell growth (Asano et al., 2016). Also, since HSF1 is a positive regulator of the *IER5* expression under control and heat stress conditions, IER5 likely operates as a positive feedback regulator of HSF1 in cancer (Ishikawa and Sakurai, 2015). Another instance when phosphorylation impacts HSF1's activity in cancer is in HER2-overexpressing breast cancer (Carpenter et al., 2015). In this type of cancer, the receptor tyrosine kinase HER2 can activate several downstream kinases, including protein kinase B (Akt), which subsequently phosphorylates Ser326 in HSF1. Importantly, the phosphorylation of Ser326 is one of the key post-translational modifications that promote HSF1's transcriptional capacity (Guettouche et al., 2005), and it is especially occurring in cancer. In HER2-positive cancer, this Akt-HSF1 signaling axis promotes invasion by HSF1-induced expression of proteins involved in various pro-tumorigenic functions (Schulz et al., 2014; Carpenter et al., 2015). In addition to Akt, several other kinases have been proven to phosphorylate and promote the activity of HSF1 in various cancer types (Ma et al., 2015; Hoj et al., 2020; Moreno et al., 2021).

2.8.2 HSF2 in cancer

The impact of HSF2 on tumorigenesis has gradually started to become unveiled (Fig. 8). The earliest finding linking HSF2 with cancer showed that it is overexpressed and constantly active under control conditions in the mouse teratocarcinoma cell line F9 (Murphy et al., 1994). Still, it is important to note that in this study, the binding of HSF2 to DNA did not increase the expression of HSPs, suggesting that HSF2 regulates the transcription of genes unrelated to heat stress in this cancer cell line (Murphy et al., 1994). In 2010, the involvement of HSF2 was further investigated, and the Wnt signaling pathway was identified as the first cancer-associated signaling pathway that regulates HSF2 expression in hepatocellular carcinoma (HCC) (Kavak et al., 2010). Moreover, analysis of xenograft samples revealed that HSF2 expression increases with the advancement of HCC, and the protein levels of HSF2 correlate with increased proliferation (Kavak et al., 2010). Fan and colleagues further

substantiated the significance of HSF2 in HCC in a recent assessment of patient data, which uncovered that HSF2 expression was significantly increased in HCC, and its knockdown significantly reduced the proliferation of HCC cells (Fan et al., 2021). Similarly, Li and colleagues found that HCC tissue displays high levels of HSF2, and the HSF2 expression level increased with the tumor stage (Li et al., 2022). Although Fan and Li's studies suggest a link between the expression of HSF2 and the progression of HCC, further research is needed to identify the genetic changes that influence HSF2 expression in HCC and characterize the mechanisms by which HSF2 promotes the advancement of the disease. To date, only one study has indicated that HSF2 may induce cell growth in HCC through a process that stimulates aerobic glycolysis (Yang et al., 2019).

Remarkably, while the first evidence connecting HSF1 to cancer was described in prostate cancer, the initial indication of HSF2's tumor-suppressive function was also identified in this type of cancer (Hoang et al., 2000; Björk et al., 2016). Björk and colleagues uncovered that the expression of HSF2 decreases during prostate cancer development, and this decrease was linked to high Gleason scores and predicted a poor prognosis for patients (Björk et al., 2016). Furthermore, investigating the mRNA expression in cancer patient samples revealed that, besides prostate cancer, HSF2 levels are significantly lower in invasive breast, small-cell lung, and ovarian serous papillary carcinomas, demonstrating that the loss of HSF2 expression is a trait of several human malignancies (Björk et al., 2016). Notably, data is lacking concerning the genetic alterations of HSF2 in cancer; however, in prostate cancer, Björk and colleagues found that the decrease in HSF2 expression in patient samples was mostly associated with heterozygous loss of HSF2.

To characterize the role of HSF2 in preserving cellular health, Björk and colleagues utilized 3D organotypic cultures, an *in vivo* xenograft model, and gene expression profiling to study how the knockdown of HSF2 in prostate cancer cells affected growth and gene expression. The results demonstrated that low levels of HSF2 promoted the growth and invasion of organotypic tumoroids, and gene set enrichment analysis indicated that this was partially accomplished by GTPase-mediated reorganization of the actin cytoskeleton (Björk et al., 2016). Additionally, analysis of protein levels revealed that a decrease in HSF2's expression coincided with an increase in mesenchymal markers, suggesting a link between Epithelial-Mesenchymal Transition (EMT) and HSF2. Therefore, it was postulated that HSF2 plays a role in maintaining the epithelial state, in which cells exhibit distinct polarity and strong intercellular adhesion.

While HSF1 has been acknowledged as a central regulator of pro-survival genes in cancer, the investigation into the regulation of these genes by HSF2 has only recently begun. For instance, a recent study by Smith and colleagues established that HSF2 could support pro-tumorigenic transcriptional programs with HSF1 (Smith et al., 2022). Based on gene expression and chromatin occupancy analyses, their results showed that HSF1 and HSF2 could interact within certain cancer types, and both bound to distinct and shared targets, which included stress-regulated genes and a novel set of genes linked to malignant transformation (Smith et al., 2022). Importantly, the downregulation of either HSF impacted distinct sets of genes, implying that each HSF modulates different pro-tumorigenic programs in cancer. Furthermore, the downregulation of either HSF in cancer cells reduced tumor

progression in xenografts, indicating that optimal levels of HSFs may be essential for cancer cell growth. It is important to note that the few studies reporting on the expression of HSF2 indicate that its expression varies depending on the cancer type, and it has been demonstrated that HSF2 influences a diverse set of functions in cancer development (Puustinen and Sistonen, 2020) (Fig. 8).

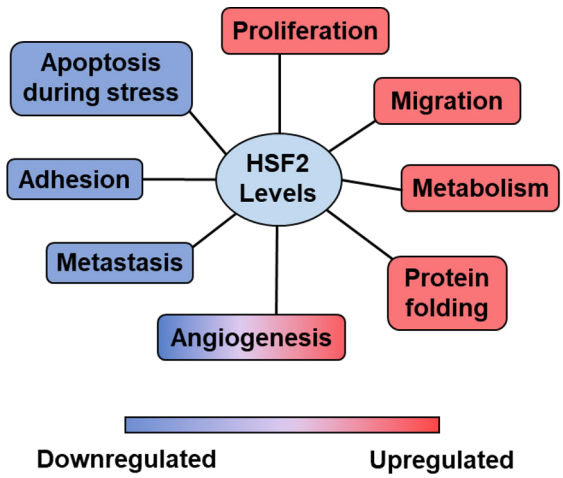

Figure 8. The protein levels of HSF2 impact different oncogenic processes in cancer. An increase or decrease in the expression of HSF2 either directly or indirectly impacts various processes in cancer. For instance, low levels of HSF2 can influence cellular adhesion, while high levels impact proliferation.

3 Epithelial-mesenchymal transition and its associated signaling pathways

Numerous cell types undergo molecular and phenotypical changes to differentiate and adapt to their surroundings. For instance, during natural developmental processes and cancer progression, epithelial cells can undergo Epithelial-Mesenchymal Transition (EMT), thereby dedifferentiating and gaining mesenchymal characteristics (Huang et al., 2022). The reversible transition process of EMT, also known as Mesenchymal-Epithelial Transition (MET), enables mesenchymal cells to transdifferentiate back to the epithelial state. The mechanisms regulating EMT/MET are complex and multifaceted, and these transitions characteristically require comprehensive rearrangement of the cytoskeleton and extracellular matrix, thus affecting cellular mobility and a plethora of processes, including wound healing and proliferation (Huang et al., 2022).

In 1958, Elizabeth Hay discovered the EMT and MET while studying forelimb regeneration of *Amblystoma punctatum* larvae (Hay, 1958). She found that following limb amputation, blastema cells had the capacity to dedifferentiate, proliferate, and then re-differentiate into cells that form cartilage, which has critical importance for development of the limb. Following this discovery, EMT was shown to have importance in wound healing, organ/tissue biogenesis, and embryonic development (Huang et al., 2022). Expanding on the research of EMT phenotypes, the focus was also placed on identifying the factors that could induce EMT. Hepatocyte growth factor (HGF) was the first identified factor uncovered to promote EMT by reducing the junction proteins between epithelial cells, thus stimulating cellular motility (Stoker et al., 1987). Subsequently, fibroblast growth factor (FGF) and transforming growth factor (TGF) were identified to stimulate EMT (Gavrilović et al., 1990; Vallés et al., 1990).

Along with the discoveries of EMT-inducing factors, several EMT-associated transcription factors (EMT-TFs) were uncovered in the 1990s. Snail was the first identified EMT-TF, and in mice, it displayed a central role during the development of the mesodermal germ layer (Smith et al., 1992). Slug and Twist are two early-identified EMT-TFs, and apart from regulating EMT-associated processes during development, they have also been shown to promote tumorigenesis (Nieto et al., 1994; Bloch-Zupan et al., 2001; Yang et al., 2004; Casas et al., 2011). Since these discoveries, the connection between EMT and cancer has increased, and today we know that EMT-TFs and their regulated networks are the fundamental drivers of EMT programs in normal and transformed cells.

3.1 Signaling pathways regulating EMT

Due to the widespread involvement of EMT in numerous biological processes, including the development of the mesoderm, neural tubes, wound healing, and inflammation, several distinct signaling pathways and transcription factors are needed to initiate and adjust the context-dependent progression of EMT (Huang et al., 2022). Adding to the complexity, crosstalk between signaling pathways influences EMT induction, demonstrating that EMT is a diverse phenotypic program. These pathways are activated by a multitude of stimuli from the local environment, including growth factors and cytokines. Importantly, once initiated, a common set of transcription factors play a key role in modulating cell- and tissue-type specific EMT-associated transcriptional programs (Gonzalez and Medici, 2014). For this reason, it is possible to use the expression of the involved factors as biomarkers for EMT progression.

3.1.1 The TGF-β signaling pathway

The TGF-β signaling pathway is the best-characterized pathway known to induce EMT (Fig. 9). This pathway is activated by ligands belonging to the TGF-β superfamily, which include three isoforms of TGF-β (1-3) and six isoforms of bone morphogenetic protein (BMP 2-7) (Morikawa et al., 2016). TGF-β1 is one of the main ligands involved in fibrosis, immune responses, and cell migration, whereas TGF-β2 and TGF-β3 modulate the development of the endocardium and palate, respectively (Shull et al., 1992; Kim et al., 2018; Camenisch et al., 2002; Nawshad and Hay, 2003). The BMPs regulate various aspects of EMT; for instance, BMP2 can induce EMT in cancer, while BMP7 can counteract EMT progression in breast cancer (Buijs et al., 2007; Kang et al., 2009).

In general, TGF-β signaling is initiated at the plasma membrane through several different isoforms of type I and type II TGF-β receptors (TGF-βRI or RII), which are single-pass serine/threonine kinases (Derynck and Feng, 1997). Upon ligand binding, these receptors form a tetrameric complex, and TGF-βRII phosphorylates the cytoplasmic domain of TGF-βRI, thus activating the kinase. Additionally, when TGF-β2 binds TGF-βRII, β-glycan, also known as TGF-βRIII, is required for forming the tetrameric receptor complex (Blobe et al., 2001). BMP-initiated signaling is mediated similarly to TGF-β-mediated signaling, but instead of TGF-βRII, BMP type II receptors form a complex with TGF-βRI to initiate the intracellular signaling cascade (Rosenzweig et al., 1995).

Upon receptor activation, TGF-β signaling can be transmitted through the canonical and the non-canonical pathways. In the canonical pathway, several members of the Smad protein family mediate the intracellular signaling cascade. The activated TGF-βRI phosphorylates and activates the group of Smads that are termed receptor-regulated Smads (R-Smads), including Smad2 and Smad3 in TGF-β signaling and Smad1, Smad5, and Smad8 in BMP signaling (Massagué et al., 2005). Once triggered, R-Smads, such as Smad2 and Smad3, translocate to the nucleus and form heteromeric complexes with Smad4, the only Smad classified as a co-Smad (Gonzalez and Medici, 2014) (Fig. 9). This R-Smad/Smad4 complex can interact with transcriptional regulators to induce or repress the expression of genes. Important genes upregulated by Smads include several EMT-TFs, such as Snail1, Snail2, Twist, and Zeb1, which are key modulators of gene expression related to EMT progression (Gonzalez and Medici, 2014). Another important group of Smads is the inhibitory Smads, which includes Smad6 and Smad7. This group of Smads attenuates signaling transduction by binding to TGF-βRI, thus preventing R-Smads from attaching to the receptor (Massagué et al., 2005).

In the non-canonical pathway, the activation of TGF-βRI triggers numerous factors involved in other signaling pathways, including various mitogen-activated protein kinases (MAPKs), phosphatidylinositol 3-kinase (PI3K), and the GTPases Ras and RhoA (Bakin et al., 2000; Horiguchi et al., 2009; Zhang, 2009; Grusch et al., 2010) (Fig. 9). The pathways work independently of Smads and regulate a diverse range of cellular processes, such as proliferation, cell migration, and remodeling of the extracellular matrix (ECM), which are processes altered during EMT progression. Apart from TGF-β, other growth factors, such as fibroblast and epidermal growth factors (FGFs, EGFs), can activate the receptor tyrosine kinases involved in regulating EMT in a cell and tissue type-specific manner (Strutz et al., 2002; Ahmed et al., 2006). For instance, FGF-2 alone can promote EMT progression in

tubular epithelial cells in part by increasing the expression of the EMT markers vimentin and fibronectin and by stimulating the activity of the matrix metalloproteinases (MMPs), which are key proteins involved in the breakdown of the ECM (Strutz et al., 2002). Furthermore, FGF-2 can enhance cell motility by altering actin cytoskeleton arrangement via Rho and PI3K (Lee and Kay, 2006). Similarly to FGF-2, EGF can activate kinases to increase cell motility. This is mechanistically achieved by increasing the activity of MMP2 and MMP9 (Ahmed et al., 2006). Taken together, the growth factors that orchestrate EMT activation act through analogous kinases and GTPases, yet the effects of a particular ligand on downstream signaling pathways are often specific to different tissues and cell types.

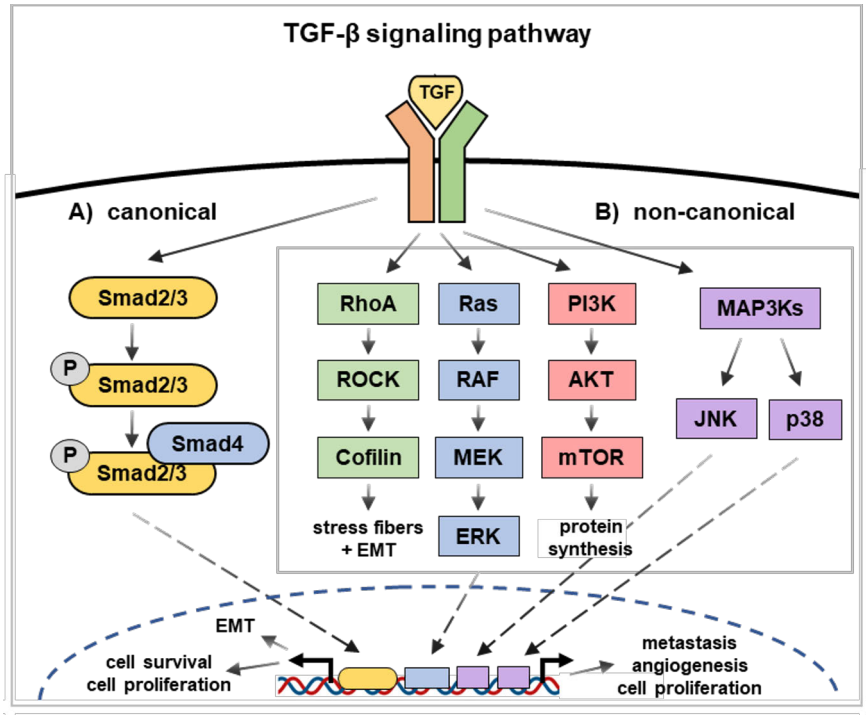

Figure 9. The canonical and non-canonical TGF-β signaling pathways. (A) The canonical TGF-β signaling pathway is activated once the TGF-β ligand binds to and activates the TGF-β receptors. Receptor activation promotes the phosphorylation of Smad2/3, which, together with Smad4, form a complex. Subsequentially, the Smad2/3/4 complex is translocated to the nucleus, where it binds to and regulates the transcription of several genes, which affects biological processes, including EMT. (B) The TGF-β receptor can activate non-canonical TGF-β signaling pathways. These signaling cascades are transmitted through numerous factors, including RhoA, Ras, PI3K, and MAP3Ks. These proteins are also modulated by other receptor tyrosine kinases, which are not indicated. Each branch of the non-canonical signaling pathway includes several downstream proteins, affecting biological processes via either transcriptional regulation or non-transcriptional mechanism. Signaling pathway crosstalk between the canonical and non-canonical pathways is not indicated. The arrows indicate the general direction of the signal transduction of the respective pathways. P = phosphorylation.

3.1.2 The WNT, Notch, and Hedgehog Signaling Pathway in EMT

Apart from the TGF-β signaling pathway, EMT is regulated via the Wnt, Notch, and Hedgehog pathways (Gonzalez and Medici, 2014) (Fig. 10). The proteins included in the Wnt family are evolutionarily conserved, and they are renowned for their importance in developmental processes (Nusse and Clevers, 2017). Wnt signaling is initiated once extracellular Wnt ligands (Wnt) bind to Frizzled receptors, which results in the phosphorylation of lipoprotein receptor–related protein (LRP) receptors (Moon, 2005). Once LRP is activated, it recruits Dishevelled (Dvl) and Axin to the membrane, indirectly preventing Axin from interacting with glycogen synthase kinase 3β (GSK-3β). The decrease in the Axin and GSK-3β interaction leads to dismantling the so-called destruction complex, wherein GSK-3β normally phosphorylates β-catenin to accelerate its degradation (Moon, 2005). The lack of destruction complexes results in the accumulation of β-catenin (Moon, 2005). Next, β-catenin translocates to the nucleus, where it binds transcription factors that help it modulate a network of genes that support EMT progression, which includes increasing the expression of the EMT-TFs: Snail2 and Twist (Gonzalez and Medici, 2014).

EMT can also be initiated via the notch receptors (Notch), which have an extracellular domain (ECD) and an intracellular domain (NICD) (Hu et al., 2012). Once neighboring cells interact via their notch receptors, they are cleaved by disintegrin metalloproteases (ADAMs) and γ-secretase. Once released from the membrane, the NICD translocates to the nucleus, where it, together with co-regulators, forms a complex that can promote the transcription of EMT-related genes (Hu et al., 2012). Some EMT-associated genes that NICD modulates include those encoding for Snail, β-catenin, NF-κB, and Akt, all demonstrating crosstalk between the oncogenic pathways driving EMT (Gonzalez and Medici, 2014).

The Hedgehog signaling pathway (Hh pathway) regulates EMT mainly during developmental processes and tissue regeneration. Additionally, during fibrosis and cancer progression, Hh signaling can alter gene expression to promote the progression of EMT (Cohen, 2010; Fabian et al., 2012; Skoda et al., 2018). The Hh pathway is activated when the various hedgehog ligands (Hh), such as sonic hedgehog (SHH), bind to their respective receptors: patched 1 and patched 2 (PTCH1/2) (Gonzalez and Medici, 2014). When ligands and receptors interact, the receptors lose the ability to inhibit the G protein-coupled receptor known as smoothened (SMO). Subsequently, the activation of SMO initiates a signaling cascade that activates the three members of the glioblastoma transcription factor family (Gli1-3) (Sabol et al., 2018). Once Gli1 and Gli2 are activated, they move to the nucleus and induce the expression of hedgehog-responsive genes that encode proteins, such as jagged canonical notch ligand 2 (JAG2), PTCH, Wnt, and Snail1 (Visbal et al., 2011; Gonzalez and Medici, 2014; Lei et al., 2022). Additionally, EMT-associated Hh signaling in gastric cancer has been demonstrated to enhance cell migration by inducing TGF-β1 secretion (Yoo et al., 2008), suggesting that Hh signaling can potentially enhance the metastatic abilities of cancer cells by activating multiple pathways related to EMT. Taken together, Hh-pathway operates independently of the TGF-β signaling pathway to induce EMT, yet its downstream targets include key proteins associated with other pathways that drive EMT progression.

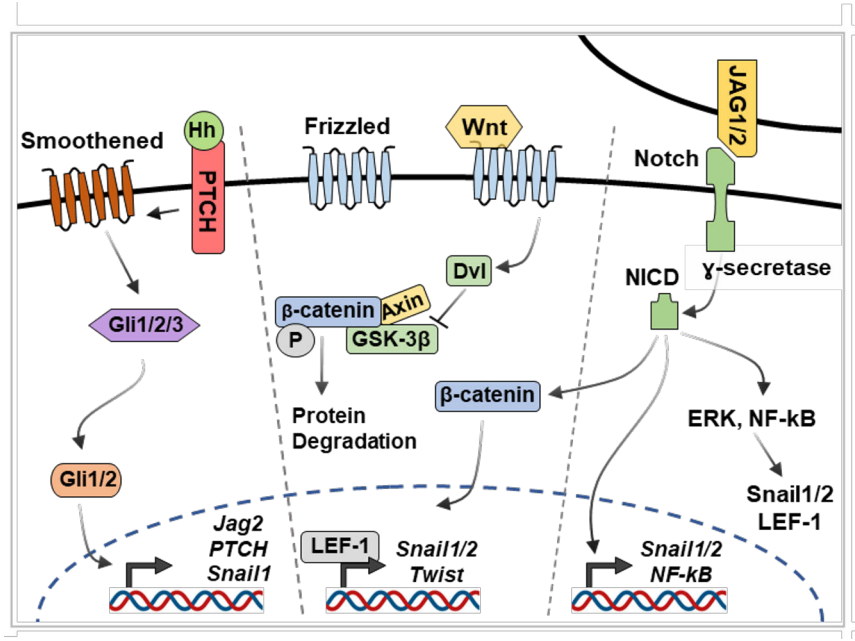

Figure 10. Wnt, Notch, and Hedgehog signaling during EMT. The Hedgehog signaling pathway modulates EMT via the patched receptors (PTCH). Signal transduction is initiated when the hedgehog ligands (Hh) bind to and inactivate PTCH. The suppression of PTCH facilitates activation of the G-protein coupled receptor smoothened, which promotes the induction of EMT-associated genes through several Gli transcription factors. Wnt ligands (Wnt) binding to the Frizzled receptors activates the Wnt signaling pathway during EMT. Activated Frizzled encourages Dishevelled (Dvl) to inhibit GSK-3β, which modulates the degradation of cytoplasmic β-catenin. Inhibition of GSK-3β results in the accumulation of β-catenin, which translocate to the nucleus where it binds additional transcription factors, including LEF-1, and induces the transcription of genes that promote EMT. The Notch signaling pathway partakes in EMT signaling via the interaction between JAG1/2 and the corresponding notch receptor on the neighboring cell. The JAG1/2-Notch interplay facilitates cleavage of the Notch's intracellular domain (NICD) by ɣ-secretase. Subsequently, NICD can directly induce the transcription of EMT-associated target genes or stabilize cytoplasmic β-catenin. Additionally, NICD can activate ERK and NF-κB, which promote EMT via their downstream targets Snail1, Snail2, and LEF-1.

3.2 Key transcription factors driving EMT

Several EMT-TFs, including Snail1/2, Twist1/2, and Zeb1/2, control the progression of EMT programs. These transcription factors share several target genes, such as E-cadherin, an important protein involved in cell adhesion, and its downregulation is a key event during the progression of EMT (Huang et al., 2022). Aside from their role in normal development and tissue biogenesis, EMT-TFs play a critical part in the progression of diseases such as cancer, particularly during the invasion process.

Two members of the Snail protein family, Snail1 and Snail2, are well-established EMT-TFs, and they are known for their ability to repress transcription (Nieto, 2002). During EMT progression, Snail1/2 typically suppress various cell-junction proteins, such as E-cadherin, occludins, and claudins, thus decreasing cell-cell adhesion. This transcriptional repression is partially achieved by recruiting the polycomb repressive complex 2 (PRC2) to the promoter region of the target genes (Herranz et al., 2008). Since PRC2 contains a subunit that operates as a histone methyltransferase, it causes histones to become methylated, thus forming a compact configuration of chromatin that inhibits transcription. Additionally, Snail1/2 can promote heterochromatin formation by interacting with HDAC1/2, and the corepressor mSin3A, which reduces the number of acetylated histones (Peinado et al., 2004). Apart from chromatin remodelers, transcriptional repression by Snail requires the scaffold protein 14-3-3, and the corepressor, LIM protein Ajuba (Hou et al., 2010).

Similarly to the Snails, the EMT-TFs Twist1 and Twist2 can repress the expression of E-cadherin and catenins; however, they can also induce the expression of vimentin, fibronectin, and N-cadherin, which are important proteins in mesenchymal cells (Huang et al., 2022). Twists utilize a plethora of chromatin remodelers to modify transcription, including the NuRD complex that contains HDAC1/2 and the methyltransferase SET8 (Yang et al., 2012; Xu et al., 2017). Twist1's interaction with SET8 promotes the mono-methylation of histone lysine 20, which in turn decreases E-cadherin expression and stimulates the expression of N-cadherin (Yang et al., 2012).

Another group of potent EMT inducers is the ZEB transcription factors. These transcriptional repressors are known for reducing the expression of key proteins implicated in maintaining the epithelial phenotype, including E-cadherin, claudin-1, and desmoplakin (Huang et al., 2022). Importantly, the two ZEB protein family members (ZEB1/2) modulate the progression of EMT via different chromatin remodelers. For instance, ZEB1 interacts with Smads and the histone acetyltransferase p300 to promote transcription, whereas ZEB2 suppresses transcription with the help of the transcriptional corepressor called C-terminal-binding protein (CTBP) (Postigo et al., 2003). Additionally, by interacting with certain chromatin remodelers, ZEB1 can also suppress transcription. For example, ZEB1 suppresses the expression of E-cadherin when it interacts with the ATPase known as BRG1, thus sequestering it away from the SWI/SNF complex and thereby reducing the complex's capacity to maintain a transcriptionally active chromatin configuration (Sánchez-Tilló et al., 2010).

The expression of EMT-TFs is modulated by both transcriptional and post-transcriptional regulation, and certain EMT-TFs can even influence the expression of other EMT-TFs (Fig. 10). For instance, Snails and Twists can regulate the transcription of ZEB1 (Dave et al.,

2011), while the expression of Snails is regulated by several transcription factors, including Gli1 and Smads, which are known for controlling embryogenesis-related pathways (X. Li et al., 2006). Additionally, in some cells, the induction of Twist1 is essential to induce the expression of Snail2 and to promote EMT and tumor metastasis (Casas et al., 2011). Taken together, the evidence suggests that EMT-TFs can modulate the expression of each other to create redundancies and interoperability within EMT-associated gene networks.

On the post-transcriptional level, phosphorylation is the major post-translational modification that affects the stability and activity of EMT-TFs. For example, the phosphorylation of Snails by GSK-3β induces their ubiquitination and proteasomal degradation (Zhou et al., 2004). In contrast, the phosphorylation of Snails by the PAR-atypical protein kinase C (aPKC) stabilizes their expression (Jung et al., 2019). Moreover, it has been demonstrated that phosphatase and tensin homolog (PTEN) can dephosphorylate Twist1 to facilitate its nuclear translocation and activation, while Akt-mediated phosphorylation increases the ubiquitination and degradation of Twist1 (C. Li et al., 2016; Hu et al., 2019). For ZEB1, it has been reported that the phosphorylation of Thr867 by ERK inhibits its activation (Llorens et al., 2016), while the phosphorylation of ZEB1 by the serine/threonine kinase ATM promotes its stability in breast cancer cells (P. Zhang et al., 2014). It is also important to note that deubiquitinases can counteract the proteasomal degradation of certain EMT-TFs. For instance, the ubiquitination-mediated degradation of Snail has been demonstrated to be mitigated by the action of deubiquitinases (W. Li et al., 2020).

3.3 EMT reorganizes the cell junctions, cytoskeleton, and ECM

EMT is typically accompanied by an extensive breakdown of cell-cell, cell-matrix junctions, as well as the ECM. The loss of cell-cell junctions can lead to the loss of apical-basal polarity, whereas the partial or complete degradation of the ECM results in open extracellular space and the release of bioactive fragments that stimulates nearby cells. Together, these processes facilitate a comprehensive cytoskeleton rearrangement, accompanied by increased cell migration, which is important in EMT-associated physiological and pathological processes.

3.3.1 Disruption of cell-junctions during EMT

Epithelial tissue is one of the four primary tissues found in humans, and it is generated by epithelial cells. Characteristically, epithelial cells form uniform sheets in layers that are tightly packed. The structure of the epithelial tissue is maintained by specialized surface proteins, creating several types of cell-cell junctions, like adherens junctions, tight junctions, desmosomes, and gap junctions (Garcia et al., 2018). Importantly, the EMT process influences all these junctions.

Adherens junctions are formed through the activity of transmembrane adhesion proteins called cadherins (Shapiro and Weis, 2009). The cadherin superfamily's two most extensively studied members are E-cadherin (CDH1) and N-cadherin (CDH2). These cadherins are calcium-dependent and typically interact with themselves on connecting cells. In addition to having an extracellular segment, cadherins contain a cytoplasmic segment that is connected to the actin cytoskeleton via cytoplasmic catenin proteins (Shapiro and Weis, 2009). Importantly, during the progression of EMT, E-cadherin expression is reduced while N-cadherin expression is upregulated, and this shift in expression is seen as one of the classical

hallmarks of EMT (Cano et al., 2000; Yang et al., 2015). The suppression of E-cadherin by EMT-TFs results in the disruption of adherens junctions, which is accompanied by the release of intracellularly bound p120-catenin/β-catenin, thus allowing β-catenin to promote further EMT progression (Ishiyama et al., 2010; Gheldof and Berx, 2013).

Another important group of cell junctions is called tight junctions. These intercellular adhesion complexes are positioned on the apical side of epithelial cells and are specialized in maintaining cell polarity and sealing the intercellular space. These complexes are primarily generated by claudins, occludins, junctional adhesion molecules (JAMs), and proteins in the occludens family (Garcia et al., 2018). Similarly to adherens junctions, the expression of proteins forming tight junctions is downregulated by EMT-TFs; thus, EMT progression characteristically reduces the amount of these junctions to promote invasion (Ikenouchi et al., 2003).

Gap Junctions (GJs) and desmosomes also operate as important cell-cell junctions. Desmosomes are formed by proteins that belong to the desmosomal cadherin family, the armadillo family, and the plakin family of cytolinkers (Chidgey and Dawson, 2007), and they specifically act as anchors for intermediate filaments. GJs differ from other types of cell-cell junctions because they are membrane-spanning channels through which ions and macromolecules pass from one cell to the other (Garcia et al., 2018). These channels are compiled by connexin proteins, which are differentially expressed in tissues. The expression of proteins that assemble desmosomes and gap GJs is suppressed by EMT-TFs, leading to the disassembly of the cell junctions during the progression of EMT (Saunders et al., 2001; Vandewalle et al., 2005; de Boer et al., 2007). However, TGF-β1 exposure has been demonstrated to promote the expression of connexin 43, an essential factor for GJ assembly in chondrocytes (Wang et al., 2019). Thus, certain cell types require some cell junctions for EMT to advance.

3.3.2 Cell-junction remodeling promotes cellular motility

During EMT, weakening of the cell-cell junctions and downregulation of E-cadherin disrupts the actin cytoskeleton, leading to a loss of cell-cell adhesion (Fig. 11). Furthermore, during the disruption of the adherens junctions and the reorganization of the actin cytoskeleton, β-catenin becomes released from the p120-catenin/β-catenin complex resulting in the repression of RhoA, which elevates active Rac1 and Cdc42, which can generate membrane protrusions called lamellipodia (Reynolds and Roczniak-Ferguson, 2004). The formation of lamellipodia facilitates cell migration, and in these membrane protrusions, the pivotal force that allows movement originates from cycles of actin filament polymerization at the leading edge and depolymerization at the rear end of the cell, resulting in a protrusive force acting on the cell membrane that allows the lamellipodia to spread (Huang et al., 2022). Furthermore, lamellipodia in mesenchymal cells also acquire small protrusions called filopodia that generate focal adhesions (FA) at the leading edge, which are subsequently dismantled at the trailing edge of the cell (Fischer et al., 2019). Hence, a combination of actin filaments polymerization, FA assembly, and disassembly is needed to generate the traction force that enables the cell to move forward.

In addition to rearranging the actin cytoskeleton, EMT alters the composition of intermediate filaments. Characteristically, EMT induction increases the production of the intermediate

filament vimentin while the levels of keratins are reduced (Kuburich et al., 2022). Keratins are important intermediate filaments in epithelial cells, and they provide strong intercellular adhesion by linking to desmosomes (Seltmann et al., 2013). Therefore, the reduction of keratins during EMT significantly affects epithelial tissue architecture. Moreover, it is unclear how EMT influences the transcription of the genes encoding keratins, yet, one study indicates that overexpression of Snail1 reduces the levels of several keratins in colorectal and breast cancer cells (De Craene et al., 2005). It is important to note that colorectal cancer cells in tumors display both variations in keratin expression and abnormal localization of keratins (Polari et al., 2020). Unlike keratins, the expression of vimentin typically increases during EMT, and it has a wide-ranging effect on cellular processes, including adhesion, migration, and cell signaling (Kuburich et al., 2022; Ridge et al., 2022). Importantly, during EMT and metastasis, cancer cells utilize vimentin as an elastic framework to position organelles in a manner that allows the cell to migrate through narrow spaces, a process that usually places mechanical stresses on the cell (Patteson et al., 2019). Similarly to actin, vimentin also regulates FAs by connecting to them via filamin A, thereby encouraging the cell to spread during migration (MacPherson and Fagerholm, 2010).

The expression of vimentin during EMT is regulated by EMT-TFs. For instance, Twist can indirectly increase the expression of vimentin through the induction of a circular RNA molecule that binds and sequester vimentin targeting miRNAs (Meng et al., 2018). The EMT-TF, ZEB1 binds directly to the *vimentin* promoter to induce its mRNA levels upon EMT induction (Qin et al., 2019), while Snail1 and Snail2 can further promote the induction of vimentin (Huang et al., 2022). Importantly, during the progression of EMT, vimentin can promote its own expression indirectly by acting as a scaffold for extracellular signal-regulated kinase (ERK) to phosphorylate Snail2, thereby enhancing the activity of the EMT-TF (Virtakoivu et al., 2015). In conclusion, both transcriptional and post-transcriptional regulation impact the expression of vimentin.

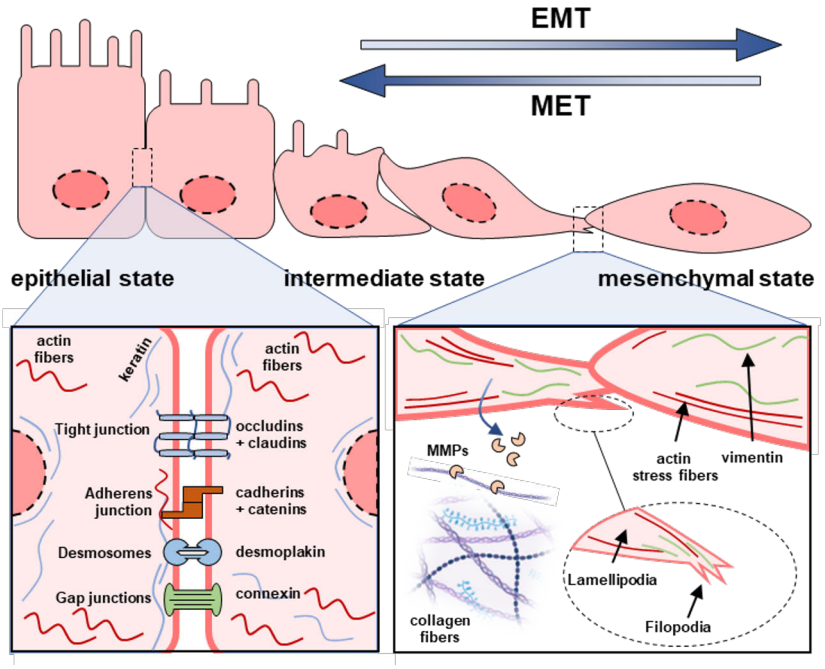

Figure 11. An overview of the shift in cellular phenotype during EMT. EMT is a reversible process encompassing epithelial, intermediate, and mesenchymal states. Several cell junctions preserve the epithelial integrity in the epithelial state. Proteins, including occludins and claudins, form tight junctions, whereas adherens junctions are generated by cadherins and catenins. Desmosomes are formed via desmoplakin and proteins of the connexin family form gap junctions. Most cell junctions are indirectly connected to actin fibers or intermediate filaments, such as keratins. Disruption of cell junctions occurs during the transition to the mesenchymal state, and following the transition, the actin cytoskeleton is rearranged, forming stress fibers. Furthermore, the expression of the intermediate filament vimentin is induced, whereas the expression of keratins is downregulated. Additionally, the transition to the mesenchymal state is also accompanied by degradation of the ECM by matrix metalloproteinases (MMPs) and the formation of lamellipodia/filopodia, which facilitates metastasis. (Modified from Huang et al., 2022)

3.3.3 ECM and cytoskeleton remodeling during EMT

The space between cells contains the extracellular matrix (ECM), a network of components including collagens, proteoglycans, elastin, and cell-binding glycoproteins. Together these components form an interlocking mesh that provides structural support to surrounding cells (Yue, 2014). The remodeling of the ECM is an essential process in healthy tissues, but cancer cells manipulate the signaling pathways governing ECM remodeling to enable them to metastasize.

The components of the ECM are secreted into the extracellular space as precursors, where they must undergo further processing to become biologically active. These precursors include elastin and different types of collagens, the latter being the most abundant component in the ECM (Bella and Hulmes, 2017). The collagens form long fibrillar structures that provide support for the cell, whereas elastin provides elasticity to tissues. In the basal lamina, collagen

fibers are connected to the cross-linked web of laminins, which aid epithelial tissue in resisting tensile forces. Another important glycoprotein is fibronectin, which, similarly to laminins, binds collagen fibers; however, it also binds to proteins called integrins (Zollinger and Smith, 2017). Integrins are membrane-spanning receptor proteins, and their binding to fibronectin facilitates the movement of cells in the ECM (Barczyk et al., 2010). Hence, these proteins are important during the wound-healing process, during which cells need to be able to migrate through the ECM.

Turnover of the ECM is an important part of normal tissue homeostasis, and processes such as EMT can elevate ECM degradation, which is vital in cancer invasion. The ECM also contains a reservoir of sequestered growth factors; hence degradation can release signaling molecules, such as vascular endothelial growth factor (VEGF), that stimulate angiogenesis (Yue, 2014). The main group of enzymes responsible for regulating the turnover of the ECM is matrix-degrading proteinases. The major families of matrix-degrading proteinases include matrix metalloproteinases (MMPs) and adamalysines (ADAMs) (Bonnans et al., 2014). Humans express 23 MMPs, divided into secreted (e.g., MMP2, MMP9) and membrane-type MMPs (e.g., MT1-MMPs). MT-MMPs are often expressed at the leading edge of filopodia, and they can breakdown ECM-associated molecules that are in contact with the cell surface (Bonnans et al., 2014). Furthermore, elevated expression of MT-MMPs modifies cell adhesion by accelerating the processing of cell adhesion molecules. Additionally, MT-MMPs can cleave and activate growth factor receptors and secreted precursor-MMPs (Itoh, 2015). The proteolytic processing of receptors is important for intracellular signaling, whereas the activation of the secreted MMPs, such as MMP2 and MMP9, promotes the degradation of elastin and numerous collagens, including fibrillar collagen (Quintero-Fabián et al., 2019). The activity of MMPs in ECM remodeling is, in part, regulated by tissue inhibitors of metalloproteinases (TIMPs) (Rapti et al., 2008).

Another group of metalloproteinases is the adamalysine family, which includes 21 ADAMs that are membrane-bound proteins and 19 ADAMs with thrombospondin motifs (ADAMTS), which are secreted proteins (Bonnans et al., 2014). ADAM and ADAMTS proteins are categorized as sheddases, meaning that they cleave the extracellular portions of several integral membrane proteins. ADAMs and ADAMTSs can shape the ECM by inducing the secretion of matrix-remodeling enzymes called meprins, which cleave ECM collagen IV and fibronectin (Geurts et al., 2012). Like MMPs, the activity of ADAMs and ADAMTSs is inhibited by TIMPs, of which TIMP3 is the main inhibitor (Rapti et al., 2008).

AIMS OF THE STUDY

Activation of the heat shock response depends on the ability of HSFs to oligomerize, translocate to the nucleus, and bind to the regulatory region of stress-inducible target genes. Additionally, activation of HSF1 is accompanied by stress-inducible hyperphosphorylation within the regulatory domain (RD). Previous studies have characterized how individual phosphorylation events impact the activity of HSF1; however, more consensus has yet to be reached regarding whether hyperphosphorylation is required for its activation. Therefore, my first study aimed to determine if phosphorylation in the RD is required for the activation of HSF1 and whether the lack of phosphorylation limits its transcriptional capacity. The focus of the second study was placed on identifying the genes and enhancers that are bound and regulated by HSF1 and HSF2 during heat and oxidative stress. This aim is particularly important because there are gaps in our knowledge regarding how HSFs cooperate in transcriptional regulation under different stress conditions. Apart from stress regulation, the pro-survival effect of HSF1 has been revealed to benefit the growth and survival of cancer cells. However, the involvement of HSF2 in cancer development is not well established. Therefore, the third study aims to uncover the pro-tumorigenic signaling pathway that influences the expression of HSF2. Furthermore, this study aims to determine if changes in the expression of HSF2 impact gene expression and pro-malignant processes in cancer cells.

The specific aims of this thesis were to:

- Determine whether stress-inducible phosphorylation within HSF1's RD is required for its activation.

- Identify which genes and enhancers are regulated and occupied by HSF1 and HSF2 during heat and oxidative stress.

- Identify the signaling pathway involved in regulating the expression of HSF2 in cancer cells and determine if HSF2 impacts pro-malignant processes.

EXPERIMENTAL PROCEDURES

Methods, cell lines, and antibodies used in each study in this thesis are listed below in separate tables. More comprehensive descriptions of each method and data analysis can be found in the original publications (I–II) and the manuscript (III).

Table 1. Methods used in this thesis.

Method	Study
Cell culture	I,II,III
Cell viability assay using Cell Counting Kit-8 (CCK8)	III
Chromatin immunoprecipitation (ChIP)	I
Chromatin immunoprecipitation sequencing (ChIP-seq)	II
Cloning of plasmids	I,III
Downregulation of HSF1 using short interfering RNA (siRNA)	III
Electromobility shift assay (EMSA)	I
Generation of stable cell lines using lentivirus	III
Immunofluorescence and confocal microscopy	I,II
In vitro vasculogenic mimicry assay	III
Luciferase and β-galactosidase assay	I,III
Measurement of GSH/GSSG ratio	II
mRNA-sequensing (RNA-seq)	III
Precision run-on sequencing (PRO-seq)	II
Quantitative reverse transcription PCR (qRT-PCR)	I,II,III
Site-directed mutagenesis	I
Transient transfection of cells	I,III
Ultra-low attachment assay (ULA)	III
Western blotting	I,II,III
Wound healing assay	III

Table 2. Cell lines used in this thesis. WT: wild-type, HSF1 KO: HSF1 knockout, HSF2 KO: HSF2 knockout.

Cell line	Type	Study
HDF	Human dermal fibroblast	III
HEK-293 T	Human embryonic kidney	III
HeLa	Human cervical cancer	I
HS578T	Human breast epithelial cancer	III
MCF10A	Human breast epithelial	III
MDA-MB-231	Human breast epithelial cancer	III
MEF HSF1 KO	Mouse embryonic fibroblast	I, II
MEF HSF2 KO	Mouse embryonic fibroblast	II
MEF WT	Mouse embryonic fibroblast	I, II

Table 3. Antibodies used in this thesis. ChIP: chromatin immunoprecipitation, ChIP-seq: chromatin immunoprecipitation sequencing, WB: Western blotting, and IF: immunofluorescence.

Antigen	Antibody	Company/Manufacturer	Application	Study
Actin	AC-40	Sigma-Aldrich	WB	I
Alexa Fluor 488	A11001	Invitrogen	IF	II
Alexa Fluor 568	A10494	Invitrogen	IF	I
CDH1	24 E10	Cell Signaling Technology	WB	III
CDK1	A17, ab18	Abcam	WB	III
CDK7	sc-7344	Santa Cruz Biotechnology	WB	III
COL3A1	22734-1-AP	Proteintech	WB	III
H2AX	05-636	EMD Millipore	IF	II
HSC70	ADI-SPA-815	Enzo Life Sciences	WB	III
HSF1	Holmberg et al., 2000; SPA-901	Sistonen's laboratory, Enzo Life Sciences	IF, ChIP, ChIP-seq, WB	I, II, III
HSF2	Östling et al., 2008; 3E2; HPA031455	Sistonen's laboratory, EMD Millipore, Sigma-Aldrich	ChIP-seq, WB	II, III
IgG	Normal rabbit IgG	EMD Millipore	ChIP-seq, IF	I,II
IgG	Normal mouse IgG	Life Technologies	IF	I
ITGA1	22146-1-AP	Proteintech	WB	III
MCM2	D7611	BioNordika	WB	III
MMP2	10373-2-AP	Proteintech	WB	III
Myc	M4439	Sigma-Aldrich	IF	I
ORC1	7A7	BioNordika	WB	III
pSmad2	138D4	Cell Signaling Technology	WB	III
Smad2/3	3102	Cell Signaling Technology	WB	III
Snail	C15D3	Cell Signaling Technology	WB	III
VP16	V4388	Sigma-Aldrich	WB	I
β-tubulin	T8328	Merck	WB	II

RESULTS AND DISCUSSION

4 Phosphorylation in HSF1's regulatory domain affects its transcriptional performance (I)

Cells are regularly exposed to various protein-damaging conditions, including elevated temperatures and oxidative reagents. To survive these harsh conditions, cells rely on stress-inducible transcription factors to increase their capability to manage denatured proteins. HSF1 is the primary stress-inducible transcription factor that activates the expression of genes that support survival and process damaged proteins. Conventionally, the activation of HSF1 is considered to require trimerization, PTMs, nuclear accumulation, and the procurement of the ability to attach to DNA. However, due to the many PTMs that HSF1 is subjected to, including phosphorylation (Fig. 7), it remains to be seen what net effect phosphorylation has on its activity. Moreover, the characterization of individual phosphorylation events has identified that modifying distinct residues in HSF1 can either stimulate or inhibit its transcriptional capacity (Anckar and Sistonen, 2011). Furthermore, mutational analyses of crucial phosphorylation sites in the RD of a chimeric Gal4-HSF1 protein caused elevated transcriptional activity both in the absence of stress and during heat shock conditions (Knauf et al., 1996). Therefore, it is plausible that phosphorylation within the RD limits HSF1's activation. Additionally, analysis of how the HSR reacts to sodium salicylate posed challenges to the understanding of how phosphorylation affects HSF1, as the anti-inflammatory drug triggered DNA-binding activity; however, it neither induced hyperphosphorylation nor transcriptional activation (Jurivich et al., 1995). This result implies that hyperphosphorylation is a prerequisite for the activation of HSF1. Hence, this study aimed to determine if hyperphosphorylation in the RD is essential for the activity of HSF1 under control conditions and acute stress.

4.1 The general features of the phosphorylation-deficient HSF1 mutant

To study how hyperphosphorylation impacts HSF1, we generated a human HSF1 mutant wherein all 15 phosphorylation sites in the RD were changed to non-phosphorylatable alanine. Using this HSF1 mutant, termed HSF1Δ~PRD, allowed us to limit our investigation to the RD, which is the region needed for HSF1's stress responsiveness (Green et al., 1995; Newton et al., 1996) and harbors most of the phosphorylation sites (Anckar and Sistonen, 2011) (Fig. 7). Considering that the loss of phosphorylation sites can modify a protein's structure, stability, and dynamics (Nishi et al., 2014), we had to verify that these features remained intact in HSF1Δ~PRD. To this end, we expressed HSF1Δ~PRD, HSF1 wild-type (HSF1-WT), and an empty vector in *Hsf1*$^{-/-}$ mouse embryonic fibroblasts (MEFs), which precludes endogenous mouse HSF1 from interfering with the experimental results.

The initial characterization of HSF1Δ~PRD showed that MEFs could express the mutant, and even though numerous phosphorylation sites were eliminated, there was elevated phosphorylation resulting from heat stress (I, Fig. 1B, lane 7 *vs.* 8 and Fig. 1C, lane 5 *vs.* 6). Importantly, the stress-induced phosphorylation was markedly greater for HSF1-WT than for HSF1Δ~PRD (I, Fig. 1B, lane 6 *vs*. 8 and Fig. 1C lane 4 *vs*. 6). Moreover, exposure of cell lysates to λ protein phosphatase did not cause the HSF1Δ~PRD to shift below the migration pattern of the non-treated control sample (I, Fig. 1C lane 5 *vs*. 11 and 12). Taken as a whole, these findings suggest that HSF1Δ~PRD is susceptible to stress-induced phosphorylation,

and these phosphorylation events occur beyond the RD. It is important to note that these results were expected since it is known that HSF1 has phosphorylation sites located outside the RD (I, Fig. 1A). While we recognized that the phosphorylation of certain residues might influence our experimental data, our setup was primarily focused at exploring the net effect of hyperphosphorylation in the RD; hence we decided to proceed with additional experiments.

Subsequently, we directed our attention to ensure that the mutations in HSF1Δ~PRD did not modify its stability. Therefore, we utilized cycloheximide, an eukaryotic translation inhibitor, to measure the turnover rate for HSF1Δ~PRD (Kao et al., 2015). The results indicate that the turnover rate of HSF1Δ~PRD was equivalent to that of HSF1-WT, thus implying that phosphorylation within the RD does not impact protein stability (I, Fig. 1D). This result was noteworthy since Kourtis and coworkers have reported that phosphorylated Ser303 and Ser307 function as a docking site for FBXW7, a substrate-targeting subunit of the SCF ubiquitin E3 ligase complex, which ubiquitinates HSF1 and directs it for proteasomal degradation (Kourtis et al., 2015). While we recognized that HSF1Δ~PRD is not likely to interact with FBXW7, it is important to note that the turnover rate of HSF1Δ~PRD is likely affected by the removal of other phosphorylation sites apart from Ser303 and Ser307. Reintroducing Ser303 and Ser307 to HSF1Δ~PRD could enable one to assess how phosphorylation impacts the stability of HSF1 more thoroughly.

The next characteristic we examined was the distribution of HSF1Δ~PRD in the cell under control and stress conditions. This was important as previous reports have revealed that phosphorylation within the RD impacts protein localization; for example, phosphorylation of Ser320 can confine HSF1 in the nucleus (Murshid et al., 2010), and phosphorylation of Ser303 and Ser307 has been shown to enable 14-3-3-mediated nuclear export (Wang et al., 2003). In addition to HSF1 relocating to the nucleus during protein-damaging stress, HSF1 has been reported to form distinct substructures in mammalian cells called nuclear stress bodies (nSBs) (Jolly et al., 1997; Sandqvist et al., 2009). To ascertain if the dynamics related to the localization of HSF1 were altered in HSF1Δ~PRD, we assessed its ability to form nSBs. To accomplish this, we expressed HSF1Δ~PRD, HSF1-WT, and an empty vector in HeLa cells, subjected them to heat shock conditions, and using immunofluorescence and confocal microscopy, we were able to inspect the subcellular localization of the HSF1 proteins.

As seen in Figure 2 (I), both HSF1Δ~PRD and HSF1-WT tended to localize in the nucleus of HeLa cells under non-stress conditions. These results were expected as prior studies have shown that HSF1 tends to mainly localize to the nucleus in HeLa cells in optimal growth conditions (Mercier et al., 1999; Sandqvist et al., 2009). Importantly, upon stress, both HSF1Δ~PRD and HSF1-WT accumulated in the nucleus and formed nSBs, thus demonstrating that HSF1Δ~PRD retains the ability to bind to DNA (I, Fig. 2). These results imply that phosphorylation within the RD of HSF1 is not essential for its capacity to relocate to the nucleus, attach to DNA, and form nSBs. While these findings indicate that HSF1Δ~PRD is a functional HSF1 mutant, it is important to bear in mind that previous studies have indicated that stress-inducible phosphorylation impacts HSF1 localization. Moreover, our experimental setup only measured the net effect of phosphorylation in the RD. To assess the possibility of a single phosphorylation event predominantly impacting the

localization of HSF1, one would need to reestablish individual phosphorylation sites in HSF1Δ~PRD before further imaging experiments.

4.2 Phosphorylation within the RD does not govern the DNA-binding activity of HSF1

While the formation of nSBs confirms that HSF1 interacts with DNA, methods such as chromatin immunoprecipitation (ChIP), which does not rely on imaging, are often used to assess transcription factor-DNA binding interactions in the chromatin *in vivo*. Additionally, endogenous HSF1 in HeLa cells might cause a problem for our experimental readout since it may affect HSF1Δ~PRD's capacity to bind to DNA and facilitate the formation of nSBs. For these reasons, we decided to use ChIP to investigate the DNA-binding activity of HSF1Δ~PRD further. For this, we expressed HSF1Δ~PRD, HSF1-WT, and an empty vector in *Hsf1*$^{-/-}$ MEFs, subjected them to heat shock, and using ChIP, we measured the HSF1 occupancy at the *Hsp70* and *Hsp25* promoter (I, Fig. 3A). The results from the ChIP assays demonstrate that both HSF1Δ~PRD and HSF1-WT bind to the *Hsp70* and *Hsp25* promoters similarly in normal and heat shock conditions. Therefore, we conclude that stress-induced phosphorylation within the RD does not impact the ability of HSF1 to bind to its targets in the chromatin.

To widen our experimental setup and include additional samples, we used electrophoretic mobility shift assay (EMSA) to assess the ability of HSF1Δ~PRD and HSF1-WT to bind DNA. While it is important to note that EMSA can measure protein-DNA interactions, this method has previously been used to assess the DNA-binding capacity of HSF1 *in vitro* (Westerheide et al., 2009; Vilaboa et al., 2017). Also, while EMSA is an *in vitro* based assay, it can allow one to assess the different stoichiometry of the HSF1 trimers, which might be a factor affecting HSF1Δ~PRD's DNA-binding activity. For EMSA, we used *Hsf1*$^{-/-}$ MEFs transfected with HSF1Δ~PRD, HSF1-WT, and an empty plasmid, and the cells were treated up to nine hours with cadmium sulfate, a heavy metal compound that activates the HSR, and one set of samples were let to recover. Following the treatments, whole-cell extracts were exposed to a purified radiolabeled HSE oligonucleotide, to which HSF1 can bind. As seen from the results, during the cadmium sulfate treatment and the recovery phase, the binding pattern of HSF1Δ~PRD to the radiolabeled probe was nearly indistinguishable from that for HSF1-WT, thus implying that the lack of phosphorylation within the RD does not affect HSF1's DNA-binding activity *in vitro* (I, Fig. 3B).

Our results and conclusions are partially supported by data from a recently published study by Jin and coworkers, who explored the biological importance of phosphorylation at Ser303 and Ser307 in HSF1 in MEFs (Jin et al., 2018b). This study used an HSF1 mutant in which Ser303 and Ser307 were changed to alanine (HSF1$^{Ser303A/Ser307A}$), and similarly to our study, EMSA was used to assess the DNA-binding activity of the mutant *in vitro*. Significantly, both HSF1$^{Ser303A/Ser307A}$ and HSF1-WT displayed a near-identical binding pattern to the HSE-probe in EMSA, both during heat shock conditions and recovery, which is in line with our results. Surprisingly, in contrast to our results, the data from the ChIP assay in Jin and colleagues' study demonstrated that the loss of phosphorylation at Ser303 and Ser307 increased HSF1's DNA-binding capacity under optimal growth conditions and modest heat shock at 40ºC (Jin et al., 2018b). Yet, during acute heat stress at 42ºC, both HSF1$^{Ser303A/Ser307A}$ and HSF1-WT showed comparable occupancy at the *Hsp70* promoter, which aligns with the

findings in our study. Hence, the experimental setup we used could have been improved by including additional samples, such as heat shock at 40ºC, which would allow one to determine if modest stress exposures affect HSF1Δ~PRD and HSF1-WT differently.

Our investigation mainly employed ChIP and EMSA to assess the interaction between HSF1 and predetermined DNA sequences; however, recent advances in high-throughput sequencing technologies have made it feasible to map the interaction between transcription factors and their target sequences on a genome-wide scale. One of these methods is chromatin immunoprecipitation sequencing (ChIP-seq), which combines ChIP to isolate protein-DNA complexes and genome-wide sequencing to identify the sequences in the complexes. Interestingly, studies using ChIP-seq have demonstrated that HSF1 binds to over 600 target loci in non-stressed human breast cancer cells (Smith et al., 2022), whereas it only binds to 45 target loci in human erythroleukemic cells (Vihervaara et al., 2013). While this discrepancy in the number of HSF1 target loci might partially be attributed to the studies employing different methodologies for normalizing the ChIP-seq data, it raises the possibility that tissue-specific factors dictate the target gene preference of HSF1. Hence, it would be intriguing to use ChIP-seq to investigate whether the phosphorylation status of HSF1Δ~PRD and HSF1-WT is a factor influencing HSF1's target gene selection.

4.3 Phosphorylation of HSF1 impacts the activation threshold of the HSR

Having discovered that removing all phosphorylation sites in the RD does not affect HSF1's stability, localization, and DNA-binding activity, we investigated if the lack of phosphorylation affects its transcriptional capacity. To accomplish this, we expressed HSF1Δ~PRD, HSF1-WT, and an empty vector in *Hsf1*$^{-/-}$ MEFs, exposed them to heat shock or cadmium sulfate, and used qRT-PCR to quantify the mRNA levels of both *Hsp70* and *Hsp25* in the samples (I, Fig. 4A and B). The results show that the expression of both *Hsp70* and *Hsp25* were similar between the HSF1Δ~PRD and HSF1-WT samples under optimal growth conditions (I, Fig. 4A and B). This important result demonstrates that lack of phosphorylation within the RD does not enhance the basal transcriptional capacity of HSF1, which implies that HSF1Δ~PRD is not spontaneously activated and therefore retains the ability to become active upon stress. Remarkably, HSF1Δ~PRD-expressing cells displayed higher levels of *Hsp70* and *Hsp25* than HSF1-WT cells upon heat stress and cadmium sulfate exposure (I, Fig. 4A and B). These results demonstrate that phosphorylation within the RD is not essential for activating HSF1 during acute stress. Furthermore, our findings suggest that stress-inducible phosphorylation in the RD suppresses HSF1's transcriptional capacity, which contradicts the notion that HSF1 requires this phosphorylation to maximize its transcriptional capacity.

Although our findings support a model in which phosphorylation mainly limits HSF1's transcriptional capacity, one needs to consider that prior studies have demonstrated that phosphorylation of certain residues can increase its activity. Therefore, to assess this possibility, future studies should examine if altering the phosphorylation sites in the RD to phosphorylation-mimicking residues, such as aspartic or glutamic acid, would reduce HSF1's transcriptional capacity during acute stress. A different approach to analyzing the effect of phosphorylation in HSF1 would involve restoring individual phosphorylation sites to HSF1Δ~PRD. For instance, restoring Ser303 and Ser307, which limit HSF1's activity, might equalize the transcriptional capacity of HSF1Δ~PRD to that of HSF1-WT. Alternatively,

reestablishing sites that promote the activity of HSF1, such as Ser326, might further enhance the transcriptional capacity of HSF1Δ~PRD. By using this systematic approach, it may be possible to discern which phosphorylation site predominantly affects HSF1's transcriptional capacity. Furthermore, contingent on how significantly the site impacts the transcriptional capacity of HSF1, it may be possible to determine if the nature of phosphorylation is mainly suppressive.

It is important to note that, aside from the mutations in the RD, HSF1Δ~PRD has intact phosphorylation sites situated in regions such as the TAD (I, Fig. 1A). Hence, it is conceivable that some of the other phosphorylation sites might have gained new functional importance in regulating HSF1Δ~PRD transcriptional capacity. For example, in the transcription factor c-Myc, it has been shown that the deletion of one phosphorylation site influences the phosphorylation of another site, thus changing c-Myc activity (Padmanabhan et al., 2013). Importantly, the possibility of mutations in the RD affecting the phosphorylation status of residues in adjacent domains was a consideration in our study; hence we performed additional experiments to address the issue, which will be discussed in section 4.4.

One potential explanation for the significant difference in transcriptional capacity between HSF1Δ~PRD and HSF1-WT may be due to a change in the interaction between HSF1 and HSF2. This interaction occurs when HSF1 and HSF2 assemble heterotrimers, and it has been demonstrated that HSF2-null MEFs have a diminished ability to induce the mRNA expression of *Hsp70* and *Hsp25* during heat shock (Östling et al., 2007). Therefore, one hypothesis is that the different composition of HSF1Δ~PRD and HSF2 heterotrimers affects the ability of HSF1Δ~PRD-expressing cells to induce the expression of *Hsp70* and *Hsp25* in heat shock environments. However, immunoprecipitation experiments would be required to assess the interaction between HSF1Δ~PRD and HSF2 *in vivo*.

To further explore HSF1Δ~PRD's impact on gene regulation, we performed additional experiments with a setup much like that in Figure 4 (I); however, we altered the temperature, cadmium sulfate concentration, and some of the treatment durations (I, Fig. 5). This modified approach allowed us to determine if the mutations in HSF1Δ~PRD affected its activation threshold, a limit at which point the basal activity of HSF1 is surpassed. To our surprise, we discovered that a modest heat shock at 40ºC for 60-minutes significantly induced the expression of *Hsp70* in HSF1Δ~PRD-expressing cells, whereas HSF1-WT-expressing cells did not display a significant induction at that time (I, Fig. 5A middle). Importantly, we noted that HSF1-WT-expressing cells required 60 minutes of heat shock at 41ºC to significantly induce the expression of *Hsp70*, whereas HSF1Δ~PRD-expressing cells only required 30 minutes (I, Fig. 5A lower). Similarly, to modest heat stress at 40ºC, a cadmium sulfate treatment of 40 µM for 3 hours also induced the expression of *Hsp70* in HSF1Δ~PRD-expressing cells, while HSF1-WT-expressing cells did not exhibit a similar response (I, Fig. 5B upper). Together, these results demonstrate that stress-inducible phosphorylation in the RD determines the activation threshold for HSF1 in different stress environments.

Although provocative, these results are supported by a recently published study by Jin and coworkers, which was mentioned briefly in section 4.2 (Jin et al., 2018b). They demonstrated that replacing the phosphorylation sites Ser303 and Ser307 in the endogenous mouse HSF1 reduced its activation threshold upon heat stress. According to ChIP assays, the decrease in HSF1's activation threshold was attributed to promoting the DNA-binding activity *in vivo*

and the recruitment of RPA1 to the chromatin. RPA1 is a chromatin remodeler that interacts with HSF1 and promotes a transcriptionally active chromatin configuration (Fujimoto et al., 2012). Given the findings in Jin and colleagues' study, using ChIP to re-evaluate the DNA-binding capacity of HSF1Δ~PRD under mild heat shock conditions would be interesting. The altered activation threshold displayed by HSF1Δ~PRD would likely manifest as an increase in the DNA-binding activity during mild stress. Additionally, since RPA1 interacts with HSF1 by binding to its wing domain, a region situated in the DNA-binding domain (Fujimoto et al., 2012), it raises the possibility that the lack of phosphorylation in HSF1Δ~PRD promotes the HSF1-RPA1 interaction, thus helping HSF1Δ~PRD to access the *Hsp70* promoter.

Moreover, the lack of phosphorylation in HSF1Δ~PRD might influence its ability to promote the release of paused Pol II at the *Hsp70* promoter, a critical step in the transcriptional induction of *Hsp70* during stress. This possibility is supported by the results of previous studies, which demonstrate that HSF1 can interact with certain components of the transcriptional machinery, thereby controlling the rate at which Pol II is released from the paused state (Fujimoto et al., 2012; Kim and Gross, 2013; Takii et al., 2019). To evaluate the ability of HSF1Δ~PRD to facilitate the release of Pol II at the *Hsp70* promoter, one would need to use assays such as nuclear run-on, a method that measures transcription initiation rates at individual genes.

4.4 Phosphorylation determines the capacity of the RD to influence HSF1's activity

To investigate the intrinsic stress responsiveness in the RD of HSF1Δ~PRD and exclude the possibility that phosphorylation sites residing outside the RD might have gained new functional importance in regulating HSF1Δ~PRD's transcriptional capacity, we made a set of different fusion proteins that we surveyed in a reporter assay (I, Fig. 6B). In the technical aspect, we cloned the RD of HSF1-WT and HSF1Δ~PRD into chimeric constructs, which comprised of the Gal4 DNA-binding domain (GAL4) fused to the herpes simplex virus 1 VP16 transactivation domain (VP16) (I, Fig. 6A). These GAL4-HSF1-VP16 constructs, and a GAL4-driven luciferase reporter gene, were transfected into HeLa cells. Next, transfected cells were either exposed to heat shock or kept under optimal growth conditions, and following a 5-hour recovery from the heat shock, the reporter activity was measured in all samples.

A significant result from our luciferase reporter assay was that inserting the wild-type RD into the GAL4-VP16 construct caused a decrease in the basal expression of the reporter gene (I, Fig. 6B), indicating that the RD suppresses the activation of the construct under non-stress conditions. It is noteworthy that a previous study of the wild-type RD, with a setup nearly identical to ours, yielded similar results (Newton et al., 1996). Another key finding was that the reporter gene expression at control conditions was comparable in the GAL4-VP16 construct containing the RD from HSF1Δ~PRD to that of the control construct without an RD (I, Fig. 6B). Taken together, these observations led us to conclude that phosphorylation is required for the RD to suppress the transactivation domain of VP16 under control conditions. This conclusion is partially supported by the work of Knauf and colleagues, who employed a similar experimental design as ours with the difference that they used several constructs containing different mutations in the RD (Knauf et al., 1996). They discovered

that the phosphorylation sites Ser303 and Ser307 allow the RD to suppress the transactivation domain under normal growth conditions; thus, the lack of these important phosphorylation sites in the RD from HSF1Δ~PRD may contribute to the altered function of the domain in a non-stress environment.

The results we obtained from the reporter assay, following the 60-minute heat shock and 5-hour recovery (I, Fig. 6B), demonstrated that the GAL4-VP16 constructs, containing either the RD from HSF1-WT or HSF1Δ~PRD, could induce the expression of the reporter gene, thus suggesting that both domains retained the ability to react to heat stress. However, the reporter gene induction was significantly higher in cells expressing the construct with the RD from HSF1Δ~PRD than with the wild-type RD (I, Fig. 6B). Based on these findings; we surmised that the phosphorylation status in the RD is an integral factor that controls the ability of HSF1 to become active during heat stress. Furthermore, because the RD from HSF1Δ~PRD could enhance the function of a viral transactivation domain (VP16) during stress, it is unlikely that the lack of phosphorylation in the RD influences the other phosphorylation sites residing outside the RD in the human HSF1.

One factor that might contribute to the stress responsiveness of HSF1Δ~PRD is SUMOylation. The main SUMOylation site in HSF1 is Lys298, and its SUMOylation is dependent on Ser303 being phosphorylated (Hietakangas et al., 2006). It is important to note that a prior investigation of these sites had demonstrated that the removal of either Lys298 or Ser303 promotes the basal activity of a GAL4-HSF1 chimeric construct (Hietakangas et al., 2006), a result similar to when the RD from HSF1Δ~PRD was inserted into the GAL4-VP16 construct in our experiments. Moreover, the same study found that removing Lys298 from HSF1 elevated its transcriptional capacity during proteotoxic stress conditions caused by proteasomal inhibition (Hietakangas et al., 2006). Considering the previous results and our findings, it is unlikely that Lys298 can be SUMOylated in HSF1Δ~PRD since the alteration of Ser303 impairs the PDSM, a motif for phosphorylation-dependent SUMO modification. Despite this, there is still a chance that Lys298 is SUMOylated in HSF1Δ~PRD via an unknown mechanism. Hence it would be interesting to investigate if mutating Lys298 would impact HSF1Δ~PRD's transcriptional capacity.

4.5 Can abnormal phosphorylation of HSF1 have implications for the development of cancer and metabolic diseases?

During the past two decades, scientists have realized the significance of HSF1 in malignant transformation. Dai and colleagues' work on the function of HSF1 in tumor development is one of many paramount studies. They showed that HSF1-null mice are resistant to cancer caused by either chemicals or gene modifications (Dai et al., 2007). It is also important to note that several studies have demonstrated that HSF1 is overexpressed in numerous types of cancer, which promotes the expression of pro-survival genes, including *Hsps* (Puustinen and Sistonen, 2020). Also, abnormal regulation of HSF1's expression has been attributed to triggering HSF1-dependent cancer-specific transcriptional programs, which promote different pro-oncogenic features, including increased proliferation and migration (Mendillo et al., 2012). Taken together, these findings justify why multiple therapeutical strategies, such as drug treatments, are currently being developed to target HSF1.

However, it is still unclear if there is a primary mechanism that directs HSF1's activities in cancer. One contributing factor credited to promoting the oncogenic function of HSF1 is the phosphorylation of Ser326, a site that is known to promote HSF1's transcriptional capacity and is therefore viewed as an important biomarker in several types of cancer (Mendillo et al., 2012; Chou et al., 2015; Pastorek et al., 2018; Vydra et al., 2019). Furthermore, the activity of HSF1 in cancer has been shown to increase due to the action of protein phosphatases that dephosphorylate key sites that would otherwise suppress HSF1's activation (Asano et al., 2016). Even though we lack comprehensive studies that address how these modifications impact tumorigenesis in animal models, one recent study has demonstrated that silencing Ser303 and Ser307 in endogenous mouse HSF1 activates an HSF1-dependent metabolic program that promotes obesity, insulin resistance, and development of fatty liver diseases (Jin et al., 2018b). While additional studies are needed to uncover how PTMs influence HSF biology in humans, Jin and colleagues' study highlights how the disruption of a post-translational regulatory mechanism may impact HSF1's function and contribute to the development of metabolic diseases.

Importantly, the results in our study demonstrate that lack of phosphorylation within the RD, which includes sites such as Ser326, Ser303, and Ser307, is not essential for the activation of HSF1, implying that cancer cells might modulate the activity of HSF1 using mechanisms other than those that rely on PTMs. While we used a focused and straightforward experimental approach to investigate the influence of phosphorylation on HSF1's function, we acknowledge that our results may depend on tissues and cell lines. Furthermore, we used transient transfections to introduce exogenous HSF1 into cells, which generates some drawbacks, including deviating transfection efficiency and short-term gene expression. Therefore, the methods we applied in our study can be improved by implementing the CRISPR/Cas9 system to precisely knockout phosphorylation sites in the endogenous HSF1. Moreover, a comprehensive selection of different cell lines that contain either single or multiple mutations in the RD would enable one to assess how hyperphosphorylation influences HSF1's activity more accurately.

5 HSF1 and HSF2 modify transcription under distinct stress stimuli (II)

Cellular stress responses have fundamental importance in counteracting various destructive conditions for macromolecules. Heat and oxidative stress are major stress conditions, and disruption of regulatory pathways that manage these forms of stress is common in cancer and neurodegenerative diseases (Gomez-Pastor et al., 2018; Dodson et al., 2019). Individual stress responses are governed by transcription factors that orchestrate complex transcriptional programs, which help cells maintain homeostasis. Transcription factors, including NRF2 and forkhead box subclass 0 proteins (FOXOs), have major roles in antioxidant defense (Soh et al., 2021; Cyran and Zhitkovich, 2022b), while HSFs primarily control gene expression upon heat stress. Remarkably, HSF1 can be activated in oxidative stress conditions (Ahn and Thiele, 2003), and some of its target genes encode HSPs, including HSP70, which can assist in both protein folding and the removal of oxidized proteins that are damaged beyond repair (Reeg et al., 2016). In contrast to HSF1, the involvement of HSF2 in oxidative stress is not well-established. However, because HSF2 and HSF1 can form heterotrimers (Sandqvist et al., 2009), and they share some target genes during heat stress (Östling et al., 2007; Vihervaara et al., 2013), HSF2 likely requires HSF1 to regulate the expression of genes in various stress environments. The cooperation between stress factors becomes more complex

in stress regulation, considering that HSF1 and NRF2 share some of their target genes (Cyran and Zhitkovich, 2022b). Additionally, the expression of HSF1 is regulated by NRF2 (Paul et al., 2018), and recently HSF1 was found to modulate the expression of FOXO3, a transcription factor involved in antioxidant defense (Grossi et al., 2018). Together these observations suggest that the interplay between transcription factors is fundamental for the cell's capacity to manage stress.

To understand how HSF1 and HSF2 regulate transcription under heat and oxidative stress, we used precision nuclear run-on sequencing (PRO-seq) to map the genome-wide induction of nascent transcripts. Therefore, this method enabled us to precisely quantify Pol II-mediated transcription throughout the entire genome, providing insight into which genomic regions are actively being transcribed (Mahat et al., 2016b). To the best of our knowledge, this technique had not previously been implemented to assess transcriptional programs linked to the oxidative stress response. Still, this method has been verified to work in analyzing the transcription of genes during heat stress (Mahat et al., 2016a; Vihervaara et al., 2017). It is noteworthy that, in difference to previous studies, our study also focused on exploring the role of HSF2 in different stress environments. Moreover, our experimental setup enabled us to identify and characterize both the genes and enhancers regulated by HSF1 and HSF2 alone or together.

5.1 Different stress environments prompt the activation of transcriptional networks that involve genes and enhancers

To be able to study how HSF1 and HSF2 regulate stress-responsive genes and enhancers, we first had to use PRO-seq to map the genome-wide transcriptional shift that occurs under heat and oxidative stress. For this, we exposed MEFs to either a 1-hour heat shock at 42°C or 30 μM menadione for 2 hours. Menadione (MD) is a synthetic naphthoquinone derivative that causes oxidative stress when the reduced intermediate reacts with oxygen, creating superoxide. Enzymatic processing of superoxide yields hydrogen peroxide and other reactive oxygen species (ROS), which oxidize and damage cellular macromolecules (Woods et al., 1997). It should be noted that MEFs tolerate MD exposure and can recover even after being exposed to a highly concentrated dose (Ghodsi Senejani et al., 2019).

The results from our PRO-seq analysis revealed that heat shock and oxidative stress both induced and repressed thousands of genes and enhancers, signifying extensive stress-specific transcriptional shifts from the normal condition (II, Fig. 1A). As expected, both stress treatments predominantly downregulated the expression of genes, which is in line with previous studies demonstrating that heat shock has this type of effect on transcription (Mahat et al., 2016a; Vihervaara et al., 2017). Importantly, this result affirms the notion that transcriptional repression is required upon stress, and while the reason for this is unknown, a reduction in general gene expression may encourage Pol II to prioritize the transcription of stress-alleviating targets.

Next, we combined data from individual stress profiles to determine which targets are affected by both stress treatments (II, Fig. 1B). The comparison demonstrates that out of the induced targets, at least 600 genes and more than a thousand enhancers were shared targets (II, Fig. 1B). However, most of the genes and enhancers do not overlap, which demonstrates that elevated temperature and oxidative stress invoke stress-specific shifts in transcription.

Subsequently, to gain mechanistic insight into how acute stress affects the release and recruitment of Pol II at promoters and enhancers, we compared the average distribution of Pol II at the stress-inducible targets. Our findings illustrate that both heat stress and MD exposure stimulate the recruitment of Pol II to the promoter-proximal region, and the dispersal of Pol II across the region exhibits a similar pattern (II, Fig. 1C). As anticipated, the stress treatments affected the recruitment of Pol II at enhancers, but in contrast to the genes, upregulated enhancers did not display paused Pol II prior to stress, and downregulated enhances lost their Pol II following stress induction (II, S5A). These Pol II binding patterns align with those previously described during heat stress (Mahat et al., 2016a; Vihervaara et al., 2017). Hence, we concluded that the mechanisms that modulate Pol II recruitment and release at genes and enhancers operate similarly during different stress conditions.

Further analysis of Pol II's distribution across genes revealed that MD treatment causes Pol II to accumulate both at the promoter-proximal regions and early gene bodies (II, Fig. 1D, 0-0.6 and 1-2 kb away from transcription start site [TSS]), whereas heat stress caused Pol II to be more equally distributed throughout distal parts of the gene body (Fig. 1D, 2-4 kb away from TSS). Since this result indicated a problem in the release of paused Pol II during the MD treatment, we did an additional analysis of the start of the genes (0.5-2.5 kb relative to the TSS) and end of the genes (-2-0 kb relative to the cleavage and polyadenylation site [CPS]) (II, Fig. 1E). Together the distribution of Pol II across these regions functions as a readout for completed transcription of a gene. This analysis revealed that MD-treated cells had less Pol II at the end of the genes when compared to heat shock, thus implying that Pol II could not complete the transcription of several MD-responsive genes, including the gene encoding calcylin-binding protein (*Cacybp*) (II, Fig. 1F). However, transcription likely reached completion in 37% of the MD-inducible genes, as estimated by Pol II occupation at the beginning and end of the gene. Therefore, MD treatments are unlikely to lead to a complete transcriptional blockage.

One potential explanation for why MD treatments disrupt transcription could be due to DNA damage, which is known to affect Pol II (H. Lu et al., 2022). To rule out this possibility, we performed additional experiments in which we exposed MEFs to heat shock, MD, or the DNA-damaging agent hydroxyurea, and subsequently investigated the expression of the DNA damage marker phosphorylated histone H2A.X (ɣH2A.X) using immunofluorescence and confocal microscopy (II, S6). Significantly, we found elevated expression of ɣH2A.X in MD-treated cells, which signifies DNA damage. In conjunction with the Pol II distribution profile (II, Fig. 1D and E), this result indicates that MD causes DNA damage in MEFs, and may stall the transcription in stress-responsive genes. Still more experiments are required to address this issue; for instance, a ChIP-seq for ɣH2A.X might be used to determine if the DNA damage sites are located within the genes that display stalled Pol II at early gene bodies following MD exposure.

Prior studies have demonstrated that MD causes modest DNA damage (Woods et al., 1997; Ghodsi Senejani et al., 2019), yet, MEFs can cope with this damage following a recovery period. Also, since ROS display high reactivity (Beckman and Ames, 1997), it would be challenging to induce oxidative stress without causing some DNA damage. Moreover, oxidative stress caused by hydrogen peroxide has been shown to stabilize promoter-proximal paused Pol II (Nilson et al., 2017), which affects the ability of Pol II to engage in

transcription. To exclude the possibility that the MD treatments we used in our study caused Pol II to stall at the promoter-proximal regions, we would need to use PRO-seq to examine transcription at multiple timepoints of MD exposure. This methodology would allow one to assay the Pol II distribution across the gene as a function of time, which directly correlates with the transcription rate. Furthermore, it is possible to promote the time-dependent clearance of Pol II from the promoter and gene body using flavopiridol and triptolide, which inhibit Pol II release and recruitment at genes, respectively (Chao and Price, 2001; Vispé et al., 2009). Thus, combining the use of these compounds, multiple timepoints, and PRO-seq would provide more insight into the advancement of transcription across the gene body of the MD-responsive genes.

5.2 HSF1 and HSF2 modulate transcription during acute stress

Having identified all targets that responded to acute stress, we proceeded with experiments to determine which genes and enhancers are directly regulated by HSF1 or HSF2 alone or together. To determine this, we exposed wild-type MEFs, HSF1-null MEFs, and HSF2-null MEFs to either 30 μM MD for 2 hours or heat shock at 42ºC for 1 hour and afterward used PRO-seq to measure transcription. Additionally, since PRO-seq alone is unable to distinguish transcriptionally active enhancers directly, we had to compare our PRO-seq data to previously published ChIP-seq data (Heintzman et al., 2007; Creyghton et al., 2010) that mapped the genomic distribution of H3K27ac and H3K4me1, which are markers for active enhancers (II, S4). Based on the analysis of the PRO-seq data, we identified hundreds of stress-responsive genes and enhancers that were either induced or suppressed in an HSF1- or HSF2-dependent manner (II, Fig. 2A and B).

Next, we performed ChIP-seq to identify the binding sites of HSF1 and HSF2 during heat and oxidative stress. The results revealed that HSF1 and HSF2 bind genes and enhancers in each stress environment. However, they bound more targets upon oxidative stress (II, Fig. 2D). Interestingly, HSF2 interacted with more sites in control conditions than HSF1, and HSF1 was the predominant HSF bound to genes and enhancers during oxidative stress (II, Fig. 2D). Although a transcription factor can bind multiple target loci, it has no guarantee to influence the transcription rate at those sites. Therefore, to distinguish which genes and enhancers are directly regulated by HSF1 or HSF2, we compared the ChIP-seq data with the PRO-seq profiles from wild-type MEFs. These comparisons demonstrate that HSF1 influenced several genes and enhancers under both stress conditions, whereas HSF2 by itself only influenced a small number of genes and enhancers under oxidizing conditions (II, Fig. 2E). It is noteworthy that HSF2 alone has no substantial effect on transcription during heat stress (II, Fig. 2D HSF2 peaks: C *vs*. HS, and Fig. 2E). Hence, one possibility is that HSF2 operates mainly as a modulator of transcription under elevated temperatures, which is in line with previous findings (Östling et al., 2007). Still, our data analysis collectively demonstrates that both HSF1 and HSF2 are important transcription factors in oxidative stress conditions.

From the comparison of the PRO-seq and ChIP-seq data, an important finding was that neither HSF1 nor HSF2 bound to most of their respective genes and enhancers upon stress (II, Fig. 2E). This implies that a significant number of the HSF1 and HSF2 controlled targets are primarily regulated by indirect mechanisms that likely include other transcription factors. This type of control has been proven to have an impact on HSF1. For instance, the

transcription factor NFATc2 has been shown to cooperate with HSF1 in regulating the expression of certain *Hsps* during stress in MEFs (Hayashida et al., 2010).

Besides regulating transcription directly at the interface of promoters and genes, HSFs likely govern some of their genes indirectly via HSF-bound enhancers. While it is possible to use computational models to approximate whether a transcription factor in an enhancer impacts transcription at a nearby gene, it is challenging to measure this interaction directly. One method that can be applied to measure HSF1-dependent promoter-enhancer contacts is paired-end tag sequencing (ChIA-PET) (Capurso et al., 2020), an adapted variation of ChIP-seq that allows for detecting chromosomal loops across the genome. Hence, the use of this method should be considered in future studies.

It is important to recognize that stress responses are conserved within a species, but it is possible that both HSFs failed to bind many targets because the MEFs used in this study originate from two different mouse strains. Hence, when comparing the sequencing data from wild-type, HSF1- and HSF2-null MEFs, any discrepancies arising from genotype differences could generate inaccurate data, thus influencing our results. A way to improve our experimental setup and minimize the impact of genotypic variation is to include transient transfections to cause short-term depletion of the HSFs in wild-type MEFs. Hence, this approach would guarantee that the transfected cells had intact stress responses pre-transfection. Remarkably, the deletion of HSFs from cancer cells has been reported to alter the transcriptome permanently; however, transient knockdowns can reduce this impact (Smith et al., 2022).

5.3 HSF1 and HSF2 regulate transcription independently and together

Given the ChIP-seq results, which showed that HSF2 primarily binds target genes during oxidative stress, as opposed to heat shock (II, Fig. 2D HSF2 peaks), additional data analysis was done to evaluate both HSF1's and HSF2's capacity to operate either independently or together. Remarkably, nearly all HSF2's heat-responsive target genes were also bound by HSF1 (II, Fig. 3B), except for *Ptges3*, a co-chaperone for HSP90 (Weaver et al., 2000). Furthermore, the finding that HSF1 also bound all enhancers bound by HSF2 upon heat stress emphasizes how HSF2 is likely dependent upon HSF1 to modulate transcription (II, Fig. 3B).

Interestingly, analysis of the ChIP-seq and PRO-seq data also revealed that HSF2 alone bound to and regulated some genes and several enhancers during MD exposure, thus demonstrating that HSF2 can independently modulate genes under oxidizing stress conditions (II, Fig. 3A), and this marks the first instance where HSF2 has been demonstrated to function independently as a transcription factor. It is also worth noting that the majority of HSF1's target genes and enhancers were governed in an HSF1-dependent manner both during heat and oxidative stress, indicating that HSF1 is the most prominent HSF during acute stress, whereas HSF2 requires HSF1 to regulate transcription at elevated temperatures (II, Fig. 3A and B). Collectively, our findings imply that both HSF can work independently and together in a synergistic manner to regulate gene expression during acute stress. This notion is partly supported by a recent report demonstrating that HSF1 and HSF2 cooperate in regulating transcriptional programs involved in tumorigenesis (Smith et al., 2022). Furthermore, it is important to keep in mind that the mechanisms governing the DNA-binding selectivity of HSFs are still unknown. Therefore, we wanted to explore the possibility that variations in the

HSE-binding motifs could influence the target specificity of HSF1. However, while HSF1 bound to a significant number of targets, our analysis found that canonical HSEs were as pervasive as non-canonical HSEs in both genes and enhancers during acute stress (II, Fig. 3F). Hence, different HSE-binding motifs are unlikely to affect the target specificity of HSFs upon stress.

Another important factor to consider that might affect the DNA-binding activity of HSFs during stress is PTMs. For instance, exposure to hydrogen peroxide has been reported to cause the oxidation of two key cysteine residues within the HSF1's DBD, prompting nuclear accumulation, trimer formation, and increased DNA-binding activity (Ahn and Thiele, 2003). While HSF2 does not appear to be subjected to similar PTMs during oxidizing stress, it is interesting to speculate that the DNA-binding activities of HSF1-HSF2 heterotrimers might be affected by oxidized HSF1. Additionally, the redox protein thioredoxin (TRX) has been shown to interact with HSF1 during oxidative stress, thereby increasing the DNA-binding capability of HSF1, but, TRX does not affect HSF2 (Jacquier-Sarlin and Polla, 1996). Taken together, it is conceivable that HSFs possess different DNA-binding capacities due to their distinct interacting partners and PTMs in various stress environments.

While acknowledging that interacting partners affect the DNA-binding activities of HSFs, it is important to remember that the intrinsic DNA-binding domain is the critical segment required for HSFs to bind to DNA. Hence, one possible cause for why HSF2 has predominantly shared targets with HSF1 during heat and oxidative stress may be attributed to the wing domain, which is situated within the DBD of each human HSF. Co-crystal structures of the DBDs in HSF1-HSF2 heterotrimers show that the wing domains help to arrange the trimers at the DNA, and the flexibility of the domain is a contributing factor to the trimer's DNA-binding specificity (Feng et al., 2021). Additionally, exchanging the wing domains between HSF1 and HSF2 has been established to impact their ability to bind their target sequences (Jaeger et al., 2016). In conclusion, since the minor variations in the wing domains influence the DNA-binding activities of each HSF, it is feasible that these differences also impact the target specificity of various HSF trimers in different stress environments.

5.4 HSF1 is a prominent driver of distinct transcriptional programs

Given that HSF1 regulates more genes and enhancers than HSF2, we conducted additional analyses of the HSF1-dependent targets. These analyses revealed that HSF1 prefers to bind to and regulate a set of genes specifically upon oxidative stress, demonstrating that a particular stress stimulus can trigger a specific HSF-driven transcriptional program (II, Fig. 3D, S1. Table). Additionally, the analyses revealed a large group of targets to which HSF1 bound during heat and oxidative stress, but HSF1 could only regulate their transcription at elevated temperatures (II, Fig. 3E, and S1. Table). Hence, one hypothesis is that PTMs or interacting partners direct the ability of HSF1 to modulate transcription in different stress environments.

Even though some of these potential regulatory mechanisms affecting DNA-binding specificity were discussed in section 5.3, oxidation of cysteines in HSF1 has not been shown to influence its transcriptional capacity, unlike PTMs such as phosphorylation (Anckar and Sistonen, 2011). It is important to note that HSF1 is likely hyperphosphorylated during the

stress treatments used in our study, as evidenced by the retarded migration of HSF1 in the SDS-PAGE (II, S.1. Fig: C *vs*. HS or MD). A similar migration pattern of HSF1 is seen in Figure 1C (I), a result discussed in section 4.1. For these reasons, the phosphorylation status of HSF1 may impact the ability of HSF1 to regulate transcription at genes and enhancers during oxidative stress. While the influence of phosphorylation on HSF1 is not established in this stress condition, some proteins attributed to impact HSF1 are activated during oxidizing conditions. For instance, hydrogen peroxide has been demonstrated to both induce the expression and promote the function of DJ-1, a protein with antioxidant activity and modulator of several signaling pathways, including ERK1/2, which modulates the activity of HSF1 via phosphorylation (Chu et al., 1996; Gu et al., 2009). Interestingly, DJ-1 can also interact with Daxx, an HSF1-interacting partner that modulates HSF1's transcriptional capacity (Boellmann et al., 2004; Junn et al., 2005). However, the nature of the Daxx-HSF1 interaction during oxidative stress is unknown. Taken together, the upstream signaling events and interacting partners that regulate HSF1 activation during oxidative stress still need to be elucidated, highlighting the necessity of further investigations.

5.5 HSF1 and HSF2 utilize enhancers to adjust transcription

Since transcription factors can utilize enhancers for the long-ranged regulation of genes, we investigated the possibility that HSF-bound enhancers influenced the transcription of surrounding genes upon heat shock conditions and oxidative stress. It is important to note that we restricted the analysis to HSF1's targets since HSF2 regulated only a few genes and enhancers (II, Fig. 3A and B, S1. Table). The analysis of the heat stress-responsive enhancers revealed that a significant number of HSF1-regulated genes resided within 100 kb of HSF1-regulated enhancers (II, Fig. 4A). Additionally, while HSF1 influenced the expression of these genes, most of them did not have HSF1 bound at the promoter, thus suggesting that it regulates these genes through enhancers. Similar results were observed during oxidative stress, in which MD-responsive genes required HSF1 for their activity, even though the nearest HSF1-binding site was at the closest HSF1-regulated enhancer (II, Fig. 4A). However, it is important to note that the distance between the genes and enhancers was not the determining factor for their HSF1-dependent regulation.

Since binding of HSF1 to the chromatin affects the recruitment and release of Pol II from the paused state (Takii et al., 2019), it was important to examine the distribution of Pol II across the gene bodies of the HSF1 indirectly-regulated genes that were located 100 kb away from HSF1-regulated enhancers (II, Fig. 4B). Our results showed that when heat stress initially promoted the binding of HSF1 to the enhancers, it consequently resulted in the increased recruitment and release of Pol II at the nearby genes (II, Fig. 4B). Next, we wanted to validate the importance of HSF1's effect on the transcription of the HSF1 indirectly-regulated genes. Therefore, we compared the distribution of Pol II at the targets in wild-type MEFs and HSF1-null MEFs. This comparison established that the lack of HSF1 disrupted the release of Pol II in all the analyzed sites (II, Fig. 4B). Therefore, based on these findings, we concluded that HSF1 modulates the expression of certain stress-responsive genes by activating enhancers rather than promoters.

To gain a general perspective of which biological processes were affected by the HSF1-dependent transcriptional programs upon heat induction, we performed gene ontology (GO) term analyses of genes directly regulated by HSF1, as well as those whose activation relied

on enhancers (II, Fig. 4C). As expected, genes regulated directly by HSF1 were associated with processes including protein folding and cellular stress responses. Remarkably, the genes located near enhancers had GO terms associated with transmembrane receptor-linked signaling pathways and focal adhesion (II, Fig. 4C). Two genes included in the latter category are filamin b (*Flnb*) and membrane-associated guanylate kinase, WW and PDZ domain containing 1 (*Magi1*) (II, Fig. 4D and S9B). Interestingly, *Magi1* displayed two sites of paused Pol II, like the promoter of the *B4galt1* gene (II, S9A). However, this type of Pol II distribution has previously been found in numerous genes containing multiple TSSs (Carninci et al., 2006).

Our findings from the GO term analyses agree with prior studies, showing that HSF1 manages the chaperone network during heat stress (Mahat et al., 2016a; Vihervaara et al., 2017). Yet, the ability of HSF1 to use enhancers to modulate a network of genes connected to the plasma membrane and adhesion is a novel finding. HSFs have been recognized to influence the expression of cell-adhesion-related genes (Joutsen et al., 2020; Smith et al., 2022); however, whether they employ enhancers to modulate this process remains to be established. For these reasons, it would be important in future studies to determine how the HSF1-dependent enhancers mechanistically modulate the expression of nearby genes. For instance, Hi-C could be used to map the enhancer-promoter interactions genome-wide (Belton et al., 2012). Thus, this technique could be utilized to determine how specific stress conditions affect the ability of HSFs to drive transcription through the enhancer-gene interfaces. Furthermore, the function of an individual stress-responsive enhancer can be assessed using a combination of CRISPR interference (CRISPRi), a technique that enables sequence-specific repression, and nuclear run-on, a method that measures transcription initiation rates (Smale, 2009; K. Li et al., 2020).

6 The decrease of HSF2 facilitates the activation of pro-tumorigenic transcriptional programs during EMT (III)

HSFs are predominantly recognized for their ability to induce the expression of chaperones during various stress conditions, and in the past two decades, this feature has become recognized to affect malignant transformation. For example, various cancer types have been evidenced to hijack HSF-regulated transcriptional programs to promote tumorigenesis, thus reprogramming metabolism and inducing the chaperone networks, and enhancing migration and proliferation (Puustinen and Sistonen, 2020). While most studies have focused on the oncogenic functions of HSF1, a few studies have included HSF2 in their research. One pivotal study conducted by Björk and colleagues demonstrated that HSF2 suppresses tumor invasion of prostate cancer cells, and the progression of cancer development correlated with a decrease in the expression of HSF2 (Björk et al., 2016). This study also demonstrated that several EMT markers were elevated in cancer cells displaying low levels of HSF2. Similarly, bone cancer cells lacking HSF2 have been shown to display abnormal cadherin expression and disrupted cell-cell adhesion, indicating that HSF2 is important for cell-cell adhesion contacts in this cell type (Joutsen et al., 2020). These findings suggest a link between HSF2 and EMT, which is the primary process destabilizing epithelial cell-cell junctions and induces the expression of mesenchymal proteins (Huber et al., 2005). Building upon the findings of these two studies, we wanted to investigate the effect of EMT-inducing stimuli on HSF2's expression. We also wanted to determine if the induction of EMT affects HSF2's ability to control gene expression in epithelial cells.

6.1 EMT-inducing cytokines cause a decrease in HSF2 levels

To assess if EMT-inducing compounds can impact the expression of HSF2, we treated two breast epithelial cancer cell lines (HS578T and MDA-MB-231) and one non-transformed breast epithelial cell line MCF10A with an EMT-inducing supplement for 24 hours. The supplement contained: the EMT-inducing cytokines TGF-β1 (hereafter referred to as TGF-β) and Wnt family member 5A (Wnt5a), as well as antibodies that target various repressors of the Wnt signaling pathway. Because the EMT-inducing supplement can activate both the TGF-β and Wnt signaling pathways, some samples were additionally treated with SB431542, a small molecule inhibitor of the TGF-β type I receptor (Inman et al., 2002). The combined use of the supplement and the inhibitor allowed us to delineate which pathway might predominantly affect the expression of HSF2. Interestingly, the results demonstrate that the EMT-inducing supplement caused the downregulation of HSF2, indicating that EMT induction suppresses the expression of HSF2 (III, Fig. 1B). Furthermore, cells treated with SB431542 expressed normal levels of HSF2, which suggests that the TGF-β signaling pathway directs the signal transduction resulting in the downregulation of HSF2.

Next, to verify that the TGF-β signaling pathway alone can suppress the expression of HSF2, we treated HS578T, MDA-MB-231, MCF10A cells, all of epithelial origin, and human dermal fibroblasts (HDFs), a mesenchymal cell line, with 10 ng/ml TGF-β for 24 hours. The results demonstrate that the TGF-β treatment caused a decrease in HSF2's expression in the epithelial cells, while the mesenchymal cell line showed normal levels of HSF2 (III, Fig. 1C). It is also worth noticing that in contrast to HSF2, the TGF-β treatment did not affect the expression of HSF1 (III, Fig. 1F), which suggests that TGF-β signaling specifically targets HSF2 in epithelial cells.

To the best of our knowledge, these findings are the first to identify a direct signaling route that regulates the expression of HSF2. However, a previous study by Kavak and colleagues did indicate that the Wnt pathway might influence the expression of HSF2 (Kavak et al., 2010). Still, they mainly utilized microarrays and reporter assays to demonstrate that Wnt5a caused a slight increase in the mRNA expression of HSF2, but the protein levels were not examined. Additionally, Kavak and coworkers used hepatocellular carcinoma cell lines for their experiments, which might have affected the results because these types of cells often display atypical TGF-β signaling (Giannelli et al., 2011). Importantly, since the Wnt signaling pathway sometimes uses the TGF-β receptors to mediate signal transduction and *vice versa* (Zhou, 2011, Murillo-Garzón et al., 2018), we cannot exclude the possibility that Wnt signaling might influence the expression of HSF2. Therefore, it would be interesting in future studies to use several pharmacological Wnt pathway inhibitors (Huang et al., 2009) to map the signaling cascade that downregulates HSF2 more precisely.

6.2 TGF-β signaling suppresses the transcription of HSF2

To assess if TGF-β-mediated downregulation of HSF2 is a result of a decrease in the transcription of *Hsf2*, we used qRT-PCR to measure the mRNA levels in HS578T, MDA-MB-231, and MCF10A cells that were treated with TGF-β for 24 hours (III, Fig. 2A). Additionally, mRNA expression of HSF1 and several EMT markers was also assessed (III, Fig. 2A and B). According to the results, the TGF-β treatment reduced the expression of *Hsf2*, whereas the treatment did not affect the expression of *Hsf1* (III, Fig. 2A). Furthermore, the induced mRNA levels of the EMT markers imply that the treatment period was sufficient to activate the TGF-β signaling pathway (III, Fig. 2B). Therefore, we concluded that TGF-β signaling downregulates the expression of HSF2 at the transcriptional level. This result is noteworthy because a prior study has demonstrated that the protein levels of HSF2 can be reduced during heat stress by the E3 ubiquitin ligase anaphase promoting complex/cyclosome (APC/C) (Ahlskog et al., 2010). Hence, given our mRNA results, it is unlikely that APC/C is capable, in this instance, of influencing the expression of HSF2.

Because a decrease in the steady-state mRNA levels does not directly correlate with a decrease in transcription due to post-transcriptional processes, we wanted to determine if TGF-β signaling influences the transcriptional activity at the *Hsf2* promoter. To examine this, we generated luciferase reporter constructs, one containing ~ 1 kb of the *Hsf2* promoter (*HSF2luc*) (III, Fig. 2C), co-expressed them in HS578T cells together with plasmids encoding β-galactosidase and the luciferase reporter gene. Next, following a 24-hour TGF-β treatment, we measured the luciferase activity. As expected, cells expressing *HSF2luc* displayed a decline in the luciferase activity following the TGF-β treatments (III, Fig. 2D). Therefore, the luciferase assay and the qRT-PCR analysis together indicate that TGF-β signaling reduces HSF2's expression at the transcriptional level.

It is important to keep in mind that the luciferase reporter constructs we used in our study contained a fragment of the *Hsf2* promoter, including the 5′ untranslated region (5′ UTR), a sequence located upstream of the translation start codon. While the 5′ UTR can stabilize the mRNA molecule, it also provides a docking site for microRNAs (miRNAs) and RNA-binding proteins, which can sometimes destabilize the mRNA molecule and cause translational repression (Meijer et al., 2013). Hence, we cannot entirely exclude the possibility that regulatory molecules, including miRNAs, impact the mRNA levels of

endogenous HSF2 and exogenous luciferase, which both have the 5′ UTR of *Hsf2*. Therefore, an important improvement to our study would be the screening of additional constructs that either lack the 5′ UTR of *Hsf2* or contain different segments of the *Hsf2* promoter. Furthermore, by identifying the segment in the *Hsf2* promoter that responds to TGF-β, it is possible to utilize oligo-pulldown and mass spectrometry to identify which transcriptional regulators bind to the *Hsf2* promoter to repress the expression of the gene.

Since HSF1 has been reported to impact the transcription of *Hsf2* (Santopolo et al., 2021) and TGF-β has been implicated in promoting the activity of HSF1 (Sasaki et al., 2002), we performed additional experiments to exclude the possibility that HSF1 is a driving factor of the TGF-β-mediated downregulation of HSF2. To accomplish this, we used a similar experimental setup to the previous luciferase assays, with the key difference being that some of the cells were co-transfected with siRNAs targeting HSF1 (siHSF1) (III, Fig. 2E). This approach allowed us to downregulate *Hsf1* prior to the TGF-β treatment. As demonstrated by the results, TGF-β treatment reduced the luciferase activity to the same levels both in the presence and absence of HSF1 (III, Fig. 2E). Therefore, we concluded that TGF-β signaling downregulates HSF2 at the transcriptional level independently of HSF1.

6.3 HSF2 aids in the maintenance of normal epithelial cell-cell adhesion and motility

Considering that TGF-β signaling can promote the loss of cell-cell junctions in epithelial cells (Grusch et al., 2010) and certain HSF2-null cells show weakened cell-cell adhesion (Joutsen et al., 2020), we postulated that a reduction in the levels of HSF2 is necessary for TGF-β signaling to be able to reduce cell-cell adhesion. To investigate this possibility, we used an adhesion assay to assess if cells overexpressing HSF2 maintain normal cell-cell adhesion upon TGF-β stimulation. Methodologically, MDA-MB-231 cells were first transfected with plasmids encoding exogenous HSF2 ($HSF2oe^T$) or GFP ($Mock^T$) and left to recover for 24 hours. Next, cells were grown in Ultra-Low Attachment (ULA) round bottom plates that promote cell-cell adhesion, and the cells were treated with TGF-β and SB431542 for 24 hours before each spheroid was imaged and analyzed (III, Fig. 3B and C).

$Mock^T$ cells generated compact spheroid-like structures in optimal growth conditions, whereas TGF-β exposure compromised the structures of the spheroids (III, Fig. 3B and C). Importantly, supplementing the TGF-β treatment with SB431542 restored the capacity of $Mock^T$ cells to generate compact spheroids, verifying that TGF-β signaling can reduce cell-cell adhesion in epithelial cells. Intriguingly, $HSF2oe^T$ cells formed dense spheroids even in the presence of TGF-β (III, Fig. 3B and C). This result implies that HSF2 can counteract the TGF-β-mediated decrease in cell-cell adhesion, which is a likely reason why HSF2 is downregulated by signaling pathways that promote EMT progression.

Considering that disrupting cell-cell adhesion can promote cellular motility, we performed additional experiments to assess if HSF2 impairs the ability of TGF-β signaling to stimulate cell migration. For this, we examined $Mock^T$ and $HSF2oe^T$ expressing MDA-MB-231 cells in a wound healing assay. The analysis of the relative wound closure rates revealed that TGF-β treatments hastened the closure of the wounds in $Mock^T$ samples, whereas the treatments did not impact $HSF2oe^T$ cells (III, Fig. 3D and E). While these results indicate that exogenous

HSF2 can counteract TGF-β-induced cell migration, it also suggests that overexpressing HSF2 reduces basal cellular motility.

Remarkably, these findings suggest that HSF2 can counteract some of the effects caused by TGF-β signaling; however, contradictory data from previous studies challenge this since high levels of HSF2 can increase cellular migration in certain cells. For example, overexpressing HSF2 in lung epithelial cells has been shown to enhance cell migration (Zhong et al., 2016). Moreover, reduced expression of *Hsf2* in the breast epithelial cancer cell line MCF7 decreases cell motility (Yang et al., 2018), while its overexpression increases the expression of the oncogenic miR-183/-96/-182 cluster, which is increased in most breast cancer cells and is known to facilitate cellular migration (Li et al., 2014). Nevertheless, in contrast to the previous studies, we utilized MDA-MB-231 cells in our functional assays, and they do not express miRNAs 183, 96, and 182 (Li et al., 2014; Riaz et al., 2013). Given that we limited the functional assays to a single cell line, there is a possibility that other cell lines might respond differently to TGF-β. Accordingly, to gain a more accurate understanding of HSF2's impact on cell migration and adhesion, future research should improve the experimental setup by including additional cell lines in the assays, as well as downregulating *Hsf2* in some samples using siRNA.

6.4 HSF2 mitigates the initiation of EMT-associated transcriptional programs

Based on the results of the functional assays (III, Fig. 3), we believed that TGF-β signaling disrupts an HSF2-regulated transcriptional program that impacts EMT-driven processes. To investigate this possibility, we performed mRNA sequencing (RNA-seq) on $Mock^T$ and $HSF2oe^T$ expressing HS578T cells under control conditions and 24-hour TGF-β exposure. The advantage of this approach was that it allowed us to induce the expression of HSF2 in some samples and reduce it in others using TGF-β treatment, thus generating the sequencing data required to adequately assess the effect of HSF2 on gene expression (III, Fig. 4B).

According to results from the RNA-seq, a cohort of 131 genes failed to react to TGF-β when HSF2 was present (III, Fig. 4C). Next, the identified target genes were grouped into four different categories according to their expression pattern (III, Fig. 4D and S2): Group I consists of genes upregulated by both TGF-β and exogenous HSF2; Group II consists of genes upregulated by TGF-β, but with an impaired response in the presence of exogenous HSF2; Group III consists of genes downregulated by both TGF-β and exogenous HSF2; Group IV consists of genes downregulated by TGF-β and with an impaired response in the presence of exogenous HSF2.

To gain a general perspective of the biological processes associated with the gene groups, we conducted a GO-term analysis on the genes that were upregulated by TGF-β exposure (Groups I and II) and those that were downregulated due to TGF-β (Groups III and IV) (III, Fig. 4E). The analysis revealed that several genes in Groups I and II were associated with vascular functions, and many genes in Groups III and IV were associated with DNA replication and cell cycle regulation (III, Fig. 4E). A closer inspection of individual genes demonstrated that exogenous HSF2 inhibited the TGF-β-mediated induction of multiple vasculogenesis-related genes (III, Fig. 4F). The expression of DNA replication and cell cycle-regulating genes was stabilized following TGF-β exposure in $HSF2oe^T$ cells (III, Fig. 4F). These findings are important as they demonstrate the presence of HSF2 during TGF-β

signaling has a significant effect on the expression of genes involved in different transcriptional programs. Therefore, we concluded that the TGF-β-mediated downregulation of HSF2 is a prerequisite for activating TGF-β-mediated pro-tumorigenic gene programs that promote vascularization and inhibit proliferation.

Although RNA-seq is often used to study the transcriptome, it cannot be used to determine whether a transcription factor regulates the expression of a gene directly. Therefore, genome-wide sequencing techniques like ChIP-seq have been developed to be used in conjunction with RNA-seq to evaluate the capability of transcriptional regulators, such as HSF2, to bind and control the expression of target genes. Hence, we could have improved our study by utilizing ChIP-seq to investigate if HSF2 is bound to the targets identified in Figure 4D and S2 (III). However, standard ChIP assays can be used in future studies to verify if HSF2 binds specific targets. Important targets to evaluate from Figure 4.F (III) would include: platelet derived growth factor receptor beta (PDGFRB), which is a pro-angiogenic factor, and cell division cycle 45 (CDC45), which is required for the initiation of DNA replication in human cells. An alternative way to evaluate whether a transcription factor is essential for regulating a set of genes is to include samples in the RNA-seq analysis where the protein of interest, such as HSF2, is downregulated before any treatments. These samples can be used to compare mRNA profiles between wild-type and knockdown cells, thus making it possible to identify genes whose regulation depends on the silenced protein. It is important to note that we attempted to include samples where siRNA was used to downregulate endogenous HSF2 in our RNA-seq analyses. Nevertheless, these samples showed considerable discrepancies in the overall mRNA expression profile between their biological repeats. Consequently, we chose not to include these samples in our final analysis.

6.5 HSF2 is a regulator of proliferation

While the role of the TGF-β signaling pathway in modulating proliferation is highly dependent on context and cell type (Y. Zhang et al., 2017), many studies have shown that during tumorigenesis, TGF-β can suppress cell growth by altering the expression of growth-promoting transcription factors, cyclin-dependent kinases (CDKs), and CDK inhibitors (Hannon and Beach, 1994; Datto et al., 1995; Chen et al., 2001). Because our RNA-seq analysis revealed that HSF2 stabilized the expression of significant gene clusters associated with DNA replication and the cell cycle (III, Fig. 4E and S2 GII+IV), we decided to broaden our RNA-seq analysis to include family members of key targets, such as the MCM complexes, GINS gene family, and cell-cycle related kinases (III, Fig. 5A). As expected, the expression of these genes diminished in response to TGF-β, and this decrease was impeded when the cells overexpressed exogenous HSF2 (III, Fig. 5A). Next, we wanted to assess if a 72-hour exposure to TGF-β had a different effect on HSF2 overexpressing cells than a 24-hour TGF-β treatment. Due to transient transfections not being able to maintain high levels of HSF2 for 72 hours (III, S3A), we generated stable HS578T cell lines that overexpressed either GFP ($Mock^S$) or exogenous HSF2 ($HSF2oe^S$). After 72 hours of exposure to TGF-β, we analyzed the levels of several proteins involved in DNA replication and cell cycle regulation in the stable cell lines (III, Fig. 5B). Remarkably, the expression of the origin recognition complex 1 (ORC1) and minichromosome maintenance protein 2 (MCM2) were increased in $HSF2oe^S$ cells regardless of TGF-β exposure (III, Fig. 5B). Another important finding was that the decrease in the protein levels of cyclin-dependent kinase 1 (CDK1) and cyclin-dependent kinase 7 (CDK7) did not occur in $HSF2oe^S$ cells during TGF-β exposure

(III, Fig. 5B). Therefore, these results suggest that a decrease in HSF2 levels is important for the correct regulation of DNA replication and cell cycle-regulating proteins during long-term TGF-β exposure.

Based on our results showing that HSF2 influences the expression of proteins associated with proliferation, we wanted to determine if TGF-β exposure affected cell proliferation in Mock^S and HSF2oe^S cells. In order to evaluate the changes in the number of cells between the samples exposed to 24 or 72 hours of TGF-β, we utilized the Cell Counting Kit-8 (CCK-8) assay. The results demonstrate that the treatments significantly reduced cell proliferation in Mock^S cells, while there was no decrease observed in HSF2oe^S cells (III, Fig. 5C). Furthermore, the cell proliferation in HSF2oe^S cells was slightly higher than that in Mock^S cells, indicating that overexpressing HSF2 may potentially promote proliferation. Taken together, the results support the GO-term analysis, indicating that the TGF-β-mediated downregulation of HSF2 is important for activating a gene program that restrains cell proliferation.

Our findings align with previous studies demonstrating that TGF-β can suppress cell proliferation (Zhang et al., 2017), whereas increased expression of HSF2 has been found to promote growth in certain cancer types (Zhong et al., 2016). In contrast, silencing HSF2 in certain cancer cells can reduce proliferation (Yang et al., 2019; Yang et al., 2018), but it should be noted that HSF2-null mice have not been reported to display abnormal cell proliferation. Moreover, it is important to consider that the results of these studies may differ due to differences in experimental conditions, assays, and cell types. Despite this, a common factor among many of the studies was that the artificial change in HSF2's expression influenced cell proliferation, implying that a stable expression of HSF2 is necessary for normal cell growth. Therefore, we cannot exclude the possibility that the increased expression of HSF2 in HSF2oe cells could have impacted our experimental results. To improve our methodology, one could use Tet-on systems (Das et al., 2016) to precisely modulate the HSF2 expression in order to maintain normal levels during TGF-β treatments.

6.6 HSF2 suppresses the induction of ECM remodelers and impedes vasculogenic mimicry

Tumor progression frequently necessitates the formation of vascular structures, which help the cancer cells access nutrients from the circulatory system and metastasize. The vascular structures formed by cancer cells differ significantly from typical blood vessels, yet, their formation relies on common processes governing normal neovascularization, including vasculogenesis, angiogenesis, and remodeling of the extracellular matrix (ECM) (Goumans and ten Dijke, 2018). While the angiogenesis process forms new blood vessels from pre-existing ones, vasculogenesis involves the formation of vascular structures from endothelial precursor cells (Coultas et al., 2005; Eguchi et al., 2007). Moreover, ECM remodeling is essential in the formation of vascular structures, and proteases, including matrix metalloproteinase (MMPs), have pivotal roles in the degradation of the ECM, whereas deposits of collagens in the ECM help maintain the shape and structure of the vessels (Quintero-Fabián et al., 2019). Importantly, the regulation of the signaling pathways that direct the formation of blood vessels is context-dependent, and many signaling molecules, such as growth factors, have a major influence on this process (Coultas et al., 2005).

Having identified that HSF2 impedes the TGF-β-mediated induction of genes related to vascular functions (III, Fig 4. E and F), we decided to investigate the expression of other gene families involved in vasculogenesis (III, Fig. 6A). Remarkably, the TGF-β-mediated induction in the expression of several genes associated with adhesion and ECM remodeling was suppressed in cells overexpressing exogenous HSF2 (III, Fig. 6A), indicating that HSF2 negatively affects the expression of genes involved in TGF-β-mediated vasculogenesis. Next, we evaluated the protein expression of some of the ECM and adhesion-associated genes in Mock^S and HSF2oe^S cells (III, Fig. 6B). Following TGF-β exposure, Mock^S cells induced the expression of MMP2, an important remodeler of the ECM (Ahmed et al., 2006), and integrin subunit alpha 1 (ITGA1), a cell surface receptor that promotes cell-matrix adhesion (Conway and Jacquemet, 2019); however, no such induction occurred in HSF2oe^S cells (III, Fig. 6B). Moreover, HSF2oe^S cells effectively decreased and blocked the TGF-β-mediated induction of collagen type III alpha chain 1 (COL3A1) and displayed an elevated E-cadherin (CDH1) expression, relative to Mock^S cells (III, Fig. 6B). Because E-cadherin is known to promote cell-cell adhesion (van Roy and Berx, 2008), and HSF2oe^S cells overexpress E-cadherin, these cells likely adhere to each other stronger than Mock^S cells, which is supported by our results from the adhesion assay (III, Fig.3 B and C). Furthermore, cells overexpressing HSF2 can neither induce the mRNA expression nor the protein levels of ECM remodelers, such as MMP2, upon TGF-β exposure (III, Fig. 6 A and B); hence, they are likely less capable than Mock^S cells of remodeling the ECM.

The ability of metastatic cancer types, such as triple-negative breast cancer, to modify the tumor ECM is fundamental for generating vasculogenic-like networks, a process referred to as "vasculogenic mimicry" (Wechman et al., 2020). Importantly, MMP2 and MMP9 are key ECM remodelers that enable vasculogenic mimicry (Wang et al., 2008; Ling et al., 2011), and EMT-TFs associated with the TGF-β signaling pathway also regulate this process (Gong et al., 2016; Sun et al., 2010). Hence, due to HSF2 overexpression impairing the induction of genes involved in TGF-β-mediated vasculogenesis, adhesion, and ECM remodeling (III, Fig. 4F, Fig. 6A), we examined the functional impact of HSF2 on the formation of tube-like structures in an *in vitro* vasculogenic mimicry assay. While this assay is particularly well-suited for studying the formation of endothelial cell-embedded vascular structures, it is also applicable for assessing the ability of cancer cells to organize and assemble into vessel-like structures independently of endothelial cells, a primary feature of vasculogenic mimicry (Martini et al., 2020). For the assay, Mock^S and HSF2oe^S cells were cultured on a basement membrane matrix both in the presence and absence of TGF-β. Subsequently, the cells formed tube-like structures that were imaged and analyzed using image processing software. As illustrated by the results, Mock^S cells displayed a significant response to TGF-β exposure by forming complex and interconnected networks, while HSF2oe^S cells exhibited impaired network formation (III, Fig. 6C and D, S4A and B). Based on these results, we concluded that HSF2 interferes with vasculogenic mimicry induced by TGF-β signaling.

Because our findings suggest that Mock^S cells are vasculogenic mimicry-competent cells, whereas this ability is reduced in HSF2oe^S cells, the pathways governing vascular mimicry are likely misregulated in cells overexpressing HSF2. It is important to note that the molecules and pathways that regulate vascular mimicry are numerous and often affect the expression of vascular endothelial cadherin (VE-cadherin), a major protein regulating intercellular junctions in endothelial cells (Wechman et al., 2020). While VE-cadherin is

predominantly expressed in endothelial cells, its expression, like other cadherins, is dysregulated in cancer cells of non-endothelial origin (Breier et al., 2014). Importantly, VE-cadherin's ability to trigger vasculogenic mimicry also promotes cell proliferation and increases the expression of ECM remodelers, such as MMP9, in breast cancer cells (Labelle et al., 2008). Our RNA-Seq analysis did not detect any changes in the *VE-cadherin* expression between the $Mock^T$ and $HSF2oe^T$ cells. However, it is conceivable that VE-cadherin could indirectly promote vasculogenic mimicry in $Mock^T$ cells through TGF-β receptors. A study by Rudini and colleagues revealed that cells forming VE-cadherin clusters at the cell membrane aid in the recruitment and assembly of TGFβ receptor complexes, which is essential for TGF-β signal transduction in endothelial cells (Rudini et al., 2008). Moreover, one study has demonstrated that the expression of VE-cadherin increases in the cell membrane of breast cancer cells following TGF-β exposure, and the downregulation of VE-cadherin impairs the TGF-β signaling transduction (Labelle et al., 2008). This study also showed that a decrease in E-cadherin expression accompanied an increase in VE-cadherin expression. Hence, the increased expression of E-cadherin in $HSF2oe^S$ cells suggests that VE-cadherin-mediated vasculogenic mimicry does not affect these cells. Our research further supports the notion that $HSF2oe^S$ cells possess a limited capacity for vasculogenic mimicry and diminished metastatic ability because of their low expression of MMP2, ITGA1, and COL3A1 (III, Fig. 6B). These are recognized proteins that augmented invasiveness in cancer cells (Gharibi et al., 2017; H. Li et al., 2020; Shi et al., 2020; Jiang and Li, 2021;), which is a hallmark of vasculogenic mimicry.

To gain a deeper understanding of HSF2's role in vascular mimicry, it would be important to evaluate the amounts and localization of VE-cadherin in $Mock^S$ and $HSF2oe^S$ cells using immunofluorescence and confocal imaging. If the expression or localization of VE-cadherin is low or atypical in $HSF2oe^S$ cells, it would suggest that TGF-β-induced suppression of HSF2 is necessary for vasculogenic mimicry. Furthermore, it would be interesting to use mouse xenograft models to examine the metastatic potential of $Mock^S$ and $HSF2oe^S$ cells *in vivo*. Xenograft models involve implanting and propagating cancer cells in the subcutaneous space of immunocompromised mice, leading to tumor formation over several months (Morton and Houghton, 2007). The histology of the tumors can be examined, and immunohistochemical analysis can be used to assess the expression of EMT-TFs, MMPs, and cadherins, which can provide information regarding the aggressiveness of the tumors. Furthermore, mouse xenografts can also be used to study cell invasion. For example, injecting cancer cells with a reporter gene into the tail vein of immunodeficient mice can cause those cells to colonize the lungs and form tumors, which can be monitored using non-invasive bioluminescence imaging (S. Yang et al., 2012). Hence, this experimental setup would allow one to assess if cells overexpressing HSF2 have a lower capacity to invade remote tissues, which would serve as an indicator of HSF2's tumor suppressive function.

6.7 A clinical perspective on HSF2's role in TGF-β signaling and cancer progression

The ability of HSFs to regulate different transcriptional programs has, in the past two decades, been demonstrated to affect the progression of several different cancer types (Puustinen and Sistonen, 2020). Our study demonstrates for the first time that the EMT-inducing cytokine TGF-β promotes the downregulation of HSF2 to enable activation of TGF-

β-induced pro-tumorigenic gene programs. These findings support the notion that HSF2 can operate as a suppressor of tumorigenesis in epithelial cells. While HSF1 is known to direct transcriptional programs that promote tumorigenesis (Mendillo et al., 2012), a recent study revealed that HSF1 and HSF2 could together modulate networks of genes in cancer (Smith et al., 2022). Therefore, we cannot entirely exclude the possibility that HSF1 influences the ability of HSF2 to suppress the TGF-β-induced pro-tumorigenic gene programs. Although this is a limitation in the study, we demonstrated that the levels of HSF1 did not change upon TGF-β exposure (III, Fig. 1F), nor did the absence of HSF1 affect the TGF-β-mediated downregulation of HSF2 (III, Fig. 2E).

While TGF-β-signaling has not previously been linked to HSF2, a few reports have implicated that HSF1 can influence various aspects of TGF-β signaling depending on the cell type. For instance, HSF1 can suppress migration by limiting the TGF-β signaling pathway in renal proximal tubular cells (Lou et al., 2019), a cell type involved in regeneration during acute kidney injury. Also, HSF1 regulates a transcriptional program in cancer-associated fibroblasts (CAFs) that stimulate TGF-β signaling, supporting the health of the tumor microenvironment *in vivo* (Scherz-Shouval et al., 2014). Thus, reflecting on our result and previous findings, it is interesting to speculate whether HSFs impact TGF-β signaling in a context-dependent manner to regulate different biological processes. In the future, it would be interesting to study if HSFs regulate transcription during various TGF-β-related processes, including fibrosis and inflammation. However, HSF-null mice would likely be required to properly duplicate the intricate model systems and environment necessary for studying these processes *in vivo*.

In the wake of the initial discovery that HSF1 can promote tumorigenesis, numerous attempts have been made to develop drugs that target HSF1 (Dong et al., 2019). Despite the advancements in developing some HSF1-targeting compounds, it remains to be established if these drugs also impact HSF2 and HSF4. Notably, HSF1 displays almost a 40 % identical amino acid sequence to HSF2 and HSF4 (Puustinen and Sistonen, 2020), and segments like the DBDs are nearly identical amongst HSFs, thus making it feasible for drugs to interact with multiple HSFs via this region. Hence, if HSF2 is affected by off-targeting effects, it can potentially lose its ability to suppress various pro-tumorigenic processes. Bearing this in mind, drug development related to HSFs should assess possible drug interactions among the different HSF family members.

CONCLUDING REMARKS

The ability to rapidly shift transcription in response to protein-damaging stress and growth factors is essential for the cell's ability to adapt to an ever-changing environment. The signaling pathways, regulatory mechanisms, and proteins that modify transcription have always garnered interest, and new methods, including genome-wide sequencing techniques, have rapidly increased our ability to study these processes. An essential pathway cells use to combat stress and adapt to the environment is the HSR, which HSFs govern. However, despite numerous studies, some aspects of the mechanisms that drive the activity of HSFs are still unknown. In the first study of this thesis, we sought to answer some of the longstanding questions surrounding the impact of hyperphosphorylation on HSF1's activity. Using a phosphorylation-deficient HSF1 mutant, we demonstrated that phosphorylation within the regulatory domain is not required for HSF1 to sense stress or stimulate transcription during the HSR, which challenges the previous paradigm. Furthermore, we revealed that the stress-inducible phosphorylation predominantly modulates the activation threshold for HSF1 during stress, which consequently alters the magnitude to which HSF1 can induce the transcription of key chaperones. Although the interacting partners responsible for HSF1's activation threshold remain unknown, this study improves our understanding of how phosphorylation impacts HSF1 activity and advances our understanding of the mechanisms that regulate the HSR.

HSF1 and HSF2 are the two major HSFs directing transcription upon protein-damaging conditions. While several studies have characterized the stress-inducible network of genes that HSF1 and HSF2 regulate during heat stress, their target genes modulated during oxidative stress remain unknown. In the second study of this thesis, we demonstrated that HSF1 and HSF2 drive distinct transcriptional programs upon heat and oxidative stress, and each HSF utilizes enhancers to direct the expression of some genes. Moreover, our investigations revealed that HSF2 could function independently as a transcription factor during oxidative stress, while it requires HSF1 to manage the transcription of genes during heat shock. Even though the molecular process that directs HSFs to their specific and collective targets remains enigmatic, our research establishes a comprehensive foundation for how they utilize networks of enhancers to modulate transcription, which will be important for research in the future.

The pathways that direct the operations of HSFs are often disrupted in diseases like cancer, leading to the initiation of the transcriptional programs that drive malignant behavior. Although multiple reports provide details of the oncogenic functions of HSF1, certain studies indicate that HSF2 can function as a tumor suppressor. However, the processes by which HSF2 does so remain unknown. Based on a previous study that implied that both suppression of HSF2 and elevated EMT signaling are linked to cancer progression, the third study of this thesis investigated how EMT-promoting cytokines impact the expression of HSF2. For the first time, we demonstrated that the pro-tumorigenic cytokine TGF-β triggers pathways that suppress the expression of HSF2 to enhance cancer cell migration. Moreover, we established that the TGF-β-mediated downregulation of HSF2 is a prerequisite for activating pro-tumorigenic gene programs that impacts vasculogenic mimicry and repress proliferation, key processes driving metastasis. Even though our findings add to the knowledge of HSF2's role as a tumor suppressor, further studies are essential to uncover both the target sites to which

HSF2 binds and identify the regulatory factors that bind to the *Hsf2* promotor to suppress its transcription. Uncovering these missing factors linked to regulating HSF2 will potentially advance the development of future pharmacological interventions.

This thesis increases our knowledge of the mechanisms driving the activation of HSF1 and the distinct transcriptional programs that HSF1 and HSF2 regulate during stress and cancer progression. Additionally, the information in this thesis unveils new mechanisms and identifies a signaling pathway that influences the expression and activity of HSFs.

ACKNOWLEDGEMENTS

The work presented in this thesis was performed during 2016-2022 at the Faculty of Science and Engineering, Åbo Akademi University (ÅAU) and Turku Bioscience Centre, University of Turku and ÅAU. I want to thank John Eriksson, Cecilia Sahlgren, and Annika Meinander for their work as heads of the Cell Biology department at ÅAU during my PhD-studies. I want to thank John Eriksson for providing an excellent scientific environment and facilities in Biocity. Also, I want to thank John for letting me broaden my research experience in the HPV Horizon project. I wish to acknowledge Cecilia Sahlgren for her outstanding effort in creating the ÅAU Center of Excellence project CellMech, which has broadened my perspective on stress biology and raised my interest in the practical application/development of regenerative medicine. Annika Meinander is acknowledged for helping me with the administrative tasks of ÅA. Additionally, I want to thank Annika for her significant input to the Turku Doctoral Network in Molecular Biosciences (MolBio), which has supported my doctoral studies educationally and financially.

I am deeply grateful to my supervisor Professor Lea Sistonen for allowing me to conduct my studies in her laboratory. Lea has supported me in countless ways, including arranging several opportunities to participate in various research projects. Lea's passion for science and the stress biology field is truly inspiring, and it was a great privilege to work and study under her guidance. I thank Kari Kurppa and Otto Kauko for reviewing my thesis and providing highly valuable and critical comments. I also thank Professor Jyrki Heino for accepting the invitation to be my opponent during my doctoral defense. Importantly, I want to thank my research colleagues and co-authors Jenny Pessa, Samu Himanen, Marek Budzyński, Jenny Joutsen, Hendrik Hästbacka, Alejandro Da Silva, Sandra Pihlström, Silvia Gramolelli, and Anniina Vihervaara.

Furthermore, I want to thank all the personnel at ÅAU who have helped me in many practical ways. A big thanks to Thomas Bymark and Jari Korhonen for providing technical assistance in the laboratory. Also, thanks to Sten Lindholm and Gunilla Henriksson for always expeditiously ordering the reagents I needed for my experiments. Moreover, the Finnish Functional Genomics Centre personnel are acknowledged for their sequencing services. Diana Toivola and Jeroen Pouwels are recognized for their work on my thesis advisory committee and their critical and helpful feedback during my doctoral studies.

I want to express my deep and sincere gratitude to all the current and past members of the Sistonen research group. I especially want to thank Jenny Pessa for her tremendous input into our study, which is included as a manuscript in this thesis. Additionally, I thank Jenny for her friendship and reliability over all these years. I am also grateful to Samu Himanen for permitting me to join him in his research on enhances, which turned out to be a fun and challenging project. However, my friend Samu also remembered to make time for me and him so we could develop our skills in the noble game called "Magic the Gathering", which likely helped both of us, for a moment, forget some problematic experiments. I will miss working with Jenny and Samu, and I sincerely wish you success in your future studies.

I also want to thank and acknowledge Marek Budzyński for mentoring me in the lab during my early years. The efforts of Marek have helped me substantially develop my analytical capacity, which is of pivotal importance in science. I am also grateful to Jenny Joutsen for

her great contributions to my research projects, and I have always been able to count on her for support in the lab. Jenny Joutsen has always kept a positive and enthusiastic attitude, which has helped me countless days in the lab. I thank Alejandro Da Silva for helping me with my research and for the great discussions surrounding philosophy. Furthermore, I thank Alejandro for letting me join him in his exciting research related to Talin. I particularly want to thank Hendrik Hästbacka for his outstanding work in the computational analysis of my third study, which was essential for completing the study. Hendrik has always had my back on computer-related stuff, which is not my strong side. Further thanks to Silvia Gramolelli for teaching me how to generate and use stable cell lines, which I will definitively try to apply in my future work. I would also like to acknowledge my gratitude to Jens Luoto, Eva Henriksson, Pia Roos-Mattjus, and Ingrid Karppi, who have helped me in numerous ways in my research.

A big thank goes to my friends Erik Niemelä and Iris Lähdeniemi for encouraging my research in many ways, and I thank you two for helping me sometimes take a break from the lab and focus on other vital parts of life, such as family and friends. I am very much thankful to my wife Satu, for her love, understanding, and continuing support in completing my PhD-studies. Also, I thank my parents, Tom and Ann-Britt and my sister Annika for being a fantastic and supportive family in every possible way.

The work presented in this thesis would not have been possible without the funding received from MolBio, ÅAU, The Swedish Cultural Foundation, Instrumentarium Science Foundation, K. Albin Johanssons stiftelse, Waldemar von Frenckells stiftelse, Lounaissuomalaiset Syöpäjärjestöt, and the Magnus Ehrnrooth Foundation.

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Uncoupling Stress-Inducible Phosphorylation of Heat Shock Factor 1 from Its Activation

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I

In mammals the stress-inducible expression of genes encoding heat shock proteins is under the control of the heat shock transcription factor 1 (HSF1). Activation of HSF1 is a multistep process, involving trimerization, acquisition of DNA-binding and transcriptional activities, which coincide with several posttranslational modifications. Stress-inducible phosphorylation of HSF1, or hyperphosphorylation, which occurs mainly within the regulatory domain (RD), has been proposed as a requirement for HSF-driven transcription and is widely used for assessing HSF1 activation. Nonetheless, the contribution of hyperphosphorylation to the activity of HSF1 remains unknown. In this study, we generated a phosphorylation-deficient HSF1 mutant (HSF1Δ~PRD), where the 15 known phosphorylation sites within the RD were disrupted. Our results show that the phosphorylation status of the RD does not affect the subcellular localization and DNA-binding activity of HSF1. Surprisingly, under stress conditions, HSF1Δ~PRD is a potent transactivator of both endogenous targets and a reporter gene, and HSF1Δ~PRD has a reduced activation threshold. Our results provide the first direct evidence for uncoupling stress-inducible phosphorylation of HSF1 from its activation, and we propose that the phosphorylation signature alone is not an appropriate marker for HSF1 activity.

The heat shock response, as characterized by inducible expression of heat shock proteins (Hsps), is an ancient, evolutionarily conserved mechanism that protects cells from various proteotoxic insults, including exposures to elevated temperatures, heavy metals, proteasome inhibition, and oxidative stress (1). Hsps function as molecular chaperones, bind to misfolded proteins, facilitate their refolding or direct them to degradation, and block the formation of protein aggregates (2, 3). The heat shock response is controlled by heat shock transcription factors (HSFs) (4). In vertebrates, four HSFs (HSF1 to HSF4) have been found, whereas yeasts, flies, and nematodes have only a single HSF. HSFs bind DNA at evolutionarily well-conserved sequences, consisting of inverted nGAAn repeats, called heat shock elements (5–8). HSF1 is considered the master regulator of the heat shock response in mammals, since mice lacking HSF1 are unable to induce Hsp expression upon exposure to protein-damaging stress (9, 10). Besides controlling the stress-inducible expression of Hsps, HSF1 plays a role in development (10–12), life span regulation (13–15), immune responses (16), and the circadian cycle (17). In addition, HSF1 is a well-recognized transcriptional regulator in malignant human cancers (18–20).

The HSF1 protein is composed of five distinguishable functional domains (see Fig. 1A). The DNA-binding domain (DBD) is located at the N terminus (21), whereas the transactivation domain (TAD) resides in the C terminus (22). A unique requirement for HSF1 activation is the process of trimerization through an intermolecular interaction of leucine-zipper-like heptad repeat domains (HR-A/B) between HSF1 monomers (23, 24). Spontaneous trimerization under normal conditions is suppressed by another heptad repeat region (HR-C), which facilitates intramolecular interactions between HR-A/B and HR-C domains (25). A centrally located part of HSF1 is called the regulatory domain (RD). Deletion of the RD results in constitutive DNA-binding activity of HSF1 and induces expression of Hsps in the absence of stress (26–28). It has also been shown that the RD is self-sufficient in its heat-sensing capacity, since a chimeric transcription factor containing $Gal4_{DBD}$-$HSF1_{RD}$-$VP16_{TAD}$ is repressed under normal conditions but is capable of activating transcription in response to stress (22).

Transient activation of HSF1 by various stresses includes accumulation in the nucleus, monomer-to-trimer transition, HSE-binding activity, and acquisition of transactivation capacity (1). During the activation-attenuation cycle, HSF1 is extensively posttranslationally modified (PTMs) and is subjected to, for example, phosphorylation, sumoylation, and acetylation (29–32). To date, 22 phosphorylation sites on serine and threonine residues have been identified within the HSF1 protein (33). Some sites, e.g., S303 and S307, appear to be constitutively phosphorylated (34–36), whereas other sites undergo inducible phosphorylation (37). Stress-inducible phosphorylation of HSF1, or hyperphosphorylation, is one of the most prominent modifications, coinciding with the acquisition of its transactivation capacity (30, 37–40). However, despite a wealth of studies on the role of single phosphorylation sites (29, 30, 34, 40–42), no direct link between hyperphosphorylation and HSF1 activation has been established.

Many pathological conditions, such as metabolic disorders, cancers, and neurodegenerative diseases, are associated with either increased or decreased activity of molecular chaperones (43, 44). Hence, modulating the heat shock response by altering HSF1 activity has been proposed as a potential therapeutic approach

Received 3 August 2014 **Returned for modification** 19 August 2014
Accepted 4 May 2015
Accepted manuscript posted online 11 May 2015
Citation Budzyński MA, Puustinen MC, Joutsen J, Sistonen L. 2015. Uncoupling stress-inducible phosphorylation of heat shock factor 1 from its activation. Mol Cell Biol 35:2530–2540. doi:10.1128/MCB.00816-14.
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doi:10.1128/MCB.00816-14

(44–46). Although a variety of compounds have been shown to affect HSF1 transactivation capacity (45–48), mechanistic understanding of how these compounds contribute to HSF1 activation is limited. Therefore, in development of drugs targeting specific phases in the HSF1 activation-attenuation cycle, emphasis should be placed on unraveling the functional impact of HSF1 PTMs. In order to investigate how the stress-inducible phosphorylation affects HSF1 activity, we generated a phosphorylation-deficient HSF1 mutant that lacks the known 15 phosphorylation sites within the RD (HSF1Δ~PRD). Our results show that phosphorylation of HSF1 RD does not affect HSF1 nuclear localization and is not required for its DNA-binding activity, suggesting that the HSF1Δ~PRD retains its properties to be accurately regulated upon exposure to stress. We conclude that the gain of HSF1 transactivation capacity is independent of the constitutive and stress-inducible phosphorylation of HSF1 within the RD, providing the first direct evidence for uncoupling HSF1 hyperphosphorylation from its activation.

MATERIALS AND METHODS

Plasmid constructs. The plasmids encoding Myc-His-HSF1 WT [in pcDNA3.1/myc-His(−)A], Gal4-VP16, β-galacatosidase, and Gal4-driven luciferase have been described earlier (31, 40, 49). The phosphorylation-deficient HSF1 mutant (HSF1Δ~PRD) was generated by replacing 15 phosphorylatable serine and threonine residues within the Myc-His-HSF1 wild-type (WT) RD with alanines (Fig. 1A). Fourteen sites (S230, S292, S303, S307, S314, S319, S320, T323, S326, S338, S344, S363, S368, and T369) were mutated by sequential rounds of site-directed mutagenesis using a QuikChange site-directed mutagenesis kit (Agilent Technologies) according to the manufacturer's instructions. Mutation 15, T367A, was performed by DNA Express, Inc. Gal4-VP16-HSF1 WT and Gal4-VP16-HSF1Δ~PRD were generated by cloning the regulatory domain (amino acids [aa] 220 to 389) of Myc-His-HSF1 WT or Myc-His-HSF1Δ~PRD into EcoRI-linearized pSGVP plasmid (pSGVP was kindly provided by Richard I. Morimoto, Northwestern University, Evanston, IL) by using an In-Fusion HD cloning kit (Clontech). The constructs were confirmed by sequencing.

Cell culture, treatments, and transfections. *hsf1*$^{-/-}$ and *hsf1*$^{+/+}$ mouse embryonic fibroblasts (MEFs) and human cervical cancer HeLa cells were cultured in high glucose Dulbecco modified Eagle medium (Sigma) containing 10% fetal calf serum (Gibco), 2 mM L-glutamine (Sigma), and streptomycin (100 μg/ml) and penicillin (100 U/ml) (both from VWR). Culture media for MEFs were supplemented with 1× MEM nonessential amino acid solution (Sigma). Heat shock treatments were conducted in a water bath at 39, 40, 41, 42, and 43°C for the indicated times. To induce heavy metal stress, $CdSO_4$ (Sigma), dissolved in sterile water, was used at a concentration of 40 and 60 μM for the indicated times. For transfections, 6×10^6 HeLa or *hsf1*$^{-/-}$ MEFs were suspended in 0.4 ml of Opti-MEM (Gibco). Cells were subjected to a single electric pulse (220 V, 975 μF for HeLa cells; 280 V, 975 μF for MEFs) in 0.4-cm gap electroporation cuvettes (BTX) using a Bio-Rad Gene Pulser II electroporator. Transfected cells were left to recover in culture medium for 48 h prior to further treatments.

Western blot. Cells were lysed in radioimmunoprecipitation assay lysis buffer (1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 0.15 M NaCl, 0.01 M sodium phosphate [pH 7.2], and 2 mM EDTA [pH 8.0]) supplemented with 0.5 mM phenylmethylsulfonyl fluoride and 1× Complete Mini-Protease inhibitor cocktail (Roche). Cell lysates, cleared by centrifugation (15,000 × *g* for 10 min at 4°C), were boiled in Laemmli sample buffer, resolved on an 8% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), and transferred to nitrocellulose membrane (Pierce). The antibodies used for Western blotting were anti-HSF1 (AB-4; Thermo Scientific), anti-HSF2 (3E2; Millipore), anti-α-actin (AC-40; Sigma-Aldrich), anti-Hsc70 (SPA-815; Enzo Life Sciences), and anti-VP16 (V4388; Sigma-Aldrich). Horseradish peroxidase-conjugated secondary antibodies were purchased from Promega, Abcam, and GE Healthcare Life Sciences, and immunocomplexes were detected by enhanced chemiluminescence (GE Healthcare Life Sciences).

Protein dephosphorylation and protein turnover analyses. For protein dephosphorylation analysis, lambda protein phosphatase (λPP; New England BioLabs) was used according to the manufacturer's instructions. Briefly, transfected MEFs were subjected to a 30-min heat shock at 43°C and lysed in buffer C (25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 20 mM HEPES [pH 8]), and λPP was used at a concentration of 50 U/μg for whole-cell lysates. Samples were incubated at 30°C for 30 min, and the reaction was stopped by boiling in Laemmli sample buffer. To measure the protein turnover, transfected MEFs were treated for up to 15 h with cycloheximide (CHX; Sigma), which was added to culture medium at a concentration of 20 μg/ml.

ChIP. Chromatin immunoprecipitation (ChIP) was performed as described by Vihervaara and coworkers (8) with minor changes to the protocol. A total of 5×10^7 transfected MEFs were cross-linked immediately after treatment for 10 min with a final concentration of 1% formaldehyde, followed by quenching in 125 mM glycine. After lysis in Joost lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl [pH 8.1]), chromatin was sonicated using a Bioruptor (Diagenode), and 1 mg of whole-cell extracts was used for each immunoprecipitation. Samples were precleared using a 50% slurry of protein G-Sepharose beads (GE Healthcare Life Sciences). Immunoprecipitation was performed overnight at 4°C using antibodies against HSF1 (SPA-901; Enzo Life Sciences). Normal rabbit serum (Jackson ImmunoResearch Laboratories) was used as a nonspecific antibody. After washing of the immunocomplexes, the remaining proteins and RNA were digested by using proteinase K and RNase A. Cross-links were reversed by incubating the samples overnight at 65°C. DNA was purified with phenol-chloroform. Samples were analyzed by quantitative PCR using StepOnePlus or QuantStudio 12K Flex Real-Time PCR Systems (both from Applied Biosystems). The following forward (f) and reverse (r) SYBR green primers were used: fHsp25 promoter, 5′-TGGGAATCGCTCCAGCT ACCG-3′; rHsp25 promoter, 5′-AAGCTTGCAAAGGGGGCGGG-3′; fHsp70 promoter, 5′-CACCAGCACGTTCCCCA-3′; and rHsp70 promoter, 5′-CGCCCTGCGCCTTTAAG-3′. Immunoprecipitation samples were normalized to values obtained for input before fold enrichment was determined by setting the HSF1 WT control sample to value 1.

EMSA. Electromobility shift assay (EMSA) was performed as described previously (50). Briefly, cell pellets from transfected *hsf1*$^{-/-}$ MEFs were lysed in buffer C, and the whole-cell extracts were incubated with ^{32}P-labeled oligonucleotide representing the proximal HSE of the human *Hsp70* promoter (forward, 5′-GAGGCGAAAACCCTGGAATAT TCCCGACCTGGCAG-3′; reverse, 5′-CTGCCAGGTCGGGAATATT CCAGGGTTTTCGCCTC-3′). Samples were resolved on a 4% native polyacrylamide gel, and the protein-DNA complexes were visualized by autoradiography.

Immunofluorescence and confocal microscopy. Transfected HeLa cells were cultured on coverslips for 48 h before treatments. Treated and untreated cells were fixed in 3.7% paraformaldehyde and permeabilized with 0.5% Triton-X in phosphate-buffered saline (PBS) for 12 min, followed by blocking with 5% bovine serum albumin (BSA) in PBS for 1 h at room temperature. The cells were incubated with rabbit anti-HSF1 (51) or mouse anti-myc (M4439; Sigma) antibodies overnight at 4°C, after which the unbound primary antibodies were washed off with PBS containing 0.1% Tween 20. After the washing step, the cells were incubated with secondary antibodies diluted 1:400 in 5% BSA-PBS for 1 h (donkey anti-rabbit antibody–Alexa Fluor 568 for anti-HSF1 and goat anti-mouse antibody–Alexa Fluor 488 for anti-myc, both from Life Technologies). Coverslips were mounted in Vectashield mounting medium with DAPI (4′,6′-diamidino-2-phenylindole; Vector Laboratories) for DNA staining. Immunofluorescence was performed with LSM 780 confocal microscope (Carl Zeiss, Inc.), and image analysis was performed using Fiji software (52).

Quantitative RT-PCR (qRT-PCR). RNA from transfected *hsf1*$^{-/-}$ MEFs was isolated using an RNeasy minikit (Qiagen) according to the manufacturer's instructions and quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). Then, 1 μg of total RNA was reverse transcribed with an iScript kit (Bio-Rad). A KAPA Probe Fast ABI Prism qPCR kit (KAPA Biosystems) and SensiFAST SYBR Hi-ROX kit (Bioline Reagents) were used for qRT-PCRs that were performed with StepOnePlus or QuantStudio 12K Flex real-time PCR systems (both from Applied Biosystems). Primers and probes were purchased from Oligomer. The following forward (f), reverse (r), and probe (pr) oligonucleotides were used in TaqMan assays: fRNA18S5, 5′-GCAATTATTCCCCATGAACG-3′; rRNA18S5, 5′-GGGACTTAA TCAACGCAAGC-3′; prRNA18S5, 5′-FAM-TTCCCAGTAAGTGCG GGTC-BHQ-3′; fHSPA1A/B, 5′-AGGTGCTGGACAAGTGCCAG-3′; rHSPA1A/B, 5′-AACTCCTCCTTGTCGGCCA-3′; prHSPA1A/B, 5′-FAM-CATCTCCTGGCTGGACTCCAACACG-BHQ-3′; fHSPB1, 5′-C ACTGGCAAGCACGAAGAAAG-3′; rHSPB1, 5′-GCGTGTATTTCCGG GTGAAG-3′; and prHSPB1, 5′-FAM-ACCGAGAGATGTAGCCATGTT CGTCCTG-BHQ-3′. The relative quantities of the target gene mRNAs were normalized against their respective 18S RNA (*RNA18S5*), and the fold induction was calculated against the respective mRNA levels in nontreated mock-transfected cells. All reactions were run in triplicate from samples derived from at least three biological replicates.

Luciferase assay. Transfected HeLa cells were snap-frozen and lysed in Passive lysis buffer (Promega) according to the manufacturer's instructions. Cell lysates were cleared by centrifugation (15,000 × *g* for 10 min at 4°C), and the firefly luciferase activity, produced by the Gal4-driven luciferase plasmid, was measured by using a Luminoskan Ascent microplate luminometer (Thermo Scientific) with luciferase assay reagent (Promega) as a substrate. The luciferase activity was normalized using Rous sarcoma virus promoter-driven β-galactosidase as an internal control by incubating the cell lysates in 100 mM phosphate buffer (pH 7.0) with 0.67 mg of *o*-nitrophenyl β-D-galactoside (ONPG; Sigma)/ml, 1 mM $MgCl_2$, and 45 mM β-mercaptoethanol at 37°C for 1 h. The absorbance was measured by a Multiskan MCC/340 (Labsystems) at 420 nm.

Statistical analysis. Statistical analyses of the data were performed in GraphPad Prism 6. The data were analyzed within each time point using independent two-way analysis of variance and corrected for multiple comparisons using the Holm-Sidak *post hoc* test, and the significance level was set to 0.05.

RESULTS

HSF1Δ∼PRD and HSF1 WT display similar turnover and subcellular localization. To study the impact of hyperphosphorylation on HSF1 activity, we generated a mutant construct of HSF1, where the known 15 phosphorylation sites residing within the RD (aa 220 to 389) were replaced with nonphosphorylatable alanines, that we designated HSF1Δ∼PRD (Fig. 1A). The RD harbors ∼70% of the known HSF1 phosphorylation sites (33) and is capable of repressing HSF1 TAD in the absence of stress, rendering HSF1 inactive under normal conditions (22, 27, 28), and we therefore mutated the phosphorylation sites within this domain.

We examined the expression, turnover, subcellular localization, and DNA-binding activity of HSF1Δ∼PRD and compared the properties to those of HSF1 WT before proceeding to the functional studies. To study specifically the properties of the mutant protein, without any interference from the endogenous HSF1, we expressed HSF1Δ∼PRD in *hsf1* knockout (*hsf1*$^{-/-}$) MEFs, derived from an *hsf1*$^{-/-}$ mouse (9, 10). To avoid generating a constitutively active HSF1, which has been observed as a result of HSF1 overexpression (26, 53), we titrated the exogenous HSF1 levels in *hsf1*$^{-/-}$ MEFs to mimic the endogenous levels in *hsf1*$^{+/+}$ MEFs (Fig. 1B). Both endogenous and exogenous HSF1 WT from stressed cells migrated more slowly on SDS-PAGE than that from untreated cells (Fig. 1B, lane 1 versus lane 2; Fig. 1C, lane 3 versus lane 4), and previous studies have shown that this effect is caused by HSF1 hyperphosphorylation (39, 40, 53). In *hsf1*$^{-/-}$ MEFs where HSF1Δ∼PRD was expressed, the retarded migration of HSF1 mutant under heat shock conditions was greatly reduced compared to the HSF1 WT (Fig. 1C, lane 4 versus lane 6), indicating that the stress-inducible phosphorylation was diminished. The residual stress-inducible phosphorylation in HSF1Δ∼PRD (Fig. 1C, lane 5 versus lane 6) was assessed with lambda protein phosphatase (λPP) treatment (54). Since the retarded migration of HSF1Δ∼PRD upon heat stress was eliminated in the presence of λPP (Fig. 1C, lane 6 versus lane 12), it is plausible that HSF1Δ∼PRD undergoes stress-inducible phosphorylation beyond the 15 phosphorylation acceptor sites that were mutated (Fig. 1A).

Multisite phosphorylation has been shown to regulate the turnover of many transcriptional regulators (37). For example, under normal conditions, p53 is targeted for rapid degradation by the E3 ubiquitin ligase Mdm2 (55), whereas stress-inducible phosphorylation of the p53 N-terminal region impairs p53-Mdm2 interaction, resulting in p53 stabilization (56, 57). In contrast, EP300 undergoes phosphorylation-mediated degradation, where hyperphosphorylation precedes its proteasomal degradation (58). To address whether phosphorylation in the RD affects HSF1 turnover, we analyzed HSF1 protein levels in *hsf1*$^{-/-}$ MEFs, expressing either HSF1 WT or HSF1Δ∼PRD, treated with the eukaryotic translation inhibitor CHX (59). The protein levels of both HSF1 WT and HSF1Δ∼PRD remained constant throughout a 15-h CHX treatment (Fig. 1D), suggesting that lack of the phosphorylation within the RD does not alter the stability of the HSF1 protein. This finding is in agreement with a recent study showing that HSF1 phosphorylation does not affect its turnover (32). In order to validate that protein translation was inhibited by CHX, we analyzed the protein levels of HSF2 which is known to have a fast turnover rate (60, 61). As expected, HSF2 was rapidly degraded and not detectable after a 3-h CHX treatment (Fig. 1D).

HSF1 accumulates in the nucleus upon exposure to stress stimuli, while under nonstress conditions it is localized both in the nucleus and in the cytoplasm (53, 62, 63). Previously, it was proposed that phosphorylation of specific serine residues in the RD affects HSF1 cellular localization (64, 65). Accordingly, phosphorylation of S320 by protein kinase A would retain HSF1 in the nucleus (65), and phosphorylation of S303 and S307 would facilitate 14-3-3ε-mediated nuclear exclusion of HSF1 (64). In primate cells exposed to various proteotoxic stresses, HSF1 forms unique subnuclear granules, called nuclear stress bodies (nSBs) (66), the formation of which requires DNA-binding competent HSF1 and coincides with HSF1 hyperphosphorylation (53, 67). Using indirect immunofluorescence and confocal microscopy, we examined the subcellular localization of Myc-His-tagged HSF1Δ∼PRD and the formation of nSBs in HeLa cells. Under control conditions, the exogenously expressed HSF1 WT and HSF1Δ∼PRD, as well as the endogenous HSF1 protein, were diffusely distributed in the nucleus (Fig. 2). In response to heat stress, both HSF1Δ∼PRD and HSF1 WT were located in the nucleus and concentrated in nSBs. These results indicate that the phosphorylation within the RD has no effect on HSF1 localization under control or stress conditions and that the formation of nSBs is independent of HSF1 hyperphosphorylation.

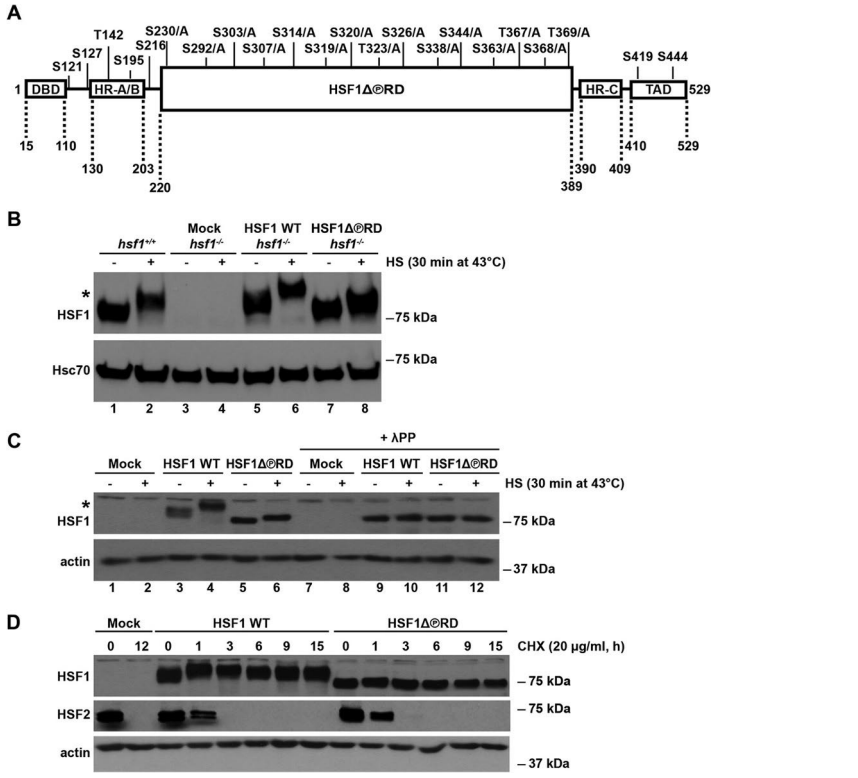

FIG 1 Characterization of the HSF1 mutant that is phosphorylation-deficient within the RD, HSF1Δ~PRD. (A) Schematic illustration of the HSF1 functional domains with the known phosphorylation sites. In HSF1Δ~PRD, 15 phosphorylation sites in the regulatory domain (RD) were mutated from serine (S) and threonine (T) residues to alanines (A) as indicated. Additional HSF1 domains include the DNA-binding domain (DBD), heptad repeat domains (HR-A/B and HR-C), and transactivation domain (TAD). Note that the figure is not drawn to scale. (B) *hsf1*$^{-/-}$ MEFs were transfected with Mock plasmid [pcDNA3.1/myc-His(−)A], Myc-His-HSF1 WT, or Myc-His-HSF1Δ~PRD. *hsf1*$^{+/+}$ represents the endogenous levels of HSF1 in MEFs. Cells were either left untreated (−) or exposed to heat shock (+). HSF1 protein levels from cell lysates were detected by Western blotting with anti-HSF1 antibody. Hsc70 is shown as a loading control. An asterisk indicates an HSF1 protein that migrates slower on SDS-PAGE due to hyperphosphorylation (53). The difference in size between the endogenous HSF1 from *hsf1*$^{+/+}$ MEFs and exogenous HSF1 WT is caused by the Myc-His tag on the human HSF1 WT construct. (C) *hsf1*$^{-/-}$ MEFs were transfected as in panel B. Cells were either left untreated (−) or heat shocked (+). Cell lysates were treated with lambda protein phosphatase (+λPP) or left untreated. Samples were analyzed by using Western blotting. α-Actin is shown as a loading control. An asterisk indicates the HSF1 protein that migrates slower on SDS-PAGE due to hyperphosphorylation (53). (D) *hsf1*$^{-/-}$ MEFs were transfected as in panel B and treated with cycloheximide (CHX) at 37°C for the indicated times. Cell lysates were analyzed with anti-HSF1 and anti-HSF2 antibodies. α-Actin is shown as a loading control.

HSF1Δ~PRD binds to DNA in a stress-inducible manner. HSF1 activation can be divided into two separate steps. First, HSF1 forms trimers, accumulates in the nucleus, and acquires DNA-binding activity (1). Second, HSF1 acquires transactivating capacity, an event that coincides with the stress-inducible phosphorylation of HSF1 (39). To study whether phosphorylation within the RD alters the DNA-binding activity of HSF1, we used ChIP to compare the occupancy of HSF1 WT and HSF1Δ~PRD at *Hsp70* (*HSPA1A* and *HSPA1B*; *HSPA1A/B*) and *Hsp25* (*HSPB1*) promoters. *hsf1*$^{-/-}$ MEFs, expressing HSF1 WT or HSF1Δ~PRD, were either left untreated or exposed to a 30-min heat shock at 43°C, followed by immunoprecipitation with HSF1 antibody or normal rabbit serum as a nonspecific antibody. Under control conditions, the signal for the occupancy of HSF1 WT and HSF1Δ~PRD at the *Hsp70* and *Hsp25* promoters was below that of the nonspecific antibody (Fig. 3A), showing that removal of the basal phosphorylation from the RD does not spontaneously induce the DNA-binding activity of HSF1. Upon heat stress, the occupancy of HSF1 WT and HSF1Δ~PRD increased similarly at the *Hsp70* and *Hsp25* promoters. Next we examined if the phosphorylation within the RD affects the DNA-binding activity of HSF1 under prolonged stress. *hsf1*$^{-/-}$ MEFs, expressing either

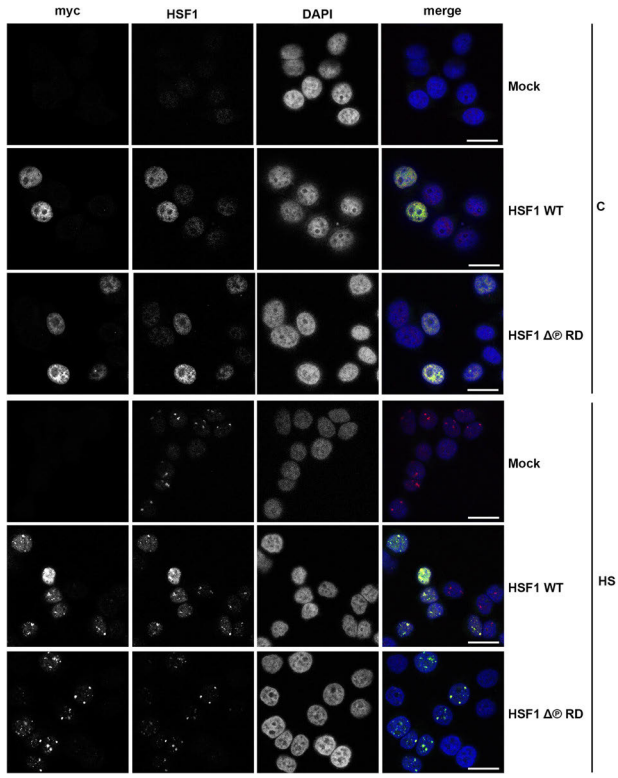

FIG 2 HSF1Δ~PRD localizes to the same subcellular compartments as HSF1 WT under normal and stress conditions. HeLa cells were transfected with Mock plasmid [pcDNA3.1/myc-His(−)A], Myc-His-HSF1 WT, or Myc-His-HSF1Δ~PRD, left untreated (C) or exposed to heat stress (HS; 1 h at 42°C), and analyzed by immunofluorescence microscopy. A monoclonal antibody against myc was used to detect exogenously expressed HSF1 protein, whereas an anti-HSF1 antibody was used to detect both endo- and exogenously expressed HSF1. DNA was stained with DAPI. The merge figure is an overlay of myc, HSF1, and DAPI signals. Scale bars, 25 μm.

HSF1 WT or HSF1Δ~PRD, were exposed to cadmium sulfate (60 μM $CdSO_4$), which in addition to promoting expression of metallothioneins induces HSF1-dependent Hsp expression (68). Whole-cell extracts were incubated with a ^{32}P-labeled oligonucleotide containing the proximal HSE of the *Hsp70* promoter and binding was studied by EMSA. During prolonged exposure to $CdSO_4$ and during recovery from stress, we did not detect any difference between HSF1 WT and HSF1Δ~PRD DNA-binding activities (Fig. 3B). Taken together, we conclude that neither basal nor stress-inducible phosphorylation within the RD is involved in the regulation of HSF1 DNA-binding activity.

Phosphorylation in the regulatory domain suppresses HSF1 transactivating capacity. To investigate the effect of phosphorylation on HSF1 transactivating capacity, we transfected HSF1 WT and HSF1Δ~PRD into *hsf1*$^{-/-}$ MEFs, exposed the cells to stress and measured the steady-state mRNA levels of *Hsp70* (*HSPA1A* and *HSPA1B*; *HSPA1A/B*), *Hsp25* (*HSPB1*), and *Hsp40* (*DnaJB1*) by qRT-PCR. Under control conditions, neither HSF1Δ~PRD nor HSF1 WT was spontaneously activated, since the levels of *Hsps* were equal to those in cells transfected with an empty plasmid (Mock) (Fig. 4A and B). Upon a 30-min exposure to heat stress at 43°C, we observed an HSF1-dependent increase in *HSPA1A/B*, *HSPB1*, and *DnaJB1* mRNAs (Fig. 4A and data not shown). Surprisingly, not only was HSF1Δ~PRD activated upon heat stress, but it exceeded the HSF1 WT in transactivating capacity, since steady-state mRNA levels of *HSPA1A/B* and *HSPB1* were 2-fold higher in the HSF1Δ~PRD-expressing cells than in HSF1 WT-expressing cells. The 2-fold difference in *Hsp* mRNA levels between HSF1 WT and HSF1Δ~PRD was observed also after 1 h exposure to heat stress. Our observation that HSF1Δ~PRD is capable of driving transcription provides the first evidence for uncoupling the stress-inducible phosphorylation from HSF1 activation.

Next, we examined whether the increased levels of *Hsps* in cells

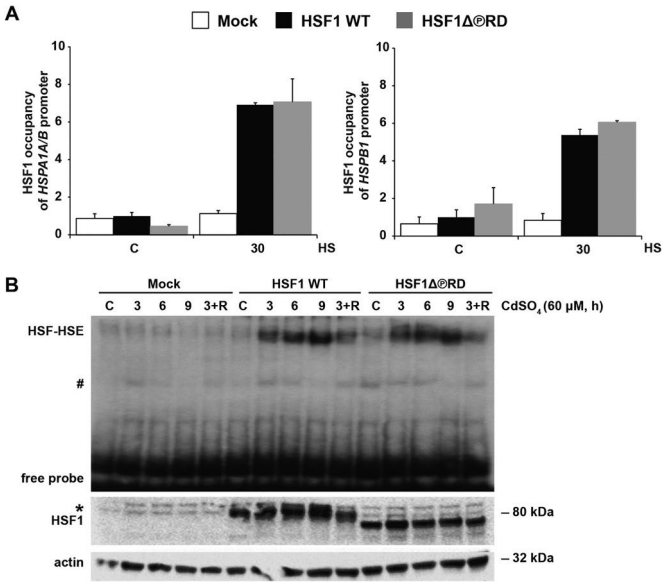

FIG 3 HSF1Δ∼PRD binds to DNA in a stress-inducible manner. *hsf1*$^{-/-}$ MEFs were transfected with Mock plasmid [pcDNA3.1/myc-His(−)A], Myc-His-HSF1 WT, or Myc-His-HSF1Δ∼PRD, and left either untreated (C) or exposed to a 30-min heat shock at 43°C (A) or heavy metal stress (B). (A) The occupancy of HSF1 at the *HSPA1A/B* (*Hsp70*) and *HSPB1* (*Hsp25*) promoters was analyzed by ChIP, followed by qPCR. The qPCR values of the immunoprecipitations were normalized to the input values and related to the HSF1 WT control sample, which was set to value 1. The data are presented as mean values from three independent experiments plus the standard errors of the mean (SEM). The values obtained for the nonspecific antibody (normal rabbit serum) are 1.07 for *HSPA1A/B* and 2.92 for *HSPB1*. (B) For assessing HSF1Δ∼PRD DNA-binding activity during prolonged stress, the cells were treated with 60 μM $CdSO_4$ for the indicated times (3+R: 3 h $CdSO_4$, followed by a 3-h recovery in fresh culture medium). The HSE-HSF complex (HSF-HSE) was analyzed by EMSA. Expression of HSF1 constructs was detected by Western blotting with anti-HSF1 antibody. α-Actin was used as a loading control. The pound sign indicates nonspecific HSE interactions, and the asterisk indicates HSF1 protein that migrates more slowly on SDS-PAGE due to hyperphosphorylation (53).

expressing HSF1Δ∼PRD were specific for heat stress only. For this purpose, we measured *HSPA1A/B* and *HSPB1* mRNA levels from cells exposed to heavy metals. We treated *hsf1*$^{-/-}$ MEFs, transfected with either HSF1 WT or HSF1Δ∼PRD, with $CdSO_4$, and found that after a 3-h exposure the mRNA levels of *HSPA1A/B* and *HSPB1* were higher in HSF1Δ∼PRD-expressing cells than in HSF1 WT-expressing cells, and the difference was maintained also after 3 h of recovery (Fig. 4B). These results demonstrate that the phosphorylation-mediated repression of HSF1 transactivating capacity is not specific for a particular type of stress.

Elevated stress-inducible *Hsp* mRNAs in cells expressing HSF1Δ∼PRD could be due to a lowered threshold of stress stimuli. To address this possibility, we exposed *hsf1*$^{-/-}$ MEFs transfected with HSF1 WT or HSF1Δ∼PRD to heat shock temperatures at 39, 40, and 41°C (Fig. 5A). Moderate heat stress can activate the heat shock response, albeit less efficiently than an exposure to 43°C (69, 70). We did not detect HSF1-mediated induction of *HSPA1A/B* mRNA within 1 h at 39°C, and at 40°C only cells expressing HSF1Δ∼PRD displayed elevated levels of *HSPA1A/B* mRNA, whereas at 41°C both HSF1 WT and HSF1Δ∼PRD were capable of inducing *HSPA1A/B* mRNA. Importantly, upon exposure to 41°C, only cells expressing HSF1Δ∼PRD displayed elevated levels of *HSPA1A/B* mRNA as early as at a 30-min time point (Fig. 5A).

To further study the activation threshold of HSF1 WT and HSF1Δ∼PRD, we treated cells with 40 μM and 60 μM $CdSO_4$ and measured *HSPA1A/B* mRNA at 1-h intervals up to 3 h. After a 3-h exposure to 40 μM $CdSO_4$, the heat shock response was activated only in cells expressing HSF1Δ∼PRD, while at 60 μM $CdSO_4$, *HSPA1A/B* mRNA was induced both in HSF1 WT- and in HSF1Δ∼PRD-expressing cells (Fig. 5B). Taken together, our results revealed that cells expressing HSF1Δ∼PRD activated the heat shock response upon moderate stress, which indicates that the activation threshold of HSF1 is lowered when the RD is not phosphorylated.

Intrinsic capacity of the regulatory domain to control transactivation depends on its phosphorylation status. The Gal4 DNA-binding domain fused to the herpes simplex virus 1 VP16 activation domain (AD) is a potent transcriptional activator capable of expressing eukaryotic genes under a promoter containing Gal4-binding sites (49). In earlier studies, where HSF1 RD was introduced into that chimeric protein, the transactivating capacity of VP16 AD became stress-responsive (22), providing evidence for HSF1 RD possessing an intrinsic ability to regulate transcription and sense heat stress. Here, we wanted to investigate whether the

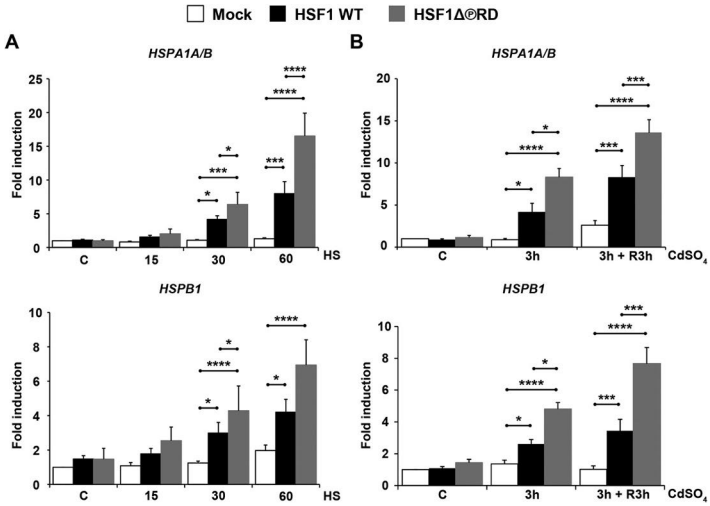

FIG 4 Phosphorylation in the regulatory domain suppresses HSF1 transactivating capacity. (A) *hsf1*$^{-/-}$ MEFs were transfected with Mock plasmid [pcDNA3.1/myc-His(−)A], Myc-His-HSF1 WT, or Myc-His-HSF1Δ∼PRD and left either untreated (C) or exposed to heat stress at 43°C up to 60 min. The mRNA levels of *HSPA1A/B* (Hsp70) and *HSPB1* (Hsp25) were quantified with qRT-PCR and normalized against *RNA18S5*. The values are shown relative to the respective mRNA levels in the Mock-transfected cells in control conditions (C), which was arbitrarily set to value 1. (B) *hsf1*$^{-/-}$ MEFs were transfected as in panel A and either left untreated (C), treated with 60 μM $CdSO_4$ for 3 h (3 h), or treated for 3 h and left to recover in fresh culture medium for 3 h (3 h + R3h). mRNA quantification and data analysis were performed as in panel A. The data are presented as mean values from at least three independent experiments plus the SEM. *, $P \leq 0.05$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

lack of phosphorylation within the RD contributes to the enhanced transactivating capacity of the heterologous VP16 AD. For this purpose, we cloned the regulatory domain (aa 220 to 389) of HSF1 WT and HSF1Δ∼PRD into the Gal4-VP16 chimeric construct (Fig. 6A). HeLa cells were cotransfected with the indicated chimeric constructs and Gal4-driven luciferase reporter gene. Luciferase activity was measured from untreated cells as well as from cells exposed to a 30-min heat shock at 42°C, followed by a 5-h recovery. To exclude the possibility that the obtained results were due to unequal expression of Gal4-VP16 chimeras, we analyzed their protein levels, and found them equally expressed (Fig. 6C). Cells expressing Gal4-VP16 displayed constitutive luciferase activity, whereas Gal4-VP16-HSF1 WT repressed the transcription of the reporter gene under nonstress conditions, reducing luciferase activity by 50% (Fig. 6B). In contrast, the luciferase activity in cells expressing Gal4-VP16-HSF1Δ∼PRD was equal to the cells transfected with Gal4-VP16. These results demonstrate that the lack of phosphorylation within the HSF1 RD reverses the repressed transactivating capacity of VP16 AD under control conditions.

Repression of Gal4-VP16-HSF1 WT was eliminated upon heat shock due to the intrinsic capacity of HSF1 RD to sense heat shock, and the luciferase activity corresponded to that observed in cells expressing Gal4-VP16 (Fig. 6B). Surprisingly, after heat shock, Gal4-VP16-HSF1Δ∼PRD was 60% more effective than Gal4-VP16 or Gal4-VP16-HSF1 WT, indicating that the phosphorylation-deficient RD is capable of further enhancing transactivation in a stress-dependent manner. Based on the obtained results, we conclude that the phosphorylation status defines the intrinsic capacity of HSF1 RD to control transactivation in response to heat stress.

DISCUSSION

Involvement of HSF1 in a plethora of cellular functions requires a sophisticated regulatory mechanism(s) that can accurately control the conditions under which HSF1 is activated. It has been suggested that phosphorylation of HSF1 would serve as an integrator of various signaling pathways triggering HSF1-driven transcription (37). The regulatory domain (RD) harbors ∼70% of the known HSF1 phosphorylation sites, and we hypothesized that HSF1 activation would primarily be controlled by phosphorylation within the RD. The results obtained here provide, to the best of our knowledge, the first direct evidence for uncoupling hyperphosphorylation from HSF1 activation. In contrast to previous studies, where disruption of the RD led to a constitutively active HSF1 (34, 35, 38), HSF1Δ∼PRD is not spontaneously active and is capable of inducing *Hsp* expression in a stress-dependent manner. These results are surprising, since phosphorylation has been regarded as an important hallmark of HSF1 activation (20, 30, 38). Although HSF1 is phosphorylated on several residues during its activation, it has been reported that only one of these residues, S326, substantially contributes to HSF1 transcriptional activity (30) and is widely used as a marker for activated HSF1 in carcinogenesis (20, 71). In agreement with the earlier report (30), the stress-inducible DNA-binding capacity of HSF1 is not dependent on S326 as an intact phosphorylation acceptor site, whereas our

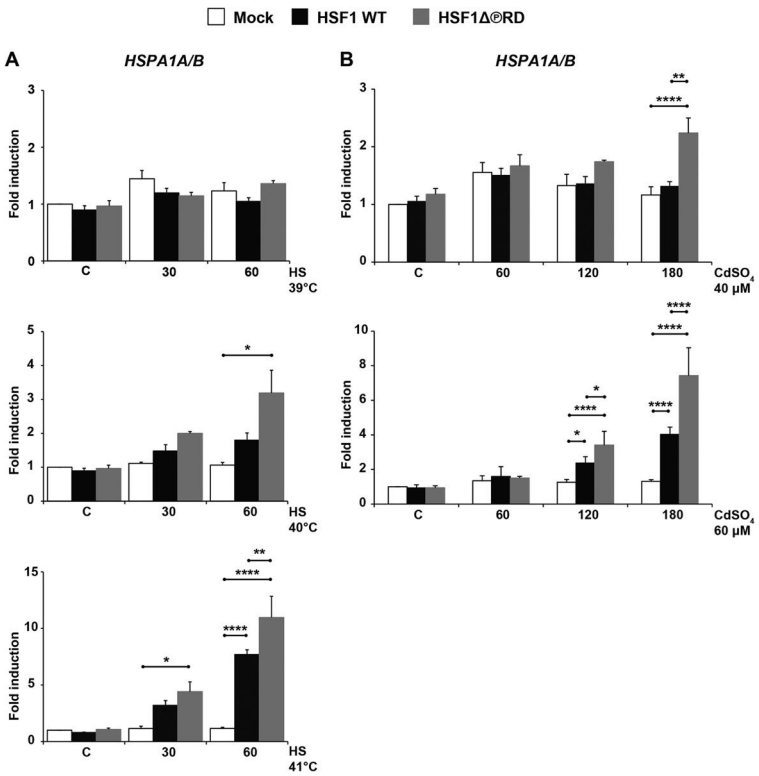

FIG 5 HSF1Δ~PRD requires a lower threshold for activation than HSF1 WT. *hsf1*$^{-/-}$ MEFs were transfected with Mock plasmid [pcDNA3.1/myc-His(−)A], Myc-His-HSF1 WT, or Myc-His-HSF1Δ~PRD and left either untreated (C) or exposed to heat stress (39, 40, and 41°C for 30 and 60 min) (A) or heavy metal stress (40 and 60 μM $CdSO_4$ for 60, 120, and 180 min) (B). The mRNA levels of *HSPA1A/B* (Hsp70) were quantified using qRT-PCR and normalized against *RNA18S5*. The values are shown relative to the respective mRNA levels in the Mock-transfected cells in control conditions (C), which was arbitrarily set to value 1. The data are presented as mean values from at least three independent experiments plus the SEM. *, $P \leq 0.05$; **, $P \leq 0.01$; ****, $P \leq 0.0001$.

results show that HSF1 can be a potent transcriptional activator without being phosphorylated on multiple sites, including S326, within the RD. This discrepancy is presumably due to a different experimental approach, i.e., single-site versus multisite mutagenesis of HSF1.

Our finding that HSF1 activity can be uncoupled from the phosphorylation events occurring in the RD, is supported by recently published studies. The results by Rossi et al. indicate that cells treated with the proteasome inhibitor bortezomib display inducible HSF1-dependent *Hsp70* expression accompanied by only a modest increase in HSF1 phosphorylation (72). Another recent study revealed that ethanol exposure leads to transcriptional activation of HSF1, which lacks hyperphosphorylation (73). Taking all of these findings together, we conclude that activation of HSF1 can occur in the absence of hyperphosphorylation. Intriguingly, hyperphosphorylated HSF1 can also be transcriptionally incompetent under certain circumstances, which was recently observed in heat-stressed mitotic cells, where *Hsp*s were not induced despite hyperphosphorylation of HSF1 (74). Thus, employing phosphorylation as a sole marker for HSF1 activation should be reconsidered.

Given that hyperphosphorylation is not required for HSF1 activation, the question of why HSF1 is hyperphosphorylated during its activation remains to be answered. The finding that HSF1Δ~PRD can be activated by milder stress than HSF1 WT (Fig. 5) indicates that phosphorylation within the RD defines the activation threshold in response to distinct stress stimuli. Furthermore, the phosphorylation-deficient HSF1Δ~PRD is a more potent transactivator than HSF1 WT (Fig. 4), suggesting that hyperphosphorylation limits the magnitude of the heat shock response. Based on these results, we propose that phosphorylation serves as a fine-tuning mechanism for regulating the transcriptionally competent HSF1. Phosphorylation can modulate transcription factor activity on at least three levels: subcellular localization, DNA-

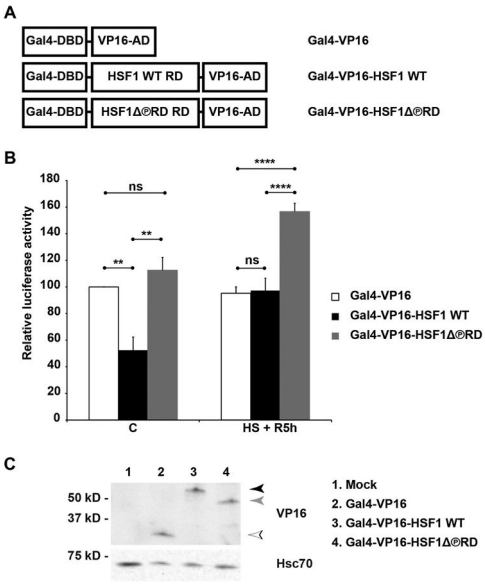

FIG 6 Phosphorylation defines capacity of the RD to control transactivation. (A) Schematic illustration of the chimeric proteins consisting of Gal4 DNA-binding domain (Gal4-DBD, aa 1 to 147), HSF1 WT or HSF1Δ~PRD regulatory domain (HSF1 WT RD and HSF1Δ~PRD RD, aa 220 to 389), and herpes simplex virus protein VP16 activation domain (VP16-AD, aa 413 to 490). Note that the figure is not drawn to scale. (B and C) HeLa cells were transfected with plasmids encoding Gal4-driven luciferase and β-galactosidase, together with indicated plasmids encoding chimeric proteins described in panel A or with an empty plasmid (Mock). Cells were left untreated (C) or heat shocked for 30 min at 42°C and let to recover for 5 h at 37°C (HS + R5h). In panel B, the relative luciferase activity was calculated against the activity in the Gal4-VP16 samples under control conditions, which was set to value 100. The data are presented as mean values from four independent experiments plus the SEM. ns, nonsignificant; **, $P \leq 0.01$; ****, $P \leq 0.0001$. In panel C, the protein levels of the Gal4-VP16 chimeras, under control conditions, were analyzed using Western blotting with anti-VP16 antibody. Hsc70 is shown as a loading control. The arrowheads indicate Gal4-VP16 (white), Gal4-VP16-HSF1 WT (black), and Gal4-VP16-HSF1Δ~PRD (gray). The difference in the migration pattern between Gal4-VP16-HSF1 WT and Gal4-VP16-HSF1Δ~PRD is likely due to phosphorylation.

binding activity, and interaction with the transcriptional machinery (75). Since we did not observe any changes in HSF1 localization (Fig. 2) or DNA-binding activity (Fig. 3), it is likely that phosphorylation within the RD modulates the interaction between HSF1 and the transcriptional machinery both via conformational changes (76) and electrostatic effects (77). It has earlier been shown that HSF1 interacts with various protein complexes required for active transcription, such as the chromatin remodeling complex SWI/SNF (78), Mediator complex (79), components of the preinitiation complex (80), and the histone chaperone FACT (81). However, the role of HSF1 phosphorylation in these protein-protein interactions has not been reported and will be investigated in our forthcoming studies. Another mechanism by which phosphorylation modulates transcription factor activity is through interconnected posttranslational modifications (PTMs) (82). It is known that HSF1 is sumoylated in a phosphorylation-dependent manner (29). In addition, HSF1 is subjected to acetylation (31), ubiquitination (32), and glycosylation (83), and it is plausible that other PTMs modulate HSF1-driven gene expression in an orchestrated manner. Thus, to fully understand how transactivation capacity of HSF1 is regulated, emphasis should be placed on phosphorylation-dependent interactions within the RD.

Our results validate hyperphosphorylation as a fundamental regulator of HSF1 transactivation capacity. Phosphorylation, in cooperation with other PTMs, creates distinct PTM signatures, which can adequately modulate the transcriptional response based on the type and severity of the stimuli. Since HSF1 is involved in a multitude of physiological processes, such as the transcriptional control of development and life span, in addition to proteotoxic stress responses (4), a specific PTM signature would provide a mechanism enabling precise temporal, spatial, and environmental transcriptional programs to ensure that a cell is able to perform its designated function. Importantly, identification and regulation of the HSF1 PTM signatures provides new possibilities to counteract the actions of HSF1 in pathological conditions, such as cancer and neurodegenerative diseases (20, 46).

ACKNOWLEDGMENTS

We thank Julius Anckar for insightful discussion in the initial stage of the project, Alexandra Elsing for expert help with the statistical analysis, Samu Himanen for help with the ChIP assay, and Emine Lundsten and Cynthia Swan for assistance with writing of the manuscript. All of the members of the Sistonen laboratory are acknowledged for their constructive comments.

This study was financially supported by the Academy of Finland, the Sigrid Jusélius Foundation, the Magnus Ehrnrooth Foundation, Åbo Akademi University Foundation (L.S.), and the Turku Doctoral Program of Biomedical Sciences (M.A.B. and J.J.).

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6102–6115 *Nucleic Acids Research, 2022, Vol. 50, No. 11* *Published online 10 June 2022*
https://doi.org/10.1093/nar/gkac493

HSFs drive transcription of distinct genes and enhancers during oxidative stress and heat shock

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Received April 26, 2022; Editorial Decision May 19, 2022; Accepted May 25, 2022

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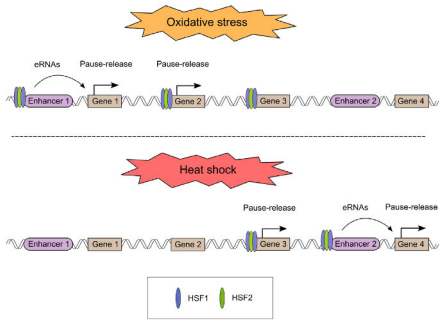
ABSTRACT

Reprogramming of transcription is critical for the survival under cellular stress. Heat shock has provided an excellent model to investigate nascent transcription in stressed cells, but the molecular mechanisms orchestrating RNA synthesis during other types of stress are unknown. We utilized PRO-seq and ChIP-seq to study how Heat Shock Factors, HSF1 and HSF2, coordinate transcription at genes and enhancers upon oxidative stress and heat shock. We show that pause-release of RNA polymerase II (Pol II) is a universal mechanism regulating gene transcription in stressed cells, while enhancers are activated at the level of Pol II recruitment. Moreover, besides functioning as conventional promoter-binding transcription factors, HSF1 and HSF2 bind to stress-induced enhancers to trigger Pol II pause-release from poised gene promoters. Importantly, HSFs act at distinct genes and enhancers in a stress type-specific manner. HSF1 binds to many chaperone genes upon oxidative and heat stress but activates them only in heat-shocked cells. Under oxidative stress, HSF1 localizes to a unique set of promoters and enhancers to *trans*-activate oxidative stress-specific genes. Taken together, we show that HSFs function as multi-stress-responsive factors that activate distinct genes and enhancers when encountering changes in temperature and redox state.

GRAPHICAL ABSTRACT



INTRODUCTION

Cells are exposed to various cytotoxic stresses including elevated temperatures and oxidative stress. While increased temperatures lead to protein misfolding, oxidative stress is caused by elevated production of reactive oxygen species (ROS) that oxidize macromolecules (proteins, lipids and nucleic acids) (1,2). Regulation of ROS levels is critical for cell survival and also for normal physiology, since basal levels of ROS activate cellular signaling pathways, while increased production of ROS promotes aging and progression of many diseases, such as cancer (1,3). To combat cytotoxic stresses, cells extensively reprogram their transcription (4). Although genome-wide transcription is repressed upon stress, certain stress-responsive transcription factors can *trans*-activate pro-survival genes, allowing cells to overcome the adverse conditions (4–6). Transcription under oxidative stress is known to be regulated by nuclear factor erythroid 2-related factor 2 (Nrf2) and forkhead box transcription factors (FOXOs), while proteotoxic stress-inducible transcription is driven by a family of heat shock factors (HSFs) (4). In addition to gene activation, cytotoxic con-

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ditions have been shown to activate transcription at numerous enhancers, which are distal regulatory elements in the DNA that can promote gene expression through loop formation (6–10). Intriguingly, active enhancers produce short and unstable enhancer RNAs (eRNAs) that regulate gene transcription by mechanisms which are not entirely understood (10). The characteristic pattern of eRNA transcription serves as a means to identify active enhancers *de novo* using methods that measure nascent transcription at a nucleotide resolution (11–13).

The master *trans*-activators in stressed cells include the HSFs, which are activated in response to various proteotoxic stresses, e.g. heat shock (14,15). Proteotoxic stress impairs proper protein folding and causes accumulation of unfolded proteins (2). To prevent and mitigate these damages, HSFs rapidly *trans*-activate genes encoding heat shock proteins (HSPs), which, in turn, function as molecular chaperones (4). HSF1 is the master regulator of chaperone expression and the most studied member of the HSF family, whereas HSF2 has been mainly characterized as a developmental transcription factor, particularly in gametogenesis and neurogenesis (15). Intriguingly, exogenous human HSF2, but not HSF1, can substitute for yeast HSF to provide thermotolerance, demonstrating that HSF2 has a capability to act as a stress-responsive transcription factor (16). There is also evidence for a context-dependent interplay between HSF1 and HSF2, either competitive or synergistic, but the functional role of HSF2 in stress-inducible transcription has remained elusive (17,18). Although HSF1 has been identified as the master regulator of the heat shock response and other proteotoxic stresses, it is also activated in response to oxidative stress (19). The biological significance of HSF1 in the regulation of redox status was previously reported in a study, where increased production of cardiac ROS was observed in the absence of HSF1 (20). Nevertheless, how HSF1 and other member of the HSF family contribute to transcriptional reprogramming upon oxidative stress is unknown.

Recently, it was shown that apart from binding promoters, HSF1 is recruited to heat-induced enhancers to activate genes, such as forkhead box O3 (*Foxo3*) and tax1-binding protein 1 (*Tax1bp1*) (6,9,21). The function of the HSF family members in the genome-wide enhancer activation under different stress conditions is, however, not known. In this study, we compared the stress-specific transcription programs by tracking transcription at genes and enhancers in cells exposed to either oxidative stress or heat shock. We used precision run-on sequencing (PRO-seq), which quantifies transcriptionally engaged RNA polymerase II (Pol II) complexes at a single nucleotide resolution across the genome (11). Unlike RNA-seq and other conventional methods that measure steady-state mRNA levels, PRO-seq allows detection of active transcription at promoter-proximal regions, upstream divergent transcripts, gene bodies, termination windows and enhancers (11,12,22). Combining PRO-seq with chromatin immunoprecipitation sequencing (ChIP-seq), we identified HSF1 and HSF2 as new regulators of oxidative stress-inducible transcription. HSF1 and HSF2 were recruited to distinct genomic sites in cells exposed to oxidative stress or heat shock, which triggered the activation of stress-specific transcription programs. Furthermore, besides functioning as conventional promoter-binding transcription factors, HSFs activate several oxidative stress- and heat-inducible enhancers. Finally, we found that in contrast to the promoter-bound HSF1, which drives the classical chaperone genes, binding of HSF1 to enhancers activates genes encoding proteins localized at plasma membrane and cell junctions. Taken together, our results show that HSFs function as multi-stress-responsive transcription factors that orchestrate stress-specific transcription programs through genes and enhancers.

MATERIALS AND METHODS

Cell lines

Wild-type (WT) and HSF1 knock-out (KO) MEFs were derived from mice generated in the laboratory of Ivor J. Benjamin (23). HFS2 KO MEFs were derived from mice generated in the laboratory of Valerie Mezger (24).

Cell culture and treatments

MEFs were grown in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 μg/ml penicillin/streptomycin, and non-essential amino acids (Gibco). Cells were maintained at 37°C with 5% CO_2. Cells were exposed to heat shock by submerging the cell culture dishes into a 42°C water bath for 1 h. This heat shock condition was used for all the PRO-seq and ChIP-seq analyzes. Oxidative stress was induced by treating the cells with freshly prepared menadione solution at 37°C. For PRO-seq and ChIP-seq, cells were treated with 30 μM menadione for 2 h, whereas for GSH/GSSG assay, cells were treated with 10, 30 and 50 μM menadione for 2 h. DNA damage was induced by exposing cells to 2 mM hydroxyurea for 17 h.

Western blotting

Cells were lysed in Laemmli sample buffer (30% glycerol; 3% SDS; 188 mM Tris–Cl, pH 6.8; 0.015% bromophenol blue; 3% β-mercaptoethanol). Equal volumes of lysates were run on SDS-PAGE, after which proteins were transferred to nitrocellulose membrane. Membranes were blocked with nonfat dried milk diluted in PBS-Tween20 for 1 h at room temperature (RT). Proteins bound to membrane were analyzed using primary antibodies against HSF1 (ADI-SPA-901, Enzo), HSF2 (3E2, EMD Millipore) and β-tubulin (T8328, Merck). Next, the membranes were incubated in secondary HRP-conjugated antibodies, and the proteins were detected with enhanced chemiluminescence.

Immunofluorescence

WT MEFs were plated on MatTek plates (P35GC-1.5-14-C, MatTek Corporation) 48 h before treatments. Cells were fixed with 4% paraformaldehyde (PFA) for 10 min, permeabilized in 0.1% Triton X-100 in PBS and washed three times with PBS. Samples were blocked with 10% FBS in PBS for 1 h at RT and incubated overnight at 4°C with a primary anti-γH2AX antibody (05-636, EMD Millipore,

1:500 in 10% FBS-PBS). Following primary antibody incubations, the samples were washed three times with PBS. Next, samples were incubated in a secondary goat anti-mouse Alexa Fluor488 antibody (A11001, Invitrogen, 1:500 in 10% FBS–PBS) for 1 h at RT. Finally, the samples were washed two times with PBS, incubated with 300 nM DAPI diluted in PBS, and covered with VECTASHIELD mounting medium (H-1000, Vector Laboratories). All images were acquired with a 3i CSU-W1 spinning disc confocal microscope (Intelligent Imaging Innovations).

Measurement of GSH/GSSG ratio

The effect of menadione on the induction of oxidative stress was determined by measuring the ratio between oxidized and reduced glutathione (GSH/GSSG) using a commercial kit by Promega (GSH/GSSG-Glo Assay, V6611).

PRO-seq

PRO-seq was performed from two biological replicates as described previously (11,25). Specifically, PRO-seq was performed in WT, HSF1 KO, and HSF2 KO MEFs that were untreated, exposed to 30 μM menadione for 2 h or heat-shocked at 42°C for 1 h. Nuclei of MEFs were isolated in buffer A (10 mM Tris–HCl pH 7.4, 300 mM sucrose, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 0.1% Triton X-100, 0.5 mM DTT) using a dounce homogenizer. The isolated nuclei were flash-frozen and stored at –80°C in a storage buffer (10 mM Tris–HCl pH 8.0, 25% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, 5 mM DTT). Run-on reactions were performed at 37°C for 3 min in the presence of biotinylated nucleotides (5 mM Tris–HCl pH 8.0, 2.5 mM $MgCl_2$, 150 mM KCl, 0.5 mM DTT, 0.5% Sarkosyl, 0.4 u/μl RNase inhibitor, 0.025 mM biotin-ATP/CTP/GTP/UTP [Perkin Elmer]). Equal amounts of nuclei extracted from *Drosophila* S2 cells were used as spike-in material in run-on reactions. Total RNA was isolated with Trizol, precipitated with ethanol and fragmented by base hydrolysis using NaOH. Biotinylated transcripts were isolated with streptavidin-coated magnetic beads (M280, Invitrogen). In the next steps, TruSeq small-RNA adaptors were ligated to the ends of nascent RNAs. Before ligating 5′adaptor, the 5′-cap was removed with RNA 5′ pyrophosphohydrolase (Rpph, NEB), after which 5′end was repaired with T4 polynucleotide kinase (NEB). Nascent RNAs containing the adaptors were converted to cDNA, amplified by PCR and sequenced using NovaSeq 6000. The raw files are available in GEO accession: GSE183245.

ChIP-seq

HSF1- and HSF2-bound DNA fragments were isolated from two biological replicates using ChIP as previously described (26). Specifically, ChIP-seq was performed in WT MEFs that were untreated, exposed to 30 μM menadione for 2 h or heat-shocked at 42°C for 1 h. Cells were crosslinked with 1% paraformaldehyde for 5 min, after which paraformaldehyde was quenched with 125 mM glycine. Cells were lysed and the chromatin was fragmented by sonication with Bioruptor Pico (Diagenode) using seven cycles (30 s on/off). Agarose gel electrophoresis was used to verify that fragment size after sonication was 300–400 bp. The following antibodies were used for immunoprecipitation: HSF1 (ADI-SPA-901, Enzo), HSF2 (26), and normal rabbit IgG (EMD Millipore). Crosslinks were reversed by incubating the samples at 65°C overnight, and the DNA was purified with phenol:chloroform. ChIP-seq libraries were generated using NEXTFLEX ChIP-seq kit and barcodes (Perkin Elmer). NovaSeq 6000 was used to sequence ChIP-seq libraries. The raw files are available in GEO accession: GSE183245.

Mapping of PRO-seq and ChIP-seq data

Adapters were removed from the sequencing reads using cutadapt (27) and the reads were mapped to mouse genome (mm10) using Bowtie 2 (28). PRO-seq reads were mapped in single-end mode with parameters: –sensitive-local. ChIP-seq reads were mapped in paired-end mode with parameters: –sensitive-local –no-mixed –no-discordant –no-unal. The raw data (GSE183245) is available in Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/).

Normalization of PRO-seq data

Various strategies are used for the normalization of PRO-seq data, including normalization to spike-ins, read counts in ends of longs genes, and read counts in genes that remain unregulated or inactive across samples (5,7,29–33). Spike-in normalization is highly recommended due to its ability to detect global changes in the level of transcription that would be left undetected with several other methods, such as sequencing depth normalization (29). To utilize spike-ins, we added equal amounts of nuclei from *Drosophila* S2 cells to each run-on reaction in the PRO-seq samples. Since transcripts produced by *Drosophila* S2 nuclei are retained in the samples through every step of PRO-seq, reads mapping to *Drosophila* genome can be used for the normalization of the sample data (9,29,34). Normalization was performed by correcting read counts from spike-in genome to library sizes, followed by calculation of final normalization factors for each sample as described earlier (29).

Normalization of ChIP-seq data

Spike-in normalization was utilized by adding equal amounts of chromatin from heat-shocked human Hs578T cells to each immunoprecipitation reaction. Hs578T cells were exposed to heat shock because it triggers the binding of HSF1 and HSF2 to chromatin, which in turn, allows simultaneous immunoprecipitation of HSF-bound DNA from the sample and spike-in material. We verified that each sample contained equal proportion of spike-in material by mapping the sequencing reads to human genome (hg38).

Quantification of transcription at genes

Actively transcribed genes were identified using discriminative regulatory elements identification from global run-on data (dREG; https://dreg.dnasequence.org) (13), which detects transcription initiation sites at genes and enhancers. Intersecting TSSs of annotated genes with dREG-called initiation sites resulted in a list of 19,350 active genes that were

retained for further analyses. Transcription was quantified from the gene bodies, which were defined as +0.5 kb from TSS to –0.5 kb from CPS. In addition, the maximum length of genes was set to 300 kb, since Pol II can only travel 240 kb during 2 h-treatments at elongation rate of 2 kb/min (35,36).

Identification of transcribed enhancers

Transcribed regulatory regions, including promoters and enhancers, were identified from the PRO-seq data using dREG gateway (https://dreg.dnasequence.org/) (13), as described in previous studies (6,9). The dREG-identified regions of divergent transcription that resided over 1 kb from the TSSs of annotated genes, were defined as transcribed enhancers. To make a unified list of enhancers across the samples, we first identified enhancers individually in each sample and then merged the coordinates of overlapping enhancers using bedtools merge with parameters: d -100 (31). The resulting list contained 44 593 enhancers, whose level of transcription was quantified in each sample from the coordinates detected by dREG. Paused Pol II can be observed at enhancers similarly to promoter-proximal regions, although pausing of Pol II is more evident at promoter-proximal regions (37). Quantification of enhancer transcription from dREG coordinates contains a possible site of Pol II pausing.

Differential expression analysis

Changes in transcription of genes and enhancers were determined using DESeq2 (38). Differential gene expression was measured in gene bodies, whose coordinates were defined as +0.5 kb from TSS to –0.5 kb from CPS. Changes in enhancer transcription were analyzed separately from plus and minus strands using the enhancer coordinates determined with dREG. To call statistically significant changes in transcription of both genes and enhancers, *P*-value threshold was set to 0.05, and fold change threshold to 1.5 for upregulated and to 0.667 for downregulated genes/enhancers.

ChIP-seq peak calling

ChIP-seq peaks were identified from two combined replicates using findPeaks tools included in HOMER program (39). For HSF1 and HSF2 peaks to be called statistically significant, we set the FDR threshold to 0.001 (default value used by HOMER) and required that the fold change over IgG was at least five. For H3K27ac and H3K4me1 peaks to be statistically significant, FDR threshold was set to 0.001 and fold change over input was required to be at least four. HSF1 and HSF2 peaks were called using parameters: -style factor -F 5 -L 7 -localSize 20 000. H3K27ac and H3K4me1 peaks were called using parameters: -region -L 0 -size 250.

GO analysis

Biological processes enriched in distinct groups of HSF target genes were identified using Metascape tool (40) (https://metascape.org/gp/index.html#/main/step1). GO terms were determined for two different heat-inducible gene groups: (i) target genes, whose promoters were bound by HSF1, (ii) target genes devoid of promoter-bound HSF1 that were located within 100 kb of enhancer-bound HSF1. GO terms were ranked in descending order based on the number of genes associated with each term.

Analysis of HSE content

Content of HSE motif in the target genes and enhancers of HSFs was analyzed using findMotifsGenome.pl tool included in HOMER program (39). HSE content was analyzed within 2 kb regions centered around the summits of HSF1 and HSF2 peaks.

Additional datasets used

H3K27ac and H3K4me1 ChIP-seq data is from GEO dataset: GSE99009.

RESULTS

Oxidative stress and heat shock reprogram transcription of distinct genes and enhancers

To examine reprogramming of transcription in response to two different types of cell stress, i.e. oxidative stress and heat shock, we tracked transcription at a nucleotide resolution in mouse embryonic fibroblasts (MEFs) utilizing PRO-seq. For determining the specific roles of HSF1 and HSF2 in orchestrating transcription under these stresses, PRO-seq was performed in HSF1 knock-out (KO) MEFs and HSF2 KO MEFs, in addition to wild-type (WT) MEFs (Supplementary Figure S1A and S1B). Oxidative stress was induced by treating MEFs with different concentrations of a commonly used ROS generator, menadione, for 2 h (41). From the concentrations tested, 30 μM was selected for transcriptional analyses, since it was the lowest concentration that caused oxidative stress, as measured by the decrease in the ratio of reduced and oxidized glutathione (GSG/GSSG) (Supplementary Figure S2). The heat shock response was induced by exposing MEFs to 42°C for 1 h. For accurate analyses of PRO-seq samples between distinct conditions and cell lines, we utilized spike-in normalization, which verified high correlation (rho $>$ 0.95) of the biological replicates (Supplementary Figure S3).

The normalized PRO-seq data was used to investigate the impact of menadione treatment and heat shock on transcription of genes and enhancers. Transcribed regulatory regions were identified using the divergent pattern of transcription that characterizes active promoters and enhancers in mammals (12,13). Enhancers were distinguished from promoters by requiring them to reside over 1 kb from any transcription start site (TSS) of annotated genes. As previously reported (6,13), the active enhancers identified from PRO-seq profiles, contained enhancer-associated histone marks H3K27ac and H3K4me1 (42,43) (Supplementary Figure S4). Both menadione and heat shock caused remarkable changes in transcription of genes and enhancers (Figure 1A). Interestingly, the changes in transcription were more prominent upon oxidative stress than upon heat shock

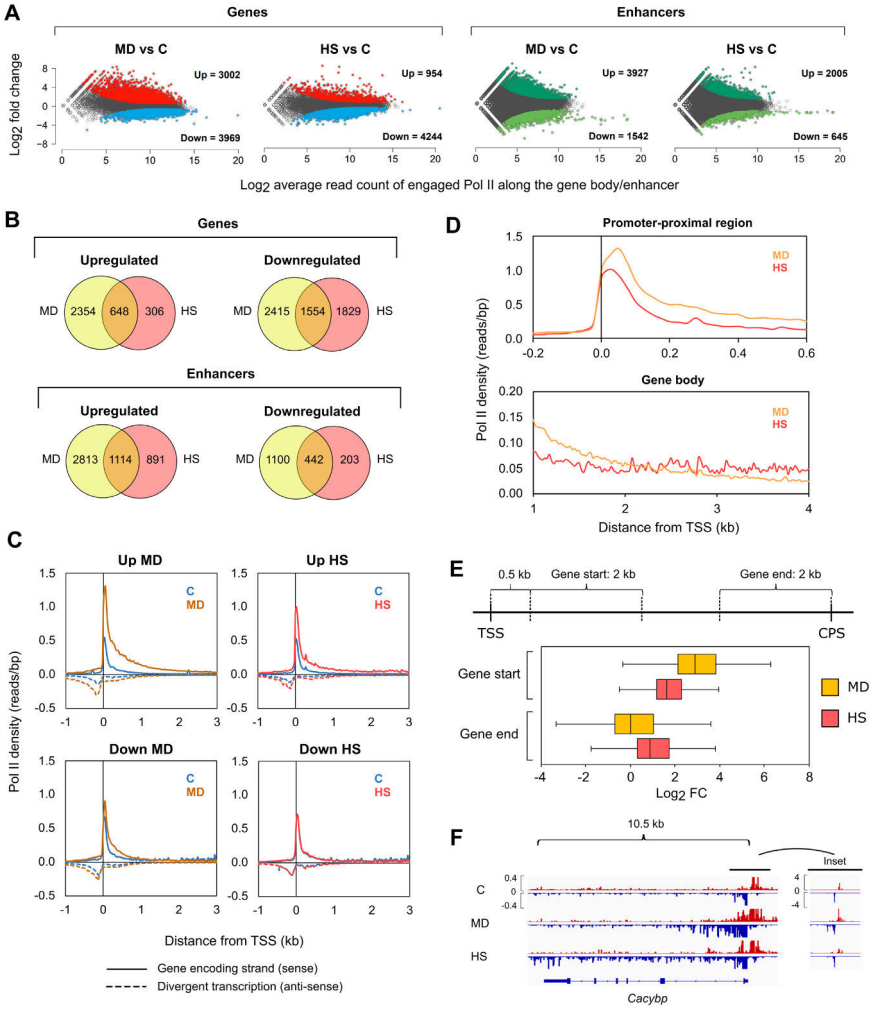

Figure 1. Oxidative stress and heat shock display distinct changes in the transcription of genes and enhancers. PRO-seq was performed in MEFs that were exposed to oxidative stress induced by menadione (MD, 30 μM, 2 h) or to heat shock (HS, 42°C, 1 h). (**A**) The number of upregulated and downregulated genes and enhancers in stressed cells was determined. Threshold for p-value was set to 0.05, and threshold for fold change was set to 1.5 and 0.667 to call statistically significant upregulations and downregulations, respectively. (**B**) Genes and enhancers with altered expression during menadione and heat shock were compared to determine the number of genes and enhancers that were upregulated or downregulated in a stress type-specific manner. (**C**) Average density of Pol II was analyzed upstream and downstream of the TSS in the genes that were upregulated or downregulated by menadione or heat shock. Pol II density was measured separately for the sense (solid line) and antisense (dotted line) strands. (**D**) Pol II densities of upregulated genes in menadione and heat shock samples were overlaid in promoter-proximal region (–0.2–0.6 kb relative to the TSS) and gene body (1–4 kb relative to the TSS). (**E**) Log_2 fold changes (FC) of upregulated genes in cells treated with menadione or heat shock were determined in start and end of the genes. Start of the gene was defined as a 2-kb window starting 0.5 kb downstream from the TSS. End of the gene was defined as a 2-kb window upstream of the CPS. (**F**) PRO-seq profile of calcylin-binding protein (*Cacybp*) gene in cells exposed to menadione and heat shock. C: control.

(Figure 1A). During both stresses, the number of downregulated genes was greater than the number of upregulated genes, whereas enhancers displayed an opposite pattern (Figure 1A). These results show a general reduction of gene transcription in response to stress, accompanied with increased residency of engaged Pol II at enhancers. Comparison of transcriptional changes at individual genes and enhancers, however, revealed a prominent stress-specific reprogramming of transcription (Figure 1B).

Pol II pause-release triggers rapid gene activation in the oxidative stress response

To gain a mechanistic understanding of transcriptional reprogramming, caused by oxidative stress and heat shock, we analyzed the distribution of Pol II along genes and enhancers. Previous studies have shown that upon induction of genes by heat shock, the paused Pol II is released from promoter-proximal regions into elongation simultaneously with the recruitment of new Pol II molecules to the promoters (6,8,44). In contrast, repression of gene transcription by heat shock occurs by reducing the pause-release, which causes accumulation of Pol II within promoter-proximal regions (6). Our results show that the distribution of Pol II in the upregulated and downregulated genes follows the same pattern at the promoter-proximal pause region upon menadione treatment and heat shock, indicating that the induction and repression of transcription is regulated at the level of Pol II pause-release during both types of stress (Figure 1C). These results demonstrate that cells activate and repress stress-specific sets of genes through universal mechanisms.

Engaged Pol II accumulates at enhancers upon oxidative stress and heat shock

The enhancers that were induced upon stress, showed an absence of Pol II under normal growth conditions (Supplementary Figure S5A). Consequently, the critical step in the upregulation of enhancers, upon both oxidative stress and heat shock, was the recruitment of Pol II, which is different from the stress-mediated activation of genes (Supplementary Figure S5A). Downregulated enhancers, in turn, displayed Pol II occupancy already under normal growth conditions, and the occupancy decreased in response to both stresses (Supplementary Figure S5A). Intriguingly, the profiles of downregulated enhancers, showed several Pol II peaks, which implies that transcriptionally active enhancer clusters, also known as super-enhancers (45), lose engaged Pol II under stress conditions.

Increased Pol II density at early gene bodies coincides with oxidative DNA damage

A detailed analysis of Pol II distribution along genes revealed that oxidative stress induced a more profound increase in Pol II density at the promoter-proximal region and beginning of the gene body (0–2 kb from TSS) than was detected at heat-activated genes (Figure 1D). In contrast, as Pol II reached more distal parts of the gene body (2–4 kb from TSS), a higher Pol II density was detected in heat-shocked cells (Figure 1D). Since productive elongation requires Pol II to transcribe through the entire gene body and beyond the cleavage and polyadenylation site (CPS), these results suggest a transcriptional hindrance after the release of paused Pol II in the menadione-treated cells. To investigate whether Pol II proceeded to the end of menadione-activated genes, we determined the fold change of engaged Pol II at the start of the gene (0.5–2.5 kb relative to TSS) and the end of the gene (-2–0 kb relative to CPS) (Figure 1E). We selected the 0.5–2.5 kb region to represent the start of the gene to avoid the paused Pol II from interfering with the measurement of the fold change in the gene body. We also discarded short genes (0–5 kb) from the analysis. Interestingly, menadione caused a greater fold change in the start of the genes than heat shock, while the fold change in the end of the genes was higher upon heat shock (Figure 1E). These results are exemplified by the calcylin-binding protein (*Cacybp*) gene, which is upregulated by both stresses, but shows elevated levels of Pol II throughout the gene body only upon heat shock (Figure 1F).

Although the average induction during menadione treatment was observed particularly in the start of the genes, we found that 37% of the menadione-inducible genes included in the analysis, displayed a fold change above 1.5 also in the end of the genes (Supplementary Figure S5B). Genes that showed increased levels of Pol II throughout the gene body in menadione-treated cells include fork head box O4 (*Foxo4*) and heme oxygenase 1 (*Hmox1*) (Supplementary Figure S5C), known to be critical in the oxidative stress response (46,47). The induction that was observed only in the start of several menadione-inducible genes could occur due to oxidative DNA damage, which has been shown to impede the elongation of Pol II (48). This is supported by our finding, which shows that the amount of DNA damage, as measured by levels of phosphorylated H2AX, was increased in response to menadione but not heat shock (Supplementary Figure S6). Furthermore, the DNA damage is likely to affect open regions, such as early gene bodies where histone acetylation increases upon transcriptional activation (6,49).

HSF1 and HSF2 direct the oxidative stress response

HSF1 is a well-known *trans*-activator of protein folding machinery under proteotoxic stress conditions, while the role of HSF2 in the regulation of stress-inducible transcription has remained elusive (17). For determining the specific roles of HSF1 and HSF2 in transcriptional activation of enhancers and genes during oxidative stress and heat shock, we quantified transcription from the PRO-seq data that we produced from WT, HSF1 KO, and HSF2 KO MEFs. To analyze the impact of HSFs on the enhancer transcription, we selected enhancers that were upregulated in WT MEFs and contained one of the two enhancer-associated histone marks, H3K27ac or H3K4me1 (Supplementary Figure S4). Previously, it has been shown that H3K27ac marks active enhancers, whereas H3K4me1 primes poised enhancers for subsequent activation (43,50,51). Similarly to heat shock, menadione treatment resulted in upregulation of hundreds of genes and enhancers in an HSF1- and/or HSF2-dependent manner (Figure 2A and B). We also found

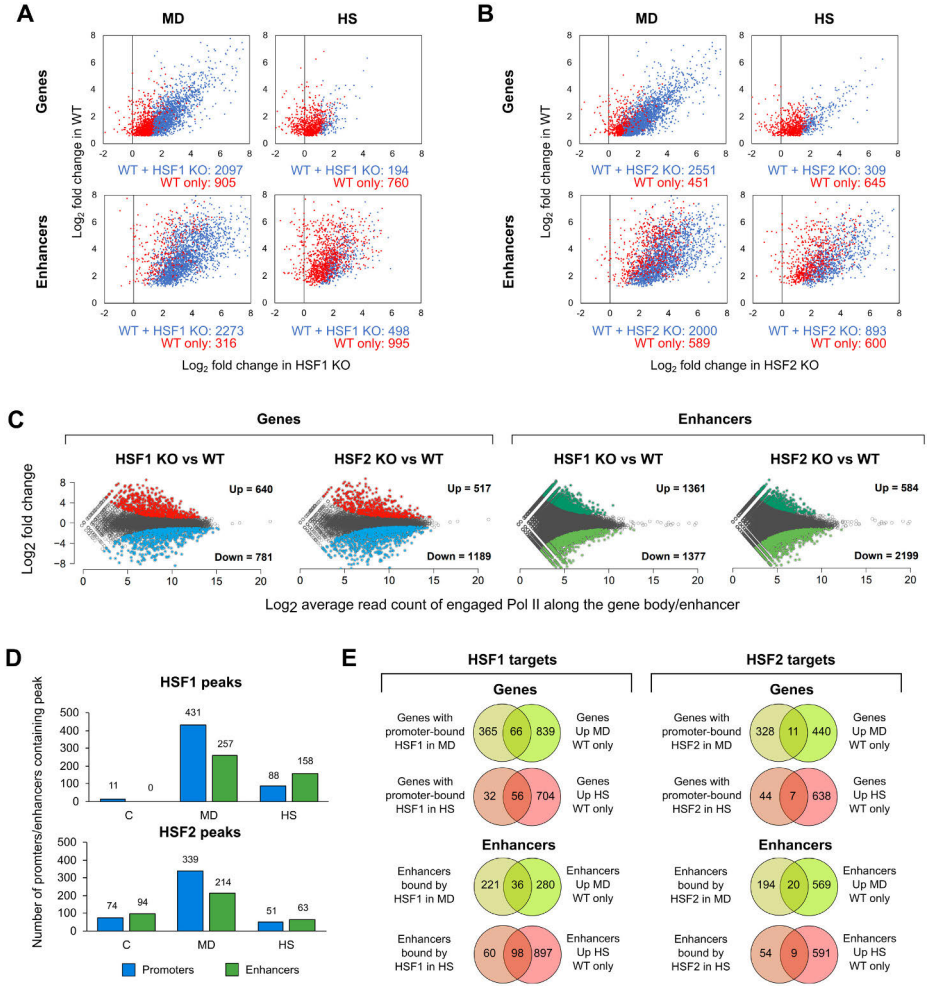

Figure 2. HSF1 and HSF2 reprogram the transcription of genes and enhancers in response to oxidative stress and heat shock. PRO-seq was performed in wild-type (WT), HSF1 knock-out (HSF1 KO) and HSF2 knock-out (HSF2 KO) MEFs that were exposed to oxidative stress induced by menadione (MD, 30 μM, 2 h) or to heat shock (HS, 42°C, 1 h). (**A, B**) Log_2 fold changes are shown for the genes and enhancers that are upregulated either in WT and KO cells (blue dots) or only WT cells (red dots). Some of the HSF-dependent genes and enhancers are likely false positives, since they displayed high fold change in both WT and KO cells (red dots towards the right side of the panels). In these cases, the fold changes in KO cells were not statistically significant and, therefore, these genes and enhancer are upregulated only in WT cells. (**C**) Comparison between KO and WT cells revealed several genes and enhancers that are upregulated or downregulated in HSF1 and HSF2 KO cells under normal growth conditions. (**D**) Antibodies against HSF1 and HSF2 were used to perform ChIP-seq in MEFs that were exposed to menadione or heat shock. The number of promoters and enhancers that contained HSF1 or HSF2 peak was determined in cells exposed to menadione or heat shock. (**E**) Target genes and enhancers regulated through direct binding of HSF1 or HSF2 were identified by comparing the targets bound by HSF1 or HSF2 with the targets that were upregulated only in WT cells. C: control.

that the transcriptional program was altered in HSF1 and HSF2 KO MEFs already under normal growth conditions (Figure 2C). This result is in line with the various roles of HSF1 and HSF2 under physiological conditions, including differentiation, development, and cell cycle control as well as in pathological states, such as cancer and neurodegeneration (14,15).

To distinguish the direct targets of HSF1 and HSF2 from the indirect ones, we identified genes and enhancers occupied by HSF1 and HSF2 in stressed cells. We treated WT MEFs with menadione (30 μM, 2 h) or heat shock (42°C, 1 h), and immunoprecipitated HSF1 and HSF2 for the ChIP-seq analysis. A clear correlation was observed between two biological ChIP-seq replicates (Supplementary Figure S7A), and thus, we combined reads from the replicates to perform peak calling. Robustness of the replicates was also evident from the profiles of HSF1 and HSF2 binding at the *Hspa1b* and *Bag3* promoters, both of which are strongly stress-inducible HSF targets (Supplementary Figure S7B). A strong stress-inducible binding of HSF1 to promoters and enhancers was evident during both stresses, and remarkably, the number of HSF1-bound promoters and enhancers was even higher upon menadione treatment than heat shock (Figure 2D). In addition to HSF1, HSF2 displayed a prominent inducible binding to both promoters and enhancers in menadione-treated cells (Figure 2D). Unlike HSF1, HSF2 bound to several targets prior to stress exposures, and the number of HSF2 targets did not increase in response to heat shock (Figure 2D). This observation could be explained by heat-induced degradation of HSF2, which occurs shortly after exposure to heat shock (52). Together, our results indicate distinct kinetics of HSF2-mediated transcription in heat-shocked and ROS-challenged cells.

Next, we identified the direct targets of HSFs whose stress-inducibility was dependent on the binding of HSF1 or HSF2 to the corresponding *cis*-acting elements in the genome. Our analysis revealed a multitude of menadione- and heat-inducible genes and enhancers, which were dependent on HSF1 binding (Figure 2E and Supplementary Table S1). Although menadione-inducible target genes of HSF1 play roles in various biological processes, many of them were related to protein folding (Supplementary Table S1). In line with our previous findings (26), HSF2-dependent heat induction was detected only for seven target genes and nine target enhancers, and HSF2 was not required for stress-inducible upregulation of *HSP* genes (Figure 2E and Supplementary Table S1). Similarly to heat shock, HSF2 was required for induction of only 11 genes and 20 enhancers during oxidative stress, implying that in both stresses, HSF1 functions as a more prominent *trans*-activator than HSF2 (Figure 2E and Supplementary Table S1).

HSF2 cooperates with HSF1 during oxidative stress and heat shock

HSF2 has been primarily described as a modulator of HSF1 activity in the heat shock response (15). In agreement, we found nearly all HSF targets in heat-shocked cells, including ST13 hsp70 interacting protein (*ST13*) gene, to be *trans*-activated in an HSF1-dependent manner (Figure 3B and C, Supplementary Table S1). However, induction of some genes, such as *Adgra3*, was dependent on HSF2, indicating that HSF2 is capable of functioning as a stress-responsive transcription factor (Figure 3B and C, Supplementary Table S1). Next, we sought to understand whether HSF2 plays an HSF1-supportive role in oxidative stress or whether it can *trans*-activate genes and enhancers independently of HSF1. In menadione-treated cells, a majority of HSF-dependent transcriptional induction was triggered by HSF1, as exemplified by an HSF1-specific target gene, solute carrier family 25 member 38 (*Slc25a38*) (Figure 3A and C, Supplementary Table S1). Although three genes and ten enhancers were HSF2-specific targets, most of them displayed equal fold changes in WT and HSF2 KO MEFs exposed to menadione (Figure 3A). Despite the minor effect of HSF2 on stress-induced transcription, HSF2 was found to co-localize to the same sites as HSF1 during both oxidative stress and heat shock, indicating that HSF2 cooperates with HSF1 to orchestrate transcription in response to different types of stress (Figure 3A–C). This is in line with a recent finding demonstrating that HSF2 occupies the same target genes with HSF1 in cancer to drive malignancy (18).

HSFs activate distinct transcription programs through stress-specific binding to chromatin

We found that HSFs regulated unique sets of genes and enhancers in cells treated with menadione or heat shock (Figure 3C and Supplementary Figure S8, Table S1). Next, we asked whether HSFs bind to stress-specific sites in the chromatin to regulate their stress-specific targets. Our results revealed a large group of genes that were occupied and activated by HSF1 only in menadione-treated cells, demonstrating for the first time that HSFs can bind unique sites in response to distinct stress stimuli (Figure 3D, Supplementary Table S1). Interestingly, we found that while heat-inducible HSF1 targets were bound by HSF1 also in response to menadione, a majority of these targets were induced in an HSF1-dependent manner only in heat-shocked cells (Figure 3E, Supplementary Table S1). This implies that HSF1 lack the full *trans*-activation capacity at certain genes during oxidative stress, which could occur either because oxidative stress represses HSF1 or because transcriptional co-activators of HSF1 are not available during oxidative stress.

Differential binding patterns of HSFs between menadione treatment and heat shock could be explained by their preference for distinct target motifs in the DNA. It is known that HSFs bind to their *cis*-acting heat shock elements (HSEs), which were originally defined to contain three inverted nGAAn sequences (53). These motifs are called canonical HSEs, but subsequent studies have identified also non-canonical HSEs, which consist of highly variable sequences (54,55). Therefore, it is plausible that oxidative stress-specific target genes of HSF1 contain primarily non-canonical HSEs that are not recognized by current motif finding algorithms. We found that canonical HSEs were equally prevalent in the menadione- and heat shock-specific target promoters and enhancers of HSF1 (Figure 3F). Taken together, our data indicate that although HSF1 binds to the same HSE motifs in both stresses, it displays

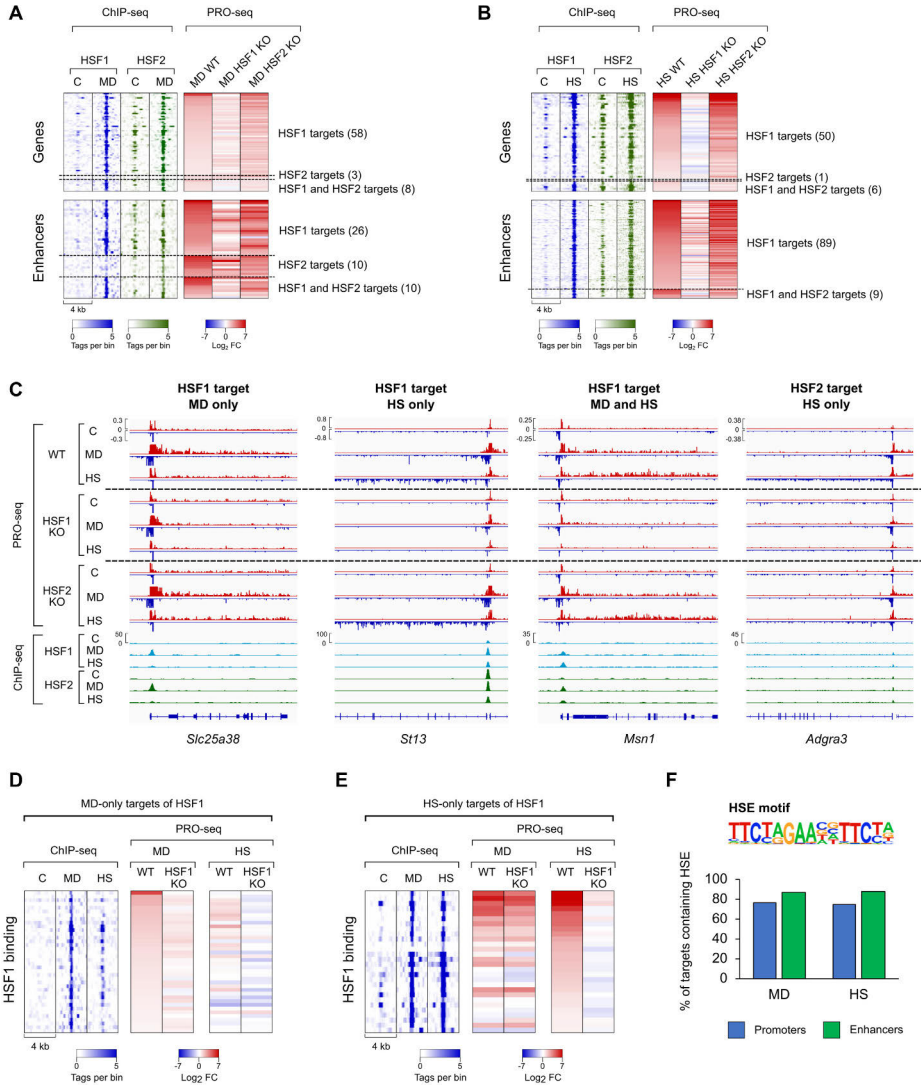

Figure 3. HSF1 and HSF2 drive distinct transcriptional programs upon oxidative stress and heat shock. (**A, B**) Heatmaps were generated from menadione- (MD, 30 μM, 2 h) (A) and heat-treated (HS, 42°C, 1 h) (B) MEFs to show genes and enhancers, which are regulated through direct binding of both HSF1 and HSF2 or only one of these factors. (**C**) PRO-seq and ChIP-seq profiles are shown for selected genes that are induced by HSF1 and HSF2 in response to menadione or heat shock. Headings above each of the four panels indicate whether the gene is regulated by HSF1 or HSF2 during menadione, heat shock or both. (**D**, **E**) Heatmaps were generated from menadione- (D) and heat shock (E) -specific target genes of HSF1. (**F**) Motif analysis was performed to determine the percentage of menadione- and heat shock-specific targets of HSF1 that contain canonical HSEs. C: control, *Slc25a38*: solute carrier family 25 member 38, *St13*: Hsp70 interacting protein, *Msn1*: meiosis specific nuclear structural 1, *Adgra3*: adhesion G protein-coupled receptor A3.

stress type-specific binding, which results in the activation of distinct transcription programs.

HSF1 and HSF2 bind enhancers to drive stress-inducible gene transcription

Since a majority of HSF1-dependent genes were not directly regulated by promoter-bound HSF1 (Figure 3E), we hypothesized that these genes could be induced through enhancers. Interestingly, we observed that during heat shock, a prominent number of HSF1-dependent genes resided within 100 kb from the direct enhancer targets of HSF1 (Figure 4A). Furthermore, most of these genes were devoid of promoter-bound HSF1, suggesting that HSF1 regulates a subset of heat-inducible genes through nearby enhancers (Figure 4A). Also, several menadione-induced genes required HSFs for activation and had the closest HSF binding-site at a nearby enhancer (Figure 4A). However, no general correlation was found between the distance of HSF-dependent genes and the enhancers activated in an HSF1-dependent manner upon menadione treatment (Figure 4A).

Since only heat-induced target enhancers and genes of HSF1 were found in the vicinity of each other, we assessed how the HSF1-activated enhancers impact distinct steps of transcription at nearby genes during heat shock. Previous studies have shown that binding of HSF1 to promoters is essential for the heat-inducible pause-release and recruitment of Pol II (56,57). Thus, we analyzed the distribution of Pol II at genes whose heat-induction was indirectly dependent on HSF1 and which were located within 100 kb from direct target enhancers. Our result showed that, similarly to the promoter-bound HSF1, binding of HSF1 to enhancers was required for the pause-release and recruitment of Pol II at nearby genes (Figure 4B). Noteworthy is that the average distribution of Pol II revealed two sites of paused Pol II in the genes that resided in the vicinity of HSF1-bound enhancers, as exemplified by the promoter-proximal region of *B4galt1* gene (Figure 4B and Supplementary Figure S9A). This pausing pattern is in line with previous results showing that certain genes display multiple TSSs (58).

Finally, we addressed whether HSF1 regulates different cellular processes through promoters and enhancers in cells exposed to cytotoxic stress, especially heat shock. For this purpose, we compared GO terms between the direct target genes of HSF1 and the indirect target genes located within 100 kb from its enhancer targets. As expected, the direct HSF1 target genes were related to processes of protein folding, and cellular stress responses (Figure 4C). On the contrary, the indirect target genes residing in the vicinity of enhancer targets were strongly associated with GO terms, such as focal adhesion and transmembrane receptor-linked signaling pathways (Figure 4C). Examples of these targets are filamin b (*Flnb*) and membrane-associated guanylate kinase, WW and PDZ domain containing 1 (*Magi1*) genes, both of which encode proteins localized to the plasma membrane (Figure 4D and Supplementary Figure S9B). Furthermore, certain genes with the highest transcriptional induction, e.g. *Hspb1*, recruited HSF1 both to the promoter and a nearby enhancer (Figure 4D).

Previous studies have shown that besides protein folding, HSFs regulate genes related to many other processes, including cell adhesion (59,60). Moreover, maintenance of cell adhesions was shown to be essential for surviving stress (60). Our results advance these studies by revealing that in contrast to the promoter-bound HSF1, which drives the classical chaperone genes, binding of HSF1 to enhancers activates genes encoding proteins localized at cell junctions and the plasma membrane. We also found that both HSFs are important for the activation of oxidative stress-inducible genes and enhancers, which are different from heat shock-inducible HSF targets. Hereby, we conclude that HSFs function as multi–stress-responsive transcription factors that activate distinct sets of genes and enhancers depending on the type of stress experienced by cells.

DISCUSSION

Mechanisms of transcriptional reprogramming in response to cellular stresses, especially acute heat shock, are well characterized, but they have remained poorly understood under other stress conditions. Here, we provide the first comprehensive study, in which we combined PRO-seq and ChIP-seq to determine the roles of HSF1 and HSF2 in the regulation of nascent transcription in cells exposed to two different types of cytotoxic stress, i.e. oxidative stress and heat shock. As illustrated in our model (Figure 5), these two stresses cause clearly stress type-specific changes to the transcription of genes and enhancers. Although the transcriptional programs differ between oxidative stress and heat shock, our results reveal that during both stresses, genes are regulated at the level of Pol II pause-release, while enhancers are regulated *via* recruitment of Pol II. Unlike heat-inducible genes, a large fraction of oxidative stress-inducible genes displayed elongating Pol II only within the early gene body (0–2 kb from TSS). This could be due to oxidative DNA damage, which has been shown to cause stalling of elongating Pol II (48). Other possible explanations are a slower movement speed of Pol II and a failure in the chromatin remodeling in front of elongating Pol II during oxidative stress.

Transcriptional regulation in oxidative stress responses has been largely devoted to nuclear factor erythroid 2-related factor 2 (Nrf2) and members of the Foxo family (46,61). Here, we expand the repertoire of transcription factors in oxidative stress by identifying HSF1 and HSF2 as new regulators of genes and enhancers in cells exposed to elevated ROS production (Figure 5). This is an important finding, since HSFs have been considered as master regulators of proteotoxic stress responses, especially the heat shock response, and they also play vital roles in cancer progression. While HSF1 triggered genome-wide changes in gene and enhancer transcription, HSF2 was found dispensable for genome-wide stress inducibility. However, HSF2 co-occupied the same promoters and enhancers with HSF1, implying that HSF1 and HSF2 cooperate to drive transcription under distinct types of stress. We also show that HSFs bind and regulate largely different targets upon oxidative stress and heat shock (Figure 5). Intriguingly, HSFs bound to the canonical HSEs in response to both stresses, suggesting that these transcription factors are recruited to their stress type-specific sites through mechanisms that are independent of the target site sequence. It is likely that HSFs

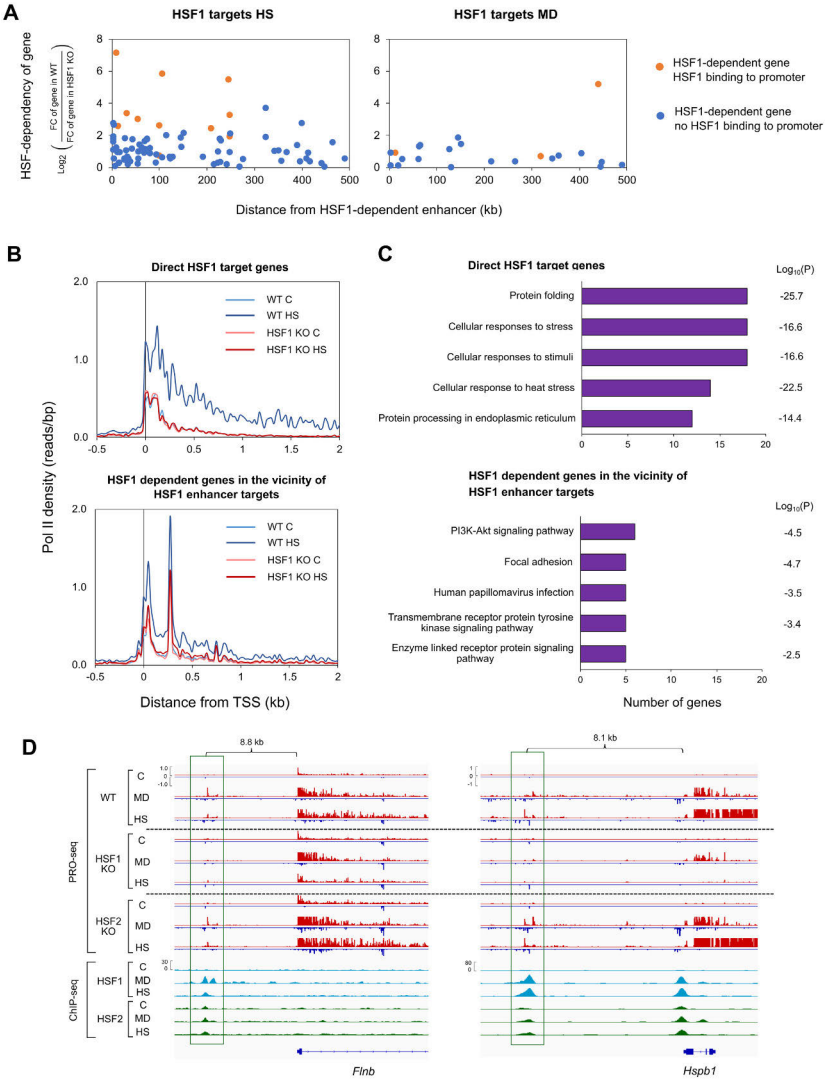

Figure 4. HSF1-dependent genes are located in the vicinity of HSF1-bound enhancers. (**A**) Distances from the target enhancers of HSF1 to the HSF1-dependent genes were measured in cells exposed to menadione (MD, 30 μM, 2 h) and heat shock (HS, 42°C, 1 h). Distances were calculated between the summit of an enhancer and the TSS of a gene. Genes were divided into two groups depending on whether their promoters were bound by HSF1 (orange dots) or not (blue dots). (**B**) Average Pol II density was analyzed in the direct HSF1 target genes and HSF1 dependent genes located within 100 kb of direct enhancer targets of HSF1. Pol II densities are shown in wild-type (WT) and HSF1 knock-out (HSF1 KO) MEFs. (**C**) GO terms of two different heat-inducible gene groups were analyzed: direct HSF1 targets and indirect HSF1 targets that were located within 100 kb of direct enhancer targets of HSF1. GO terms were ranked in descending order based on the number of genes associated with each term. (**D**) PRO-seq and ChIP-seq profiles of selected target enhancers and genes of HSF1 that were found in the vicinity of each other. Enhancers are framed with green rectangles. All the enhancers and *Hspb1* gene are regulated through direct binding of HSF1, while *Flnb* gene is devoid of promoter-bound HSF1. C: control, *Flnb*: filamin b, *B4galt1*: beta-1,4-galactosyltransferase.

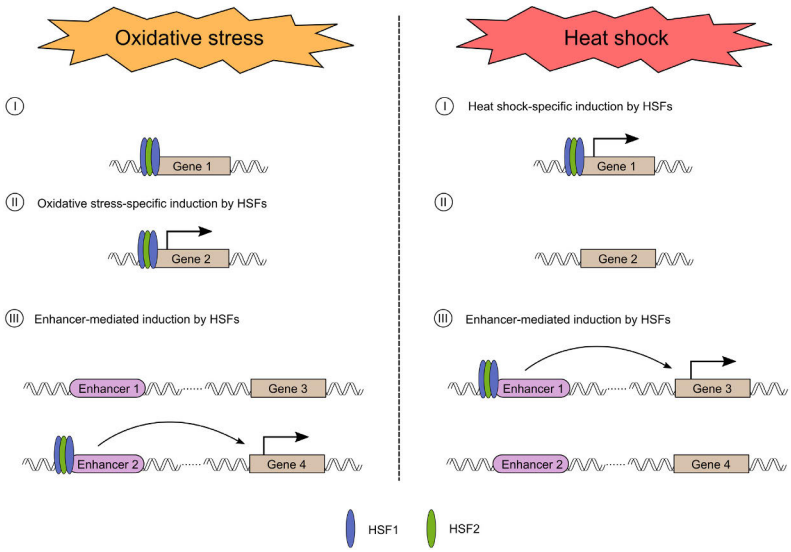

Figure 5. Schematic model of how HSF1 and HSF2 drive stress-specific transcriptional programs through activation of genes and enhancers. (I) HSF1 and HSF2 co-occupy several gene promoters during oxidative stress and heat shock. However, many of these HSF1 and HSF2 -bound genes are only induced in response to heat shock, in an HSF1-dependent manner. (II) Increased levels of ROS trigger HSF1 and HSF2 to bind to their oxidative stress-specific target genes. (III) HSF1 and HSF2 bind stress-inducibly to a large number of enhancers. The HSF-bound enhancers differ in heat shock *versus* oxidative stress, but during both conditions HSFs can trigger the release of paused Pol II from the promoter-proximal region of a nearby gene. Please note, in this model co-occupancy of HSF1 and HSF2 is drawn as a heterotrimer.

bind to their oxidative stress-specific targets by interacting with cofactors that are activated by changes in the cellular redox status. Formation of these interactions, in turn, could involve stress-specific protein modifications, since HSFs are known to undergo extensive post-translational modifications, including the oxidation of two redox-sensitive cysteines within the DNA-binding domain of HSF1 (17,19). In addition, chromatin environment likely undergoes different changes upon oxidative stress and heat shock, which could allow HSFs to access unique sites depending on the type of stress.

Our data uncover a new regulatory level of stress-inducible transcription that is mediated through enhancers, which in turn are activated by HSFs (Figure 5). We found that unlike promoter-bound HSF1, which activates classical chaperone genes, enhancer-bound HSF1 was required for the transcriptional induction of cell type-specific genes, including genes that encode proteins localized in the plasma membrane and cell junctions. Enhancer-mediated induction of genes by HSFs is likely not restricted to stress, since HSFs are important transcription factors in a wide variety of physiological processes, including development, differentiation, and metabolism, as well as pathologies, especially cancer and neurodegeneration (14,15). Furthermore, enhancers play key roles in determining cell fate during development and differentiation, while cancer cells hijack oncogenic enhancers to promote malignancy (62). In future studies, it will be fundamental to determine the functional relevance of HSF-activated enhancers in physiology and pathology.

DATA AVAILABILITY

Collection of PRO-seq and ChIP-seq raw data has been deposited to Gene Expression Omnibus (GEO) database with accession number GSE183245. In addition to raw data, accession contains bedgraph files that are used for the visualization of the data.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We thank all the members of Sistonen laboratory for constructive discussions during the preparation of the manuscript. The Finnish Functional Genomics Centre (Turku Bioscience, University of Turku, Åbo Akademi University and Biocenter Finland) is acknowledged for services, instrumentation and expertise.

FUNDING

Academy of Finland [to A.V., L.S.]; Sigrid Jusélius Foundation [to A.V., L.S.]; Åbo Akademi University [to L.S.]; Can-

cer Foundation Finland [to L.S.]; Magnus Ehrnrooth Foundation [to L.S]; Finnish Cultural Foundation [to S.V.H.]; Alfred Kordelin Foundation [to S.V.H.]; Swedish Cultural Foundation, Finland [to M.C.P.]; Science for Life Laboratory, Sweden [to A.V.]; Joe, Tor and Pentti Borg Foundation [to A.V.]; Svenska Tekniska Vetenskapsakademien i Finland [to A.V.]; South-West Finland's Cancer Foundation [to A.V.]. Funding for open access charge: Sigrid Juselius Foundation.

Conflict of interest statement. None declared.

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HSF1 and HSF2 in Stress and EMT-Associated Transcriptional Networks

This thesis investigates how cells react to protein-damaging stress and a growth factor that can support cancer development. Heat shock and oxidizing compounds damage proteins and other macromolecules, necessitating cells to rely on proteins that increase their survival ability. An important protein family is the heat shock factors (HSFs), stress-sensitive transcription factors that can induce the expression of proteins that help the cell deal with damaged proteins. This work investigates how phosphorylation (binding of phosphate groups) in HSF1's regulatory domain affects its ability to induce transcription of genes, which support cell survival. The results show that phosphorylation determines the threshold at which HSF1 is activated under stress. The work also investigated how HSF1 and HSF2 independently and together control transcription under heat and oxidative stress. The results reveal new groups of HSF-regulated genes and suggest that enhancers, which are regulatory sequences near genes, have an important role in this regulation. In cancer, HSFs have shown deviant expression and activities. Exploring the connection between pro-tumorigenic growth factors and HSFs, this work characterizes how transforming growth factor (TGF) affects the expression and activity of HSF2. The results showed that activating the TGF signaling pathway significantly reduces the amount of HSF2 in breast cancer cells, preventing HSF2 from constraining several cancer-promoting processes. These results provide new insights into HSF-related biology and create a foundation for further research into gene regulation.

ISBN 978-952-12-4308-0