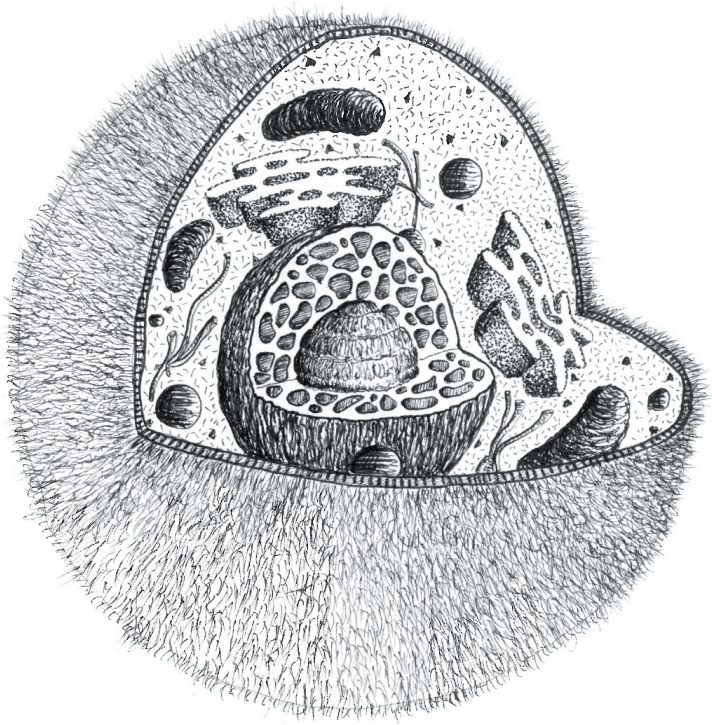
Åbo Akademi University

Jenny Joutsen (*née* Siimes)

Regulation of Cellular Stress Proteins in Physiology and Disease



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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications and a manuscript, which are referred to in the text by Roman numerals (I-IV). In addition, unpublished data is included. The original publications have been reproduced with the permission of the copyright holders.

I Blomster HA*, Imanishi SY*, **Siimes J**, Kastu J, Morrice NA, Eriksson JE & Sistonen L (2010) *In vivo* identification of sumoylation sites by a signature tag and cysteine-targeted affinity purification. *The Journal of Biological Chemistry* 285:19324-19329.

II Budzyński MA, Puustinen MC, **Joutsen J** & Sistonen L (2015) Uncoupling Stress-Inducible Phosphorylation of Heat Shock Factor 1 from Its Activation. *Molecular and Cellular Biology*. 35:2530-2540.

III Björk JK*, Åkerfelt M*, **Joutsen J**, Puustinen MC, Cheng F, Sistonen L & Nees M (2016) Heat-shock factor 2 is a suppressor of prostate cancer invasion. *Oncogene*. 35:1770-1784.

IV **Joutsen J**, Da Silva AJ, Luoto JC, de Thonel A, Concordet JP, Mezger V, Sabéran-Djoneidi D, Henriksson E & Sistonen L. HSF2 protects human osteosarcoma U2OS cells against proteotoxicity by maintaining cell-cell adhesion. (manuscript)

* Equal contribution

Other publications:

V Niskanen EA, Malinen M, Sutinen P, Toropainen S, Paakinaho V, Vihervaara A, **Joutsen J**, Kaikkonen MU, Sistonen L & Palvimo JJ. (2015) Global SUMOylation on active chromatin is an acute heat stress response restricting transcription. *Genome Biology*. 16:153

ABBREVIATIONS

AD	Activatory domain
ATP	Adenosine 5´-triphosphate
BTZ	Bortezomib
CAM	Chicken chorioallantoic membrane assay
ChIP	Chromatin immunoprecipitation
CTD	C-terminal domain
DBD	DNA-binding domain
DUB	Deubiquitinating enzyme
ECM	Extracellular matrix
EMSA	Electromobility shift assay
EMT	Epithelial-to-mesenchymal transition
FASD	Fetal alcohol spectrum disorder
HDAC	Histone deacetylase
HR-A/B/C	Heptad repeat A/B/C
HSE	Heat shock element
HSF	Heat shock transcription factor
Hsp	Heat shock protein
KO	Knock-out
MEF	Mouse embryonic fibroblast
MEF2	Myocyte enhancer factor 2
NDSM	Negatively charged amino acid-dependent sumoylation motif
nSB	Nuclear stress body
PCDH	Protocadherin
PDSM	Phosphorylation-dependent sumoylation motif
PIAS	Protein inhibitor of activated STAT
PML	Promyelocytic leukemia protein
PTM	Post-translational modification
RanGAP1	Ran GTPase-activating protein 1
RD	Regulatory domain
SENP	SUMO-specific protease
shRNA	Short hairpin RNA
SIM	Sumo interacting motif
STUBL	Sumo targeted ubiquitin ligase
SUMO	Small ubiquitin-like modifier
Ub	Ubiquitin
UBD	Ubiquitin-binding domain
UBL	Ubiquitin-like
ULD	Ubiquitin-like domain
UPR	Unfolded protein response
WT	Wild-type

ABSTRACT

All cells in a human body are constantly exposed to environmental fluctuations to which the cells must respond. Most of the cellular responses are mediated by proteins that are specifically regulated according to the distinct requirements of the cell. Among various protein regulatory mechanisms, modulation by post-translational modifications (PTMs), such as phosphorylation or sumoylation, provides an efficient and rapid means to alter protein function. PTMs have an incredibly vast selection of target substrates and thus are involved in nearly every aspect of cells´ life. The fast modulation of cellular response pathways is particularly important during stress, which often poses a threat to cell survival unless mitigated appropriately. One of the most extensively studied cellular stress response pathways is called the heat shock response, which is initiated upon exposure to protein damaging conditions. Heat shock response is mediated by heat shock transcription factors (HSFs), which assist in the restoration of protein homeostasis, by activating the transcription of genes encoding for molecular chaperones. Of the mammalian HSFs, HSF1 is considered the main regulator of heat shock response. During its activation-attenuation cycle, HSF1 is extensively modified by PTMs, which accompany the acquisition of transcriptional activity. For this, the modifications are considered as a prerequisite for HSF1 activity, though their exact importance has not been conclusively examined. Due to its ability to potently enhance cell survival during stress, HSF1 is regarded as a powerful enabler of carcinogenesis and high HSF1 expression has been detected in multiple human cancer types. HSF2, on the other hand, is mainly recognized as an important regulator of other differentiation and developmental programs, but its role in proteotoxic stress and in human malignancies is currently uncharacterized.

In the first study of this thesis, I focus on sumoylation as a post-translational regulator of protein function and describe a novel method to examine sumoylated proteins *in vivo* in cells. The method is based on engineered human SUMO1 and can be utilized in the identification of novel sumoylation substrates. The second study examines HSF1 phosphorylation and aims at understanding the importance of this modification as a regulator of HSF1 activity. The results presented in this thesis show that hyperphosphorylation is not required for HSF1 activity, but functions more as a fine-tuning mechanism for heat shock response. In the third study, we aimed at expanding our understanding on the role of HSFs in human malignancies. The study demonstrates that in contrast to oncogenic HSF1, HSF2 functions as a suppressor of prostate cancer invasion and shows that HSF2 downregulation promotes metastatic behavior of prostate cancer cells. Finally, the fourth study examines the role of HSF2 in proteasome inhibition-induced prolonged proteotoxic stress and establishes HSF2 as an essential cell survival factor in these conditions. Moreover, the work identifies HSF2-dependent expression of cadherins as a key determinant of cellular sensitivity to proteotoxic stress and thus greatly expands our knowledge regarding HSFs as factors promoting cell survival. Taken together, the work presented in this thesis elaborates on HSF-mediated cellular survival pathways and lays a ground for future studies regarding HSFs as important regulators of human physiology and disease.

TYÖN TIIVISTELMÄ (ABSTRACT IN FINNISH)

Jokainen elimistömme solu elää jatkuvasti erilaisten stressitekijöiden ympäröimänä. Säilyäkseen elossa vaihtelevassa ympäristössä, solun on kyettävä tunnistamaan suuri määrä erilaisia viestejä ja osattava käynnistää kulloiseenkin viestiin sopiva solunsisäinen vaste. Solunsisäiset vasteet ovat usein proteiinien eli solun valkuaisaineiden välittämiä, joiden toimintaa säädellään muun muassa erilaisten translaation jälkeisten muokkausten, kuten fosforylaation tai sumolaation, välityksellä. Translaation jälkeisillä muokkauksilla on soluissa lukuisa joukko kohdeproteiineja ja niiden kautta voidaankin säädellä lähes jokaista solun toimintaa. Useiden proteiinien yhtäaikainen säätely on erityisen tärkeää erilaisten solustressien aikana, jolloin solun on nopeasti käynnistettävä useita solua suojelevia geeniohjelmia. Eräs tärkein solujen stressivasteista on nimeltään lämpösokkivaste, ja sen tehtävä on suojella solua sen altistuessa proteotoksisille eli proteiineja vahingoittaville ympäristötekijöille, kuten korkeille lämpötiloille tai raskasmetalleille. Lämpösokkivastetta säädellään lämpösokkitekijöiden (engl. heat shock factors, HSFs) välityksellä, jotka aktivoituvat proteotoksisen stressin seurauksena ja käynnistävät solua suojelevien esiliinaproteiinien geenien luennan. Esiliinaproteiinit estävät vahingoittuneiden proteiinien virheellisen laskostumisen ja siten varmistavat solujen elossa säilymisen stressistä huolimatta. Ihmisellä esiintyvistä lämpösokkitekijöistä HSF1:n ajatellaan olevan tärkein esiliinaproteiinien ilmenemistä säätelevä tekijä. Aktivoitumisen yhteydessä HSF1 käy läpi useita translaation jälkeisiä muokkauksia. Näistä merkittävin on fosforylaatio, jonka ajatellaan vaikuttavan HSF1:n kykyyn säädellä esiliinaproteiinien ilmenemistä, vaikka tarkkaa tutkimusaineistoa tästä ei vielä ole. Säätelemällä esiliinaproteiinien ilmenemistä vaikeissakin olosuhteissa HSF1:llä on erinomainen kyky suojella solua ohjelmoidulta solukuolemalta. Tämä solua suojeleva ominaisuus heijastuu HSF1:n taipumukseen edistää syövän etenemistä poikkeavissakin olosuhteissa ja HSF1:n yli-ilmeneminen onkin havaittu useissa ihmisen syöpätyypeissä. Jokainen ihmisen syöpäsolu ilmentää kuitenkin myös HSF2:ää, joskin se toiminta syövässä on vielä täysin tuntematon.

Tämän väitöskirjan ensimmäisessä osatyössä tarkastelen sumolaatiota proteiinien toimintaa säätelevänä tekijänä ja kuvailen uuden menetelmän sumoloituneiden proteiinien tutkimiseen soluissa. Menetelmä perustuu ihmisen SUMO1-muunnelmaan, jota voidaan tehokkaasti hyödyntää uusien sumolaation kohdeproteiinien tunnistamisessa. Väitöskirjan toinen osatyö keskittyy fosforylaatioon ja sen merkitykseen HSF1:n säätelyssä. Työ osoittaa aikaisempien käsitysten vastaisesti, että fosforylaatio ei määrää HSF1:n aktiivisuutta vaan toimii pääasiassa lämpösokkivasteen voimakkuuden hienosäätelyssä. Kolmas osatyö tarkastelee HSF2:n merkitystä eturauhassyövässä ja osoittaa, että toisin kuin syöpäsoluja suojeleva HSF1, HSF2 hidastaa eturauhassyövän etenemistä. Työssä osoitetaan, että alhainen HSF2:n määrä edistää eturauhassyövän etenemistä. Väitöskirjan neljännen ja viimeisen työn tarkoitus on ollut määritellä HSF2:n tehtävät proteotoksisissa stresseissä. Työssä selvisi, että HSF2 säätele soluissa kadheriinien ilmenemistä ja siten on tärkeä solujen elinkykyä määrittävä tekijä pitkäkestoisen proteasomi-inhibition yhteydessä. Kaiken kaikkiaan tässä väitöskirjassa esitetyt osatyöt laajentavat merkittävästi tuntemustamme lämpösokkitekijöiden säätelystä ja tehtävistä erilaisissa fysiologisissa ja patofysiologisissa konteksteissa ja siten toimii tärkeänä pohjana tuleville jatkotutkimuksille.

SAMMANFATTNING (ABSTRACT IN SWEDISH)

Varje cell i människan är konstant utsatt för olika stressförhållanden. För att kunna överleva varierande omgivningar, måste cellen ha förmågan att reagera och anpassa sig till varierande miljöer och initiera försvarsmekanismer när det behövs. De flesta cellskyddande signalräckorna är styrda av proteiner som är reglerade via olika mekanismer beroende på cellens behov. Ett av de snabbaste sätten att påverka proteinernas funktion är genom posttranslationella modifieringar, såsom fosforylering eller sumoylering. Posttranslationella modifieringar har en stor mängd målproteiner och följaktligen framträder i majoriteten av cellbiologiska program. Reglering av proteiners funktion genom posttranslationella modifieringar är speciellt viktig under cellulär stress som kräver en snabb aktivering av skyddsprogrammen. En utav de viktigaste försvarsmekanismerna kallas för värmechockresponsen, som aktiveras vid proteinskadande stress. Värmechockresponsen är karakteriserad av stressinducerad aktivering av värmechockfaktorer, vilka styr genuttrycket av molekylära chaperoner som i sin tur hjälper andra proteiner att upprätthålla sin struktur och funktion. Utav de fyra värmechockfaktorerna som förekommer hos däggdjur (HSF1-4), är HSF1 den viktigaste för värmechockresponsen. Under aktiveringen av värmechockresponsen utsätts HSF1 för omfattande fosforylering, vilket ansetts krävas för dess aktivering, fast obestridbart bevis ännu saknas. Med tanke på att HSF1 kan skydda celler i varierande omgivningarna, kan cancerceller utnyttja denna egenskap i syfte att anpassa sig till cellulärstress och hög HSF1 uttryck har upptäckts i många cancertyper. Funktionen av HSF2 i cancer är dock ännu okänd även om det har rapporterats att HSF2 kan också modulera värmechockresponsen.

I den första studien av denna avhandling, har jag fokuserat på sumoylering som en posttranslationell regulerare av proteinfunktion och beskriver hur ett specifikt konstruerat SUMO1-protein uttrycks i cellen och kan användas för identifiering av nya målproteiner för sumoylering *in vivo*. I den andra studien undersökte jag fosforylering som en regleringsmekanism för HSF1:s aktivitet. I motsatts till de tidigare rapporterade resultaten, visar denna studie att fosforylering inte är nödvändig till HSF1:s aktivering, utan modifikationen styr tröskelvärdet för aktivering av värmechockresponsen. Den tredje studien fokuserade på att karakterisera HSF2:s funktion i prostatacancer. Resultaten visar att i motsats till den cellskyddande inverkan av HSF1, har HSF2 en hämmande effekt på tumörutveckling och nedreglering av HSF2 uttrycket ökar cancercellernas invasion. Den fjärde studien undersöker HSF2:s funktion under proteasominhibition, vilket förorsakar proteinskadande stress. Studien visar att HSF2 är en essentiell faktor under kronisk proteinskadande stress och den styr uttrycket av cellulära adhesionsmolekyler, kadheriner, som kan skydda celler mot stress. Resultaten som presenteras i denna avhandling ökar vår förståelse om värmechockfaktorernas funktion i olika fysiologiska och patofysiologiska förhållandena och utgör grunden till vidare undersökningar i framtiden.

INTRODUCTION

The human body is a collection of tissues and organs that are all composed of different types of cells. Regardless of the tissue type, cells are continuously surrounded by diverse environmental signals that, for example, direct the maintenance of distinct tissue phenotypes. Sometimes the environmental signals are damaging to the cells and can severely compromise cell survival. In such cases, the damaged cell initiates a survival response, which aims at restoring cellular homeostasis. Nearly every cellular survival response is characterized by activation of distinct DNA-binding proteins that translocate to the nucleus and induce the production of specific mRNA species in a process called transcription. The mRNA is subsequently exported from the nucleus to the cytoplasm, where the RNA-code is translated into a peptide chain that functions as the building material for new proteins. The newly produced proteins then assist in repairing the damage and prevent the induction of cell death. As proteins are the key factors mediating cellular functions, cells are particularly vulnerable to signals that damage the proteins. Accordingly, cells have acquired a variety of survival responses that can be launched to protect the proteome. Adequate regulation of survival pathways is essential, as their misregulation can lead to severe human diseases, including cancer, type II diabetes, and neurodegenerative disorders.

This thesis focuses on the cellular responses that are elicited particularly upon protein damaging stresses. The first study examines a specific post-translational modification, called sumoylation, which can be used as a rapid regulatory mechanism to steer protein function during stress and other conditions. The study presents a novel method to analyze sumoylated proteins in cells and thus promotes our understanding on the complexity of sumoylation as a regulator of diverse cellular functions. The second study begins the exploration of a distinct cellular survival pathway, called the heat shock response. Heat shock response is activated in response to cytosolic protein damage and it is directed by heat shock transcription factors (HSFs). In response to stress, HSFs accumulate in the nucleus and activate the transcription of their target genes. The canonical HSF target genes encode molecular chaperones that by restoring the intracellular protein folding environment promote cell survival during stress.

This thesis investigates how HSF1 and HSF2 are regulated in proteotoxic stress conditions. The second study reveals that in contrast to previous assumptions, post-translational modification of HSF1 with phosphorylation is not required for HSF1 activity but rather fine-tunes the threshold of HSF1-mediated stress response. Due to the ability to promote cell survival during stress, HSF1 is considered as a strong enabler of cancer progression. However, the importance of HSF2 in cancer progression has remained unknown. Thus, the third study aimed to elucidate whether HSF2 has an impact on cancer progression. Intriguingly, the study shows that, unlike HSF1, HSF2 suppresses prostate cancer development *via* genes that are related to invasion and metastasis formation. In contrast to HSF1, which is essential for cell survival during acute stress, HSF2 has been shown to be dispensable to acute heat shock response and its role in proteotoxic stresses has remained enigmatic. In the fourth study of this thesis, HSF2 is presented as an essential survival factor during prolonged proteotoxic stress, such as that induced by long-term proteasome inhibition. The study shows that HSF2 protects cells against proteotoxicity by maintaining cadherin-mediated cell-cell adhesion and thus defines a completely novel HSF2-dependent transcriptional program in cell protection. Taken together, this thesis provides novel information regarding the regulation of cellular survival responses and implicate distinct roles for HSF1 and HSF2 in human physiology and disease.

REVIEW OF THE LITERATURE

1 POST-TRANSLATIONAL MODIFICATIONS

The life of a cell is characterized by the plasticity of its proteome, which is achieved through multilevel protein regulation. For example, the existence of a protein can be regulated at the level of transcription or translation, through which the cell can shift its protein composition. More rapid way to generate functional diversity is, however, regulation through post-translational modifications (PTMs), where already existing proteins are modified by covalently conjugated chemical groups or small proteins or subjected to other structural changes. Perhaps the most well-known examples of chemical modifications are phosphorylation, acetylation, and methylation, whereas conjugation of ubiquitin-like (UBL) proteins is a classic example of protein post-translational modifications. Phosphorylation is by far the most common PTM and it has been estimated that the human proteome contains over 230 000 distinct phosphorylation sites (Vlastaridis *et al*., 2017). Not surprisingly, the importance of phosphorylation has been demonstrated in a vast variety of cellular signaling pathways, all the way from cell division to cell death. Acetylation and methylation [also with thousands of target sites (Khoury *et al*., 2011)], are specifically recognized for their role as histone modifications affecting gene expression, whereas ubiquitination is the main modification regulating protein degradation. Signaling through PTMs is often mediated by a selection of effector proteins, which recognize the modification *via* modification-specific interaction domains. For example, SH2 (Src homology domain 2) and Polo-box domains can bind phosphorylated tyrosine and serine/threonine residues, respectively, and thereby transduce the signal forward in the signaling pathway. Common to all the above-mentioned modifications is, that they are all covalently conjugated to substrates *via* specific enzymes that mediate the attachment in an ATP-dependent manner. Moreover, these modifications are almost exclusively transient in their nature and their deconjugation from substrates is biologically equally essential as their conjugation.

In this section of *1 Post-translational modifications*, I will first describe ubiquitin and the ubiquitin conjugation pathway. After that I will proceed with the small ubiquitin-like modifier, SUMO, which is in the center of the first original publication (I). Moreover, I will briefly describe the best-studied chemical modification, phosphorylation, which has a key role in the second original publication (II). Finally, the interplay between sumoylation and ubiquitination will be reviewed, as that was the focus of my early PhD work and will be presented in the *Results and discussion*.

1.1 Ubiquitin

In 1975, Gideon Goldstein and co-workers isolated a novel polypeptide from bovine thymus that had thymocyte-differentiating properties (Goldstein *et al*., 1975). Curiously, the same protein appeared to be expressed in all possible tissues they studied and could be identified from phylogenetically distant organisms such as mammals and plants. Inspired by these findings, Goldstein and co-workers courageously ended the article by proposing that the polypeptide is a universal feature of a living cell. And they were right. Goldstein and co-workers had isolated ubiquitin, a 76-amino acid protein, which is highly conserved all across the Eukaryota (Hochstrasser, 2009). Intriguingly, analogously functioning prokaryotic ubiquitin-like (Pup) protein has been identified in Eubacteria, which as an example of convergent evolution, further highlights the importance of ubiquitin in biological systems (Delley *et al*., 2017).

In humans, ubiquitin is encoded by a multigene family, composed of *UBB*, *UBC*, *UBA52*, and *RPS27A*, from which it is transcribed and translated as a precursor protein polyubiquitin or as a protein fusion with ribosomal proteins. From these precursors, single ubiquitin moieties are cleaved by endopeptidases and folded to their natural conformation in the cell. Ubiquitin is structurally characterized by a β-grasp fold, where antiparallel β-sheets fold around the central α-helix (Figure 1). This structure, also called the ubiquitin fold, is common to all ubiquitin-like (UBLs) proteins and ubiquitin-like domains (ULDs) (Winget & Mayor, 2010). More specific to ubiquitin itself, is a hydrophobic patch on the surface of the protein, which is generated by the closely landing hydrophobic amino acids in the globular state of the protein. This patch mediates many of the protein-protein interactions and hence is essential for the correct function of ubiquitin.

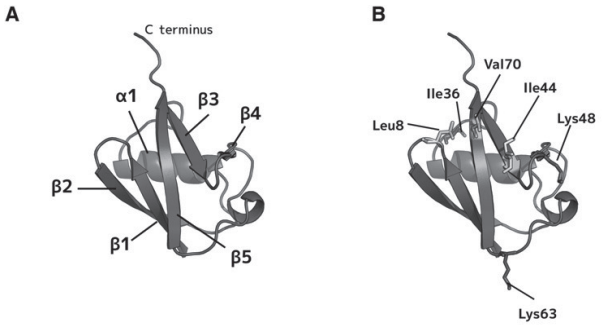

Figure 1. Ubiquitin molecule. (A) Ubiquitin is a globular protein where antiparallel β-sheets (β1-β5) surround the core α-helix (α1). **(B)** Hydrophobic patch on the surface of ubiquitin is formed by the proximity of hydrophobic amino acids. Adapted from (Winget & Mayor, 2010).

1.1.1 Ubiquitin conjugation pathway

Ubiquitin is covalently conjugated to its target proteins through a stepwise enzymatic cascade that consists of E1-activating enzyme, E2-conjugating enzyme and E3-ligase (Figure 2). The current literature estimates that the human genome encodes two E1-activating enzymes, 40 E2-conjugating enzymes, and at least 600 E3-ligases (Bett, 2016). The large number of enzymes creates enormous functional diversity, as specific combination of enzymes can be utilized to induce ubiquitination in response to distinct stimuli. In the activation step, ubiquitin first forms an adenylate intermediate *via* E1 in an ATP-dependent reaction after which it is linked to the active site cysteine on E1 in a reaction that releases AMP. In the conjugation step, activated ubiquitin is transferred from the E1 to the active site cysteine on the E2-conjugating enzyme. Finally, the E3-ligases responsible for substrate selection mediate the conjugation of the ubiquitin moiety to the target lysine on the substrate. The isopeptide linkage conjugating ubiquitin to the substrate is formed between the carboxyl group of the C-terminal glysine residue (G76) on ubiquitin and the ε-amino group of the target lysine of the substrate. Recurrent repetition of the cascade creates a ubiquitin chain on the substrate protein, which dictates the biological outcome of the modification. Ubiquitin can be removed by deubiquitinating enzymes (DUBs), which function as isopeptidases and break the linkage between the substrate and the ubiquitin or ubiquitin moieties in a ubiquitin chain. After this, ubiquitin is recycled and can enter yet another round of conjugation.

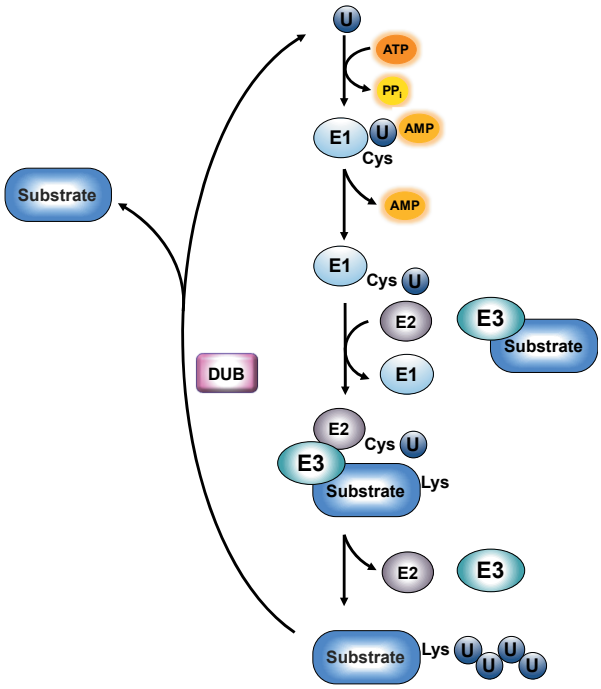

Figure 2. Ubiquitin conjugation pathway. Ubiquitin (U) is conjugated to target proteins through an enzymatic cascade that consists of E1-activating enzyme, E2-conjugating enzyme and E3-ligase. Ubiquitin is first activated by E1 in an ATP-dependent reaction after which it is transferred to E2 and conjugated to the target lysine with the assistance of substrate-recognizing E3 ligases. Deubiquitinating enzymes, DUBs, deconjugate ubiquitin from the target proteins and mediate the recycling of ubiquitin in the cell.

1.1.2 The ubiquitin code

Ubiquitination was originally identified as a modification regulating protein degradation by Avram Hershko, Aaro Ciechanover, and Irwin Rose, who in 2004 were awarded with a Nobel Prize in Chemistry for their discovery on the essential cellular degradation pathway, the ubiquitin-proteasome system (UPS). Since their original observations, it has become evident that the ubiquitin signal, here also referred to as ubiquitin code, is extremely versatile and regulates several cellular pathways in addition to protein degradation. The molecular diversity of the ubiquitin code is based on the notion that ubiquitin can function as a signaling moiety in practically every conformation that it occurs in a cell. For example, monoubiquitination, multi-monoubiquitination, and polyubiquitination all function as specific signaling signatures with distinct biological roles (Figure 3, Table 1). Moreover, polyubiquitin chains can adopt diverse linkage type dependent conformations (Figure 3), which increases significantly the complexity of the ubiquitin code. Ubiquitin can also signal as free chains or even as free diubiquitin, which also can adopt several different conformations and thus influence the signal that it mediates (Ye *et al*., 2012). Similar to all PTMs, versatile signaling through ubiquitination requires recognition of the modification by specific ubiquitin binding motifs (UBDs) on larger effector proteins, which transform the covalent modification to molecular actions (Hurley *et al*., 2006).

The structural basis for the ubiquitin signal variety is the amino acid sequence of ubiquitin itself, which contains multiple target sites for additional PTMs (Swatek & Komander, 2016). For example, ubiquitin has seven internal lysine residues (K6, K11, K27, K29, K33, K48, and K63) and an N-terminal methionine (M1), which can all function as acceptor amino acids for ubiquitin moieties enabling the formation of polyubiquitin chains (Figure 3). These chains can be either homogeneous (only one linkage type) or heterogeneous (multiple linkage types) and thus they can generate chains with diverse conformations and branched structures (Figure

3). The variability in the linkage types is, however, only one layer of the ubiquitin code as ubiquitin can be modified also by other PTMs. For example, many of the recent mass spectrometric screens have identified ubiquitin as a target protein for other ubiquitin-like modifiers, such as SUMO or NEDD8, and thereby have provided evidence for the existence of mixed UBL-chains (Hendriks *et al*., 2014; Lamoliatte *et al*., 2014). Moreover, ubiquitin is also modified with small chemical modifications, such as acetylation and phosphorylation (Swatek & Komander, 2016), indicating that ubiquitin signaling is far more complex than originally expected. Indeed, it appears that the linkage type, chain length, conformation, and decoration, altogether provide an essentially unlimited spectrum of signal combinations relaying the biological effects of ubiquitination according to the specific cellular requirements.

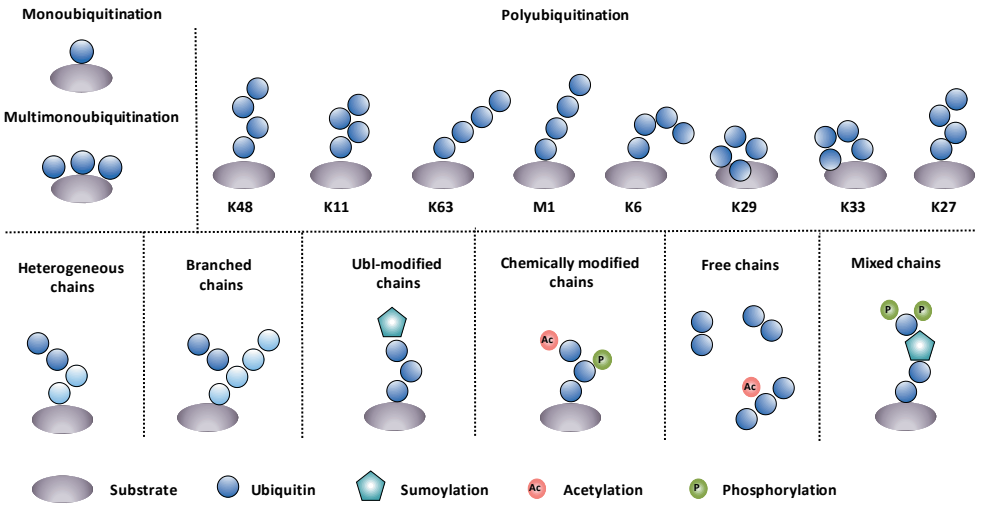

Figure 3. Ubiquitin code diversity. Ubiquitin is a 76-amino acid protein that together with other PTMs creates enormous signal diversity. Target substrates (grey ovals) can be monoubiquitinated or polyubiquitinated by ubiquitin chains with different linkage types and conformations. Ubiquitin chains can also be formed *via* heterogeneous linkages and can be branched. Furthermore, other PTMs, such as other ubiquitin-like proteins (*e.g.* SUMO, turquoise pentagon) can further modify ubiquitin chains. Modification through acetylation (red) and phosphorylation (green) has also been reported. Free ubiquitin chains, or modified free ubiquitin chains, have also cellular signaling functions. Altogether, these modifications can create essentially an unlimited number of signal variation when mixed according to the requirements of the cell.

The differentially linked ubiquitin chains are the most extensively studied form of ubiquitin code variation. As identified in the original studies, many of the differentially linked ubiquitin chains target proteins to proteasomal degradation, which still is the best examined function of polyubiquitination. Typically, the degradation signal is composed of K48-linked ubiquitin chains, though K6-, K11-, and K29-linked chains can also direct proteins to the proteasome in specific circumstances (Swatek & Komander, 2016). For long, the central dogma of the field proposed that K48-linked tetraubiquitin chains are the minimal requirement for proteasomal recognition. However, recent discoveries questioned the prevalent view by showing that diubiquitin chains distributed in multiple lysine residues are more efficient degradation signals than the tetraubiquitin, indicating that versatile determinants can regulate the decision of protein degradation (Lu *et al*., 2015). In addition to K48-linked chains, K63-linked polyubiquitin chains are considered as the second canonical

ubiquitin chain type. K63-linked chains are not directly involved in protein degradation, but function in other important cellular processes of which regulation of the NF-κB (nuclear factor κB) signaling and DNA repair are probably the most extensively studied (Swatek & Komander, 2016).

All the other chain linkage types (M1, K6, K11, K27, K29 and K33) are often referred to as "atypical" ubiquitin chains and only a few specific cellular functions are known to require formation of these atypical chains. For example, similar to K63-linked chains, the M1-linked ubiquitin chains have an important role in the NF-κB-signaling, where they regulate the formation of a signaling hub eventually leading to the phosphorylation and subsequent degradation of the NF-κB inhibitor IκB (Iwai *et al*., 2014). K6-linked ubiquitin chains are involved in the mitochondrial quality control, whereas K11-linked ubiquitin chains are especially important during cell cycle progression (Matsumoto *et al*., 2010). Interestingly, the mitotic regulator APC/C (metazoan anaphase promoting complex/cyclosome) utilizes the only known K11-specific E2 enzyme, UBE2S, to generate K11-linked chains on specific proteins, which appear to function as additional degradation signals enhancing the proteasomal destruction of the targets (Wickliffe *et al*., 2011). The K27-linked chains are the least understood ubiquitin chain type. Few of the recent studies suggests that K27-linked chains are involved in the epigenetic regulation of DNA damage response through histone ubiquitination and together with K29-linked chains might be involved in the neuronal protection in Parkinson´s disease (Gatti *et al*., 2015; Nucifora *et al*., 2016). K29-linked chains have also been shown to be negative regulators of the Wnt-signaling pathway by creating non-degradable ubiquitin chains that disrupt the interaction between Wnt and its co-receptors (Fei *et al*., 2013). Finally, K33-linked chains appear to be relevant for TCR (T-cell antigen receptor) and AMPK (AMP-activated protein kinase) signaling as well as intracellular protein trafficking (Akutsu *et al*., 2016). The importance of mixed ubiquitin chains is only emerging and at least mixed K48/K11-chains have been reported to be extremely efficient proteasomal degradation signals (Meyer & Rape, 2014). The current knowledge regarding the physiological role of various ubiquitin chain types is summarized in the table below (Table 1).

Table 1. Ubiquitin code diversity specifies the physiological impact of ubiquitin conjugation. Table generated according to (Akutsu *et al*., 2016) and (Swatek & Komander, 2016).

Ubiquitin linkage type	Physiological role
Monoubiquitination	Gene expression; protein interaction; autophagy; trafficking
Multimonoubiquitination	Endocytosis
K6	DNA damage response; Parkin-mediated mitophagy; proteasomal degradation
K11	Cell cycle control; proteasomal degradation
K27	Nuclear translocation; DNA dmage response
K29	Ub-fusion degradation; Wnt/b-catenin signaling; proteasomal degradation
K33	TCR signaling; post-Golgi trafficking; AMPK-related kinase signaling
K48	Proteasomal degradation
K63	Endocytosis; protein trafficking; innate immunity; NF-kB signaling; DNA repair
M1	Innate immunity; NF-kB signaling; angiogenesis; selective autophagy
Unanchored chains	Second-messenger-like signaling
Mixed chains	K48/K11 in proteasomal degradation; K63/M1 in scaffolding; Lys11/K63 endocytosis

1.2 Small ubiquitin-like modifier, SUMO

As the name directly implies, the 12 kDa SUMO belongs to the superfamily of ubiquitin-like (UBL) proteins that resemble ubiquitin in their structure, function, and conjugation pathway. Similar to all the family members, SUMO is characterized by the globular β-grasp fold and the C-terminal di-glycine motif required for the covalent attachment. The SUMO protein was initially found in yeast two-hybrid assays as a binding partner of seemingly unrelated proteins associated with acute promyelocytic leukemia (Boddy *et al*., 1996), apoptosis (Okura *et al*., 1996), and DNA repair (Shen *et al*., 1996). Almost simultaneously with these findings, the covalent attachment of SUMO was discovered by two separate laboratories, who both identified it as a modification regulating the localization of RanGAP1 to nuclear pores (Matunis *et al*., 1996; Mahajan *et al*., 1997). Today, nearly 20 years after the initial discoveries, SUMO conjugation, or sumoylation, has been connected to an extensive number of essential biological processes such as DNA replication and repair, chromatin organization, transcription, ribosome biogenesis and protein degradation (Pichler *et al*., 2017). Moreover, recent advancements in substrate identification methods have revealed thousands of novel sumoylation substrate proteins, further highlighting the biological importance of this modification in diverse cellular functions (Hendriks *et al*., 2014).

The human genome encodes five distinct SUMO isoforms (SUMO1-5), that differ in their amino acid sequences, target selection, and tissue distribution. In most contexts, SUMO2 and SUMO3 are collectively called SUMO2/3 since they share 97% sequence homology (Figure 4) and cannot be experimentally separated with the current methodologies. SUMO1 on the other hand, shares only 47% sequence similarity with SUMO2/3 and is considered as an independent SUMO paralog (Figure 4). Intriguingly, only SUMO2 is essential to mouse development, whereas SUMO1 and SUMO3 are dispensable (Evdokimov *et al*., 2008; Zhang *et al*., 2008). SUMO1-3 proteins are ubiquitously expressed in a wide range of tissue types, whereas SUMO4 and SUMO5 have been cloned only from specific tissues (Guo *et al*., 2004; Liang *et al*., 2016). Moreover, it has not been conclusively shown that SUMO4 or SUMO5 would be translated to functional proteins in cells and hence their biological relevance remains elusive (Pichler *et al*., 2017).

```
SUMO1   1 MSDQEAKPSTEDLGDKKEG-EYI KLKVI GQDSSEI HFKVKMTTHLKKLKESYCQRQGVPMNSLRFLFEGQRI ADNHTPKE 79
SUMO2   1 MADE--KP---KEGVKTENNDHI NLKVAGQDGSVVQFKI KRHTPLSKLMKAYCERQGLSMRQI RFRFDGQPI NETDTPAQ 75
SUMO3   1 MSEE--KP---KEGVKTEN-DHI NLKVAGQDGSVVQFKI KRHTPLSKLMKAYCERQGLSMRQI RFRFDGQPI NETDTPAQ 74

SUMO1  80 LGMEEEDVI EVYCEQTGGHSTV - 101
SUMO2  76 LEMEDEDTI DVFQQQTGGVY 95
SUMO3  75 LEMEDEDTI DVFQQQTGGVPESSLAGHSF 103
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Figure 4. SUMO sequence alignment. Homologous sequences between human SUMO isoforms are indicated with green. The diglycine motifs required for SUMO conjugation are highlighted in red.

Due to the sequence variation, SUMO1 and SUMO2/3 have both common and unique selection of substrate proteins and thus are biologically partially different (Saitoh & Hinchey, 2000; Vertegaal *et al*., 2006). For example, RanGAP1 is preferentially sumoylated with SUMO1, whereas SP100 nuclear antigen prefers SUMO2/3 (Saitoh & Hinchey, 2000; Vertegaal *et al*., 2006). One of the most striking structural differences is the presence of sumoylation consensus motif (discussed below) on the SUMO2/3 N-terminus, which enables efficient formation of SUMO2/3 chains through K11 both *in vitro* and *in vivo* conditions (Matic *et al*., 2008; Tatham *et al*., 2001). Upregulation of SUMO2/3 chain formation is detected upon a variety of cell stress conditions and it is widely considered as a modification

promoting cell survival during stress (Saitoh & Hinchey, 2000; Blomster *et al*., 2009; Golebiowski *et al*., 2009). Although SUMO1 is mainly recognized for its role as a cap regulating SUMO2/3 chain elongation, it can also form chains through non-consensus lysine residues (Matic *et al*., 2008). Unlike the diverse signaling mediated by versatile ubiquitin chain types, analogous functions for SUMO chains have not been reported.

1.2.1 SUMO conjugation pathway

The SUMO conjugation pathway resembles closely the ubiquitin conjugation pathway as it similarly consists of E1-activating enzyme, E2-conjugating enzyme and E3-ligase (Figure 5) (Gareau & Lima, 2010). Prior to conjugation, the immature SUMO precursors are cleaved from their C-terminal end by SUMO-specific proteases (SENPs) revealing the diglycine motif (GG) required for covalent substrate attachment. After maturation, SUMO is activated by the E1-activating enzyme, which in an ATP-dependent reaction first forms an adenylate intermediate with SUMO and then binds to the cleavage-exposed C-terminal glycine residue through a high-energy thioester bond. The SUMO pathway utilizes only one heterodimeric E1-activating enzyme, which in humans is composed of subunits SAE1 and SAE2 (Aos1-Uba2 in yeast). After activation, SUMO is transferred to the E2-conjugating enzyme Ubc9, which further transfers the SUMO to the target lysine residues. Distinct to the ubiquitination pathway, where dozens of E2-conjugating enzymes have been identified, Ubc9 is the only known SUMO E2 enzyme. Ubc9 is essential to sumoylation and to organismal development as Ubc9 knock-out is lethal to most eukaryotes (Hayashi *et al*., 2002; Nacerddine *et al*., 2005; Nowak & Hammerschmidt, 2006). Interestingly, and also opposite to hundreds of substrate-recognizing ubiquitin E3-ligases, Ubc9 can be solely responsible for recognizing the sumoylation target lysine (Bernier-Villamor, Sampson, Matunis, & Lima, 2002). However, the substrate-Ubc9-SUMO complex needs to be stabilized to enable efficient SUMO conjugation and this stabilization can be mediated by SUMO-specific E3 ligases or additional Ubc9-binding (Revievewd in Pichler *et al*., 2017). Finally, the covalent isopeptide bond is formed between the G97 of SUMO and the ε-amino group on the target lysine. The fast SUMO deconjugation is mediated by SUMO-specific proteases (SENPs), which hydrolyze the covalent isopeptide bond between the substrate and the SUMO, maintaining the free pool of SUMO in the cell (Figure 5).

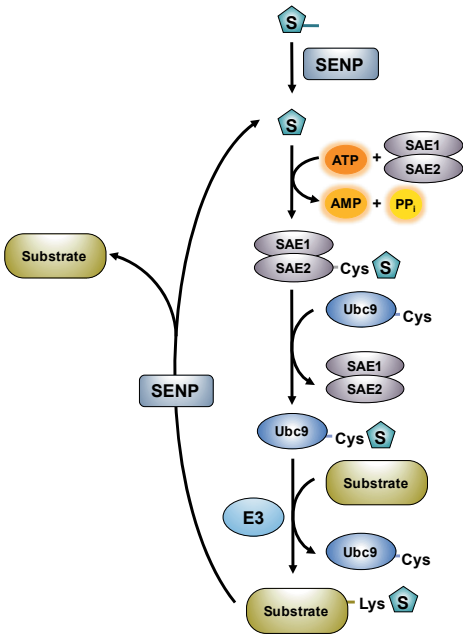

Figure 5. SUMO-conjugation pathway. Before entering the conjugation pathway, precursor-SUMO (S) is cleaved by SUMO-specific proteases, SENPs, to reveal the C-terminal GG-motif. After that, SUMO is activated by heterodimeric E1 in an ATP-dependent manner. From E1, SUMO is transferred to the catalytic cysteine residue on the E2-conjugating enzyme Ubc9. With the assistance of E3-ligating enzymes, SUMO is covalently conjugated to the target lysine residues on the substrate protein. SUMO can be removed from substrate proteins by SENPS, after which SUMO can re-enter the conjugation pathway.

1.2.2 Sumoylation consensus motif and SUMO-interaction motif

The sumoylation target lysine on substrate proteins, is often embedded in the sumoylation consensus motif (SCM), denoted by ΨKxE/D (Ψ is a hydrophobic residue, often I or V). This consensus motif is found in approximately half of the identified sumoylation substrates. The SCM is directly recognized by Ubc9 (Bernier-Villamor *et al*., 2002) and the interaction can mediate E3-independent sumoylation *in vitro*. However, in cells this interaction requires additional stabilization, which can be acquired through multiple ways. Such stabilizing mechanisms are, for example, the SCM-extensions that provide additional interface between the substrate and Ubc9 thereby enhancing the affinity. In 2006, we identified in human heat shock transcription factor 1 the first SCM-extension, the phosphorylation-dependent sumoylation motif (PDSM) (Hietakangas *et al.*, 2006). The characterized motif consists of the consensus tetrapeptide and an adjacent phosphorylatable serine residue at position +5 from the target lysine (ΨKxExxSP) and was identified from many transcriptional regulators. Subsequently, an analogous motif, termed negatively charged amino acid-dependent sumoylation motif (NDSM), was described, where negatively charged amino acids, such as glutamic acid (E) or aspartic acid (D), were identified adjacent to the sumoylation target lysine (ΨKxExx(E/D)$_4$) (Yang *et al*., 2006). Mechanistically these motifs function similarly, as the negative charge provided by the phospho group or the amino acids E and D, enhance the affinity to the basic patch on Ubc9 and thereby stabilize the interaction (Yang *et al*., 2006; Mohideen *et al*., 2009). Notably, biologically these extensions are distinct as the transient nature of phosphorylation and subsequent sumoylation provides additional flexibility to the complex stabilization and thus can be regulated according to cellular requirements.

Although many of the identified sumoylation targets contain the sumoylation consensus tetrapeptide, it is important to note that sumoylation can also occur on non-consensus binding sites or inverted sumoylation motifs (ExK). In these cases, the structural environment surrounding the sumoylation target lysine, determines the occurrence of the modification. An

example of such regulation is the sumoylation of ubiquitin ligase E2-25K on non-consensus lysine 14, which only occurs in the context of specific α-helix (Pichler *et al*., 2005). Similarly, a specific loop structure on the DNA-binding domain of human heat shock factor 1 (HSF1) restricts the sumoylation of a nearby consensus lysine though the same lysine in different structural environment can be efficiently sumoylated (Anckar *et al*., 2006). Interestingly, non-consensus sumoylation seems to respond to cell environmental changes, since stress conditions, such as heat shock, are known to increase specifically non-consensus sumoylation.

In addition to covalent non-consensus/consensus sumoylation, SUMO can exert its regulatory effects also through non-covalent protein-protein interactions. These interactions are conveyed by hydrophobic core regions called SUMO-interaction motifs (SIM), which are characterized by an array of V/I-V/I-x-V/I or V/I-x-V/I-V/I amino acids flanked by acidic residues (Song *et al*., 2004). The SIMs are analogous to the ubiquitin binding domains (UBDs), but instead of multiple well-defined UBDs, only one type of SIM has currently been identified. SIMs are embedded in a highly versatile set of host proteins, including sumoylation enzymes, sumoylation target substrates, and other SUMO-binding proteins (Gareau & Lima, 2010), and hence can mediate multiple biological effects.

1.2.3 Identification of sumoylation sites

Since sumoylation is widely accepted as an essential modification in a multitude of biological processes, the growing interest in characterizing the whole mammalian sumoylated proteome, or sumoylome, is not surprising. After identification of the sumoylation consensus motif (SCM), analysis of protein amino acid sequence was used as the primary method to identify novel sumoylation target substrates. However, existence of the motif is insufficient to determine the susceptibility for SUMO-modification, as the general structural environment created by the surrounding amino acids is also essential (Anckar *et al*., 2006). Furthermore, as sumoylation can also occur on non-consensus sumoylation sites, it is obvious that more advanced methods to identify *in vivo* sumoylation sites are mandatory.

Sumoylation is a very similar modification to ubiquitin and therefore the experimental approaches to study sumoylated proteins are also highly similar. Like ubiquitination, global sumoylation of cellular proteins is often studied through proteomics, which currently is the most reliable method for such analysis. A typical SUMO proteomics experiment utilizes Histidine-tagged (His_6 or His_{10}) exogenous SUMO, which is enriched from the cells with metal-affinity resins based on divalent cations (*e.g.* Ni^{2+} or Co^{2+}) (Hendriks & Vertegaal, 2016a). Subsequently, the purified sample is digested, and the peptides are analyzed with mass spectrometry, which ionizes the peptide sample and sorts the ions according to their mass-to-charge ratio. The challenge in this kind of approach is the nature of the branched SUMO remnants that yield complex fragmentation patterns and thus are complicated to analyze. A common way to overcome this is to generate additional tryptic digestion site to the C-terminus of SUMO proteins (*e.g.* T95R on human SUMO1), which truncates the long peptide remnant and consequently alleviates the analytical challenge (Hendriks & Vertegaal, 2016a). Moreover, sumoylation is a very transient and low-stoichiometry modification, which further challenges the purification and detection sensitivity. Thereby the sample preparation usually requires inhibition of desumoylating enzymes, *e.g.* with NAM (N-ethyilmaleimide), which enhances the accumulation of sumoylated proteins inside the cell.

1.3 Interplay between SUMO and ubiquitin

Though being independent modifications, SUMO and ubiquitin pathways are known to converge in a variety of ways (Denuc & Marfany, 2010). For example, SUMO and ubiquitin can have antagonistic effects on protein function through shared lysine residues or SUMO can modify enzymes in the ubiquitination pathway. Sumoylation of a protein can also cooperatively stimulate ubiquitination and direct proteins to degradation. An example of antagonistic SUMO and ubiquitin effects is the regulation of NF-κB, a key factor involved in immune responses and cell survival. To become activated, NF-κB requires efficient function of the ubiquitination machinery that promotes the degradation of its inhibitor IκBα. However, the ubiquitinated lysine (K21) on IκBα can also be sumoylated by SUMO-1, which inhibits IκBα degradation and consequently results in the inactivation of NF-κB signaling (Desterro *et al*., 1998). Examples of ubiquitination enzymes regulated by SUMO are E2-25K (ubiquitin conjugating enzyme) (Pichler *et al*., 2005) and USP25 (ubiquitin-specific protease 25) (Meulmeester *et al*., 2008), which both are inhibited by sumoylation. Furthermore, the cooperative crosstalk is best exemplified by the function of SUMO-targeted ubiquitin ligases (STUBLs), which recognize polysumoylated proteins through SIMs and ubiquitinate the target through a separate ubiquitin E3 ligase domain. Most extensively studied STUBL is RNF4 (RING finger protein 4), which was identified as the first enzyme mediating SUMO-dependent degradation of the oncogenic PML-RARα fusion in acute promyleocytic leukemia (APL) (Lallemand-Breitenbach *et al*., 2008; Tatham *et al*., 2008). RNF4 has two N-terminal SIMs through which it recognizes sumoylated PML, whereas a separate C-terminal RING ubiquitin E3 ligase domain mediates the ubiquitination and subsequent degradation of PML. APL is mainly treated with arsenic trioxide, which RNF4-dependently stimulates PML-RARα degradation and consequent differentiation of the leukemic cells, often resulting in clinical remission of the patients. Interestingly, STUBLs can also ubiquitinate SUMO itself (Tatham *et al*., 2008), which further increases the versatility of the SUMO-ubiquitin crosstalk.

Despite the above-mentioned observations regarding SUMO and ubiquitin crosstalk, there is only mass spectrometric evidence regarding direct regulation of ubiquitin by SUMO *i.e.* sumoylation of ubiquitin. Since SUMO-1 can regulate SUMO-2/3 chain length by acting as a cap in the end of the chain (Matic *et al*., 2008), the possibility that it could also regulate ubiquitin chains should not be excluded. Considering that ubiquitin chains have a central role in multiple cellular pathways, the hypothesis that SUMO could regulate ubiquitin biology is intriguing and will be addressed in more detail in this thesis in the chapter *1.2 Sumoylation of ubiquitin* in *Results and discussion*.

1.4 Phosphorylation

Protein phosphorylation is the most common PTM and used to regulate proteins in all organisms, including animals, plants, fungi, bacteria, and archaea (Khoury *et al*., 2011). Phosphorylation refers to a chemical modification of a protein, where negatively charged phosphate group is conjugated to the target amino acid on the substrate proteins. Usually the modification is targeted to serine (S), threonine (T), or tyrosine (Y) residues, although basic amino acids such as histidine (H), lysine (K), and arginine (R) have been also reported to undergo phosphorylation (Cieśla *et al*., 2011). Similarly to other PTMs, phosphorylation is ATP-dependent (though in some cases other nucleoside triphosphates can also provide the phosphate) and regulated by specific enzymes, called kinases and phosphatases that mediate the phosphorylation and dephosphorylation events, respectively. Mechanistically, the kinases function by transferring the terminal phosphate group from ATP to the target amino acid on

the substrate, whereas the removal of the phosphorylation is catalyzed by phosphatase-mediated hydrolysis.

From the cellular point of view, protein phosphorylation is an essential PTM that is involved in an extensive amount of regulatory pathways and thereby affects nearly every aspect of a cells´ life (Cohen, 2000). Phosphorylation of a protein can modulate protein function in a variety of ways, such as changing the conformation of the protein or by altering its activity, localization, or stability. Phosphorylation can also create novel interaction sites for diverse phosphopeptide binding motifs on effector proteins and thus can regulate the whole interactome of the protein. Phosphorylation-regulated signaling pathways typically function through step-wise kinase cascades, where the modification and subsequent activation of a kinase is sequentially repeated. One example of such kinase cascades, is the MAP-kinase cascade, which is activated by external stimuli of transmembrane receptors and leads to activation of MAP3K (*e.g* Raf) that activates MAP2K (*e.g* MEK1/2) that activates MAPK (*e.g* ERK1/2) that finally activates the effector proteins, which transform the signal to, for example, transcriptional response (Keshet & Seger, 2010). Many of the transcriptional effectors undergo multi-site phosphorylation (*i.e.* phosphorylation of multiple amino acids on the same target protein), and thereby can integrate various signaling inputs before transforming the modification into biological action. Moreover, phosphorylation often interplays with other post-translational modifications, which further increases the signal diversity. Interestingly, a very recent study by Hendriks and co-workers revealed that especially sumoylation is often preceded by phosphorylation (Hendriks *et al*., 2017), suggesting that these modifications are closely connected in various cellular signaling pathways.

2 CELLULAR RESPONSES TO PROTEOTOXIC STRESS

Every cell in the human body is dependent on the correct function of its proteins. When encountering environmental variations disturbing protein homeostasis (or proteostasis), the cell responds to the stress by initiating specific transcriptional programs to counteract the damage. Depending on the type and location of the damage, the transcriptional selection of genes is optimized according to the requirements of the cell. For example, accumulation of misfolded proteins in the endoplasmic reticulum or mitochondria initiates an unfolded protein response (erUPR or mtUPR, respectively) that activates genes leading to normalization of organelle homeostasis (Haynes and Ron, 2010; Walter and Ron, 2011). However, when the protein damage occurs in the cytosol, the counteractive response is called the heat shock response and it leads to induction of molecular chaperones, which help to mitigate the damage by restoring the cytosolic protein folding environment. The importance of maintaining proteostasis is highlighted by multiple human diseases, such as Huntington´s disease, Alzheimer´s disease, or cancer, which all stem from imbalances in protein homeostasis (discussed in more detail in section *3 HSFs in pathologies*).

In this section of *2 Cellular responses to proteotoxic stress*, I will first focus on the cytosolic proteotoxic stress response, the heat shock response, and introduce the heat shock transcription factors that mediate this response. After that, I proceed with a more detailed description of HSF1 and the PTMs regulating its activity, a key question addressed in the second study of this thesis (II). I will end this section by reviewing the current knowledge about HSF2, as that is in the main focus in the third and fourth study of this thesis (III and IV).

2.1 Heat shock response

In 1962, an Italian geneticist Ferruccio Ritossa accidentally discovered the heat shock response, while studying the chromatin structure of the salivary gland polytene chromosomes of fruit fly *Drosophila busckii* (Ritossa, 1962). During one of the experiments, the fly cells were non-intentionally cultured at elevated temperatures, which resulted in appearance of specific chromosomal puffs associated with newly synthesized RNA (Ritossa, 1962). Nearly a decade later, this heat-inducible RNA synthesis was shown to coincide with the synthesis of specific proteins, which consequently were designated as the heat shock proteins (Hsps) (Tissières *et al*., 1974). Yet another decade later, genomic mapping and comparative sequence analysis of the puffed *loci* led to the identification of a common promoter element, the heat shock element (HSE), which was shown to be essential for the heat-induced activation of the *loci* (Mirault *et al*., 1982; Pelham, 1982). Subsequently, the HSE, consisting from inverted nGAAn pentamers, was used as a bait to purify the HSE-binding proteins, eventually leading to the identification of heat shock transcription factors, HSFs, first from *Drosophila* (Parker *et al*., 1984; Wu, 1984) and later on also from yeast (Sorger & Pelham, 1987) and human cells (Kingston *et al*., 1987). Hence, though not knowing it himself, Ritossa had serendipitously described a phenomenon that together with the subsequent studies defined the fundamental attributes of heat shock response; namely the heat-inducible activation of specific factors, which through distinct genomic elements regulate the mRNA synthesis of heat shock proteins.

Despite originally identified in the fly, the heat shock response is an evolutionarily extremely well conserved stress protective mechanism that is activated upon exposure to variable environmental stresses (Figure 6). In fact, the ability to respond to proteotoxic stress is so

vital to an organism, that analogous protective pathways have been identified in every living organism, all the way from unicellular bacteria and yeast to multicellular plants and animals. And as one of the pioneers of the modern stress biology, Susan Lindquist, once said "It may be that some creature living in the depths of the ocean does not have a heat shock response, but that is doubtful" (Lindquist, 1986). In all studied organisms, the response is characterized by a transcription factor (or factors), which stress-inducibly binds to its target promoters and activates the transcription of its target genes. Most of these target genes code for molecular chaperones, such as the heat shock proteins, which are essential in promoting protein homeostasis in both unstressed and stressed cells (Hartl *et al*., 2011). The superfamily of molecular chaperones consists of six major Hsp subfamilies: HSPH (Hsp110), HSPC (Hsp90), HSPA (Hsp70), DNAJ (Hsp40), HSPB (Small Hsps), and chaperonins HSPD/E (HSP60/10) and CCT (TRiC) (Kampinga *et al*., 2009). In non-stressed cells, the main role of these chaperones is to assist in the folding of newly synthesized polypeptides and it is the HSPCs, HSPAs (especially the constitutively expressed HSPA8) and CCT chaperonins that are mainly responsible for this *de novo* folding. These chaperones function by forming multiprotein complexes with co-chaperones and utilize ATP to assist the acquisition of correct 3D conformation. The mechanism by which chaperones recognize their target, is based on their ability to bind hydrophobic amino acid residues exposed either on the nascent polypeptide chain or on the stress-inducibly unfolded proteins.

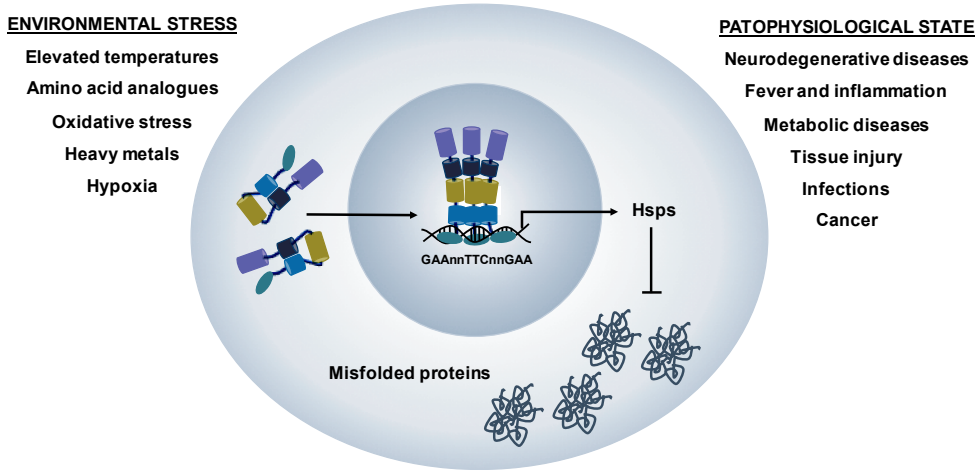

Figure 6. Heat shock response is activated in a variety of conditions. During stress or specific pathologic conditions, cytosolic HSFs are activated, trimerized and localized to the nucleus. In the nucleus, HSFs bind to their target elements and activate the transcription of the target genes. Many of the target genes encode molecular chaperones, such as the heat shock proteins (Hsps), which ameliorate the toxicity of damaged proteome.

The majority of the chaperone superfamily proteins are upregulated during proteotoxic stress, but by far the best characterized is the massive upregulation of the HSPA (Hsp70) family proteins, such as HSPA1A, HSPA1B, and HSPA6. During stress, these chaperones prevent the aggregation of damaged proteins and help the refolding of unfolded proteins. The ATP-bound HSPAs bind to non-native proteins with the help of DNAJ co-chaperones, which deliver the substrates to HSPAs and stimulate the ATPase activity. With further help of additional co-factors such as a nucleotide exchange factor (NEF), Bcl2-associated athanogen-1 (Bag1), or Hsp70-binding protein 1 (Hspbp1), ADP and the client substrate are released and the machinery can enter yet another round of folding cycle. The ATP-

independent HSPBs, the small Hsps, are also essential for the heat shock response, as they bind with high affinity to exposed hydrophobic patches and form soluble protein deposits inside the cell. With the assistance of Hsp70 and/or Hsp90 machineries, these aggregates are resolved and refolded back to their native conformations or directed to protein degradation machineries (Richter *et al*., 2010).

2.2 Mammalian heat shock transcription factor family

One of the most striking features of the heat shock response is the extremely rapid transcriptional activation of genes required for cellular survival. Both in prokaryotes and eukaryotes, the rapid activation is achieved through specific transcription factors that regulate gene expression in a signal dependent manner. In the bacteria *Escherichia coli,* this stress-inducible transcription factor is called σ^{32}, which can heat-inducibly activate transcription of DnaK, a prokaryotic homologue of Hsp70 (Grossman *et al*., 1984). In yeast *Saccharomyces cerevisiae*, nematode *Caenorhabditis elegans*, and fruit fly *Drosophila melanogaster*, the heat shock response is mediated by a single heat shock factor (ScHSF, CeHSF, and DmHSF, respectively), whereas multiple HSFs exist in fish, birds, mammals and plants. In fact, the existence of several HSFs was primarily reported in *Lycopersicon peruvianum* (tomato), which led to the hypothesis that more than one HSF exists also in other organisms (Scharf *et al*., 1990).

The mammalian HSF family consists of seven members (HSF1-5, HSFX, and HSFY), of which HSF1 and HSF2 are the most extensively studied. Cloning of these factors from human cells was reported simultaneously in 1991 by two independent studies, both utilizing a probe produced according to the conserved amino acids in the DNA-binding domain of fly and yeast HSFs. The first study screened a human B cell lymphoma cDNA library and identified human HSF1 (Rabindran *et al*., 1991). The second study isolated two distinct HSF cDNAs, with significant homology to the conserved HSF-regions, from HeLa cells and thus indicated for the existence of another HSF in human cells (Schuetz *et al*., 1991). These factors were named HSF1 and HSF2 and already in that original study, the factors were hypothesized to have separate functions in human cells (Schuetz *et al*., 1991). Later on, homologous factors were also cloned from mouse liver (mHSF1 and mHSF2) (Sarge *et al*., 1991) and chicken (cHSF1, cHSF2, cHSF3) (Nakai & Morimoto, 1993), which conclusively confirmed the existence of multiple HSFs in different vertebrates.

HSF1 is the most studied member of the mammalian HSFs and the main factor regulating inducible gene expression during stress (McMillan *et al*., 1998). HSF1 is the functional counterpart of ScHSF, CeHSF, DmHSF, and the avian HSF3, and essential for the cellular and organismal protection against heat-induced damage (McMillan *et al*., 1998). Similarly to HSF1, HSF2 has the ability to bind DNA stress-inducibly, though its role in the stress response is considered modulatory (Östling *et al*., 2007). Instead, HSF2 is especially important in specific developmental processes, such as gametogenesis and corticogenesis, and it has been implicated in neuronal migration and fetal alcohol spectrum disorder (FASD) (El Fatimy *et al*., 2014). Both HSF1 and HSF2 will be discussed in more detail in the forthcoming chapters.

The third member of the mammalian HSFs, HSF3, was discovered relatively late, and currently, it has been identified only in mouse (Fujimoto *et al*., 2010). In the experiments conducted by Fujimoto and co-workers, exogenously expressed mHSF3-GFP (green fluorescent protein) did accumulate in the nucleus in response to stress, but failed to activate

the Hsp70 promoter. However, mHSF3 appeared to be important in the regulation of non-classical heat shock genes, such as *PDZK1* and *PROM3* (Fujimoto *et al*., 2010), indicating that the protein can function as an active transcription factor. Interestingly, overexpression of mHSF3 in *Hsf1*$^{-/-}$ MEFs partially restores the cellular resistance to heat shock, suggesting that mHSF3 might be important during inducible heat shock response. Similarly to HSF2, HSF4 is also highly important in differentiation and developmental processes and more specifically in the development of mammalian sensory organs. In humans, a point mutation in the HSF4 DBD leads to cataract, linking HSF4 directly to human diseases (Bu *et al*., 2002). Studies performed with HSF4 knock-out mice have revealed that the cataract is at least partially caused by decreased expression of γ-crystallins and increased expression of fibroblast growth factors (FGFs) that leads to accumulation of inclusions and premature differentiation of the lens epithelial cells, respectively (Fujimoto *et al*., 2004). Lastly, the biological importance of HSF5, HSFX and HSFY is currently uncharacterized, but they are all hypothesized to be involved in gametogenesis (Widlak & Vydra, 2017).

2.2.1 Structural features of HSFs

All HSF family members are characterized by functional domains that contain both common and unique features. The most conserved domain is the N-terminal winged helix-turn-helix DNA-binding domain (DBD) (Figure 7), which is present in all eukaryotic HSFs (Vuister *et al*., 1994). The crystal structure of DBD was reported in 1994 by Harrison and co-workers, who by studying the yeast *Kluyveromyces lactis* revealed the core structure of the domain: four-stranded antiparallel β-sheet capping three α-helices (Harrison *et al*., 1994). More recently, the mammalian DBD was co-crystallized with DNA, uncovering the positioning of DBD and the remaining HSF on the opposite sites of the DNA strand in a so called DNA-embracing structure (Jaeger *et al*., 2016; Neudegger *et al*., 2016). Furthermore, Jaeger and co-workers extended the structural knowledge by crystallizing also the DBD wing-domain from human HSF2 (Jaeger *et al*., 2016). This wing-structure is distinct among other winged helix-turn-helix transcription factors, as it is not in contact with the DNA minor groove or with the DNA backbone (Littlefield & Nelson, 1999). In addition, the wing-domains between hHSF1 and hHSF2 differ both structurally and functionally and provide essential isoform-specific protein-protein interaction sites important for DNA-binding affinity and binding-specificity in distinct cellular states (Ahn *et al*., 2001; Jaeger *et al*., 2016).

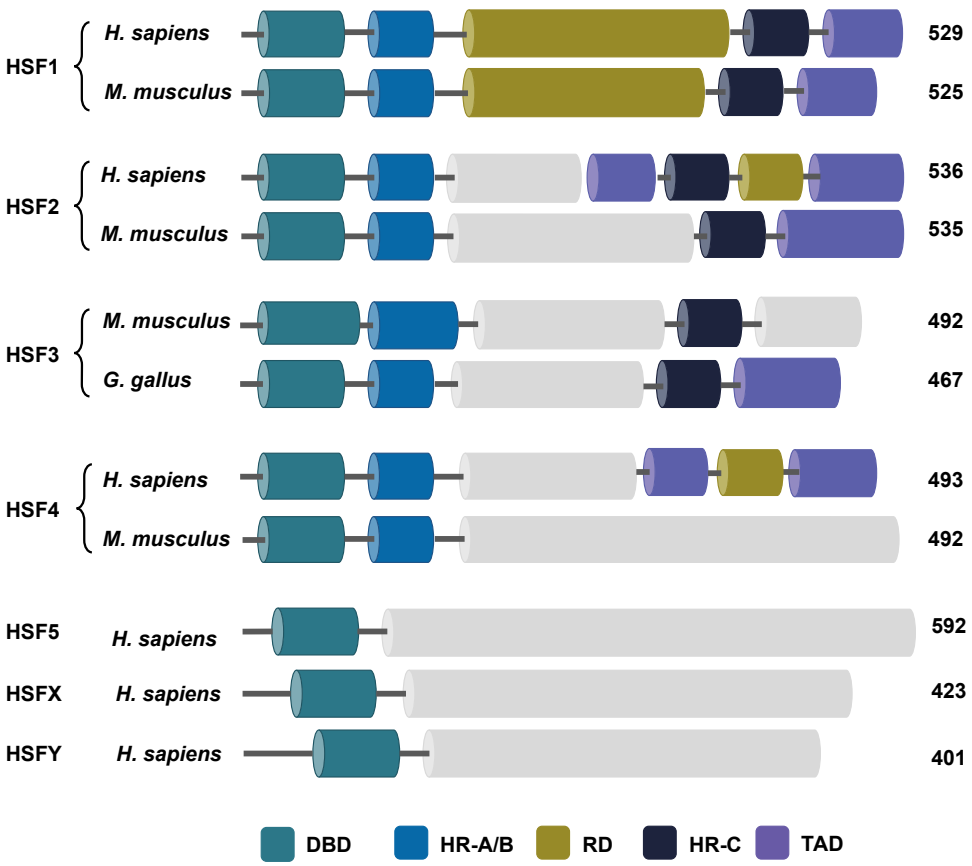

Figure 7. Heat shock factor family. Out of the seven mammalian HSFs (HSF1-5, HSFX and HSFY) HSF1, HSF2, and HSF4 are expressed ubiquitiously in human and mouse, whereas expression of HSF3 is only detected in mouse. The chicken HSF3 is depicted only to highlight the structural similarity between hHSF1, mHSF1 and the cHSF3 that likely results in the detected functional similarity of these proteins in acute stress responses. Domain abbreviations: DBD, DNA-binding domain; HR-A/B/C heptad repeat domains required for oligomerization; RD, regulatory domain; TAD, transactivation domain. Adapted from Dayalan Naidu & Dinkova-Kostova (2017).

Unlike many other transcription factors that often exist as dimers, HSFs are trimerized upon activation, which is thought to improve the DNA-binding affinity. This intermolecular oligomerization is mediated by overlapping arrays of hydrophobic leucine-zipper-like heptad repeats (HR-A/B) located directly adjacent to the DBD (Sorger *et al*., 1989) (Figure 7). In addition to the highly conserved DBD, this region is also largely similar between the HSF isoforms. Indeed, already in the initial studies that recognized multiple HSF isoforms in mammalian cells, the region was hypothesized to provide an "inter-isoform" interaction site and thus suggested for the existence of HSF heterotrimers. Excitingly, this hypothesis was later confirmed experimentally (Östling *et al*., 2007; Sandqvist *et al*., 2009) and is currently considered as one of the mechanisms enabling cell- and developmental-state specific HSF target gene selection. Yet another heptad repeat (HR-C) resides closer to the C-terminus and exists only in specific HSF isoforms. This domain confers an intramolecular interaction site and represses spontaneous oligomerization and subsequent acquisition of DNA-binding activity. Interestingly, neither mammalian HSF4 nor ScHSF contains HR-C, resulting in

constitutively trimeric and DNA-binding competent forms of these HSFs (Sorger *et al*., 1987; Nakai *et al*., 1997).

Though trimerized and DNA-bound, HSFs do not necessarily induce target gene expression (Hensold *et al*., 1990; Jurivich *et al*., 1992). For that they require the C-terminal transactivation domain (TAD), which provides an interaction site for co-factors and chromatin remodelers thus potentiating transcription (Sullivan *et al*., 2001; Boellmann *et al*., 2004). In the absence of stress, the TAD is negatively regulated by a centrally located regulatory domain (RD) (Shi *et al*., 1995, Zuo *et al*., 1995). The RD is self-sufficient in its heat-sensing capability and can provide heat-sensitivity also to heterologous activatory domains (Newton *et al*., 1996). The RD is subjected to multiple PTMs, such as phosphorylation, sumoylation, and acetylation, but the exact role of these modifications is still partially unknown.

2.2.2 HSE, target sequence for HSF binding

Similarly to many other sequence-specific transcription factors, HSFs act through a conserved upstream regulatory DNA-element, known as the heat shock element, HSE (Amin *et al*., 1988). These elements consist of inverted repeats of the pentameric nGAAn consensus sequence and are highly variable in their primary sequences, length, and orientation (Mendillo *et al*., 2012; Riva *et al*., 2012; Vihervaara *et al*., 2013). A typical HSE contains two to six inverted nGAAn repeats and heat-responsive promoters harbor often more than one HSE (Littlefield & Nelson, 1999). Since HSFs bind DNA as trimers, the optimal HSE consists of three consecutive inverted nGAAn repeats, to which all the HSF subunits can be in contact with. This is not, however, always required and HSF oligomers can also bind HSEs with only two repeats, presumably maintaining one subunit unattached to the DNA. Mechanistically this sequence-specific binding of HSFs occurs through hydrogen bonding between a conserved arginine residue (R63 in human HSF2) in the recognition α-helix (α3 in the DBD core structure) and the guanine nucleotide (n<u>G</u>AAn) located in the major groove of DNA (Jaeger *et al*., 2016).

Due to the inverted nature of the element, two contiguous nGAAn repeats can exist either in so called head-to-head orientation (nGAAnnTTCn) or in tail-to-tail orientation (nAAGnnCTTn) (Jaeger *et al*., 2014). Consequently, in HSEs with more than two repeats, the orientation of contiguous nGAAn repeats can vary. This orientation variability can result in a complex HSF binding patterns where affinity of the trimers, orientation of trimer subunits and interactions between adjacent trimers can be affected. For example, HSFs can recognize the orientation of the nGAAn repeats and bind with higher affinity to head-to-head oriented repeats (Bonner *et al*., 1994). Because of the conserved binding mechanistic described above, the orientation of the repeat also affects the HSF monomer orientation, as the DBD always forms a contact with the consensus guanine. Furthermore, HSEs with several repeats often promote cooperative binding of multiple HSF trimers, and depending on the orientation of distinct repeats the adjacent trimers can interact and create specific interfaces for additional regulatory inputs (Jaeger *et al*., 2014). Altogether, these HSE features result in variations in HSF binding to specific promoters, which likely is reflected in the observed differences in HSF target gene selection between stress (Vihervaara *et al*., 2013), cancer (Mendillo *et al*., 2012), and neurodegenerative diseases (Riva *et al*., 2012).

2.3 Heat shock factor 1, HSF1

Mammalian HSF1 is the functional counterpart of single HSF found in yeast, nematode and fruit fly and the best characterized member of the HSF family. During stress, HSF1 is the fundamental and irreplaceable activator of Hsp gene expression and hence generally accepted as the master regulator of the heat shock response. The expression of HSF1 among tissues is ubiquitous (Fiorenza *et al*., 1995) and as evidenced by *Hsf1*$^{-/-}$ mice studies, the factor is essential for acquisition of both cellular and organismal thermotolerance as well as protection against heat-induced apoptosis (McMillan *et al*., 1998; Xiao *et al*., 1999). In addition to elevated temperatures, HSF1 is activated in response to a vast repertoire of proteotoxic stress signals, proposing a key role for the factor in the overall cellular response to damaged proteins.

The first step in the HSF1 activation-attenuation pathway (Figure 8) is the conversion of inert HSF monomers to DNA-binding competent trimers. In the absence of stress, HSF1 monomers shuttle between the cytoplasm and the nucleus, whereas upon stress the nuclear export is restricted, which leads to accumulation of HSF1 monomers in the nucleoplasm and thereby enables oligomerization (Budzyński & Sistonen, 2017). Trimerization increases HSF1 affinity for DNA and precedes transcriptional activation, which leads to Hsp production. Transcriptional attenuation occurs in response to stress withdrawal or in prolonged stress conditions and involves repression of transcriptional activity through negative feedback from Hsps as well as decrease in the DNA-binding affinity due to DBD acetylation (Westerheide *et al*., 2009). Despite still being under a debate, the current view proposes that rather than being reverted back to monomers, the attenuated trimers are subjected to ubiquitin-mediated proteasomal degradation, which ends the stress induced HSF1 activation-attenuation pathway (Budzyński & Sistonen, 2017). During the whole pathway, HSF1 is subjected to multiple PTMs, such as phosphorylation, sumoylation and acetylation, which will be discussed in more detail in the following section *2.3.2 Post-translational regulation of HSF1*.

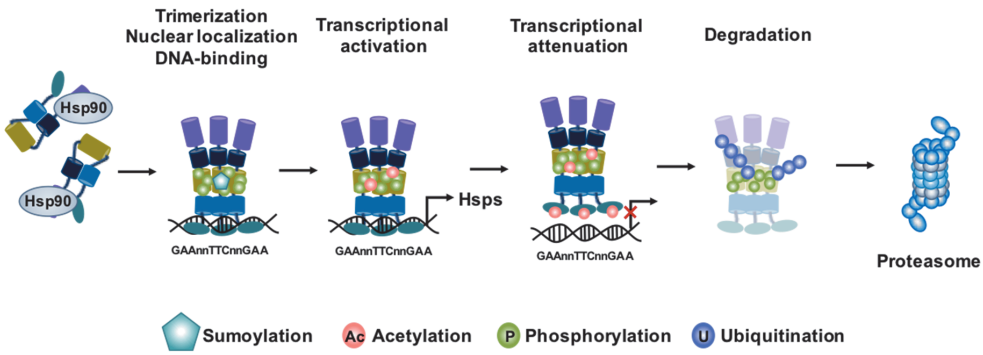

Figure 8. HSF1 activation-attenuation pathway. In the absence of stress, HSF1 exists primarily as monomers in chaperone-bound inactivated state. Heat shock and other forms of stress, induce HSF1 oligomerization, accumulation in the nucleus and DNA-binding activity. Simultaneously, HSF1 is extensively modified by phosphorylation and sumoylation. Transcriptional activation of HSF1 co-occurs with acetylation of specific sites and leads to upregulation of molecular chaperones, such as the Hsps. Transcriptional attenuation is regulated through acetylation of the DBD, which releases HSF1 trimers from target DNA. Subsequently, acetylated HSF1 trimers are ubiquitinated and directed to proteasomal degradation.

The role of HSF1 as the master regulator of heat shock response has evoked large interest towards the sensory mechanisms that recognize the stress and regulate stimulus-dependent HSF1 activity. Interestingly, HSF1 itself appears to be one such molecular sensor as it has an intrinsic ability to sense stress and can form DNA-bound trimers when subjected to versatile proteotoxic stresses *in vitro* (Mosser *et al*., 1993; Goodson & Sarge, 1994; Larson *et al*., 1995). Recently, particularly the RD and the HR-C were shown to undergo temperature-dependent conformational alterations (Hentze *et al*., 2016). However, in cells this property is suppressed by negative regulators, which maintain HSF1 monomers in inactive state and inhibit spontaneous trimerization and subsequent DNA binding. Perhaps the best known of such regulators is Hsp90, which interacts with HSF1 in the absence of stress and retains it monomeric (Figure 8) (Zou *et al*., 1998). During proteotoxic stress, accumulation of denatured proteins sequester Hsp90 and the interaction between HSF1 and Hsp90 rapidly decreases, thereby allowing HSF1 trimerization and induction of HSF1 DNA-binding and transcriptional activities (Zou *et al*., 1998, Guo *et al*., 2001). Upon recovery from the stress, upregulated Hsps restore the protein homeostasis, which leads to Hsp liberation from their substrates and re-association with HSF1. Thus, Hsp90 provides the cell with a negative feedback mechanism sensitive for the cellular folding state.

In addition to regulating the initial monomer-to-trimer transition, Hsps interact also with the trimeric HSF1 during stress and the interaction is considered as one of the mechanisms important for the response attenuation. For example, the Hsp90-FKBP2-p23 chaperone complex has been reported to interact with the HSF1 regulatory domain, which appears to be required for the appropriate transcriptional attenuation, as inhibition of the interaction prolongs HSF1 DNA-binding activity (Bharadwaj *et al*., 1999; Guo *et al*., 2001). Moreover, trimeric HSF1 can also interact with Hsp70 and its co-chaperone Hsp40, which in contrast to Hsp90-mediated regulation of HSF1 DNA-binding activity, seems to be more important in the regulation of its transcriptional activity (Shi *et al*., 1998). Indeed, already in 1982, DiDomenico and co-workers reported that accumulation of Hsp70 is required for the transcriptional repression of the heat shock response (DiDomenico *et al*., 1982). Therefore, though not functioning directly as the initial sensor for the stress, Hsp interaction with the trimeric HSF1 can generate an essential self-regulating step during the heat shock response, which helps to coordinate HSF1 activity according to the requirements of the cell.

2.3.1 Post-translational regulation of HSF1

Phosphorylation of HSF1

HSF1 is a stress-inducible transcription factor that undergoes multiple PTMs during its activation-attenuation pathway. The significance of PTMs in HSF regulation was revealed already in 1987 by Zimarino and Wu, who studied the heat shock response kinetics by analyzing HSF binding in *Drosophila* cells and revealed that the inducible HSF binding is not dependent on newly synthesized proteins. Simultaneous translation inhibition and heat-treatment did not hamper the heat shock response, indicating that the HSF must pre-exist in *Drosophila* cells and undergo stimulus-specific PTMs to gain its activity (Zimarino & Wu, 1987). Later during the same year, Sorger and co-workers studied the properties of human and yeast HSFs, and observed that in contrast to heat-inducible binding of human HSF1, the yeast HSF is constitutively DNA-bound and exhibits stress-specific migration pattern on a polyacrylamide gel. Treating the heat-shocked yeast cells with calf intestinal phosphatases eliminated the pattern difference, demonstrating that the factor was phosphorylation in response to stress (Sorger *et al*., 1987). Following year, also the human HSF1 was reported to undergo heat-inducible phosphorylation (Larson *et al*., 1988), altogether confirming that

the post-translational modification of HSFs through phosphorylation is a general phenomenon in eukaryotic cells.

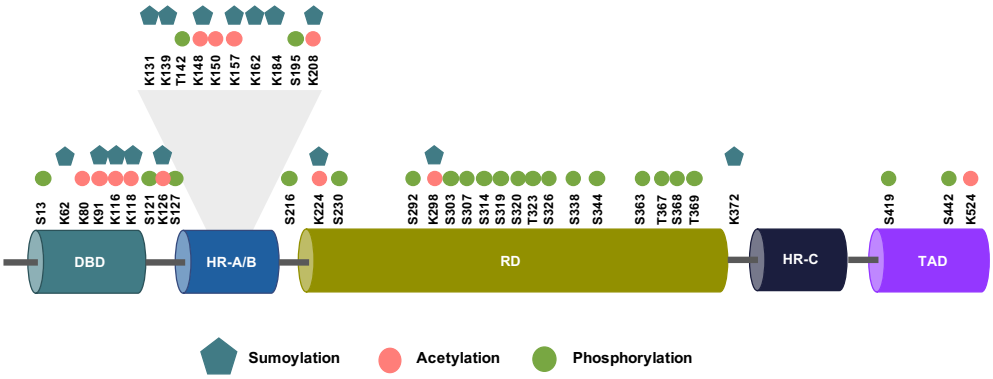

Figure 9. Schematic overview of identified post-translational modification sites on human HSF1. HSF1 is phosphorylated on multiple serine (S) and threonine (T) residues, mainly located in the regulatory domain (RD). DNA-binding domain (DBD) and the oligomerization domain (HR-A/B) harbor most of the acetylatable and sumoylatable lysine (K) residues. HSF1 is also ubiquitinated, but the exact lysine residues are not thus far identified. Additional abbreviations: C-terminal heptad repeat domain (HR-C) and transactivation domain (TAD).

Today, altogether 23 phosphorylatable serine (S) and threonine (T) residues have been identified on human HSF1, of which most reside within the RD (Guettouche *et al*., 2005, (Figure 9). Although the prominent hyperphosphorylation is strongly induced upon heat shock and coincides with the acquisition of DNA-binding and transcriptional activities (Cotto *et al*., 1996), most of the studied phosphorylation sites are in fact repressive. For example, MAPK (mitogen-activated protein kinase) -driven phosphorylation of S303, GSK3β (glycogen synthase kinase 3 beta) -driven phosphorylation of S307, and PKC (protein kinase C) -driven phosphorylation of S363 have all been shown to repress HSF1 activation in non-stress conditions (Chu *et al*., 1996; Knauf *et al*., 1996; Kline & Morimoto, 1997; Chu *et al*., 1998). Moreover, pro-inflammatory protein kinase MAPKAPK2 (MAPK-activated protein kinase 2) inhibits HSF1 transcriptional activity through phosphorylation of S121, which promotes HSF1-Hsp90 interaction and consequently inhibits HSF1 trimerization and DNA-binding activity (Wang *et al*., 2006). More recently, AMPK-mediated phosphorylation of S121 was shown to suppress proteotoxic stress response specifically during metabolic stress, suggesting that HSF1 repression *via* phosphorylation is important not only in the absence of proteotoxic stress but also during specific cellular stress conditions (Dai *et al*., 2015).

Besides repressing spontaneous HSF1 activation, phosphorylation appears to repress HSF1 function also by regulating its degradation during stress. As shown by Kourtis and co-workers, the ubiquitin ligase FBXW7 (a substrate-targeting subunit of the Skp1-Cul1-F box E3 ubiquitin ligase) interacts with HSF1 in a phosphorylation-dependent manner, resulting in proteasomal degradation of the factor (Kourtis *et al*., 2015). Interestingly, mutation of S303/307 on HSF1 abolished the interaction with FBXW7 and prolonged heat shock response, indicating that the degradation is required for proper attenuation of the response. Moreover, inhibition of GSK3β and MEK kinases decreased the interaction between FBXW7 and HSF1, proposing that the previously identified kinases are responsible for S303 and S307 phosphorylation and subsequent degradation of HSF1 (Kourtis *et al*., 2015). More recently,

Gomez-Pastor and co-workers elegantly confirmed these results by showing that CK2α′(casein kinase II alpha prime) -mediated HSF1 phosphorylation promotes HSF1 interaction with FBXW7 in Huntington´s disease, which leads to abnormal HSF1 degradation and supports the disease phenotype (Gomez-Pastor *et al*., 2017).

In addition to somewhat counterintuitive repressive phosphorylation sites, HSF1 is also subjected to activatory phosphorylation events. The best-known example among activating phosphorylation sites is S326, which is generally accepted as a hallmark of HSF1 activation (Boellmann *et al*., 2004; Guettouche *et al*., 2005; Shah *et al*., 2016) and has been shown to be the target amino acid for MEK and p38 MAPK kinases (Tang *et al*., 2015, Dayalan Naidu *et al*., 2016). Furthermore, stress-inducible phosphorylation of S230 by CaMKII (calcium/calmodulin-dependent protein kinase II) similarly potentiates HSF1 transactivation capacity, as evidenced by decreased Hsp70 levels in cells expressing mutant HSF1 with S121A substitution (Holmberg *et al*., 2001). Yet another type of activating phosphorylation signals are conveyed by PKA (pleiotropic protein kinase A) and PLK1 (polo-like kinase 1), which through S320 and S419 phosphorylation, promote the nuclear accumulation of HSF1 upon heat stress (Kim *et al*., 2005; Murshid *et al*., 2010). Interestingly, during mitosis PLK1 phosphorylates S216, which has been shown to be important for mitotic progression by contributing to HSF1 ubiquitination and degradation (Lee *et al*., 2008). Despite a wealth of studies examining the importance of single phosphorylation sites in the regulation of HSF1, the role of hyperphosphorylation still remains to be elucidated and will be assessed in the second study of this thesis.

Sumoylation and acetylation of HSF1

In addition to phosphorylation, HSF1 is also stress-inducibly sumoylated, which was initially described as a modification required for proper DNA-binding and induction of Hsp genes (Hong *et al*., 2001). Soon after, the importance of sumoylation was, however, contradicted by another report, which showed that HSF1 sumoylation is dispensable for the induction of heat shock response (Hietakangas *et al*., 2003) and thereby suggested that the biological outcome of the modification might be context-dependent. According to both of the original studies, HSF1 is mainly sumoylated on lysine 298, though an additional minor sumoylation site on lysine 126 was also identified (Hong *et al*., 2001, Hietakangas *et al*., 2003). The HSF1 sumoylation target lysine resides within an extended SCM, called phosphorylation-dependent sumoylation motif (PDSM) (discussed in more detail in *1.2.2 Sumoylation consensus motif and SUMO-interaction motif*), where phosphorylation of the nearby S303 is a prerequisite for the SUMO-modification on K298 (Hietakangas *et al*., 2006). The PDSM has also been identified in various other transcription factors (Hietakangas *et al*., 2006) and appears to be a common mechanism to integrate different intracellular signaling pathways. Reporter gene assays and RT-PCR analysis of HSF1 target genes, have demonstrated that sumoylation is a negative regulator of HSF1 transactivation capacity during proteotoxic stress (Hietakangas *et al*., 2006), albeit the mechanistic understanding of this process is still scarce. Interestingly, Hendriks and co-workers recently identified 14 putative sumoylation sites on HSF1, mainly residing on the DBD and the HR-A/B domains (Hendriks *et al*., 2017) (Figure 9), further highlighting the requirement of future work regarding HSF1-SUMO interactions.

HSF1 is acetylated on 12 lysine residues that appear to be important in different phases of the HSF1 activation-attenuation pathway (Westerheide *et al*., 2009; Raychaudhuri *et al*., 2014). In non-stressed cells, HSF1 is acetylated by histone acetyl transferase EP300 on three

individual lysine residues (K118, K208 and K298), of which K208 and K298 are essential for HSF1 stabilization by preventing the proteasomal degradation of the factor (Raychaudhuri *et al*., 2014). Interestingly, though lysine residues are subjected to ubiquitination, blocking ubiquitination seems not to be the main mechanism behind acetylation-mediated stabilization, since only acetylation-mimicking mutation K208Q, but not K208R, prevents HSF1 degradation (Raychaudhuri *et al*., 2014). In contrast to the stabilizing effect in untreated cells, increased HSF1 acetylation upon HS contributes to attenuation of heat shock response, through at least two acetylatable lysine residues in the DBD (K80, K118) (Westerheide *et al*., 2009; Raychaudhuri *et al*., 2014). Acetylation of DBD during stress releases HSF1 from the DNA, which is generally accepted as a key step required for the response attenuation. The acetylation status of the K80 can be regulated by the deacetylase SIRT1 as well as histone deacetylases HDAC7 and HDAC9, which all have been shown to deacetylate HSF1 and prolong the heat shock response (Westerheide *et al*., 2009; Zelin & Freeman, 2015).

It is worth mentioning that the novel sumoylation sites identified by Hendriks and co-workers raise an important question about the interplay between distinct PTMs, as many of the identified lysine residues appear to be subjected to both sumoylation and acetylation. Most of those residues are located in DBD and HR-A/B, and it is tempting to speculate that alternating lysine modification could provide a cell state-specific mechanism to regulate oligomerization or the DNA-binding activity of HSF1. Particularly intriguing residue is lysine 298 in the RD, which is the only lysine within a PDSM consensus extension and has been implicated in the regulation of HSF1 transactivation capacity. Previous studies have demonstrated in two distinct PDSM-containing substrates, MEF2A (myocyte enhancer factor 2A) and tumor suppressor HIC1 (hypermethylated in cancer 1), that acetylation and sumoylation of the same lysine within a PDSM provides a regulatory switch, where acetylation of the lysine blocks sumoylation and thereby counteracts the repressing effect of SUMO conjugation (Shalizi, 2006; Stankovic-Valentin *et al*., 2007). Therefore, future studies on post-translational regulation of HSF1 should perhaps not only focus on single modifications, but examine the combinatorial effect of various HSF1 PTMs.

2.3.2 HSF1 in physiological context

In addition to its prominent role in the heat shock response, HSF1 is essential also in multiple physiological processes, such as development, immune responses and organismal aging (Figure 10). For example in yeasts, the single HSF is absolutely essential for cell viability already in normal growth conditions, as genetically disrupted cells lacking HSF are unable to produce viable spores (Sorger & Pelham, 1988; Gallo *et al*., 1993). Although the yeast HSF has been reported to bind multiple gene *loci* in non-stressed conditions, the lethal knock-out phenotype can be rescued with overexpression Hsp70 and Hsp90, implying that the main role of yeast HSF is the maintenance of proteostasis (Solis *et al*., 2016). In contrast to yeast, the DmHSF is not required for adult fly viability, but is essential for larval development and oogenesis (Jedlicka *et al*., 1997). Intriguingly, larval lethality and impaired oogenesis is not caused by defective Hsp expression, indicating that unlike in yeast, HSFs in multicellular organisms maintain an additional genetic program essential for organismal development. Supporting the view, Li and co-workers recently revealed that CeHSF targets a distinct selection of genes during nematode development and regulates a genetic program different from heat shock response (Li *et al*., 2016). Intriguingly, the developmental activation of CeHSF was shown to depend on binding of another transcription factor E2F, proposing that

the stimulus specific HSF activation might be regulated at the level of complex transcription factor interplay.

In mammals, HSF1 is expressed in a variety of tissue types, and though not directly required for the survival of the organism, *Hsf1*$^{-/-}$ mice have severe developmental defects (Xiao *et al*., 1999). One of the most visible alterations detected in *Hsf1*$^{-/-}$ mice is the reduced body and organ size of both female and male mice. Intriguingly, by showing that interaction between HSF1 and JNK (c-Jun N-terminal kinase) is required for the maintenance of mTORC1-governed translation, it was proposed that the reduced body size is at least partially caused by hampered translation in *Hsf1*$^{-/-}$ mice (Su *et al*., 2016). In addition to reduced size, the *Hsf1*$^{-/-}$ female mice suffer from multiple reproductive shortcomings, such as placental insufficiency and infertility, which appear to be caused by deficiencies in zygotic divisions (Xiao *et al*., 1999; Christians *et al*., 2000). These deficiencies are not solely caused by alterations in the basal Hsp expression (Xiao *et al*., 1999). Although Hsp90α does support the meiotic maturation of the oocytes (Metchat *et al*., 2009), HSF1 also regulates genes associated with synaptonemal complex, cohesin, and spindle assembly checkpoint (Le Masson *et al*., 2011). The *Hsf1*$^{-/-}$ male mice produce less sperm and show disorganized layering of the seminiferous epithelium (Salmand *et al*., 2008), which surprisingly does not result in infertility (Izu *et al*., 2004). However, HSF1 is required for brain development as both female and male mice exhibit enlarged ventricles, neurodegeneration, demyelination, astrogliosis and accumulation of ubiquitinated proteins in the brain (Santos & Saraiva, 2004; Homma *et al*., 2007). Interestingly, the developing mouse brains are considered normal until E18.5 (Xiao *et al*., 1999), which indicates that the abnormalities likely occur postnatally and that HSF1 activity is regulated in a developmental stage-specific manner.

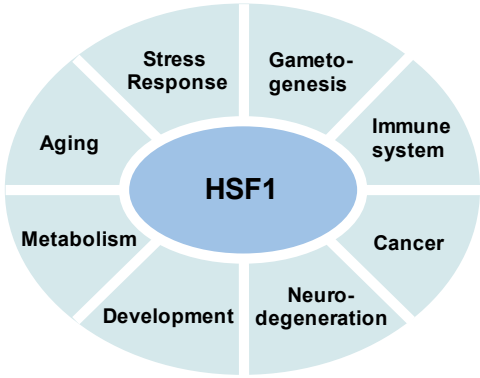

Figure 10. Versatile functions of human HSF1. The role of HSF1 is not restricted to its essential function in the cellular stress response, but it is important also in other physiological and pathophysiological contexts. Involvement of HSF1 in cancer and neurodegeneration will be discussed in more detail in section *3 HSFs in pathologies*.

HSF1 provides protection also at the organismal level through modulation of immune responses. Accordingly, *Hsf1*$^{-/-}$ mice exhibit increased mortality during pathological challenges, partly caused by exaggerated production of pro-inflammatory cytokine TNF-α (Xiao *et al*., 1999). Moreover, the mice have significantly lower constitutive expression of cytokines and chemokines, as well as impaired induction of serum immunoglobulins, such as IgG_{2a}, all important for proper immune responses (Inouye *et al*., 2004). Organismal protection is also demonstrated by HSF-mediated life-span regulation, which was originally demonstrated in *C. elegans* (Garigan *et al*., 2002). By using a RNAi-screen to search for factors affecting the animal life-span, CeHSF was identified as one of the proteins important

for preserving youthfulness (Garigan *et al*., 2002). The following year CeHSF was reported to promote life together with a forkhead family transcription factor Daf-16 (Hsu *et al*., 2003), previously well-recognized for enhancing nematode longevity (Kenyon *et al*., 1993). Upon activation of the insulin/IGF-1-like receptor Daf-2, Daf-16 is phosphorylated and retained in the cytoplasm, which prevents it from activating its anti-aging target genes (Hsu *et al*., 2003). However, when the signaling is impaired for example by RNAi-mediated downregulation of the transmembrane Daf-2 receptor, Daf-16 localizes to the nucleus together with HSF1 and initiates a transcriptional program required for life-span extension (Hsu *et al*., 2003; Morley & Morimoto, 2004). The cooperation between Daf-16 and HSF1 is essential, as downregulation of either of the factors suppresses the longevity phenotype (Hsu *et al*., 2003; Morley & Morimoto, 2004). Interestingly, these transcription factors share a selection of their target genes, such as hsp-12 and hsp-16 encoding for small Hsps. Though downregulation of these target genes reduce longevity, the effect is minor compared to downregulation of CeHSF (Morley & Morimoto, 2004), suggesting that CeHSF promotes life-span extension through a more complex transcriptional network. Moreover, inactivation of IGF-1R, a mouse homologue of Daf-2, results in 26% increase in the animal life-span, indicating that the insulin/IGF-signaling is crucial also in the regulation of mammalian aging (Holzenberger *et al*., 2003). However, it remains to be examined, whether the mammalian HSF1 plays any role in promoting longevity in an analogous manner.

2.4 Heat shock factor 2, HSF2

Already at the time of its discovery in 1991, HSF2 was found profoundly different to its close homologue HSF1. For example, albeit both factors were able to bind consensus HSE sequences, they exhibited distinct binding preferences and differential activation in response to stress. Unlike HSF preferring arrays of four to five nGAAn consensus pentamers, HSF2 favors HSEs with two to three repeats, which results in negligible cooperativity between adjacent trimers upon DNA-binding (Kroeger *et al*., 1993; Kroeger & Morimoto, 1994). Moreover, in contrast to prominent heat-inducible activation of HSF1, HSF2 is DNA-binding competent already in non-stressed conditions (Sarge *et al*., 1991) and shows constitutive DNA-binding in mouse embryonic carcinoma cells (Murphy *et al*., 1994) and during mouse embryogenesis (Mezger *et al*., 1994a; Mezger *et al.*, 1994b; Rallu *et al*., 1997). More importantly, constitutive HSF2 binding appears to be uncoupled from the classical activation of *Hsp* gene expression, suggesting that the factor is not necessarily important for the acute heat stress responses (Mezger *et al.*, 1994a; Mezger *et al.*, 1994b; Rallu *et al.*, 1997 Eriksson *et al.*, 2000). Consistently, *in vitro* transcription assays have revealed that HSF1 is a more potent transcriptional activator of Hsp70 promoter than HSF2 (Kroeger *et al*., 1993). Prominent HSF2 binding can, however, be detected during hemin-induced differentiation of K526 cells (Sistonen *et al*., 1992) or proteasome inhibition (Mathew *et al*., 1998; Rossi *et al*., 2014), suggesting that the factor is perhaps more important during differentiation and development or in other types of proteotoxic stresses.

Unlike monomeric HSF1, HSF2 exists primarily as cytosolic dimers in non-stressed cells, whereas upon appropriate stimuli, it trimerizes and associates with DNA (Sistonen *et al*., 1994; Mathew *et al*., 1998). Interestingly, in contrast to HSF1, only exogenously expressed hHSF2 is constitutively trimerized in yeast and can functionally complement the viability defects detected in *S. cerevisiae* lacking the HSF gene (Liu *et al*., 1997). HSF1 on the other hand, can complement the defects only when HR-C is disrupted, indicating that the trimerization is an essential regulatory step in HSF activation. Though the regulatory mechanism behind the trimerization difference is not fully elucidated, it is at least partially

dictated by the distinct structural features of HSF1 and HSF2. In HSF1, the DBD resident wing-domain accounts for the temperature-dependent trimerization of HSF1 and defines the target gene selectivity (Ahn *et al*., 2001). Intriguingly, when transposed to HSF2, the wing-domain confers analogous stress-responsiveness and DNA-binding specificity, suggesting that the structural features provide an essential stress-sensing mechanism differentially regulating HSF1 and HSF2 activation in specific cellular contexts.

2.4.1 Regulation of HSF2 activity

In contrast to the prominent PTMs that regulate HSF1 transcriptional activity, HSF2 activity is mainly regulated on the level of its expression. HSF2 is a very short-lived protein, with half-life of approximately 60 min (Mathew *et al*., 1998) and shows diverse tissue type, cell type, stress stimulus, and cell cycle phase dependent expression patterns (Sarge *et al*., 1994; Fiorenza *et al*., 1995; Mathew *et al*., 1998, Elsing *et al*., 2014). High HSF2 expression in cells and tissues correlates with prominent HSF2 DNA-binding activity (Mathew *et al*., 1998; Sarge *et al*., 1994), suggesting that the activity is indeed mainly dependent on HSF2 expression levels. In further support of the prevalent view, overexpression of HSF2 has been shown to activate Hsp70 reporter constructs and endogenous genes (Sarge *et al*., 1993; Sandqvist *et al*., 2009). Moreover, treatments such as hemin or proteasome inhibition lead to concomitant upregulation of HSF2 protein expression and DNA-binding activity. (Sistonen *et al*., 1994; Mathew *et al*., 1998, Rossi *et al*., 2014).

As the levels of HSF2 are important in the regulation of its activity, cells have developed efficient mechanisms to modulate HSF2 expression according to their specific requirements. For example, when exposed to elevated temperatures, HSF2 is ubiquitinated by the E3-ubiquitin ligase anaphase-promoting complex/cyclosome (APC/C), which leads to HSF2 degradation in the proteasome (Ahlskog *et al*., 2010). During heat shock, APC/C co-factor cdc20 and the proteasome subunit α2 localize to the Hsp70 promoter, which engages HSF2 in proteasome-mediated degradation and allows for prominent Hsp70 expression during stress. Although the importance of this type of regulation remains to be fully elucidated, it seems that HSF2 degradation is required to direct selective target gene expression during stress in freely cycling (Ahlskog *et al*., 2010) and in mitotic (Elsing *et al*., 2014) cells. The stress-specific ubiquitination sites on HSF2 have not been experimentally validated, but five lysine residues have been identified as ubiquitination target sites in unbiased mass spectrometric screens (Kim *et al*., 2011; Wagner *et al*., 2011) (Figure 11), though their regulatory functions remain to examined. In testis, HSF2 expression is regulated at the mRNA level by a specific microRNA, miR-18, which belongs to the Oncomir-1 or miR-17~92 cluster of microRNAs (Björk *et al*., 2010). During spermatogenesis, miR-18 is expressed in a cell-type specific manner and downregulates HSF2 expression through binding to the HSF2 mRNA 3′-UTR (Björk *et al*., 2010). Specific downregulation of HSF2 results in decreased expression of testis-specific HSF2 target genes, Speer4 and Ssty (Åkerfelt *et al*., 2008), suggesting that by targeting HSF2, miR-18 orchestrates stage-specific gene expression during spermatogenesis. Altogether, these regulatory mechanisms provide elegant examples on how HSF2 expression can be modulated during stress and in development.

Similarly to HSF1, HSF2 is also subjected to post-translational modification by sumoylation, which appears to be important in controlling HSF2 DNA-binding ability. Sumoylation of HSF2 was initially proposed by Goodson and co-workers, who detected interaction between HSF2 and Ubc9 in yeast-two-hybrid studies and subsequently identified lysine 82 as the main

sumoylation site on HSF2 (Goodson *et al*., 2001). K82 resides in the HSF2 DBD (Figure 11) and was first reported to be required for efficient HSF2 DNA-binding both *in vitro* and *in vivo* in *Xenopus laevis* (Goodson *et al*., 2001; Hilgarth *et al*., 2004). Later on, however, our laboratory re-examined HSF2 sumoylation and contradicted the previous studies by showing that sumoylation of K82 in fact inhibits HSF2 DNA-binding ability (Anckar *et al*., 2006). The results have been later confirmed by other laboratories (Tateishi *et al*., 2009; Feng *et al*., 2016) and HSF2 sumoylation is generally considered as an inhibitory modification. When contemplating the putative explanations for the observed differences, it is important to note that all the previous studies regarding HSF2 sumoylation have been conducted either with exogenous overexpression constructs (of HSF2 and SUMO1 or SUMO2/3) or in diverse *in vitro* assays, which greatly underestimates the delicate biology of a living cell. Indeed, when Hendriks and co-workers utilized mass spectrometry based identification methods to study protein sumoylation in cells, they identified 20 novel sumoylation sites on HSF2 DBD and HR-A/B (Figure 11), suggesting that the post-translational regulation of HSF2 by SUMO is perhaps much more complex than originally expected (Hendriks *et al*., 2016). Thus, it is possible that the previously observed contradictions are in fact caused by secondary-site sumoylation events, which were not fully detectable with the methods used at that time. Moreover, as K82 resides within the HSF2 wing-domain, suggested to be involved in the regulation of HSF-specific protein interaction network and target-gene selectivity (Jaeger *et al*., 2016), over-expression of the regulatory components can result in biased observations regarding the functional role of HSF2 sumoylation. Thereby, it would be of great interest to re-examine HSF2 sumoylation in biologically more relevant contexts.

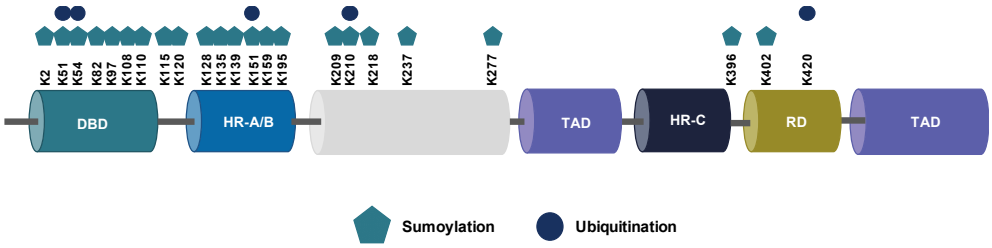

Figure 11. Schematic overview of identified post-translational modification sites on human HSF2. HSF2 is sumoylated on multiple lysine (K) residues, mainly located in the DNA-binding domain (DBD) and oligomerization domain (HR-A/B). Ubiquitination of five lysine residues has been reported. Additional domain abbreviations: C-terminal heptad repeat domain (HR-C), regulatory domain (RD) and transactivation domain (TAD).

2.4.2 HSF2 as a developmental regulator

The significance of HSF2 in biological processes other than stress responses (Figure 12), was initially proposed by demonstrating that HSF2 activity increases during hemin-induced differentiation of K562 erythroleukemia cells (Sistonen *et al*., 1992). Subsequently, spatiotemporally variable HSF2 expression was detected in multiple mouse tissues in both developing and adult animals, which further supported the emerging view of HSF2 as a developmentally important transcription factor. However, it was not until the generation of *Hsf2*$^{-/-}$ mouse by three independent research laboratories, which finally provided compelling evidence for the involvement of HSF2 in developmental processes (Kallio *et al*., 2002; McMillan *et al*., 2002; Wang *et al*., 2003). In none of these studies, disruption of HSF2 gene led to robust morphological abnormalities of the animals. However, two laboratories

observed nearly identical phenotypic defects, mainly related to corticogenesis and gametogenesis in both genders (Kallio *et al*., 2002; Wang *et al*., 2003). The lack of similar observations in the third mouse model is likely due to the selection of different genetic approaches to disrupt the HSF2 gene as well as utilization of distinct mouse strains as the knock-out background.

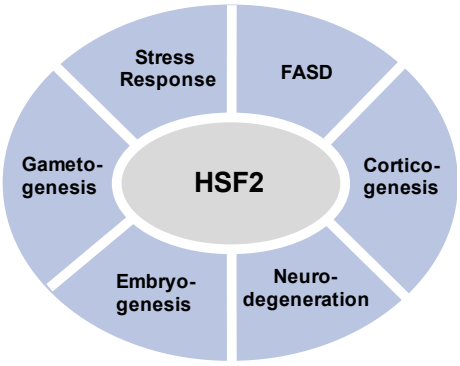

Figure 12. Versatile functions of human HSF2. In contrast to the prominent role of HSF1 in stress, HSF2 is mainly related to differentiation and developmental processes. Moreover, HSF2 is important in specific human pathologies, such as fetal alcohol spectrum disorder (FASD) and neurodegeneration (will be discussed in more detail in *3 HSFs in pathologies).*

During mouse embryogenesis, HSF2 expression is mainly detected in the central nervous system and more specifically in the ventricular layer containing the highly proliferating neural precursors (Rallu *et al*., 1997; Kallio *et al*., 2002, Wang *et al*., 2003). In adult *Hsf2*$^{-/-}$ mice both lateral and third ventricles are significantly dilated (Kallio *et al*., 2002, Wang *et al*., 2003), indicating that developmentally regulated expression of HSF2 might be important in the tissue homeostasis during ventricle formation. Interestingly, when analyzing the expression of specific Hsps during embryogenesis, Wang and co-workers observed equivalent Hsp levels in both *Hsf2*$^{-/-}$ and wild-type mice, suggesting that the neuronal defects are not caused by misregulated Hsp expression. In addition to ventricular defects, the *Hsf2*$^{-/-}$ mice display neuronal mispositioning at the cortical layers of the brain (Chang *et al*., 2006). Neuronal migration is architecturally supported by two different cell types, namely the radial glia cells and the Cajal-Retzius cells, which both are less abundant in the *Hsf2*$^{-/-}$ mouse brain. Moreover, in search of the molecular mechanism behind the defective neuronal migration, p35 was identified as a novel HSF2 target gene in brain (Cheng *et al*., 2006). p35 is a well-established activator of Cdk5 (cyclin-dependent kinase 5), which is one of the essential factors required for cortical lamination. In *Hsf2*$^{-/-}$ mice, p35 expression and subsequently also Cdk5 activity is reduced, suggesting that the observed migratory defects are at least partly mediated by lack of HSF2 target gene expression (Chang *et al*., 2006). Interestingly, HSF2 binding to the *p35* promoter is inhibited during fetal alcohol exposure, which results in neuronal migratory defects similar to those detected in the children diagnosed with fetal alcohol spectrum disorder (FASD) (El Fatimy *et al*., 2014). Altogether, these studies imply that HSF2 is an essential regulator of neuronal migration, which could potentially function as a target molecule in the development of novel strategies to prevent fetal brain damage.

Among all the studied tissue types, HSF2 is most prominently expressed in adult testes (Sarge *et al*., 1994, Fiorenza *et al*., 1995) and particularly in the pachytene spermatocytes as well as round spermatids (Sarge *et al*., 1994; Alastalo *et al*., 1998; Björk *et al*., 2010). Consequently, the *Hsf2*$^{-/-}$ mice display multiple testicular defects, including reduced testis size, morphologically abnormal seminiferous tubules, and increased apoptosis of the spermatocytes, which does not affect male fertility (Kallio *et al*., 2002, Wang *et al*., 2003). *Hsf2*$^{-/-}$ female mice are also challenged with reproductive insufficiencies, as they show

increased embryonic lethality and reduced fertility caused by defective ovulation, meiotic problems, and diverse hormonal abnormalities (Kallio *et al*., 2002). During spermatogenesis, HSF2 occupies the male-specific region of the mouse Y chromosome long arm (MSYq) and is required for the transcriptional induction of the MSYq-resident multicopy genes, such as *Ssty2*, *Sly*, and *Slx* (Åkerfelt *et al*., 2008). Altered expression of multicopy genes leads to conformational abnormalities in the sperm heads, suggesting that HSF2 is involved in the quality control of male gametes. Interestingly, the combined disruption of both HSF1 and HSF2 exacerbates the *Hsf2*$^{-/-}$ phenotype and the double knock-out mice are infertile due to stalled spermatogenesis at the pachytene stage (Wang *et al*., 2004). By examining HSF1 and HSF2 DNA-binding sites in mouse testes with ChIP-seq (Chromatin immunoprecipitation combined with sequencing), Korfanty and co-workers revealed that these factors indeed share target binding sites during spermatogenesis (Korfanty *et al*., 2014), suggesting that HSF1 and HSF2 cooperatively regulate gene expression during sperm development.

2.4.3 HSF2 regulates gene expression together with HSF1

Due to the significant homology of the oligomerization domains, the existence of HSF1-HSF2 heterotrimers was hypothesized already in the first study that identified the factors in human cells (Schuetz *et al*., 1991). Quite a few years later, the direct interaction between HSF1 and HSF2 was revealed by co-immunoprecipitation analyses and was shown to be dependent on the oligomerization domain of HSFs (Alastalo *et al*., 2003; He *et al*., 2003). HSF1 and HSF2 were also demonstrated to co-localize on the *clusterin* gene promoter containing only one HSE with three nGAAn repeats, which strongly suggested that the factors bind DNA as a heterotrimer (Loison *et al*., 2006). Finally, by utilizing the nuclear stress bodies (nSBs, discussed more below) as a model system to study HSF1-HSF2 interplay, it was revealed that the factors can indeed bind DNA in a heterotrimeric complex (Sandqvist *et al*., 2009). Experimental manipulation of the relative abundance of HSF1 and HSF2 affected the transcriptional properties of the trimers, indicating that the composition of heterotrimers might serve as an integrator of cellular signals and assist in the fine-tuning of HSF-mediated transcription (Sandqvist *et al*., 2009).

The interplay between HSF1 and HSF2 has been shown to modulate stress-induced activation of transcription. Although both HSF1 and HSF2 occupy nearly identical *loci* during heat shock (Vihervaara *et al*., 2013), HSF2 localization to the *hsp* promoters is dependent on HSF1 and affects transcription gene-specifically (He *et al*., 2003; Östling *et al*., 2007). For example, lack of HSF2 in mouse embryonic fibroblasts results in impaired heat-induced expression of *hsp70* and *hsp25*, whereas *hsp40* and *hsp110* are induced in the absence of HSF2 (Östling *et al*., 2007). However, in stressed and mitotic K562 cells, HSF2 interferes with HSF1 binding and subsequent transcriptional activation of the *hsp70* promoter (Elsing *et al*., 2014), suggesting that the effect of HSF1-HSF2 heterotrimers depends on cell type and growth conditions.

In addition to regulating the transcription of protein coding genes, HSF1 and HSF2 jointly participate in the regulation of the non-coding *SatIII*-transcripts, produced from the 9q12 *locus* during proteotoxic stresses (Sandqvist *et al*., 2009). Accumulation of HSF1 and HSF2 to the *locus* produces characteristic nuclear structures, termed nuclear stress bodies (nSBs), which only appear in the primate cells (Jolly *et al*., 2002). The structures were initially identified already in the early 90s, by demonstrating that HSF1 stress-inducibly localize to specific nuclear granules (Sheldon & Kingston, 1993). Later on, the sites were reported to be transcriptionally active and separate to *hsp70* and *hsp90 loci* (Jolly *et al*., 1997), which led

to the identificaiton of specific repetitive satellite III transcripts (Jolly *et al*., 2002). Upon exposure to elevated temperatures, HSF2 localization to nSBs is HSF1-dependent and lack of HSF2 results in increased heat-inducible expression of the *SatIII*-transcripts (Sandqvist *et al*., 2009). However, experimental upregulation of HSF2, mimicking the high HSF2 expression during development, activates *SatIII loci* in the absence of stress, indicating that the biological consequence of HSF1-HSF2 interplay is dependent on the relative amount of the factors. Intriguingly, a recent study by Goenka and co-workes demonstrated that the *SatIII*-transcripts are involved in stress-dependent transcriptional repression and required for cellullar protection against damaging conditions (Goenka *et al*., 2016). Since the mechanisms behind the heat-induced transcriptional repression are not elucidated, it is tempting to speculate that through non-coding transcription sites, HSFs could participate in the comprehensive transcriptional reprogramming of the cell during stress. Moreover, as the importance of heterotrimers in developmental processes is currently unknown, it remains to be established whether HSF2 could function as the main driver of gene expression in specific developmental processes.

3 HSFs IN PATHOLOGIES

The obvious importance of HSFs as regulators of protein homeostasis and cell survival has evoked large interest towards their putative roles in severe human pathologies such as cancer and neurodegenerative diseases. Intriguingly, these two diseases (or rather umbrella terms for highly variable groups of diseases) represent two extreme opposites of misregulated tissue homeostasis. Cancers are usually described by unrestrained cell growth leading to neoplasms (for more details see *4 Cancer)*, whereas neurodegenerative diseases are always associated with aberrant cell death and tissue damage. Such diversity undeniably poses specific challenges to the cellular survival mechanisms, which from the organismal point of view are too active in cancer and malfunctional during degeneration.

In this section of 3 *HSFs in pathologies* I first review the current knowledge regarding the roles of HSFs in human cancers as that is the main topic further examined in the third study of this thesis (III). After that, I will describe the contributions of HSFs in protein aggregation diseases and neurodegeneration, since the final study of this thesis (IV) might have implications relevant to such conditions.

3.1 HSF1 in cancer

Upregulation of Hsps is an evolutionarily conserved stress protective mechanism that guards the cellular proteome in times of damage. In cancer, the proteotoxic damage is caused by both intrinsic and extrinsic factors, as the tumor cells characteristically harbor multiple genetic mutations and maintain growth in adverse environments. Due to the unstable proteome, abnormally high expression of Hsps has been reported in a wide range of human cancers and is currently considered as a general feature of neoplastic cells (Whitsell and Lindquist, 2000). However, it was not obvious in the beginning whether the increased expression of Hsps is the cause or the consequence of malignant transformation. In the 1990s, the question was examined by multiple individual research laboratories, who provided several lines of evidence supporting the causality between Hsps and tumorigenicity. For example, ectopically induced overproduction of Hsp70 or Hsp27 was reported sufficient to promote metastatic potential of cancer cells in host organisms (Jäättelä, 1995; Garrido *et al*., 1998). Moreover, it was demonstrated that abnormally high Hsp70 expression can induce malignant transformation and metastatic T-cell lymphoma in transgenic mice (Seo *et al*., 1996). Furthermore, overproduction of Hsp70 in immortalized Rat-1 cells resulted in features characteristic to oncogenic transformation, including loss of contact inhibition, growth on soft agar, and tumor formation in nude mice (Volloch & Sherman, 1999). Altogether, these results indicated that overproduction of molecular chaperones is sufficient to drive tumorigenesis and sustain cancer progression.

Though Hsp levels in normal cells are maintained by various transcription factors (Stephanou & Latchman, 2011), in stress HSF1 is the primary factor responsible for inducible expression of Hsps. Thus, after recognizing the importance of Hsps in cancer formation, it became simply impossible to ignore the putative role of HSF1 in the malignant transformation. The relationship between HSF1 and cancer begun to emerge through few important studies published in the early 2000s. First, analysis of the differentially expressed genes in metastatic variants of prostate adenocarcinoma cell lines, PC-3 and PC-3M, revealed that HSF1 is strongly upregulated in the more metastatic PC-3M line (Hoang *et al*., 2000). A few years later, expression of dominant negative HSF1 (DN-HSF1) was reported to inhibit aneuploidy in the same PC-3 model system (Wang *et al*., 2004), suggesting that HSF1 could be involved

in regulating genomic instability. HSF1 was also related to cancer-specific growth phenotypes by demonstrating that heregulin β1 (HRGβ1), an oncogenic growth factor, HSF1-dependently promotes anchorage-independent growth and inhibits apoptosis in breast and cervical carcinoma cell lines (Khaleque *et al*., 2005). HSF1 was also related to the migratory behavior of cells by showing that MEFs lacking HSF1 display reduced migration upon EGF stimulation (O´Callaghan-Sunol & Sherman, 2006).

It was not until the two groundbreaking studies from the Mivechi and Lindquist laboratories in 2007, when the door for HSF1-related cancer research finally became wide open. By utilizing *Hsf1*$^{-/-}$*p53*$^{-/-}$ (Min *et al*., 2007) and *Hsf1*$^{-/-}$*p53*$^{R172H/+}$ (Dai *et al*., 2007) mouse models both laboratories demonstrated that loss of HSF1 protects mice from spontaneous tumor formation often associated with p53-deficiency (Min *et al*., 2007; Dai *et al*., 2007). The p53 R172H mutation (missense mutation affecting the p53 DBD structure) had been found from many types of spontaneously arising human tumors (Olive *et al*., 2004), which already then highlighted the putative clinical relevance of the findings by Dai and co-workers. Moreover, the protective effect of HSF1-deficieny was not limited to p53-mutant background, as *Hsf1*$^{-/-}$ mice exposed to mutagenic dimethylbenzanthracene (DMBA) and tumor promoting 12-O-tetradecanoylphorbol-13-acetate (TPA), were also shown less susceptible to skin carcinogenesis induced by the chemicals (Dai *et al*., 2007). Chemical-induced tumors are often associated with activating *H-RAS* mutations and interestingly all sampled tumors from WT and *Hsf1*$^{-/-}$ mice harbored *H-RAS* mutations. These results indicated that HSF1 provides protection independently of the oncogenic driver identity. The observation was further supported by studies in cell culture systems, which demonstrated that *Hsf1*$^{-/-}$ MEFs are more resistant against spontaneous transformation when transfected with either *H-RAS* or *PDGF-B* proto-oncogenes (Dai *et al*., 2007). To study the importance of HSF1 in maintaining the transformed phenotype, Dai and co-workers depleted HSF1 with shRNA from a wide selection of well-established human cancer cell lines and measured the cell viability after four days in culture. Intriguingly, nearly every malignant cell type exhibited reduced cell viability at the experimental endpoint altogether proposing that HSF1 is not only required for the cancer initiation but supports the transformed cell phenotype independently of the underlying genetic defects. Thereby, it appeared that HSF1, conventionally considered as a survival factor, had an opposite role in cancer; as a strong supporter of the malignant phenotype, it challenges the survival of the organism.

Due to the original findings, the ability to maintain high Hsp expression was generally considered as one of the key mechanisms by which HSF1 enhances cancer cell survival. The concept is often presented as “chaperone addiction” model, underlining the requirement of chaperones as buffers against genetic instabilities. However in 2012, Mendillo and co-workers demonstrated that the transcriptional program that HSF1 regulates in malignant cells, is in fact surprisingly diverse and extends far beyond the molecular chaperones (Mendillo *et al*., 2012). By comparing the HSF1 binding sites in heat shocked and transformed mammary epithelial cells, the study revealed that HSF1 is not only activated during transformation but occupies promoters distinct from the heat shock response. The genes regulated by HSF1 in malignant cells were shown to be related to translation, energy metabolism, adhesion, immune processes, and cell cycle, suggesting that HSF1 comprehensively impacts the physiology of the cancer cell (Figure 13). Similar gene occupancy was detected in a variety of established human cancer cell lines and human patient material, indicating that HSF1 has a specific transcriptional cancer signature (Mendillo *et al*., 2012). Importantly, this HSF1 cancer signature was associated with reduced survival in breast, colon and lung cancer patients (Mendillo *et al*., 2012).

Reflecting the previous results, the molecular mechanisms by which HSF1 promotes cancer progression have been reported to be versatile and follow closely the proposed repertoire of cancer hallmarks (Hanahan & Weinberg, 2011) (Figure 13). One of the key features of cancer cells is, for example, their ability to avoid apoptotic signals and HSF1 participates in this process by inducing the expression of Hsp70 co-chaperone BAG3 (Bcl-2-associated athanogene domain 3), which stabilizes the anti-apoptotic Bcl-X_L, Mcl-1, and Bcl-2 proteins (Jacobs & Marnett, 2009). Moreover, HSF1 transcriptionally represses the expression XAF1 (XIAP associated factor 1), a factor antagonizing the cytoprotective XIAP (X-linked inhibitor of apoptosis) (Wang *et al*., 2006). Another feature of cancer cells is the acquisition of metastatic behavior, which allows the cells to escape from the original tumor site and colonize distant tissues (see *4 Cancer* for more details). Intriguingly, an unbiased screen identified HSF1 as one of only six essential drivers of melanoma invasion (Scott *et al*., 2011). HSF1 has also been shown to reduce adherence and enhance metastatic spread of other cell types *in vivo* in mice (Khaleque *et al*., 2005; Toma-Jonik *et al*., 2015). The role of HSF1 in angiogenesis was demonstrated by studying the slowly growing tumors in *Hsf1*$^{-/-}$ mice and HSF1-depleted MCF7 xenografts that were both found to exhibit reduced angiogenesis associated with suppressed HIF-1 40 expression (Gabai *et al*., 2012). Moreover, HSF1 appears to be important in reprogramming the cellular energy metabolism that is required to fuel the uncontrolled proliferation characteristic to neoplastic growth. By reshaping the transcriptional network upon alterations in translational flux, HSF1 functions as an integrator of translational and transcriptional inputs and supports the malignant anabolic state (Santagata *et al*., 2013). HSF1 has been also shown to control chemoresistance in MCF7 and RKO cells by enhancing cytoprotective autophagy *via* upregulation of ATG7 and p62/SQSTM1 expression in response to carboplatin and Hsp90 inhibitors, respectively (Desai *et al*., 2013; Samarasinghe *et al*., 2014). Importantly, HSF1 is an essential downstream effector protein of mitogenic signaling pathways and thereby is required for e.g. HER2-related tumorigenesis (Xi *et* al., 2012).

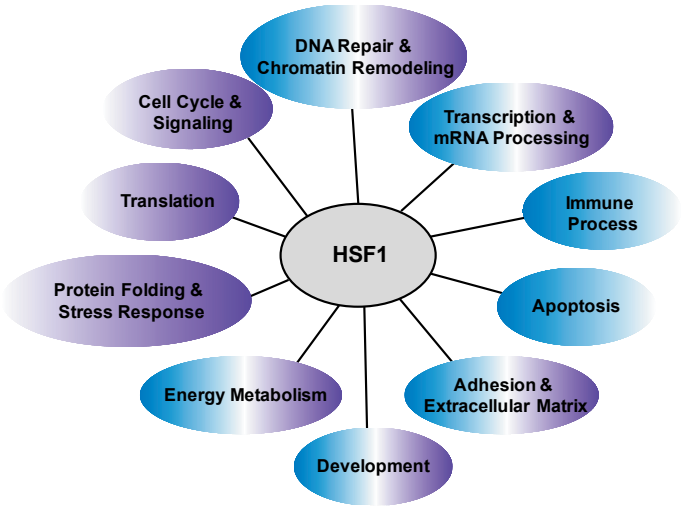

Figure 13. In cancer HSF1 drives a transcriptional program distinct to the classical heat shock response. In addition to regulating the chaperone expression, HSF1 binds to several other genes and affects their expression both negatively (blue) and positively (purple). Modified from (Mendillo *et al*., 2012).

Malignant cancer cells in a tumor are often surrounded by extensive tumor stroma, which consists of other cell types, such as cancer associated fibroblasts (CAFs), endothelial cells, and immune cells, as well as extracellular matrix components. Signaling between the cancer cells and the stroma is continuous and currently considered as one of the cancer hallmarks required to support tumor progression and metastasis (Hanahan &Weinberg, 2011). Intriguingly, by examining mouse xenografts composed of breast cancer cells and either HSF1 WT or HSF1-null MEFs, it was demonstrated that HSF1 impacts the signaling between stromal and cancerous cells and lack of HSF1 in CAFs significantly reduces tumor growth (Scherz-Shouval *et al*., 2014). Comparison of the transcriptional programs induced in cancers cells co-cultured with either WT or HSF1-null MEFs revealed that HSF1-expressing CAFs promote expression of genes associated with extracellular matrix (ECM) organization and adhesion. In contrast, lack of HSF1 in CAFs resulted in upregulation of genes encoding pro-inflammatory cytokines (Scherz-Shouval *et al*., 2014). Observed gene expression alterations were shown to be mediated by CAF-secreted TGF-β (transforming growth factor β) and SDF1 (stromal-derived factor 1), of which SDF1 was identified as a direct HSF1 target gene. Altogether the results indicated that the tumor progression is affected by HSF1-regulated chemokine signaling in the heterogeneous tumor cell population. Importantly, the study established that HSF1 is upregulated in stromal cells in a selection of human cancers (lung, skin, esophageal, colon, gastric, prostate cancers) and that high HSF1 expression in stroma is associated with poor outcome in breast and lung cancer patients (Scherz-Shouval *et al*., 2014).

What then causes HSF1 activation in cancer? The pathways regulating HSF1 activation are not fully elucidated even in the contexts of heat shock response and thereby our knowledge regarding cancer-specific HSF1 regulation is also limited. However, several oncogenic pathways have been reported to alter HSF1 activity, which suggests that the regulation can be mediated through multiple signaling mechanisms. For example, activation of HER2 receptor tyrosine kinase (also known as Erbb2 and Neu) has been shown to increase HSF1 trimer formation and protein expression in MCF7 and MDA-MB-435 breast cancer cell variants (Zhao *et al*., 2009), whereas activation of RAS/RAF/MAPK signaling pathway results in increased HSF1 activity in transformed human embryonic kidney cells (HEK293T) (Tang *et al*., 2015). Also HSF1 protein levels seem to be deregulated in cancer. In a panel of melanoma cell lines (WC00125, WM3862, WM39) ubiquitin ligase FBXW7-deficiency resulted in stabilization of nuclear HSF1 and promoted activation of invasion-supportive HSF1 transcriptional program (Kourtis *et al*., 2015). Furthermore, in the same study, negative correlation between FBXW7 and HSF1 nuclear localization was observed in metastatic melanoma patient samples, indicating that regulation of HSF1 protein stability might be essential in HSF1-mediated cancer progression (Kourtis *et al*., 2015). These results are further supported by the observations of Santagata and co-workers, which conclusively showed that high expression and nuclear localization of HSF1 in a cohort of breast cancer samples derived from the Nurses´ Health Study (NHS) correlate with poor patient outcome (Santagata *et al*., 2011).

Lastly, though the role of HSF1 in various human malignancies begins to be well-acknowledged and the mechanisms leading to HSF1 activation are emerging, the role of other HSFs in these processes is completely unknown. For example, the expression of HSF2 in tumor samples has not been examined in any of the above-mentioned studies and the importance of HSF2 for cancer progression is thus far unknown. Therefore, the role of HSF2 in cancer is examined in the third study of this thesis (III) and further discussed in the *Results and Discussion*.

3.2 HSFs in neurodegenerative diseases

Neurodegenerative diseases, such as Alzheimer´s disease (AD), Parkinson´s disease (PD), and amyotrophic lateral sclerosis (ALS), are all characterized by toxic intracellular protein aggregates that disturb neuronal homeostasis and eventually lead to cell death. Toxic protein aggregates are caused by misfolding of distinct diseases-specific proteins, such as Amyloid-β or Tau in the case of AD or α-synuclein in the case of PD, that associate with important cellular effectors and thereby disturb normal cell physiological functions (Sweeney *et al*., 2017). In healthy cells, misfolding-induced proteomic imbalances are counteracted by molecular chaperones as well as the ubiquitin-proteasome and autophagy systems, which regulate protein folding and degradation in order to maintain proteostasis. In neurodegenerative diseases, many of these regulatory pathways are misregulated and the diseases typically exhibit overall decline in the protein quality control pathways, which results in proteostasis collapse and subsequent propagation of the disease.

Several lines of evidence have suggested that enhancing the protein folding capacity *via* increased expression of molecular chaperones can prevent proteostasis collapse and thus have neuroprotective effects. For example, overexpression of Hsp70 and other chaperones, has been shown to suppress aggregation related toxicity in mouse models of PD (Klucken *et al*., 2004), spinocerebellar ataxia type 1 (Cummings *et al*., 2001), amyotrophic lateral sclerosis (Nagy *et al*., 2016), and AD (Magrane *et al*., 2004). Moreover, similar results have been observed in various *D. melanogaster*, *C. elegans, S. cerevisiae,* and *in vitro* models for neurodegeneration and amyloid formation (Warrick *et al*., 1999; Muchowski *et al*., 2000; Auluck *et al*., 2002; Takeuchi *et al*., 2002; Nollen *et al*., 2004; Evans *et al*., 2006), altogether indicating that chaperones are important regulators of toxic aggregates. Due to the importance of HSF1 as a regulator of chaperone gene expression, its role in neurodegenerative diseases has rightfully been hypothesized. One of the first unbiased evidence regarding HSF1 as an important suppressor of aggregation was provided by a genome-wide RNAi screen in a polyQ expressing *C. elegans*, which identified CeHSF1 as one of the factors required to suppress age-related protein aggregation (Nollen *et al*., 2004). In *Hsf1*$^{-/-}$ MEFs, lack of HSF1 was shown to result in increased accumulation of ubiquitinated proteins and polyQ-aggregates and similar observations were made in *Hsf1*$^{-/-}$ mice (Fujimoto *et al*., 2005; Homma *et al*., 2007). The importance of HSF1 in specific diseases was confirmed more recently by showing that ectopically expressed HSF1 suppresses the disease phenotypes of Alzheimer´s disease as well as spinal and bulbar muscular atrophy (Jiang *et al*., 2013; Kondo *et al*., 2013). Consequently, HSF1 activation *via e.g.* Hsp90 inhibitors or small HSF1 activating molecules (HSF1-A1) has been documented to have positive effects in several cell culture models of neurodegenerative diseases as well as in fly and mouse models of PD, SCA (spinocerebellar ataxia), and Huntington´s disease (Bose & Cho, 2017).

Albeit proposing for prominent therapeutic potential, increasing HSF1 activation with small molecules might not be sufficient to rescue the degenerative disease phenotypes. As evidenced by two recent studies, HSF1 expression levels are specifically downregulated during aggregate accumulation, suggesting that also HSF1 stability is affected in neurodegenerative diseases. By examining α-synuclein aggregation in neuroblastoma cells, Kim and co-workers observed aberrant NEDD4-mediated HSF1 ubiquitination, which led to HSF1 degradation *via* the ubiquitin proteasome system (Kim *et al*., 2016). In both mouse and human tissues of α-synucleopathy, NEDD4 and HSF1 presented reciprocal expression levels, indicating that the whole molecular pathway is misregulated during aggregation (Kim *et al*., 2016). Analogously, FBXW7-mediated HSF1 ubiquitination and degradation was

shown to result in decreased HSF1 expression in Huntington´s disease (Gomez-Pastor *et al*., 2017). These results propose that regulation of HSF1 stability is of key importance during disease progression, to which the current literature presents no therapeutic interventions.

It is not also exactly clear, how HSF1 promotes cell survival during neurodegeneration. Active HSF1 has been shown to suppress polyQ-aggregation more efficiently than any combination of Hsps (Fujimoto *et al*., 2005), suggesting that also other HSF1-regulated genes are essential in preventing the disease phenotype. In accordance, Pat-10 was identified as a novel HSF1-regulated gene in aging *C. elegans*, which by maintaining cytoskeletal integrity was shown to promote stress resistance (Baird *et al*., 2014). Moreover, HSF1 has been demonstrated to protect neurons independently of its trimerization (and thereby also DNA-binding) capacity, proposing that the factor might have a more extensive role in these diseases than originally anticipated. Intriguingly, also HSF2 is implicated as an essential factor required to maintain proteostasis against polyQ-aggregation (Shinkawa *et al*., 2011). By crossing the *Hsf2*$^{-/-}$ mouse with R6/2 Huntington´s disease mouse, it was revealed that lack of HSF2 accelerates the accumulation of polyQ-aggregates in mouse brain and shortens the organismal lifespan (Shinkawa *et al*., 2011). The observations were partially attributed to the cellular inability to regulate αB-crystallin (Hspb5) expression together with HSF1, indicating that the interplay between HSF1 and HSF2 can also function as an important determinant of neuronal degeneration.

4 CANCER

The tissues in multicellular organisms are composed of organized collections of cells, which through constant cell-to-cell signaling regulate the life of their neighbors to maintain proper tissue homeostasis. When a cell acquires a capability to escape these surveillance systems it may become a cancer cell and function as a foundation for a neoplasm, an abnormal growth of new tissue. Such "cell-of-origin", the last normal ancestor of a neoplasm, can arise in whatever adult tissue, which together with an endless amount of mutation combinations results in a highly variable disease spectrum called cancer.

Despite the heterogeneity of the disease, a great majority of human cancers are characterized by specific features, which in the current literature are referred to as the hallmarks of cancer (Hanahan & Weinberg, 2011). One of the most fundamental hallmarks is the ability to sustain constant proliferative signaling that can be acquired in a variety of ways. For example, the cancer cell might overexpress growth factor receptors, contain activating mutations in the downstream effector proteins, or even be able to produce growth factor ligands itself. Simultaneously, however, the cell must be able to avoid proliferation inhibiting messages, such as those conveyed by TP53 (tumor protein 53; p53) or Rb (retinoblastoma-associated protein, pRb). Yet another hallmark of cancer is the ability to escape signals that induce programmed cell death, apoptosis. Initiation of apoptosis is controlled by balancing the amount of pro- and anti-apoptotic factors, such as those belonging to the Bcl2 (B-cell lymphoma 2) -family of proteins (Adams & Cory, 2007). In approximately half of the human cancers the anti-apoptotic Bcl-2 is found overexpressed (Weinberg, 2014). In healthy tissues, cells with proliferative capacity eventually undergo replicative senescence and cease into a state where they no longer can divide. This phenomenon is caused by cell division dependent shortening of chromosomal ends, called telomeres, and predisposes the cell to DNA damage and subsequent activation of apoptosis. In cancer, replicative senescence is circumvented by expression of a specific DNA-polymerase, telomerase, that has the ability to add repetitive telomeric sequences to telomeres and thereby induce cell immortalization (Hanahan & Weinberg, 2011).

Similarly to all the cells in a human body, cancer cells require nutrients and oxygen to fuel their growth and proliferation. In fact, inadequate oxygen supply is one of the key factors restricting tumor growth and thus bypassing such limiting barrier, by *e.g.* inducing angiogenesis, is essential for cancer progression (Weinberg, 2014). In this respect, the tumors greatly resemble sites of wound healing, where new vasculature is rapidly formed to support the regeneration of damaged tissue. Perhaps the most clinically relevant of the cancer hallmarks is, however, the cancer cells´ ability to activate invasive migration to the surrounding tissues. Acquisition of invasive potential allows cells to intravasate into blood or lymphatic microvessels that can transport the cells to distant sites in the human body. At these sites, cells may extravasate the microvessels, colonize the tissue, and eventually form large macrometastases. This multi-step process is known as invasion-metastasis cascade (Talmadge & Fidler, 2010) and approximately 90% of cancer related deaths are caused by metastases, evolved *via* this process (Lambert *et al*., 2017).

Considering the diversity of the above-mentioned cancer hallmarks, it is hardly a surprise that the acquisition of novel features is regulated by a vast variety of molecular pathways. Albeit the literature describing these pathways is impressive, in this section of 4 *Cancer* I will only describe the key molecular events regulating cancer cell invasion, since that will be in the focus of the third (III) study of this thesis.

4.1 Molecular effectors of cancer cell invasion

Since nearly 80% of diagnosed human cancer are of epithelial origin, that is carcinomas, the molecular details regulating carcinoma cell invasion are perhaps the most extensively studied (Lambert *et al*., 2017). In many cases, the initial steps of carcinoma cell dissemination are thought involve activation of a key program, called EMT (epithelial-to-mesenchymal transition). EMT is a normal developmental program particularly important during gastrulation, when epiblasts detach from embryonal ectoderm and migrate to the center of the embryo to form the mesoderm (Weinberg, 2014). During EMT, cancer cells, as well as the normal epiblasts, lose epithelial cell polarity, cytokeratin expression, and E-cadherin-mediated adherens junctions, and gain mesenchymal cell properties, such as fibroblast-like shape, motility, and expression of vimentin. Many of these alterations are coordinated by a selection of so called EMT-transcription factors (*e.g.* Snail, Slug, Twist, and Zeb1), which orchestrate the transition from epithelial to mesenchymal gene expression programs and eventually allow the initiation of cell migration (Lambert *et al*., 2017; Lamouille *et al*., 2014).

During EMT cells reorganize their cortical actin cytoskeleton (typical for epithelial cells) and gain front-to-rear polarity to enable directional actin driven protrusions, such as lamellipodia and filopodia (Pollard, 2003; Mayor & Etienne-Manneville, 2016). This actin cytoskeleton reorganization is mainly regulated by the Rho-family of small GTPases, which through their downstream effector proteins modulate actin polymerization (Parri & Chiarugi, 2010). The Rho-family of small GTPases consist of Rho, Rac and Cdc42 subfamilies, of which RhoA, Rac1 and Cdc42 are the most well-studied. The general consensus is that the role of RhoA is to regulate actin stress fibers and focal adhesions, whereas Rac1 and Cdc42 mainly function in formation of lamellipodia and filopodia, respectively (Lamouille *et* al., 2014). The activity of all Rho-GTPases is tightly regulated by various guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs) that all modulate GTP-binding status of the GTPases. The GEFs, GAPs, and GDIs act downstream of many growth factor receptors and transmembrane adhesion molecules and thus can integrate multiple cellular signals into distinct Rho-GTPase activity profiles (Parri & Chiarugi, 2010).

The process of cellular movement can be divided into distinct general steps. In the beginning, the cell generates finger-like actin-rich structures, called filopodia, which are used to probe the cellular surroundings for appropriate growth environment (Jacquemet *et al.*, 2015). This step is regulated mainly by Rac, which is compartmentalized to the leading edge through lipid anchoring (Parri & Chiarugi, 2010). Once activated, Rac interacts with WAVE-complex (member of the Wiskott-Aldrich Syndrome Protein family) and subsequently activates actin nucleation through Arp2/3 protein complex (actin-related proteins 2/3). This creates a membrane protrusion that pushes the cell forward (Parri & Chiarugi, 2010; Ridley, 2015). Simultaneously, the cell forms cell-ECM interactions by nascent integrin attachments, which through formation of focal adhesions help to organize actin bundles. It has been suggested that Cdc42, acting also in the front of the cell, would assist in the formation of these focal adhesions (Weinberg, 2014). In addition, Cdc42 has been shown to be important in regulating the cell polarity through Par polarity complex and microtubules (Ridley, 2015). An essential element of the cell movement is also the production of ECM-degrading MMPs (matrix metalloproteinases) in the front of the cell, which enable the cell movement through the ECM. Degradation of the ECM also releases matrix-bound growth factors creating a positive feedback loop for the migratory behavior of the cell. The MMPs are produced by the tumor-surrounding stromal cells as well as the cancer cells, and there are indications that Rac activity would be required for adequate MMP production (Parri & Chiarugi, 2010). The final

requirement for the cell to move is to create contractile forces in the lagging edge of cell, which help to push the cell body forward and assist in the detachment of cell-ECM contacts at the rear of the cell. This step is mainly regulated by Rho and one of its effector proteins ROCK (Rho-associated serine/threonine kinase) that phosphorylates myosin light chains when activated. Moreover, Rho promotes the formation of actin bundles, which together with the phosphorylated myosin creates contractile forces.

In addition to their key roles in cell movement, the functions of Rho-family of GTPases in cancer extends far beyond the regulation of invasion and involves cellular processes such as gene expression, proliferation, and cell survival (Sahai & Marshall, 2002). Interestingly, the cancer-specific abnormalities in Rho GTPase function appear to be mainly at the level of deregulated expression and activity, rather than at the level of genetic mutations (Sahai & Marshall, 2002; Parri & Chiarugi, 2010). Considering the multi-functional nature, Rho GTPases are intriguing targets for molecular cancer therapy and therefore detailed knowledge about the factors affecting their expression and activity is warranted. A summary of observed abnormalities in Rho GTPases in human cancer can be found in the table below (Table 2).

Table 2. Aberrant regulation of Rho-family of GTPases in cancer. Table modified from (Sahai & Marshall, 2002; Parri & Chiarugi, 2010).

Rho-GTPase subtype	Abnormality	Cancer type
RhoA	High expression, High activity	Liver, skin, colon, ovarian, bladder, gastric, esophageal squamous cell (SCC), testicular, breast
RhoB	High expression, Low expression	Breast (high exp.), lung (low exp.)
RhoC	High expression, High activity	Melanoma metastases, breast, squamous cell (SCC), pancreas, liver, ovarian, head and neck, prostate, non-small cell lung (NSCLC), gastric cancer
RhoH	Rearragement, Mutations	Non-Hodgkin´s lymphoma, multiple myeloma
Rac1	High expression, High activity	Testicular, colon, breast, squamous cell (SCC)
Rac1B	Alternative splicing	Colon, breast
Rac2	High expression	Head, neck squamous-cell carcinoma (SCC)
Rac3	High expression, High activity	Breast (high exp.), lung (low exp.)

AIMS OF THE STUDY

At the beginning of this work, the methods that were used to identify novel sumoylation substrates mainly relied on protein sequence analysis for the existence of sumoylation consensus motifs. Already then, it was well recognized that SUMO can be conjugated to non-consensus lysine residues, which at that time were laborious to identify. To alleviate the experimental challenge, one of the aims in this thesis was to develop a novel biochemical method to study sumoylated proteins in cells. Simultaneously, HSF1 had been identified as a target for various PTMs, though their regulatory roles were still partially uncharacterized. Phosphorylation was considered as the most prominent HSF1 modification, and multiple laboratories were interested in the stress-inducible phosphorylation of HSF1, which was mainly considered as a prerequisite for HSF1 activation. However, most of the studies had focused on single site phosphorylation events, whereas we wanted to conclusively examine how hyperphosphorylation affects HSF1 transactivation capacity.

More recently, I have investigated the significance of HSFs, and more specifically HSF2, in human malignancies. Prior to this work, only HSF1 had been established as a key transcription factor in cancer progression, whereas the role of HSF2 was completely unknown. For this reason, this thesis work aimed at investigating if HSF2 has a role in cancer progression. Moreover, although the prominent role of HSF1 in cell survival upon proteotoxic stress is well-acknowledged, the importance of HSF2 in such conditions has remained enigmatic. Since the identification of HSF2, the factor has been considered as a dispensable bystander of the stress response with only modulatory role in the regulation of heat shock gene expression. However, HSF2 is responsive to distinct types of proteostasis alterations, such as those created by proteasome inhibition, suggesting that the factor might be important in particular types of stress. Thereby, the final aim of this thesis has been to examine the role of HSF2 in prolonged proteotoxic stress conditions.

The specific aims of this thesis work were to:

- Develop novel methods to identify sumoylated proteins in cells.
- Study the role of phosphorylation in the regulation of HSF1.
- Examine if HSF2 has a function in prostate cancer progression.
- Define the role of HSF2 during proteotoxic stress.

EXPERIMENTAL PROCEDURES

The experimental procedures used in this thesis are provided here in a table format. More detailed information about specific methods can be found from the original publications (I-III). In addition, materials and methods used in the unpublished work included in thesis are described in the end of this section.

Table 3. Methods used in this thesis work.

Methods	Original work
3D cell culture	III, IV
Cation exhange chromatography	I
Cell aggregation assay	IV
Cell culture	I, II, III, IV
Cell viability assay through Calcein	IV
Chicken chorioallantoic membrane assay	III
Chromatin immunoprecipitation (ChIP)	II
CRISPR-Cas9	IV
Cys-tag enrichment	I
Electrophoretic mobility shift assay	II
HE-staining	III
His-purification	I
Image analysis	I, II, III, IV
Immunofluorescence	II, III, IV
Immunohistochemistry	III
In vitro sumoylation assay	Results and Discussion
LC-MS/MS	I
Luciferace assay	II
LysC-digestion	I
Microscopy	II, III, IV
mRNA Microarray	III
Protein dephosphorylation	II
Protein turnover analysis	II
Quantitative RT-PCR	II, III
RNA extraction	II, III
RNA interference	III
RNA sequencing	IV
SDS-PAGE and immunoblotting	I, II, III, IV
Site-directed mutagenesis	II
Statistical analysis	II, III, IV
Transient transfections	I, II, III

Table 4. Cell lines used in this thesis work.

Cell lines	Type	Original work
DU145	Human prostate carcinoma	III
EP156T	Human prostate epithelial	III
HeLa	Human cervical carcinoma	I,II
LNCaP	Human prostate adenocarcinoma	III
Mef WT	Mouse embryonic fibroblast	II
Mef *Hsf1* -/-	Mouse embryonic fibroblast	II
PC3	Human prostate carcinoma	III
PC3-M	Human prostate carcinoma	III
PrEC	Human primary prostate epithelial	III
U2OS WT	Human osteosarcoma	IV
U2OS 2KO	Human osteosarcoma	IV

Table 5. Plasmids used in this thesis work. These expression plasmids were used to express the indicated wild-type and mutant proteins or reporter constructs.

Plasmids	Original work
GAL4-luciferase	II
Gal4-VP16	II
Gal4-VP16-HSF1 WT	II
Gal4-VP16-HSF1 ΔPRD	II
HA-CKAP2L K198R	I
HA-CKAP2L WT	I
His-SUMO1 C52S H75K V87K V90C Q92C T95R	I
His-SUMO1 C52S V87K V90C Q92C T95R	I
His-SUMO1 E89K T95R	I
His-SUMO1 E93C T95R	I
His-SUMO1 I88K T95R	I
His-SUMO1 Q92C T95R	I
His-SUMO1 T95R	I
His-SUMO1 V87K T95R	I
His-SUMO1 V87K V90C Q92C T95R	I
His-SUMO1 V90C T95R	I
His-SUMO1 WT	I
hsp70-prom-luciferase	II
Myc-His-HSF1 WT	II
Myc-His-HSF1ΔPRD	II
Myc-PARP-1 H53R	I
shRNA HSF1	III
shRNA HSF2	III
shRNA res HSF2	III
shRNA scr	III
β-galacatosidase	II

Table 6. Antibodies used in this thesis work. Abbreviations ChIP, Chromatin immunoprecipitation; IHC, Immunohistochemistry; IP, Immunoprecipitation; WB, Western blotting.

Antigen	Cat#/Clone	Company	Application	Original work
ARHGAP1	HPA004689	Sigma-Aldrich	WB	III
Actin	AC-40	Sigma-Aldrich	WB	II
Cdc42	ACD03	Cytoskeleton	WB	III
CDH1	GTX125890	Gentex	WB	III
CDH2	EPR1792Y	Millpore	WB	III
FK2	PW8810	Biomol International Inc.	WB	Results and Discussion
GAPDH	ab9485	Abcam	WB	IV
GNA13	HPA010087	Sigma-Aldrich	WB	III
HA	PRB-101C	Covance	IP, WB	I
Hsc70	SPA-815	Stressgen	WB	I, III, IV
HSF1	SPA-901, AB-4	Stressgen, Thermo Scientific	WB	II, III
HSF2	HPA031455/3E2	Sigma-Aldrich, Millipore	WB	III, IV
MMP14	MAB3328	Sigma-Aldrich	IHC	III
Myc	M4439	Sigma-Aldrich	WB, ChIP	I, II
PARP-1	sc-8007	Santa Cruz Biotechnology	WB	IV
Snail2	9585	Cell Signaling	WB	III
SUMO1	ab58424	Abcam	WB	I
Tubulin	T8328	Sigma-Aldrich	WB	III, IV
Vimentin	5741	Cell Signaling	WB	III
VP-16	V4388	Sigma-Aldrich	WB	II

1 In vitro sumoylation

For *in vitro* sumoylation reactions, 10 µM of monoubiquitin (WT or mutants) or 1 µM of tetraubiquitin chains were incubated in the presence of 0.14 µM Aos1/Uba2 (E1), 0.05 µM Ubc9 (E2), 0.05 µM BP2ΔFG/IR1+M/Pias1 (E3), 10 µM SUMO1 or SUMO2, and 10 µM ATP in final volume of 20 µl of sumoylation assay buffer (SAB) [transport buffer (200 mM HEPES, 110 mM KOAc, 20 mM $Mg(OAc)_2$, 10 mM EGTA, pH 7.3) containing 10 µg/ml ovalbumin, 2 % Tween20, 1 µg/ml LP (leupeptin/pepstatin), AP (aprotinin), and 1 mM DTT]. Samples were incubated at 30˚C for 1 h after which the reaction was stopped by boiling the samples in Laemmli-sample buffer before subjecting them in to SDS-PAGE and immunoblot analysis. The purified ubiquitin proteins were purchased from Boston Biochem [monoubiquitin (WT, K0, K6 only, K11 only, K27 only, K29 only, K33 only, K48 only, K63 only), K48-linked tetraubiquitin, K63-linked tetraubiquitin] or from Enzo Life Sciences (linear tetraubiquitin). The E1 (Aos1/Uba2), E2 (Ubc9), and E3 enzymes (BP2ΔFG, IR1+M, Pias1), as well as purified SUMO1, SUMO2 and HA-tagged monoubiquitin were a kind gift from Dr. Andrea Pichler (Max-Planck Institute of Immunobiology and Epigenetics, Freiburg, Germany).

Since sumoylation is an ATP-dependent reaction, a sample without ATP was prepared to be used as a negative control. Additionally, a sample without ubiquitin was used to distinguish the unspecific signal from ubiquitin. To study the possibility of E3-independent sumoylation of ubiquitin, two samples with high (12.5 µM) or low (0.05 µM) concentration of Ubc9 without any E3-ligase, were also prepared. As sumoylation of ubiquitin has not been experimentally examined in the literature before, the ubiquitin specific SUMO-ligases have not been identified. Therefore, I used three different E3-ligases, with specific properties to

study the ubiquitin sumoylation. The BP2ΔFG is a C-terminal fragment of SUMO E3-ligase RanBP2 and contains the SUMO E3-ligase activity that has been shown to be sufficient in promoting sumoylation *in vitro* (Pichler *et al*., 2002). BP2ΔFG further consists of two central IR-regions, IR1 and IR2, that are separated by a short M-region. Together with the short M-region, IR1 (IR1+M) has SUMO-ligase activity. IR1+M lacks completely substrate specificity and for that was included in these experiments. As the third ligase, I used full-length Pias1, which belongs to the SP-RING like family of SUMO E3-ligases that are the most studied SUMO-ligases (Pichler *et al*., 2017). For the experiments studying the SUMO-isoform specificity of ubiquitin sumoylation, similar samples were prepared where only SUMO2 was used instead of SUMO1. To study the sumoylation target lysine on ubiquitin, samples were prepared as the IR1+M sample (see table 7), but with different ubiquitin constructs (HA-Ub, WT, K0, K6 only, K11 only, K27 only, K29 only, K33 only, K48 only, K63 only) as substrates (Table 7).

Table 7. *In vitro* sumoylation sample preparation. In the *in vitro* reactions, 13 different ubiquitin constructs were used as sumoylation substrates. For all the monoubiquitins used (HA-Ub, WT, K0, K6 only, K11 only, K27 only, K29 only, K33 only, K48 only, K63 only) the concentration in the reaction was 10 μM. Tetraubiquitin chains (K48-linked chains, K63-linked chains, linear chains) were used in concentration of 1 μM.

	no ATP	no ubiquitin	High Ubc9	Low Ubc9	BP2ΔFG	IR1+M	Pias1
Aos1/Uba2	0,14 μM	0,14 μM	0,14 μM	0,14 μM	0,14 μM	0,14 μM	0,14 μM
Ubc9	0.05 μM	0.05 μM	12.5 μM	0.05 μM	0.05 μM	0.05 μM	0.05 μM
BP2ΔFG	-	-	-	-	0.05 μM	-	-
IR1+M	0.05 μM	-	-	-	-	0.05 μM	-
Pias1	-	-	-	-	-	-	0.05 μM
SUMO1/SUMO2	10 μM	10 μM	10 μM	10 μM	10 μM	10 μM	10 μM
Ubiq.	1 μM/10 μM	-	1 μM/10 μM	1 μM/10 μM	1 μM/10 μM	1 μM/10 μM	1 μM/10 μM
ATP	-	10 μM	10 μM	10 μM	10 μM	10 μM	10 μM

RESULTS AND DISCUSSION

1 IDENTIFICATION OF *IN VIVO* SUMOYLATION SITES (I)

Sumoylation is a post-translational modification that targets thousands of substrate proteins in eukaryotic cells (Hendriks *et al*., 2014). Through the versatility of its targets, sumoylation has been connected to many essential processes, such as DNA repair, chromatin remodeling, and cellular stress responses. Moreover, the importance of sumoylation has been demonstrated in severe human diseases like cancer and neurodegeneration, further highlighting the fundamental role of sumoylation in organismal health.

The obvious importance of sumoylation has raised the interest in identifying the sumoylated proteome in mammalian cells, which is often challenged by the biological nature of the modification. For example, low abundance of specific sumoylation substrates complicates the detection as classical methods, such as immunoblotting, are not sensitive enough. In addition, the dynamic nature of the modification caused by highly active SUMO-isopeptidases that maintain their activity in a wide range of buffer conditions results in fast SUMO deconjugation and thereby hampers the purification of sumoylated substrates. The amino acid sequence of the protein itself also poses a challenge. Nearly all current methods that are used to study protein sumoylation, rely on different proteomics strategies for the final peptide identification. An essential part of that kind of procedure is the cleavage of the purified proteins with specific enzymes, such as LysC and trypsin. However, cleavage of endogenous SUMO with such enzymes produces a large signature tag on the target lysine residues, resulting in such a complex pattern in tandem MS/MS spectra that the target peptides are nearly impossible to identify. To that end, we developed a novel method that is based on an engineered human SUMO1 and can be used to alleviate the above-mentioned issues.

1.1 Generation of engineered SUMO1

To facilitate the *in vivo* identification of sumoylated substrates, we generated an engineered human SUMO1 protein with various single site mutations in its C-terminus. The aim of these mutations was to improve both purification of sumoylated peptides and subsequent target site identification. We introduced two cysteine residues at positions V90 and Q92 to enable cysteine-targeted affinity purification, a method previously used to examine human plasma proteome (Liu *et al*., 2006). In this method, peptides are enriched with specific affinity beads (Thiopropyl-Sepharose 6B in this study) that bind cysteine residues covalently. During the development of our method, SUMO1 peptides containing an internal cysteine at position 52 (C52) were also found to bind to the affinity beads. For that, C52S mutation was included in our mutant SUMO1 protein. H75K and V78K were introduced to enhance peptide digestion with the endoproteinase LysC, which cleaves proteins specifically on the C-terminal side of lysine residues and thus produces shorter peptides required for MS/MS analysis. In most of the proteomic approaches, the peptides are further digested with trypsin, a serine protease, that cleaves proteins at both lysine and arginine residues. Neither LysC nor trypsin can cleave at lysine residues that are post-translationally modified with ubiquitin or ubiquitin-like proteins, which results in branched peptides with intact lysine cleavage site and covalent attachment of the specific signature tag (Knuesel *et al.,* 2005). In the case of ubiquitin, the signature tag consists of two glycine residues (GG), since the 76-amino acid ubiquitin contains arginine at position 74 (R74) and thus is cleaved by trypsin at that site. In the case of 97-amino acid human SUMO1 the first tryptic digestion site (counting from the C-

terminus) is lysine 78 (K78), whereas in 93-amino acid human SUMO2/3 the first site is arginine 61 (R61). This results in signature tags of 19 or 32 amino acids for SUMO1 and SUMO2/3, respectively, and thereby challenges the target identification by generating complex mass spectra. Diglycine (GG) increases the mass of the peptide with only ~114 Da, which can be more readily identified with MS/MS. To facilitate the identification of sumoylated peptides, we substituted the threonine at position 95 in SUMO1 with arginine (T95R). Such mutation creates an additional tryptic cleavage site to SUMO1 C-terminus (-RGG) and allows generation of a short diglycine tag at the target lysine after tryptic digestion. Importantly, introduction of the T95R mutation has been previously shown to be dispensable for SUMO1 conjugation efficiency (Knuesel *et al*., 2005). Finally, the construct was His_6-tagged to enable metal affinity purification of the sumoylated proteins from cell lysates. Metal affinity purification was specifically used, as it tolerates strong denaturing conditions (8 M urea in this study) that are required to disrupt all the non-covalent protein interactions. Altogether, these mutations resulted in His-SUMO-1C protein with C52S, H75K, V87K, V90C, Q92C and T95R substitutions.

The functionality of the generated His-SUMO-1C was examined by co-expressing the mutant protein in HeLa cells together with the well-established SUMO-target PARP-1 (Martin *et al*., 2009) and analyzing PARP-1 sumoylation with immunoblotting. In this study, we used a DNA-binding mutant PARP-1 (Myc-PARP-1 H53R) since it can be sumoylated already at normal growth conditions (unpublished observations by Henri Blomster). As evidenced by Figure 1C (in I), similar higher molecular weight bands representing the sumoylated PARP-1 were detected in the samples expressing His-SUMO-1C and SUMO1 WT, indicating that His-SUMO-1C can be efficiently conjugated to target substrates.

1.2 Identification of sumoylation sites in cells

For the *in vivo* identification of novel sumoylation substrates, His-SUMO-1C was transiently expressed in HeLa cells. Cells were lysed and the sumoylated proteins were purified with metal affinity purification in denaturing conditions. Purified proteins were digested with LysC and the sumoylated peptides were enriched from the sample with cysteine-targeted affinity purification. Covalently bound peptides were treated with trypsin, which released the diglycine modified peptides (Figure 1B in I). These peptides were then subjected to LC-MS/MS (liquid chromatography coupled with tandem mass spectrometry) with QSTAR Pulsar and LTQ-Orbitrap XL mass spectrometers. The received data were analyzed with Mascot against the SwissProt Human database. As a result, 14 sumoylated peptides originating from 12 proteins were identified (Table 1 in I). For example, we identified two sites (K8 and K524) from RanGAP1 and K11 from SUMO2/3, which all have previously been identified as sumoylation target sites (Mahajan *et al*., 1998: Tatham *et al*., 2001; Matic *et al*., 2008), thereby confirming the functionality of our method. To further validate the method, we examined sumoylation of one of the identified non-consensus targets CKAP2L (cytoskeleton-associated protein 2), which had not been previously shown to be sumoylated. We mutated the identified lysine (K198) on CKAP2L to arginine, after which the mutant and WT CKAP2L proteins together with SUMO1 were expressed in HeLa cells. As shown in Figure 2C (in I), mutation of K198 abolished specific bands from the CKAP2L K198R migration pattern, demonstrating that CKAP2L is indeed sumoylated on lysine 198. These results confirmed that the His-SUMO-1C-based method can be used as a novel tool to study sumoylation in cells and is applicable for the identification of both consensus and non-consensus sumoylation substrates.

Albeit presenting a functional tool to identify sumoylated proteins in cells, our method significantly suffers from low enrichment efficiency and low number of identified sumoylation substrates. This could be caused by utilization of the mutant SUMO1 protein, which might not be conjugated to all substrates as efficiently as the endogenous WT SUMO1. In our study, the functionality of the protein was only examined with exogenously expressed mutant PARP-1 (Myc-PARP-1 H53R), which can serve as an excellent model system to study sumoylation, but represents only one mutant example of a sumoylation target. Therefore, it is possible that the construct is conjugated only to a specific subset of target proteins and thus results in an overall reduced number of sumoylated proteins in the sample. In addition, as this method is based on transiently expressed His-SUMO-1C, it is also possible that the construct is not sufficiently expressed in HeLa cells. As we did not monitor the expression levels of our construct, we cannot exclude the possibility that the low peptide yield is due to low protein expression.

Yet another explanation for the low number of identified proteins could be provided by our experimental procedure, in which sumoylated proteins are first His-purified and digested with LysC, after which the peptides are enriched with cysteine-targeted affinity purification and trypsinized when bound to Thiopropyl-Sepharose 6B beads. In contrast, the most efficient currently used method uses both fewer experimental steps and inverse order of the key purifications (Hendriks *et al*., 2014). This method is called the K0-method and it is based on the His-K0-SUMO2 Q87R mutant protein, where all the internal lysine residues in SUMO2 as well as the glutamine at position 87 are substituted with arginines (8K>R/Q87R) (Hendriks *et al*., 2014; Hendriks & Vertegaal, 2016b). Similarly to our approach, this protein can be expressed from exogenous plasmid construct, though a cell line stably expressing the His-K0-SUMO2 Q87R has been developed (Hendriks *et al*., 2014). After induction of the His-K0-SUMO2 Q87R expression, sumoylated substrates are purified and peptides analyzed with MS/MS. They key difference between our and the K0-method is the eight lysine mutations (8K>R), which render SUMO2 resistant to the first digestion with LysC. This allows digestion of the unpurified whole cell lysate and results in a sample, where all other proteins are digested into peptides except for SUMO2 itself. Digestion of the sample into peptides considerably reduces the sample complexity, whereas the intact SUMO2 enables utilization of efficient metal affinity purification for the sample enrichment. Subsequently, sumoylated peptides are trypsinized when bound to the metal affinity resin, which due to the C-terminal Q87R mutation results in branched peptides more compatible with MS/MS (Hendriks *et al*., 2014). Thus, in comparison to our method, both the number and order of experimental steps in the K0-method is different, which likely results in more efficient enrichment of sumoylated peptides. Consequently, the K0-method has been successfully used to identify thousands of novel sumoylation substrates (Hendriks *et al*., 2014) (Table 8) and is currently considered the best method to examine sumoylated proteins in cells (Hendriks & Vertegaal, 2016).

One obvious drawback of the above-mentioned methods is that they both rely on mutant SUMO proteins, which might be differentially regulated than the endogenous SUMOs. For example, mutating all SUMO2 lysine residues to arginines in His-K0-SUMO2 Q87R inhibits SUMO2 chain formation, which has been shown to promote cell survival during cell stress and thus appears essential for the correct function of the protein (Saitoh & Hinchey, 2000; Blomster *et al*., 2009; Golebiowski *et al*., 2009). To surpass the usage of mutant constructs, a method called Protease-Reliant Identification of SUMO Modification (PRISM) has been developed, which can be used to identify sumoylated proteins modified with wild-type SUMO (Hendriks *et al*., 2015). In this method, sulfosuccinimidyl acetate (SNHSA) is used

to block all free lysine residues in a SUMO-purified sample, after which SUMO is enzymatically removed with SENPs. This exposes free lysine residues that are not blocked by SNHSA or SUMO, thereby allowing for identification of the lysines with MS/MS. The PRISM method still relies on epitope-tagged SUMO (His10-SUMO) for the sample purification and thus is not suitable for studying endogenous sumoylation sites. For that, Lumpkin and co-workers have recently developed a method called WaLP, which utilizes wild-type α-lytic protease (WaLP) instead of trypsin to enzymatically digest the protein sample into peptides. WaLP can specifically cleave after threonine amino acid residues and as all human SUMO isoforms contain threonines directly adjacent to the C-terminal di-glycine motif (Figure 4), the WaLP-cleavage generates a GG signature tag on target substrates. Resulting peptides can subsequently be identified with methods previously developed to identify ubiquitinated substrates with GG-tag (Lumpkin *et al.*, 2017). Albeit not as efficient as the K0-method, WaLP is the only currently available method that can be used to identify endogenous sumoylation sites and is therefore the only method that is applicable for the examination of sumoylation on tissue and patient material (Table 8).

Table 8. Various methods used for identification of sumoylation sites. IP, Immunoprecipitation; PRISM, Protease-Reliant Identification of SUMO Modification; WalP, wild-type α-lytic protease. Both NQTGG- and Diglycine-immunoprecipitations are methods where monoclonal antibodies produced against the tryptic remnant are used to immnopurify sumoylated peptides from cell samples (Impens *et al*., 2014; Lamoliatte *et al*., 2014; Tammsalu *et al*., 2015). Table modified from (Hendriks & Vertegaal, 2016a).

			SUMO properties		SUMO sites identified		
Publication	Year	Method	Mutation	Tag	Control	Stress	Total
Tammsalu *et al*.,	2014	Diglycine IPa	T90K	His6	-	1002	1002
Impens *et al*.,	2014	Diglycine IPa	T90R	His6	295	-	295
Lamoliatte *et al*.,	2014	NQTGG-IP	Q87R/Q88N	His6	123	936	954
Hendriks *et al*.,	2015	PRISM	-	His10	392	556	751
Hendriks *et al*.,	2014	K0	8>R/Q87R	His10	1069	4317	4361
Xiao *et al*.,	2015	K0	8>R/Q87R	His10	1043	-	1043
Hendriks *et al*.,	2015	K0	8>R/Q87R	His10	755	-	755
Lumpkin *et al*.,	2017	WaLP	-	-	1209	-	1209

In our study, we found that the majority of the identified peptides were modified on non-consensus sumoylation sites (*i.e.* sites with no SCM). Previously, SIM-directed sumoylation has been proposed as one of the mechanisms directing non-consensus sumoylation (Zhu *et al*., 2008), and six of the 11 identified non-consensus peptides did contain SIM in close proximity to the target lysine (data not shown). The remaining five peptides, however, suggested that other determinants can also direct substrate sumoylation. By manually inspecting the MS/MS spectrum, we detected that many of the peptides were also modified with other PTMs, such as phosphorylation and acetylation (Table 1 in I). Based on this finding we hypothesized that perhaps other PTMs are important in directing a subset of sumoylation evens. Intriguingly, a recent study examined the peptide profile of 6,747 human proteins and defined phosphorylation as one of the key mechanisms directing substrate sumoylation (Hendriks *et al*., 2017). These results indicate that our method could also be valid to examine complex PTM interactions directing sumoylation events in cells.

Taken together, we developed a mass-spectrometric method to identify sumoylated proteins in cells. The method is based on mutant human SUMO1 protein, which through its specific features facilitates both the purification of sumoylated peptides and the identification of sumoylation target sites. The results presented in this study validate the utilization of our

method in sumoylation research and provide a novel tool to examine both consensus and non-consensus sumoylation events in cells.

1.3 Sumoylation of ubiquitin

In addition to the 14 peptides published in Study I of this thesis, one of the putative non-consensus sites identified was lysine 48 on ubiquitin. At that time, this lysine was only known to serve as a conjugation site for another ubiquitin moiety, but not for SUMO. To examine whether ubiquitin can function as a sumoylation target substrate, I performed *in vitro* sumoylation assays and used four different purified ubiquitin constructs as sumoylation target substrates; monoubiquitin, K48-linked tetraubiquitin, K63-linked tetraubiquitin and linear tetraubiquitin. Ubiquitin constructs were mixed with the sumoylation machinery supplemented with ATP and the samples were incubated at 30°C for 1 h. The samples were resolved in SDS-PAGE and subjected to immunoblotting with ubiquitin-specific antibodies. Surprisingly, these experiments revealed that ubiquitin can indeed be sumoylated *in vitro* in the presence of substrate unspecific IR1+M ligase fragment of RanBP2 (Figure 14). Importantly, not only monoubiquitin, but also all the tested tetraubiquitin chains were sumoylated *in vitro*, suggesting that SUMO can target ubiquitin in different conformations (Figure 14). Furthermore, two higher molecular weight bands were detected with linear tetraubiquitin chains (Figure 14). This might be caused by modification of two individual lysine residues on the linear ubiquitin chain and result in differential separation on the SDS-PAGE.

Due to the finding that higher molecular weight ubiquitin signal was only detected in the sample with unspecific IR1+M fragment, and not with BP2ΔFG fragment or full-length Pias1 (Figure 14), it is likely that neither RanBP2 nor Pias1 is the cellular SUMO-ligase targeting ubiquitin. Sumoylation has been reported to occur independently of an E3-ligase *in vitro* in high Ubc9 concentrations (Bernier-Villamor *et al*., 2002). However, I did not detect a higher molecular weight signal in the sample with high (12.5 µM) Ubc9 concentration (Figure 14). This is likely due to the observation that E3-independent *in vitro* sumoylation often requires the target lysine to be embedded in the SCM (Pichler *et al*., 2017), whereas none of the lysine residues of ubiquitin resides within the consensus motif. These results provide for the first time direct evidence for ubiquitin sumoylation and demonstrated that not only monoubiquitin but also ubiquitin chains of variable linkage types can be sumoylated *in vitro*.

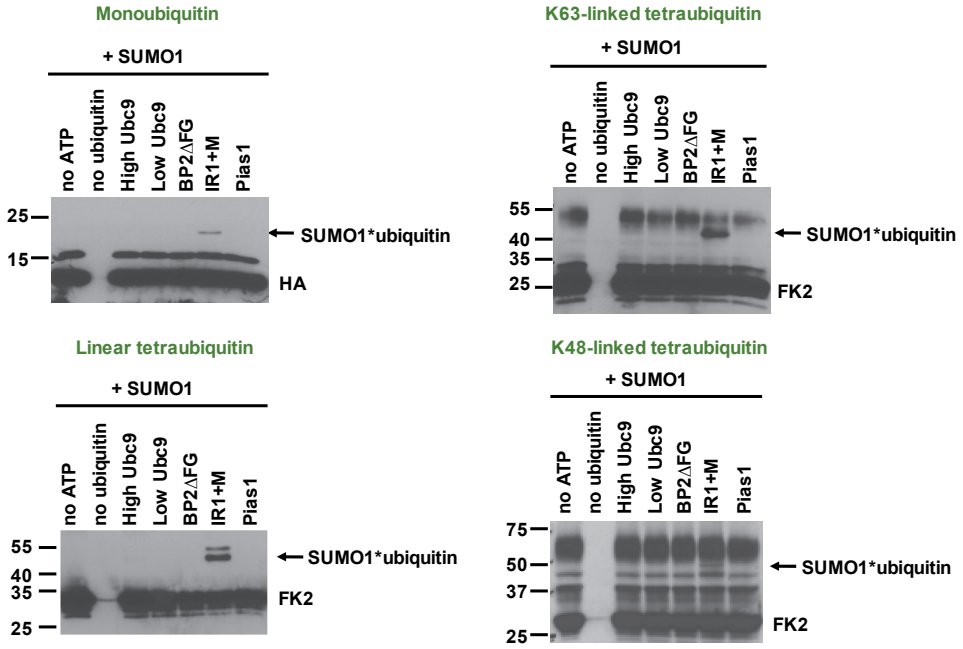

Figure 14. Monoubiquitin and ubiquitin chains with variable linkages are sumoylated *in vitro*. *In vitro* sumoylation reactions were performed in the presence of 0.14 μM Aos1/Uba2, 0.05 μM Ubc9 or 12.5 μM Ubc9 only for the "High Ubc9" sample, 0.05 μM BP2ΔFG/IR1+M/Pias1, 10 μM SUMO1, and 10 μM monoubiquitin or 1 μM of tetraubiquitin chains in 20 μl of reaction volume. Samples were incubated at 30°C for 1 h after which they were subjected to SDS-PAGE. Ubiquitin was detected with specific antibodies (FK2). Representative results from three independent experiments are shown.

Ubiquitin contains seven internal lysine residues as well as the N-terminal methionine, which can all serve as conjugation sites for another ubiquitin moiety (Swatek & Komander, 2016). By using the WT monoubiquitin or ubiquitin chains as sumoylation substrates, it is impossible to separate which one of these lysines is the actual sumoylation target lysine. To study the sumoylation site specificity, *in vitro* reactions were performed in the presence of specific ubiquitin constructs, where all except for one lysine was replaced with arginine (*e.g.* K6 only = all the other six lysines are replaced with arginine, only K6 is functional). These experiments revealed that lysine 11 (K11) is the predominant target lysine for sumoylation (Figure 15). A weak signal at lysine 63 (K63) was also detected, suggesting that it can also function as a sumoylation target lysine, albeit to a lesser extent. No higher molecular weight signal was detected with the K0-construct, where all the lysines were abolished, indicating that the M1 of ubiquitin cannot function as a sumoylation target site. These results are consistent with the recent results by Hendriks and co-workers (2017), who revealed in an unbiased proteomics screen that K11 and K63 are the main sumoylation target lysines on ubiquitin, whereas no M1-targeted sumoylation was detected.

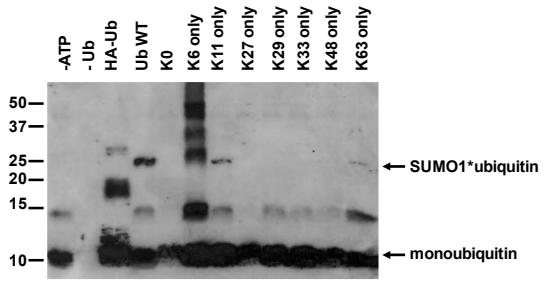

Figure 15. Sumoylation of ubiquitin shows site specificity. *In vitro* sumoylation reactions were performed in the presence of 0.14 μM Aos1/Uba2, 0.05 μM Ubc9, 0.05 IR1+M, 10 μM SUMO1, and 10 μM monoubiquitin (HA-Ub, WT, K0 only, K6 only, K11 only, K27 only, K29 only, K33 only, K48 only, K63 only) in 20 μl of reaction volume. Samples were incubated at 30°C for 1 h after which they were subjected to SDS-PAGE and immunoblotting. Ubiquitin was detected with specific antibodies (FK2).

Since the initiation of this project in 2010, multiple MS screens have identified ubiquitin as a sumoylation target substrate in cells (Hendriks *et al*., 2015; Lamoliatte *et al*., 2014; Lumpkin *et al*., 2017; Xiao *et al*., 2015). These studies did not directly address the biological role of ubiquitin sumoylation, but the obtained results suggested that sumoylation of ubiquitin could function as a modification regulating ubiquitin chain formation. Modification of K11 on SUMO2/3 by SUMO1 has been shown to limit SUMO2/3 chain elongation (Matic *et al.*, 2008), and it is tempting to speculate that SUMO1 could also regulate ubiquitin chain elongation. Such a hypothesis could be examined by *in vitro* reactions assessing ubiquitin chain length in the presence of variable SUMO1 concentrations. Increased appearance of short ubiquitin chains in samples with high SUMO1 concentrations would suggest a role for SUMO1 in the regulation of ubiquitin chain length. Recently, Wauer and co-workers reported that phosphorylation of S65 of ubiquitin affects ubiquitin chain structure, chain assembly, and hydrolysis (Wauer *et al*., 2015). Their study confirms that ubiquitin biology is indeed regulated by other post-translational modifications and it would be extremely exciting to perform analogous experiments with SUMOs to examine the functional role of ubiquitin sumoylation.

Although many experiments can be performed in *in vitro* conditions, the *in vivo* characterization of ubiquitin sumoylation is essential for the biological validation of the PTM-interplay. Unfortunately, this appears not to be straightforward. SUMO and ubiquitin share a large number of target substrates (Lumpkin *et al*., 2017), which means that immunoprecipitation with *e.g.* ubiquitin-specific antibodies and immunoblotting with SUMO-specific antibodies is not sufficient to examine the phenomenon. For this purpose, we generated a selection of mutant ubiquitin proteins that were unable to bind to substrate proteins. Most of these constructs contained a G75/76A double mutation, which abolishes the ubiquitin C-terminal di-glycines and thereby inhibits ubiquitin conjugation. We predicted that such proteins could still function as initiator ubiquitin for chain formation and by immunoprecipitating these proteins we would be able to detect ubiquitin sumoylation *in vivo*. Unfortunately, despite multiple efforts, I was not able to detect ubiquitin sumoylation in cells and the project was terminated. However, I still find the original *in vitro* observations very intriguing, since sumoylation-mediated ubiquitin regulation has not thus far been reported. Moreover, as MS/MS is unable to detect modifications in protein multimers, such as

ubiquitin chains, the results presented in this thesis are novel and advocate the continuation of this fascinating project.

2 POST-TRANSLATIONAL REGULATION OF HSF1 (II)

During its activation and attenuation pathway, HSF1 undergoes multiple post-translational modifications, such as phosphorylation, sumoylation and acetylation (Budzyński & Sistonen, 2017). By far the most extensively studied of these modifications is phosphorylation which, due to its correlation with HSF1 transcriptional activity, has been proposed as the main modification regulating HSF1 transactivation capacity. Nearly 70% of the known HSF1 phosphorylation sites occur within the regulatory domain (RD), which is the domain responsible for heat-inducibility and repression of transactivation domain (TAD) in non-stressed conditions (Shi *et al*., 1995; Zuo *et al*., 1995). Previous studies addressing the importance of phosphorylation for HSF1 function have mainly utilized single-site HSF1 mutants, which disregards the existence of prominent multi-site hyperphosphorylation of the RD. To elucidate the role of hyperphosphorylation in HSF1 function, we generated a phosphorylation-deficient HSF1 mutant and analyzed effect of phosphorylation loss on key HSF1 features: stability, DNA-binding activity, transactivation capacity and stress-inducibility.

2.1 Characterization of the phosphorylation-deficient HSF1 mutant

To study the importance of hyperphosphorylation on HSF1´s ability to function as a stress-inducible transcription factor, we generated a human HSF1 mutant, called HSF1Δ~PRD, where 15 known phosphorylation sites within the RD were mutated to non-phosphorylatable alanines. Before proceeding into functional studies, the expression and stability of the mutant construct was examined in *Hsf1*$^{-/-}$ MEFs. These cells were used throughout the study to exclude the interference of the results by endogenous HSF1. HSF1 WT and HSF1Δ~PRD constructs were transfected in *Hsf1*$^{-/-}$ MEFs and expression of the proteins in control and upon a 30-min heat shock was analyzed by immunoblotting. These experiments demonstrated that HSF1Δ~PRD is expressed in *Hsf1*$^{-/-}$ MEFs and migrates faster on SDS-PAGE in both control and stress conditions than HSF1 WT (Figure 1C in II). Observed migration differences were abolished by treating the cell lysates with lambda protein phosphatase (λPP) (Figure 1C in II), confirming that the proteins are differentially phosphorylated in both control and heat shock conditions. The stability of HSF1Δ~PRD in relation to WT HSF1, was examined by treating the HSF1 WT and HSF1Δ~PRD transfected *Hsf1*$^{-/-}$ MEFs with eukaryotic translation inhibitor cycloheximide (CHX). The protein levels of both constructs remained stable throughout the CHX time course, indicating that the phosphorylation within the RD does not regulate HSF1 turnover in MEFs treated with CHX (Figure 1D in II). It is plausible that this result reflects the finding by Raychaudhuri and co-workers, who showed that HSF1 is stabilized through EP300-mediated acetylation, not phosphorylation (Raychaudhuri *et al*., 2014). However, our observations could also be cell type dependent, as it has been shown that phosphorylation of serines 303 and 307 (both sites mutated in HSF1Δ~PRD) precedes HSF1 degradation in medium spiny neurons of Huntington´s disease mouse model and in HEK293 cells (Kourtis *et al.*, 2015; Gomez-Pastor *et al.*, 2017). This suggests that lack of phosphorylation might stabilize HSF1 in specific cell

types and possibly also during distinct stresses. Thereby, it would be interesting to examine whether HSF1Δ~PRD would be more protected against degradation in such conditions.

To be able to induce transcription, HSF1 must accumulate in the nucleus and gain DNA-binding capacity. To examine whether HSF1 hyperphosphorylation is required for the nuclear localization and DNA-binding of HSF1, we analyzed the appearance of nuclear stress bodies (nSBs) in HSF1 WT and HSF1Δ~PRD transfected HeLa cells using indirect immunofluorescence. nSBs are primate-specific subnuclear structures that are formed at *satIII*-repeats during heat shock and correspond to active transcription at these sites (Biamonti & Vourc'h, 2010). Localization of HSF1 to *satIII*-repeats depends on HSF1´s ability to trimerize and bind DNA (Jolly *et al.*, 2002) and thus nSBs can be used as an indicator of HSF1 DNA-binding ability. In our experiments, both HSF1 WT and HSF1Δ~PRD localized to the nucleus and formed nSBs upon stress (Figure 2 in II), indicating that the phosphorylation within the RD is not required for the stress-induced accumulation of HSF1 to the nucleus or for HSF1´s ability to bind DNA. Considering the notions that HSFs form trimers while binding to DNA and that these experiments were performed in HeLa cells, it is possible that the detected nSBs could be formed by heterotrimeric HSF consisting of both exogenous and endogenous HSF1 monomers. To exclude the possibility of interference by endogenous HSF1, we examined the HSF1Δ~PRD DNA-binding ability in *Hsf1*$^{-/-}$ MEFs. *Hsf1*$^{-/-}$ MEFs were transfected with HSF1 WT and HSF1Δ~PRD and the heat-induced binding of the proteins to the endogenous *HSPA1A/B* and *HSPB1* promoters was assessed with chromatin immunoprecipitation (ChIP). As shown in figure 3A (in II), both HSF1 WT and HSF1Δ~PRD equally occupied the *HSPA1A/B* and *HSPB1* promoters after a 30-min heat shock, indicating that phosphorylation within the RD does not affect HSF1´s ability to bind chromatin upon acute stress.

The DNA-binding activity of HSF1 has been previously shown to depend on stress duration (Sarge *et al*., 1993). To be able to examine the DNA-binding activity of HSF1 WT and HSF1Δ~PRD in more prolonged stress conditions, we used electrophoretic mobility shift assay (EMSA), which is technically more suitable than ChIP for such experiments. *Hsf1*$^{-/-}$ MEFs were transfected with HSF1 WT and HSF1Δ~PRD and treated with 60 μM $CdSO_4$ for times indicated in Figure 3B (in II). *Hsf1*$^{-/-}$ MEFs are highly sensitive to continuous heat stress (unpublished observations) and for that we used cadmium for the induction of prolonged stress conditions. Moreover, cadmium has been previously shown to induce HSF1 DNA-binding activity and Hsp expression (Koizumi *et al*., 2007). After cadmium treatments, whole-cell lysates were incubated with ^{32}P-labeled oligonucleotides containing the proximal HSE from the *HSPA1A* promoter and the protein-DNA complexes were visualized with autoradiography. At the 9-h time point, both HSF1 WT and HSF1Δ~PRD showed equal *in vitro* binding capacity to the radiolabeled oligonucleotides (Figure 3B in II) and thus suggested that the phosphorylation within the RD is not required for HSF1 DNA-binding activity during acute or prolonged stress conditions.

Considering the notion that DNA-binding activity of HSF1 WT and HSF1Δ~PRD was only studied with a distinct set of target promoters and with pure DNA in the form of oligonucleotides, it is plausible that hyperphosphorylation impacts HSF1 DNA-binding on a subset of target promoters. HSF1 has a versatile selection of target genes, which varies depending on the cell type and growth conditions (Mendillo *et al*., 2012; Riva *et al*., 2012.; Vihervaara *et al*., 2013). A specific PTM signature has been frequently proposed as one of the mechanisms regulating HSF1 target gene selection, though the question has not been

directly addressed in mammalian cells. In yeast *S. cerevisiae,* the importance of phosphorylation on target gene selection was recently examined by comparing the genome-wide binding of WT ScHSF and phosphorylation-deficient ScHSFΔpo4 mutant in control and heat shock conditions with ChIP-seq (Zheng *et al*., 2016). The obtained results demonstrated that the heat stress-induced chromatin occupancy of ScHSF is not affected by lack of phosphorylation (Zheng *et al*., 2016), suggesting that hyperphosphorylation does not regulate HSF1 binding to specific promoters during stress.

2.2 Lack of phosphorylation potentiates HSF1 transactivation capacity

Activation of the mammalian HSF1 is a multistep process evidenced by the observation that HSFs can localize to the nucleus and bind DNA without activating transcription (Hensold *et al*., 1990; Jurivich *et al*., 1992). Acquisition of HSF1 transactivation capacity coincides with the inducible hyperphosphorylation, which has been suggested as an elementary step in the initiation of transcription (Cotto *et al*., 1996). To establish whether hyperphosphorylation within the RD is required for HSF1 transactivation capacity, we transiently transfected *Hsf1*$^{-/-}$ MEFs with HSF1 WT and HSF1Δ~PRD and measured the steady-state mRNA levels of *HSPA1A/B* and *HSPB1* upon heat stress with qRT-PCR. At control conditions, neither expression of HSF1 WT nor HSF1Δ~PRD induced upregulation of mRNA levels of the studied genes, indicating that the proteins are not constitutively active (Figure 4A and B in II). Upon exposure to acute heat stress (30 min or 60 min at 43°C) both proteins induced prominent increase in *HSPA1A/B* and *HSPB1* mRNA levels (Figure 4 A in II). Intriguingly, HSF1Δ~PRD exceeded the transactivation capacity of WT HSF1 by inducing even higher mRNA levels at both time points (Figure 4A in II). These results show that the stress-inducible hyperphosphorylation does not define HSF1 transactivation capacity during heat stress. To examine whether the observed results are stress type dependent, we performed the same experiments in the presence of heavy metal cadmium (60 μM $CdSO_4$). Similarly to heat shock, HSF1Δ~PRD induced more pronounced expression of *HSPA1A/B* and *HSPB1* in cadmium treatment and the effect persisted throughout a 3-h recovery period (Figure 4B in II). Together these results show that instead of being a modification required for transactivation capacity, hyperphosphorylation rather represses HSF1 activity. Moreover, this effect appears to be general, as the phosphorylation-mediated repression of HSF1 transactivation capacity was detectable in both heat shock and heavy metal induced stress. Intriguingly, similar observations have been obtained also in human cancer cell lines, where IER5-PP2A-mediated dephosphorylation of HSF1 was shown to increase HSF1 transactivation capacity (Asano *et al*., 2016)

The difference between the levels of Hsps induced by the HSF1 WT and HSF1Δ~PRD, could be due to a decreased activation threshold of HSF1Δ~PRD. Previously, lowered heat-induced activation has been observed with HSF2 (Shinkawa *et al*., 2011). To study the propensity of the proteins to induce the heat shock response, we transfected *Hsf1*$^{-/-}$ MEFs with HSF1 WT and HSF1Δ~PRD, exposed the cells to different heat shock temperatures (39°C, 40°C, and 41°C) or cadmium concentrations (40 μM and 60 μM), and measured the induction of *HSPA1A/B* with qRT-PCR. We found that HSF1Δ~PRD indeed has a lower activation threshold than WT HSF1, as stronger HSF1Δ~PRD-mediated induction of *HSPA1A/B* was detected in milder stress conditions (Figure 5A and B in II). Moreover, HSF1Δ~PRD induced an increase in *HSPA1A/B* mRNA levels in shorter stress exposures (30 min at 41°C). These results show that the lack of phosphorylation within the RD lowers the HSF1 activation

threshold and produces faster induction of the heat shock response in various stress conditions.

We can only hypothesize on the possible reasons why HSF1Δ~PRD is a more potent transcription factor than WT HSF1. It is, however, widely accepted that phosphorylation modulates transcription factor activity often in concert with other PTMs (Filtz *et al*., 2014), and HSF1 RD has been shown to be subjected to multiple PTMs, including sumoylation and acetylation (Budzynski & Sistonen, 2017). Sumoylation of lysine 298 occurs only if the adjacent S303 residue is phosphorylated (Hietakangas *et al*., 2003) and is considered as a repressive modification (Hietakangas *et al.*, 2006). Thus, lack of phosphorylation within the RD might inhibit stress-induced sumoylation of HSF1 and thus could retain HSF1Δ~PRD more active. Therefore, it would be interesting to examine whether reintroduction of S303 to HSF1Δ~PRD restores sumoylation and normalizes the transactivation capacity to the level of WT HSF1. Furthermore, K298 is also subjected to acetylation, which has been indicated as a stabilizing modification during stress (Raychaudhuri *et al*., 2014). Because we did not assess the stability of HSF1 WT or HSF1Δ~PRD during the heat shock time course, we cannot exclude the possibility that lack of S303 phosphorylation and subsequent impairment in K298 sumoylation enables more persistent acetylation on K298 and hence increases the stability of HSF1Δ~PRD.

Another possibility is that phosphorylation regulates the protein interactome that is required for the normal HSF1 activation. As one of many examples of HSF1 interacting partners, HSF1 forms heterotrimes with HSF2 (Sandqvist *et al*., 2009) and this trimerformation has been shown to have an inhibitory effect on the expression of specific target genes during stress (Östling *et al*., 2007; El Fatimy *et al*., 2014) and in mitosis (Elsing *et al*., 2014). Though not directly mediating HSF oligomerization, lack of phosphorylation could prevent HSF1 and HSF2 interaction and thereby enable unrestrained HSF1 activation. Co-immunopurification experiments, assessing the interaction between endogenous HSF2 and exogenously expressed HSF1 WT and HSF1Δ~PRD, would provide insight into such a hypothesis. Heterotrimerformation has been implicated as a regulatory mechanism affecting HSFs activity in various stress, differentiation, and developmental conditions (Sandqvist *et al*., 2009). However, the mechanisms controlling the trimerformation are currently unknown and the proposed experiments could potentially provide important information about the post-translational regulation of HSF1-HSF2 trimers.

2.3 Phosphorylation-deficient HSF1 can sense stress

HSF1 RD has an intrinsic capacity to sense stress and, thus, can provide stress-inducibility to heterologous activatory domains, such as the activatory domain of herpes simplex virus 1 VP16 (Newton *et al*., 1996). To assess whether hyperphosphorylation within the RD is necessary to provide heat-sensitive transactivation capacity also to heterologous activatory domains, the RDs of WT HSF1 and HSF1Δ~PRD were cloned into a Gal4-VP16 chimeric construct (Figure 6A in II). The constructs, as well as Gal4-VP16, were expressed together with a Gal4-driven luciferase reporter gene in HeLa cells and the relative luciferase activity in control and heat shock treatments was measured. In control conditions, cells expressing the Gal4-VP16-HSF1 WT displayed lower luciferase activity than cells expressing Gal4-VP16 (Figure 6B in II). No statistically significant difference was observed between the luciferase activities of Gal4-VP16 and Gal4-VP16-HSF1Δ~PRD expressing cells (Figure 6B in II). After a 30-min heat shock and 5 h of recovery, cells expressing Gal4-VP16-HSF1 WT displayed equal luciferase activity to Gal4-VP16 expressing cells. In the same conditions,

however, Gal4-VP16-HSF1Δ~PRD expressing cells showed 60% higher luciferase activity denoting even more pronounced stress-inducible activation of transcription.

These results are in line with a previous study and show that in control conditions, phosphorylation within the HSF1 RD represses the transactivation capacity of heterologous VP16 in a constitutively DNA-bound chimera, whereas lack of phosphorylation reverses this repression (Kline & Morimoto, 1997). Interestingly, the study further showed that the repression in control conditions is mediated mainly through phosphorylation of S303 and S307 residues, which could be confirmed by reintroducing those sites into HSF1Δ~PRD. By gaining a repressed phenotype in the control conditions, such protein could demonstrate that the intrinsic ability of the regulatory domain to sense stress is dependent on phosphorylation of these two serine residues. The observed difference in the stress-induced luciferase activity between HSF1 WT and HSF1Δ~PRD expressing cells cannot, however, be exclusively explained by reduced repression at the control conditions. Instead, the observation could also be related to more persistent activation. It has been reported that in *K. lactis* HSF phosphorylation promotes the return of the activated HSF to an inactive state (Høj & Jakobsen, 1994). Considering the time scale of our experiments (5 h after initial exposure to stress), it is fully possible that the increased luciferase activity in HSF1Δ~PRD expressing cells is indeed caused by impaired transcriptional attenuation. Interestingly, HSF1 hyperphosphorylation is detected also during mitosis, where HSF1 DNA-binding activity is significantly reduced compared to freely cyclin cells (Vihervaara *et al.*, 2013: Elsing *et al*., 2014). These observations suggest that phosphorylation might regulate HSF1 displacement from the chromatin.

Altogether, our findings show that phosphorylation within the RD is not required for nuclear localization or acquisition of DNA-binding and transactivation capacities of HSF1. Phosphorylation was, however, shown to regulate the HSF1 activation threshold and affect the magnitude of the heat shock response. Based on these results, we propose that phosphorylation might function as a fine-tuning mechanism and together with additional PTMs, create a distinct HSF1 signature that provides the factor with functional variability in a multitude of stress and disease conditions. Altered HSF1 activity has been particularly implicated in pathologies such as cancer and neurodegenerative diseases (see *3 HSFs in pathologies*). Increased HSF1 activity often leads to more prominent cancer growth and HSF1 phosphorylation at S326 has been proposed as a marker for HSF1 activity in human cancers (Mendillo *et al*., 2012; Shah *et al*., 2016). In the study II of this thesis, we showed that HSF1 phosphorylation within the RD, including S326, is not required for HSF1 transactivation capacity (Figure 4 in II). However, the role of phosphorylation was only examined in a non-transformed mouse cell line and thus we cannot fully conclude that HSF1 activity is independent of phosphorylation in malignant human cells. It is plausible that the observed S326 phosphorylation denotes a cancer-specific HSF1 phosphorylation signature that is required for the distinct transcriptional program that HSF1 drives in malignant cells (Mendillo *et al*., 2012). To date, the importance of specific PTMs in HSF1 target gene selection or transactivation capacity has not been assessed in mammalian cells in a genome-wide manner (*e.g.* with ChIP-seq or RNA-seq). Therefore, it would be interesting to examine in the fortcoming studies whether HSF1 has a specific PTM signature in human cancer cells and define whether specific modulation of HSF1 PTMs could serve as a novel platform for drug development.

3 HSF2 IN PROSTATE CANCER (III)

Prostate cancer is the most commonly diagnosed cancer in males and the second leading cause of cancer-related deaths in Finland as well as in other western countries (Finnish Cancer Registry, Jemal *et al*., 2011). Since the introduction of the PSA (prostate specific antigen) -test in early 1980´s (Kuriyama *et al*., 1980), prostate cancer incidence has increased rapidly and the cancers are often diagnosed while still localized to the prostate tissue. Most of such tumors can be effectively treated by combining androgen deprivation therapies (ADT) (Huggins, 1967) to radical prostatectomy (*i.e.* surgical removal of the prostate gland), although such measures do not necessarily cure the disease. In approximately 7-10 % of the diagnosed prostate cancer cases, the disease progresses into castration resistant prostate cancer (CRPC), which no longer responds to anti-hormonal therapies (Ceder *et al*., 2016; Feldman & Feldman, 2001). This is mainly due to AR (androgen receptor) overexpression that has been shown to be sufficient for the disease to turn into CRPC (Chen *et al*., 2004). At this stage, the disease progression often includes metastasis formation in distant tissues, which eventually might cause patient death.

Prostate cancers are a heterologous group of diseases and the patient outcome largely depends on good stratification systems and subsequent therapy decisions. Gleason scoring, a histology-based grading system, is currently used as the main tool to predict prostate cancer progression, though it is insufficient in classification of all prostate cancer subtypes. Gleason score (2-10) is assigned by combining the histological grades (1-5) of the most common tumor pattern with the most aggressive pattern observed in a given tumor biopsy (*e.g.* grade 3 + grade 5 = Gleason score 8) (Gleason, 1966; Epstein *et al*., 2017). Grade 1 pattern tumors resemble closely the normal prostate tissue, with well differentiated and organized glandular structure. Appearance of degenerated glandular structures increases the grade and in the most aggressive grade 5 pattern the prostate tissue has none or only a few recognizable glands and mainly consists of neoplastic cells. The tumors with Gleason score ≤6 are considered indolent and have low risk of progressing into CRPC. In contrast, tumors with Gleason score ≥8 are highly aggressive and predictive for poor patient outcome. The most challenging group of tumors consists of Gleason 7 scored tumors that show intermediate tumor phenotype. Correct stratification of such tumors into prognostic groups is difficult and has significant implications for the patient outcome (Uemura *et al*., 2009). Thereby, detailed molecular characterization of mechanisms involved in prostate cancer progression is essential as identification of novel therapeutic targets and biomarkers is in high demand.

HSF1 is a well-recognized promoter of cancer progression and pronounced HSF1 expression has been detected in a large selection of human cancers, including prostate cancer (Jiang *et al*., 2015). In breast cancer, high HSF1 level correlates with poor patient outcome and utilization of HSF1 expression has been proposed as a prognostic marker of specific breast cancer types (Santagata *et al*., 2011). In contrast, HSF2 has not been related to cancer progression and the importance of HSF2 in malignancies is unknown. Thereby, in the study III of this thesis, we investigated whether HSF2 has a role in cancer progression.

3.1 HSF2 mRNA expression is decreased in aggressive prostate cancers

This study was initiated by examining HSF2 mRNA expression *in silico* from Memorial Sloan-Kettering Cancer Centre (MSKCC) prostate cancer data set consisting of 216 prostate cancer specimens originating from both primary and metastatic tumors (Taylor *et al*., 2010). By comparing HSF2 mRNA expression in normal and cancerous prostate tissue, we found

that HSF2 levels are lower in primary prostate tumors and further decreased in metastatic tumors (Figure 1A in III). Specifically, HSF2 mRNA expression negatively correlated with the Gleason grading, as lower HSF2 levels were detected in tumors denoted with high Gleason grades (≥8) (Figure 1A in III). Furthermore, decreased HSF2 expression was associated with lymph node invasion, positive surgical margins (*i.e.* cancer cells can still be found in patients after surgical removal of the cancerous tissue), and seminal vesicle invasion, all indicative for aggressive and advanced prostate cancer (Figure 1A in III). These results indicated that a decline in HSF2 expression associates with aggressive prostate cancer phenotype.

Since HSF2 was not previously connected to cancer progression, we next wanted to examine the relevance of our observations by comparing the observed HSF2 alterations to those detected with well-established tumor-suppressors PTEN and p53 as well as Myc oncogene and HSF1. We used a subset of 85 tumor specimens that had been analyzed for transcriptomic (mRNA and miRNA) and copy-number alterations as well as subjected to focused exon resequencing (157 high interest genes) (Taylor *et al*., 2010). HSF2 alterations were observed in 20% of the studied specimens and these alterations mainly consisted of HSF2 mRNA downregulations (~18% of tumors) (Figure 1B and Supplementary Figure S1A in III). PTEN and p53 alterations were found comparable to those of HSF2, as 18% and 29% of the tumors displayed changes in these genes, respectively. Interestingly, HSF1 was also found altered in 20% of the tumors so that 70% of those exhibited HSF1 mRNA upregulation (Figure 1B in III). Putative copy-number alteration assessment revealed that the decreased HSF2 mRNA expression was mainly associated with loss of heterozygosity, whereas PTEN alterations were associated with both loss of heterozygosity and homozygous deletions (Figure 1C in III).

To explore if our findings have clinical relevance, we analyzed 23 metastatic prostate cancer samples and correlated HSF2 expression to patient survival. Intriguingly, the analysis showed that decreased HSF2 expression is predictive of poor patient outcome, albeit performed with limited number of tumor samples (only 23 tumors) (Supplementary Figure 1B in III). Taken together, these results showed that the HSF2 expression is downregulated in aggressive prostate cancers in a frequency comparable to other tumor suppressors and that the decline in HSF2 levels correlates with poor patient survival. This indicated for the first time that, in addition to HSF1, also HSF2 might have a role in cancer progression.

3.2 HSF2 expression is dynamic and reflects initiation of invasion

The findings shown in Figure 1 (in III) proposed that HSF2 levels vary according to the severity of the disease and a decline in HSF2 expression correlates with aggressive cancer phenotype. To verify our observations in cells, we assessed HSF2 expression at both mRNA and protein levels in a selection of human prostate cell lines with variable degrees of malignancy. As shown in Figure 2A (in III), HSF2 expression was low in cells with low malignant potential (primary prostate epithelial cells, PrEC; immortalized prostate epithelial cells, EP156T), whereas high expression was observed in the cancerous cell types LNCap (prostate adenocarcinoma) and Du145 (prostate carcinoma) (Figure 2A in III). Importantly, HSF2 mRNA expression in the highly malignant and invasive PC-3 (prostate adenocarcinoma derived from bone metastasis) and PC-3M (PC-3 variant with high metastatic potential) cells was clearly reduced when compared to the less invasive cancerous cell lines. Similar results were obtained from HSF2 protein expression analysis (Figure 2B

in III), implying that a decline in HSF2 expression correlates with invasive growth phenotype also in cell lines.

Cancer development is a multi-step process, during which the cancer must acquire novel properties, such as sustained proliferation and ability to avoid apoptosis, to enable the unrestricted neoplastic growth (Hanahan & Weinberg, 2011). A hallmark of cancer development is the ability to activate invasion and metastasis, which is a process strongly suppressed in normal epithelial tissue. To analyze whether the observed decrease in HSF2 expression occurs concomitant with invasive behavior, we used 3D cell culture method to examine prostate cancer cell growth. The method presents a biologically more relevant model system to study cancer progression than conventional 2D cultures. In the 3D growth model, cells are placed between layers of gel (Matrigel in this study), which usually is composed of a selection of extracellular matrix constituents, such as collagens, laminins, and heparan sulfate proteoglycans (Hughes *et* al., 2010). This mimics the natural 3D cell environment and thus provides the cells with physiologically relevant cell-cell and cell-matrix contacts (Lee *et al*., 2007; Pampaloni *et al*., 2007). Previously, both normal and cancerous prostate epithelial cells have been shown to grow and form organoid structures in 3D cultures in a process often referred to as organoid development (Härmä *et al*., 2010). Moreover, gene expression profiling has demonstrated that the organoids undergo cancer-relevant epithelial differentiation and can activate invasion (Härmä *et al*., 2010). Multiple cell types are currently used as models to study prostate cancer and we chose the widely used PC-3 cells. When cultured in 3D, PC-3 cells first form well-differentiated and polarized organoids (around days 4-7), after which the organoids spontaneously de-differentiate (around days 8-12) and give rise to invasive stellate structures (Härmä *et al*., 2010) (see also Figure 2C in III). We analyzed HSF2 mRNA expression in 3D-cultured PC-3 cells at days 4, 8, 13 and 15, as the days comprehensively cover the steps of organoid development. As shown in figure 2D (in III), HSF2 mRNA levels were upregulated during spheroid polarization (day 4) but decreased concomitantly with de-differentiation and appearance of invasive structures (days 8, 13, and 15). Similar results were obtained from protein analysis, which clearly demonstrated that HSF2 expression also decreases during PC-3 organoid development and co-occurs with the activation of invasion.

3.3 Lack of HSF2 promotes invasive behavior of prostate cancer organoids

Since fluctuating HSF2 expression has been previously connected to various differentiation and developmental processes, such as spermatogenesis and corticogenesis (Björk & Sistonen, 2010), it is possible that HSF2 downregulation only reflects the organoid differentiation state but does not directly regulate it. Therefore, we next examined the importance of dynamic HSF2 expression during PC-3 organoid development. HSF2 was silenced with siRNA and the cells were cultured in 3D for eight days and monitored for their morphology and invasive properties (Figure 3 in III). HSF2 silencing efficacy during the experimental course was examined with qRT-PCR (Supplementary Figure S3 in III). During the 8-day experiment, cells transfected with control siRNA progressed into well-differentiated organoids and initiated invasive behavior similarly as reported earlier (Härmä *et al*., 2010) (Figure 3B in III). Surprisingly, HSF2 silencing accelerated the differentiation process, as invasive structures were observed already at day 7 of the organoid development (Figure 3B in III). In contrast, PC-3 cells where HSF1 was silenced failed to develop into well-differentiated organoids and exhibited apoptotic and non-invasive phenotype, supporting the previous results demonstrating that HSF1 is essential for cancer progression

(Dai *et al*., 2007). These results indicated that HSF2 directly impacts the organoid development.

The observed differences between control and HSF2-depleted organoids were quantified with high-content automated morphometric image data analysis (AMIDA) software, which allows quantitative measurement of thousands of organoids with variable shapes and sizes (Härmä *et al*., 2014). AMIDA has been successfully used to examine growth phenotypes of a large selection of prostate and breast cancer cell lines (Härmä *et al*., 2014). AMIDA analysis of the spheroids confirmed that HSF2-depleted PC-3 cells exhibited decreased organoid roundness, increased roughness and length of the invasive structures, all indicative for pronounced metastatic potential (Figure 3E in III) (Härmä *et al*., 2014). In stark contrast, HSF1 silencing produced round and small organoids with decreased roughness, only a few invasive appendages and overall decrease in cell number (Figure 3E in III), suggesting that HSF1 and HSF2 might have an opposite effect on cancer progression. The HSF2-dependent phenotypic differences were further validated by rescue experiments, where shRNA-mediated HSF2 downregulation was combined with overexpression of shRNA-resistant exogenous HSF2. As presented in Figure 4 (in III), re-introduction of HSF2 reversed the invasive phenotype detected with HSF2 silencing, strongly implying that HSF2 functions in a developmental switch involved in the maintenance of the well-differentiated epithelial phenotype and suppressing the initiation of invasive behavior of PC-3 organoids.

Considering that the role of HSF2 in PC-3 organoid development was examined by silencing HSF2 expression with siRNA (Figure 3 in III), it is important to note that such a method can only induce transient changes in the expression of the desired gene. This means that in long-term 3D cultures, *e.g.* the 8-day cultures used in this study, downregulation of HSF2 levels does not persist (Supplementary Figure S3 in III) but begins to be modulated according to the requirements of the cell. To be able to examine the importance of HSF2 during the whole course of organoid development, the HSF2 gene would have to be knocked out from the PC-3 cells *e.g.* by CRISPR-Cas9-based gene-editing methods. However, in this study, we observed that HSF2 is dynamically regulated during the organoid development (Figure 2 in III). This indicates that PC-3 cells both upregulate and downregulate HSF2 expression when grown in 3D, which in turn suggests that high HSF2 expression is required for the initial development of well-differentiated organoids (at day 4), after which the levels decline to allow activation of metastatic behavior. Complete abolishment of HSF2 expression would thereby likely result in misregulated organoid development and disable the investigations addressing the role of HSF2 as a switch regulating the initiation of invasion. In fact, by using the transient siRNA-mediated downregulation of HSF2, we likely shift the invasive switch to be activated at an earlier time point, which results in accelerated development of invasive structures. A more accurate approach to study the impact of HSF2 in this invasive switch could be generation of a stable cell line, where HSF2 expression would be under an inducible promoter (*e.g.* Tet-ON, tetracycline-responsive promoter). Such a cell line would allow for a more precise upregulation of HSF2 expression at a specific time point in 3D cultures and thus could more precisely investigate whether HSF2 has the ability to maintain specific epithelial differentiation state.

Dynamic HSF2 expression during the PC-3 organoid development suggests that cancer cells have an inherent ability to downregulate HSF2 levels. In fact, the regulation appears to be very precise, as disruption of the expression pattern resulted in accelerated appearance of invasive structures (Figure 3 in III). Moreover, forced HSF2 upregulation suppressed the invasive phenotype, altogether implying that, similarly to the normal mammalian

developmental programs, fluctuating HSF2 expression is a key determinant also during malignant progression. These results imply that the detected change in HSF2 levels is perhaps secondary to the initial developmental signal, which raises the question about the regulatory mechanisms behind dynamic HSF2 expression. As the alterations in HSF2 expression were detected also at the mRNA level, it is reasonable to hypothesize that the mechanism functions through regulating the synthesis or stability of mRNA. In testis, HSF2 expression is regulated through an mRNA-targeting miR-18 (Björk *et al*., 2010), which could be one of the mechanisms behind fluctuating HSF2 expression also in cancer. However, many developmental processes are characteristically driven by transcription factors acting in sequence, and it is tempting to speculate that HSF2 expression, during organoid development, is also regulated by modulating the activity of the *HSF2* gene. Currently, little is known about *HSF2* gene regulation and only a few factors have been shown to bind to the *HSF2* promoter in mammalian cells. In rat, USF2 (upstream stimulatory factor 2) has been shown to bind to an E-box element 200 bp upstream of the transcription start site and appears critical for the *HSF2* promoter activity in C6 rat neuronal cell line (Lee *et al*., 2003). In addition, by studying the target genes of the activated Wnt/β-catenin/TCF4 pathway, it was found that β-catenin can bind to the promoter of *HSF2* and activate its transcription in Huh7 (hepatocyte carcinoma), HEK293T (human embryonic kidney cells), and glioblastoma cells (Kavak *et al*., 2010). Interestingly, USF2 has been shown to have a suppressive role in prostate carcinogenesis (Chen *et al.*, 2006), whereas the Wnt/β-catenin-signaling is a well-recognized positive regulator of prostate cancer development (Kypta & Waxman, 2012). Nevertheless, it remains to be established, whether these factors are responsible for the fluctuating HSF2 expression detected in this study.

3.4 HSF2 affects genes related to cell invasion

HSF2 is a potent DNA-binding factor that regulates gene expression in a stimulus-dependent manner. High expression of HSF2 has been shown to correlate with its DNA-binding activity and modulation of HSF2 levels is considered the main mechanism regulating HSF2 transcriptional activity (see *2.4.1 Regulation of HSF2 activity*). Therefore, we wanted to establish whether the observed phenotypic differences between control and HSF2-depleted organoids are mediated by HSF2-regulated alterations in the organoid gene expression profile. For this, control and HSF2 depleted PC-3 cells were cultured in 3D and organoids were collected at days 5 and 8 representing the critical time points of epithelial differentiation and initiation of invasive behavior. From the collected samples, total RNA was isolated, amplified, and used for *in vitro* synthesis of biotinylated cRNA (complementary RNA). Subsequently, cRNA was subjected to whole-genome microarray analysis through hybridization on Illumina Sentrix HumanHT-12 v4 Expression BeadChips and the relative gene expression changes were analyzed with limma and lumi R/Bioconductor packages. Gene set enrichment analysis (GSEA) is a bioinformatics tool that can be used to examine whether specific classes of genes are overrepresented in a given gene set. In this study, GSEA-analysis of the differentially expressed genes demonstrated that genes affected by HSF2 silencing were related to gene ontology (GO) terms associated with key cellular functions, such as RNA processing, ribosome biogenesis, and organelle homeostasis (Figure 5A in III). Importantly, HSF2 silencing was also shown at both time points to affect genes associated with GO terms such as GTPase activity and signaling as well as actin cytoskeleton organization (Figure 5A and Supplementary Table in III). GTPases are a large family of proteins that can catalyze hydrolysis of GTP to GDP and have an important role functioning as molecular switches in cells. In addition to the heterotrimeric G-proteins associated with G-protein coupled receptors, the most well-known subclass of GTPases is the Ras

superfamily of GTPases, which is further divided into multiple subfamilies. From the subfamilies, especially the Rho family small GTPases have been implicated as important regulators of actin cytoskeleton dynamics and cancer cell motility (Parri & Chiarugi, 2010). Intriguingly, among the genes differentially expressed at day 5, members from the Rho-family of GTPases (*e.g.* RhoA and Rnd3) were found affected by HSF2 downregulation.

We used a DAVID analysis tool (Dennis *et al*., 2003) to perform a functional cluster annotation of the 300 most significantly changed genes at both time points. At day 5, enriched clusters were related to extracellular matrix interactions and cell adhesion and included genes from laminin, collagen, and integrin families (Figure 5C left panel in III). At day 8, the clusters were associated with small GTPase signaling, and more specifically to Ras and Rho-family of small GTPases, as well as actin cytoskeleton organization, and included genes such as ROCK2 (Rho-associated protein kinase 2) and WASF2 (Wiskott-Aldrich syndrome protein family member 2) and Cdc42BPB (Cdc42 binding protein kinase β), all important in regulating actin cytoskeleton dynamics (Olson & Nordheim, 2011) (Figure 5C in III). Moreover, ingenuity pathway analysis (IPA) showed alterations in pathways affecting, for example, focal adhesion assembly and actin polymerization in lamellipodia, suggesting for multi-level pathway alterations in HSF2-depleted cells at day 8 (Figure 5D in III). Taken together, these results indicated that by affecting genes related to small GTPase-mediated regulation of actin cytoskeleton, HSF2 is involved in the initiation of invasion in PC-3 organoids.

The Rho-family of small GTPases are a well-established family of proteins regulating actin cytoskeleton and cancer cell movement (Lamouille *et al.*, 2014). In contrast to Ras, which is often found mutated in human cancers (Simanshu *et* al., 2017), mutations in Rho GTPases are rarely found. Instead, the detected alterations in Rho GTPases are mainly related to increased expression or increased activity (Parri & Chiarugi, 2010; Sahai & Marshall, 2002). To directly link HSF2 to GTPase-signaling, we analyzed the pathways at the molecular level. Protein expression analysis with immunoblotting demonstrated that HSF2 silencing leads to a prominent increase in the expression of GNA13, a stimulatory G-protein subunit α13 (Figure 6A in III), which has been previously implicated as a factor essential for PC-3 invasion, migration, and RhoA activation (Rasheed *et* al., 2013). In addition, expression of ARHGAP1 (Rho GTPase-activating protein 1) was decreased in HSF2-depleted cells, suggesting that lack of HSF2 may impact the GTP-binding and activation state of the Rho GTPases, rather than the expression of these signaling modulators (Figure 6A in III). Accordingly, Cdc42, another Rho-family GTPase, levels were not affected in HSF2 downregulated cells (Figure 6A in III).

To examine whether the putative alterations in GTPase activity are related to the observed HSF2-affected organoid development, a selection of chemical inhibitors were used to regulate the activity of key small GTPase effector proteins. In accordance with previous observations (Härmä *et al*., 2014), Y27632-mediated inhibition of the RhoA downstream effector protein ROCK resulted in highly invasive organoids as evidenced by increased roughness and loss of round shape (Figure 6B and 6C in III). Moreover, inhibition of the Rac1 downstream effector PAK (p21 activated serine/threonine kinase 1) with IPA3, prevented the initiation of the invasive behavior (Figure 6B and 6C in III). Intriguingly, when applied to the HSF2-depleted PC-3 organoids, IPA3 reversed the aggressive phenotype and resulted in organoids with increased roundness and reduced roughness (Figure 6B and 6C in III). These results propose that during PC-3 organoid development, downregulation of HSF2

expression affects genes associated with GTPase activity regulation and thereby impacts the organoidal ability to activate invasion.

The dynamic reorganization of actin cytoskeleton *via* small GTPases is essential for the epithelial-mesenchymal transition (EMT) program, during which the cancer cells escape from organized epithelial structures and gain migratory mesenchymal properties (Parri & Chiarugi, 2010 and Lamouille *et al*., 2014). As the analysis of the HSF2-affected genes demonstrated prominent alterations in GTPase signaling and actin cytoskeletal reorganization (Figure 6 in III), we assessed whether the invasive phenotype observed with HSF2-downregulated organoids is associated with EMT. We analyzed a panel of EMT markers during the organoid development in 3D. One of the hallmarks of EMT is the downregulation of E-cadherin (CDH1) that reinforces the de-stabilization of adherens junctions characteristic to epithelial structures. Downregulation of E-cadherin is balanced by increased expression of N-cadherin (CDH2), through which the cells acquire affinity for other mesenchymal cells (Lamouille *et al*., 2014). As shown in Figure 6E (in III), HSF2 silencing in PC-3 organoids resulted in decreased expression of CDH1 at day 8, whereas increase in CDH2 was detected at day 5 (Figure 6E in III). In addition, expression of Snail2 (SNAI2), an important transcription factor inducing EMT, exhibited increased expression in HSF2 depleted organoids at day 5 (Figure 6E), indicative for accelerated EMT progression. Analyzing the mRNA levels of critical EMT-mediators further supported our observation, as significant downregulation of *CDH1* and upregulation of the mesenchymal markers *S100A4*, fibronectin (*FN1*), and vimentin (*VIM*) was detected at the mRNA level (Figure 6B in III). Taken together, our results indicate that HSF2 downregulation is involved in the epithelial disintegration of tumor cells leading to a motile and invasive mesenchymal phenotype.

Transcriptional regulation of EMT involves complex cooperation of signaling pathways that together function to repress epithelial and activate mesenchymal phenotypes *via* multiple different target genes (Lamouille *et al*., 2014). In prostate cancer, the molecular events leading to EMT are mainly mediated by activation of androgen receptor and TGF-β-signaling pathways and may lead to metastasis formation and acquisition of therapeutic resistance (Montanari *et al*., 2015). Our results show that HSF2 is tightly connected to the gene expression changes related to prostate cancer invasion and EMT, and thereby it would be interesting to examine the impact of these signaling pathways in the regulation of HSF2 expression. At this point, it is important to note that the PC-3 cells used in this study are aggressive prostate cancer cells that have been originally isolated from patient bone metastasis and, thus, have likely undergone EMT. Nevertheless, PC-3 cells are a widely used model system to study prostate cancer EMT and it has been proposed that the cell line has also undergone MET (mesenchymal to epithelial) program (reviewed Kong *et al*., 2011). To conclusively show that HSF2 levels are specifically regulated during EMT, it would be important to assess how non-tumorigenic cell lines regulate HSF2 expression *e.g.* in response to TGF-β. Moreover, as TGF-β is one of the main signaling molecules secreted by the cancer associated fibroblasts (CAFs) (Scherz-Shouval *et al*., 2014) and CAFs have been shown to promote invasive properties of prostate cancer organoids (Åkerfelt *et al*., 2015), the importance of trans-cellular tumor signaling as a regulator of HSF2 expression would be intriguing to elucidate.

3.5 Lack of HSF2 promotes tumor growth and invasion in vivo

In the human body, the tumor microenvironment contains a selection of different cell types, such as the above-mentioned CAFs, endothelial cells, and immune cells. Hence, cancer

progression is by no means a sole outgrowth of a single cell type, whereas it requires a coordinated network of complex cell-cell interactions. Various *in vivo* model systems are often used in cancer research to more accurately mimic the tumor microenvironment in patients. In this study, we used the chicken chorioallantoic membrane (CAM) model system as a platform to examine HSF2 capacity to affect tumor development *in vivo*. The CAM is based on the ability of the highly vascularized chorioallantoic membrane to support cancer cell growth, and due to the natural immunodeficiency of chicken embryos, it has been successfully used as a model for tumor growth and invasion (Dagg *et* al., 1956; Kunzi-Rapp *et al*., 2001). In contrast to immunocompromised mice (*e.g.* Nude, RAG, NOD/SCID strains), which currently are the most frequently used as *in vivo* models, the CAM presents a more rapid and cost-efficient growth model, albeit being of non-mammalian origin.

For the CAM experiments, HSF2-depleted PC-3 cells were mixed with Matrigel and implanted on the CAM. Cell growth was followed for three days. During this time, control transfected PC-3 cells had developed into large cell masses with uneven borders (Figure 7B in III), reflecting the high metastatic capacity of PC-3 cells. Intriguingly, cells lacking HSF2 exhibited enhanced tumor growth and clearly larger tumors nearly covering the whole implantation area (7 mm) were detected. Moreover, in line with the previous results presented in this and other studies, HSF1 silencing inhibited PC-3 tumor growth, as significantly smaller tumors were observed with HSF1-depleted cells (Figure 7B, C in III). Histological analysis of the paraffin embedded hematoxylin-eosin-stained tumors revealed that HSF2-depleted cells, similarly to the control transfected cells, developed into adenocarcinoma-like tumors with abnormally large and uneven nuclei (Figure 7D in III). More cells escaping the primary implantation site were detected in HSF2-depleted tumors when compared to the non-transfected or control transfected PC-3 tumors (Figure 7D in III). In stark contrast, tumors derived from HSF1-depleted cells were fibrotic, had only a few cancer cells, and mainly consisted of chicken tissue (Figure 7D in III). Increased metastatic potential of the HSF2-depleted cells was confirmed by immunohistochemical staining of MMP-14, which is one of the metalloproteinases required for extracellular matrix degradation and prostate cancer invasion (Littlepage *et al*., 2010). In HSF2 depleted tumors, MMP-14 localized to the tumor edges and exhibited strong staining at cell membranes, whereas more cytoplasmic localization was detected in the control cells (Figure 7E). Since strong MMP-14 expression has been detected in aggressive prostate cancers (Wang *et al*., 2009), these results imply that HSF2-depleted PC-3 tumors mimic the aggressive tumor phenotype *in vivo* in CAM.

3.6 Expression of HSF2 target genes is decreased in human cancers

Having established that HSF2 silencing from 3D cultured PC-3 cells affects a specific set of genes related to GTPase signaling, focal adhesion, and actin cytoskeleton reorganization, we next wanted to analyze whether the same genes are also altered in the MSKCC prostate cancer data set (Taylor *et al*., 2010). Analysis of selected genes affected by HSF2 downregulation in PC-3 organoids, including *COL6A3* (collagen α6), *FLNA* (filamin A), *WASF2*, and *ROCK2,* revealed a correlation between HSF2 and the putative target gene expression, as similar mRNA patterns with lower expression levels in more advanced disease were detected (Figure 8A in III). In fact, all of these genes were clearly downregulated in metastatic prostate cancer samples *vs.* normal prostate tissue. More detailed analysis of the individual tumors (Taylor *et al*., 2010) demonstrated a strong co-occurrence or mutual exclusivity between HSF2 and the target genes, indicating that HSF2 directs similar genetic programs also in the clinical prostate cancer samples (Figure 8B in III). Finally, we extended the study by analyzing HSF2 mRNA expression in other human malignancies (Rhodes *et al*.,

2004) of both epithelial and non-epithelial origin. Excitingly, decreased HSF2 expression was observed in a large variety of human malignancies, such as breast cancer, small-cell lung carcinoma, embryonal carcinoma, and glioblastoma (Figure 9 in III). Therefore, low HSF2 expression is not only restricted to prostate cancer, as it appears as a characteristic feature of a large variety of human cancer types.

Taken together, this study expands our knowledge on HSFs in cancer by revealing for the first time that HSF2 is an important regulator of cancer progression. In contrast to HSF1, HSF2 suppresses prostate cancer progression, which indicates that the factors have opposing roles during malignant growth. In this study, HSF2 expression was found to be dynamically regulated during PC-3 organoid development, whereas HSF1 expression remained stable (Figure 2 in III). HSF2 interacts with HSF1 through heterotrimer formation and modulating the relative abundance of HSF1 and HSF2 has been implicated as a mechanism regulating transcription in response to distinct stimuli (Sandqvist *et al*., 2009). Thus, it is likely that the functions of HSF1 and HSF2 in cancer are not entirely separate and it would be interesting to examine, whether the dynamic expression of HSF2 has a role in the regulation of HSF1 activity. Dynamic HSF2 expression also suggests for inherent HSF2 regulation in PC-3 cells and proposes that HSF2 acts as a switch maintaining the epithelial phenotype and suppressing activation of invasive behavior. These results are, however, limited by the utilization of a single highly malignant PC-3 cell line and should be further confirmed with other cell lines.

The whole-genome microarray analysis demonstrated that HSF2 affects genes related to GTPase signaling and actin cytoskeleton plasticity, and thus connected HSF2 to the key cellular pathways required for cell motility (Figure 5 in III). Modulation of the GTPase effector proteins implicated that HSF2 affects the activity of GTPases during the invasive switch, though the direct mechanism remains to be uncovered. As this study only examined the gene profile at the level of mRNA expression, utilization of more direct methods to assess HSF2-regulated genes during cancer progression would be beneficial. For example, performing HSF2 ChIP-seq at different stages of PC-3 organoid development would yield more information on the genes directly regulated by HSF2 in cancer. To date, only two individual studies have reported for ChIP-seq-based analysis of genome-wide HSF2 binding sites. One of these was performed in non-adherent K562 cells (Vihervaara *et al*., 2013), whereas the other examined HSF2 binding in mouse testis (Korfanty *et al*., 2014). Albeit revealing essential information about the importance of HSF2 in those cells and tissues, the results cannot be directly applied to adherent human organoids.

In this study, we found that lack of HSF2 alters the expression of specific EMT-markers (Figure 6 in III). This places HSF2 in the very center of cancer-specific de-differentiation and suggests that HSF2, among other factors, impacts the transition from epithelial to mesenchymal phenotype. *In vivo* growth analysis in CAM further confirmed that lack of HSF2 promotes invasive properties of PC-3 cells (Figure 7 in III). CAM has been previously used as an *in vivo* model for prostate cancer growth (Kunzi-Rapp *et al.*, 2001). However, CAM is a non-mammalian model system and thus it would be important to confirm our findings in *e.g.* mice xenografts. For such experiments, generation of a stable cell line with inducible HSF2 expression would be essential, since transient downregulation is not sufficient to maintain low HSF2 levels in long-term mouse experiments.

Finally, by analyzing *in silico* the HSF2 mRNA expression in clinical prostate cancer specimens, we showed that low HSF2 expression correlates with high Gleason score (Figure 1 in III). Together with the other findings reported in study III, this indicates that HSF2

functions as a suppressor of prostate cancer progression. Downregulation of putative HSF2 target genes was also detected in the clinical samples, proposing that the HSF2 affected genetic program might serve as a starting point for the development of novel therapeutics. Considering the challenge of correct patient stratification presented in the very beginning, our results propose that similarly to the HSF1 expression that can be used as a biomarker to predict the outcome of specific breast cancer types (Santagata *et al*., 2011), detecting HSF2 levels in prostate cancer biopsies might have a prognostic value in the clinics.

4 HSF2 IN PROLONGED PROTEOTOXIC STRESS (IV)

From the mammalian HSFs, HSF1 is considered as the master regulator of inducible HSP expression and it has been shown to be essential for cell viability upon a variety of acute proteotoxic stress conditions (Gomez-Pastor *et al*., 2018). In contrast, HSF2 is negatively regulated during acute heat stress (Ahlskog *et al*., 2010), and albeit localizing to the *HSP70* promoter, its impact on gene expression is only modulatory (Östling *et al.*, 2007). Interestingly, in response to proteasome inhibition, HSF2 is activated and acquires DNA-binding capacity (Kawazoe *et al.*, 1998; Mathew *et al.*, 1998), indicating that the factor can respond to distinc types of proteotoxic stressors. However, it has been clearly shown that only HSF1 is required for proteasome inhibition induced upregulation HSP70 (Pirkkala *et al.*, 2000; Rossi *et al.*, 2014) and thus the role of HSF2 during proteotoxicity has remained elusive.

The eukaryotic 26S proteasome is a multiprotein complex that consists of a 20S core particle, responsible for peptide cleavage, and a 19S regulatory particles required to recognize the ubiquitinated substrate proteins (Tomko & Hochstrasser, 2013). The ubiquitin-proteasome system is one of the main cellular mechanisms regulating protein turnover and thus it affects multiple aspects of cell physiology, such as signal transduction and apoptosis (Hershko *et al.*, 1998; Varshavsky, 2012). Due to its key role in cell physiology, the proteasome complex has emerged as an important target for anti-cancer therapy and subsequently led to the identification of multiple proteasome inhibiting drugs (Deshaies, 2014). One of the most well-known drug to inhibit proteasome activity is called Bortezomib (PS-341, VELCADE®), which is currently used as a standard treatment in multiple myeloma and other hematological malignancies (Chen *et al.*, 2011). Bortezomib is a dipeptide boronic acid derivative that targets the chymotrypsin-like activity of the 26S proteasome (Kisselev *et al.*, 2006). Bortezomib does not fully inhibit the activity of the proteasome and thereby causes prolonged proteotoxic stress upon long-term treatments (Chen *et al.*, 2011; Goldberg, 2012). Intriguingly, in human blood-derived primary cells Bortezomib treatment has been shown to result in a remarkable upregulation of HSF2 at both mRNA and protein levels (Rossi *et al.*, 2014), suggesting that HSF2 plays a role in the cellular response to prolonged Bortezomib-induced proteotoxicity. However, although HSF2 was shown to localizes to distinct stress-responsive promoters together with HSF1, only HSF1 was required for the Bortezomib-induced expression of these genes (Rossi *et al.*, 2014). Nevertheless, proteasome inhibition has been shown to be severely toxic to both HSF1- and HSF2-deficient mouse embryonic fibroblasts (Lecomte *et al.*, 2010), proposing that HSF1 and HSF2 have unique roles in the cellular protection against proteasome inhibition. Thereby, this study aimed at identifying the role of HSF2 and the unique HSF2-dependent gene expression profile during prolonged proteasome inhibition.

4.1 HSF2 is specifically regulated during proteasome inhibition

This study was initiated by examining how human osteosarcoma U2OS cells respond to prolonged proteotoxic stress induced by Bortezomib. U2OS wild-type (WT) cells were treated with different concentrations of Bortezomib (BTZ) for 6 and 22 h and the HSF2 protein expression levels were examined with immunoblotting. As shown in figure 1A (in IV) HSF2 protein levels were modestly upregulated already after 6 h treatment, whereas a prominent increase in HSF2 levels was detected after 22 h treatment (Figure 1A in IV). HSF2 localization was examined with indirect immunofluorescence, which showed that HSF2 is predominantly nuclear already under normal growth conditions and that BTZ treatment further enhances the nuclear accumulation of HSF2 (Figure 1B in IV). These results are well in line with the observations made by Rossi and co-workers (2014) in human blood-derived primary cells and indicate that HSF2 is a specific responder to BTZ treatment, as both its protein expression levels and nuclear accumulation are enhanced in cells exposed BTZ-induced proteotoxic stress.

4.2 Lack of HSF2 predisposes U2OS cells to Bortezomib

Due to the marked upregulation of HSF2 in cells exposed to prolonged BTZ treatment, we asked if HSF2 is required for cell survival during BTZ-induced proteotoxic stress. To study that, we generated, to the best of our knowledge, the first human HSF2 knockout (KO) cell line, by mutating the first exon of HSF2 gene in human osteosarcoma (U2OS) cells with CRISPR-Cas9 mediated genome editing. This genetic ablation led to a full depletion of HSF2 protein in the U2OS HSF2 KO (2KO hereafter) cell line, whereas the expression of HSF1 remained intact (Figure 1C in IV). The sensitivity of these cells to proteasome inhibition was examined by treating both WT and 2KO cells with different concentrations of Bortezomib (BTZ) and analyzing the effects with microscopy. Already at this stage, we observed dramatic differences in the viability of WT and 2KO cells, as cells lacking HSF2 exhibited de-attached and apoptotic appearance in concentrations were the WT cells were still adherent (Figure 1D in IV). The detected differences were quantified by measuring the cell viability with Calcein AM based fluorometric assay, which confirmed that the 2KO cells are more sensitive to BTZ and die in concentrations ineffective to their WT counterparts (Figure 1E in IV). Moreover, the 2KO cells accumulated more cleaved PARP-1 upon BTZ treatment, indicating that the cells die through apoptosis (Figure 1F in IV). To confirm that the observed decrease in the 2KO cell survival is caused by proteasome inhibition, we repeated the above-described experiments with a well-established proteasome inhibitor MG132 (Figure Supplement 1A-C). The obtained results revealed that the 2KO cells are extremely sensitive also to MG132-induced proteotoxic stress (Figure Supplement 1A-C) and thus verified that HSF2 is required to protect cells during prolonged proteasome inhibition. Importantly, as lack of HSF2 has been previously shown to sensitize HSF2-deficient mouse embryonic fibroblasts (MEFs) to MG132 and to thermal stress of febrile-range (Lecomte *et al.*, 2010; Shinkawa *et al*., 2011), we propose that HSF2 is an important cell survival factor during prolonged proteotoxic stress in both murine and human cells.

Although an efficient and sequence-specific genome editing tool, the guide RNA combined CRISPR-Cas9 nuclease can have off-target effects at sites that closely resemble the on-target sequence (Kim *et al.*, 2015). Thus, to verify that the decreased survival of the 2KO cells is not caused by CRISPR-Cas9 off-target effects, HSF2 was transiently downregulated from the WT cells by transfecting the cells with HSF2 shRNA encoding expression plasmids (described in Östling *et al.*, 2007). Intriguingly, transient downregulation of HSF2 also sensitized the WT cells to BTZ and resulted in impaired cell viability (Figure 1G in IV).

Moreover, HSF2 downregulation enhanced the BTZ-induced accumulation of cleaved PARP-1 (Figure 1H in IV), indicating that the cells are more apoptotic than the WT cells. These results strongly imply that HSF2 is a key cellular survival factor during prolonged BTZ-induced proteotoxic stress.

It is, however, important to note that our results only describe the effect of HSF2 downregulation on one single U2OS cell line. To elucidate if HSF2 downregulation also sensitizes other cell types to BTZ-induced proteotoxic stress, we transfected K562 and PC-3 cells with HSF2 shRNA encoding plasmids and exposed the cells to BTZ. However, we were not able to completely disrupt the expression of HSF2 in these cells (data not shown) and thereby did not observe any impairment of cell viability upon BTZ treatment. These observations likely reflect the strong ability of BTZ to induce upregulation of HSF2 at the mRNA level (Rossi *et al*., 2014), which might override the capacity of shRNA to fully deplete HSF2 mRNA molecules. Thereby, targeting HSF2 at the mRNA level might not be suitable for demonstrating the importance of HSF2 during proteotoxicity in all cell types. Interestingly, Clift and co-workers recently described a novel method to downregulate protein expression at the protein level (Clift *et al*., 2017). The method is called Trim-Away and it is based on TRIM21 E3 ubiquitin ligase, which has high affinity for the Fc-domains of antibodies and thus can rapidly direct the degradation of antibody-bound complexes through the ubiquitin-proteasome system. Since TRIM21 is widely expressed in a variety of cell types, including U2OS and HEK2893T cells (Clift *et al*., 2017), the key requirement of this method is the transfection of target-specific antibodies to the cells *e.g.* by electroporation. Thus, it would be useful to examine, whether Trim-Away-based transient depletion of functional HSF2 protein can sensitize a broader selection of cell types to BTZ treatment.

Proteasome inhibition is a well-known inducer of heat shock response, which is mounted to alleviate the proteotoxic damage through HSF1-mediated expression of HSPs (Gomez-Pastor *et al.*, 2018). Recently, Shah and co-workers reported that expression of HSF1 as well as its phosphorylation on serine 326 are required for the survival of myeloma cells during BTZ-induced proteasome inhibition (Shah *et al*., 2016). To determine whether the decreased viability of 2KO cells is caused by impaired HSF1 expression or phosphorylation on S326, we treated the WT and 2KO cells with BTZ and MG132 and analyzed the levels and the phosphorylation status of HSF1 S326 by immunoblotting with specific antibodies. Intriguingly, we observed no differences in HSF1 expression levels or the phosphorylation status of serine 326 between WT and 2KO cells (Figure Supplement 1D in IV). These results suggest that albeit HSF1 is essential for HSP expression and cell survival during acute stress, it is unable to solely protect cells from apoptosis during prolonged BTZ-induced proteotoxicity. Furthermore, the results propose that HSF1 and HSF2 might provide protection against proteotoxic stress through two different mechanisms.

4.3 HSF2 disruption does not compromise inducible survival programs

Similarly to other surveillance transcription factors, such as the DNA-damage sensitive p53 (Kubbutat *et al.*, 1997) or the oxidative stress sensitive Nrf2 (Kobayashi *et al.*, 2004), HSF2 is an unstable protein under normal growth conditions (Ahlskog *et al.*, 2010) and regulation of its protein expression levels is considered the key mechanism determining its DNA-binding activity (Mathew *et al.*, 1998; Sarge *et al.*, 1994). Due to the prominent upregulation of HSF2 and localization to the nucleus upon BTZ-treatment, we next asked if the decreased survival of HSF2-depleted cells could be caused by misregulation of HSF2 target genes. To study that, we decided to perform a whole genome transcriptome analysis with RNA-seq, on

samples generated form U2OS WT and 2KO cells treated with DMSO or with 25 nM BTZ for 6 or 10 h (Figure 2A in IV). These time points were selected to represent such proteotoxic conditions, where the cell viability is not yet compromised (Figure Supplement 2A in IV). Before proceeding into RNA-extraction the 2KO phenotype and the functionality of the treatment was confirmed by analyzing the expression levels of HSF2, HSF1, and Hsp70 with immunoblotting. As evidenced by Figure Supplement 2B (in IV), we observed no HSF2 in samples generated from 2KO U2OS cells. Moreover, the 2KO cells exhibited equal HSF1 levels and inducible Hsp70 expression compared to WT cells in response to BTZ treatment (Figure Supplement 2B in IV). From the verified samples, the poly-A containing mRNA molecules were purified with poly-T oligo magnetic beads and fragmented under elevated temperatures. RNA fragments were reverse transcribed to cDNA after which the cDNA molecules were ligated to Illumina TrueSeq indexing adapters to generate the cDNA library. Samples were sequenced with the Illumina HiSeq 3000 and the raw sequences were aligned against the hg38 human genome assembly. After normalization, the statistical testing between the sample groups was performed with Bioconductor R package Limma (Ritchie *et al.*, 2015) and the differentially expressed genes were filtered using fold change (FC) ≥3 and false discovery rate (FDR) <0.001 as cutoff. Correlations between the quadruplet samples were determined with Spearman´s metrics (Figure Supplement 2C in IV). According to the analysis, both WT and 2KO U2OS cells responded to BTZ treatment with significant upregulation and downregulation of distinct genes (Figure 2B and Table supplement 1 in IV). The table supplements 1 and 2 can be accessed through the following link, which will be available until June 18th, 2018: https://bit.ly/2EQKyyu.

To identify the putative HSF2-mediated transcriptional survival program, we first compared the genes that were inducibly upregulated and downregulated in WT and 2KO cells in response to BTZ. To our surprise, the inducible gene expression profiles were highly similar between the cell types (Table supplement 1 in IV) and the GO-term analysis of biological processes did not reveal any processes that would be significantly misregulated in the 2KO cells (data not shown). To specifically search for the survival programs that would be altered in the 2KO cells, we next addressed whether induction of autophagy or unfolded protein response would be affected in the 2KO cells. Both processes are known to be induced in response to BTZ (Hideshima & Anderson, 2012) and inhibition of their activity has been linked to reduced cell survival during BTZ treatment (Chang & Wang, 2016). Immunoblot analysis of LC3B-II, a ubiquitin-like molecule widely used as a cellular readout for autophagy levels (Barth *et al*., 2010; Hansen & Johansen, 2011), revealed nearly equal levels of accumulating LC3B-II (Figure 16), indicating that the BTZ-induced autophagy is not hampered in the 2KO cells. Moreover, the normalized gene expression data was used to examine the expression kinetics of distinct target genes associated with the UPR (Walter & Ron, 2011), which clearly showed that the lack of HSF2 does not impair the inducible expression of ER-stress related target genes (Figure 17).

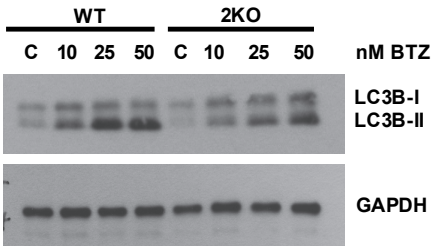

Figure 16. Lack of HSF2 does not affect BTZ-induced autophagy. U2OS WT and 2KO cells were treated with indicated concentrations of BTZ for 22 h and the expression of LC3B-II was examined with immunoblotting. GAPDH was used as a loading control.

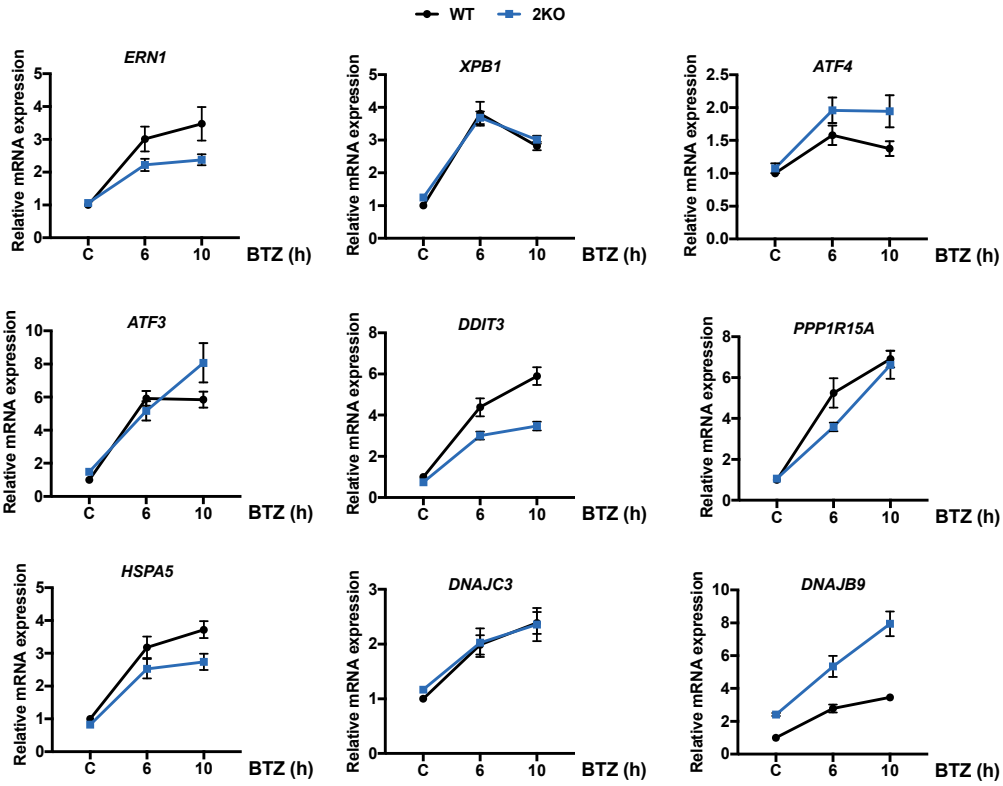

Figure 17. HSF2 disruption does not compromise the induction of the unfolded protein response upon BTZ treatment. mRNA expression levels of selected ER-stress related genes. Normalized ene expression data for *ERN1* (IRE1)**,** *XPB1* (XPB1), *ATF4* (ATF4), *ATF3* (ATF3), *DDIT3* (CHOP), *PPP1R15A* (GADD34), *HSPA5* (GRP78), *DNJAC3* (p58IPK), and *DNAJB9* (ERdj4) was used to analyze the expression kinetics of these genes in control (C) and BTZ-treated (6 or 10 h) WT (black lines) and 2KO (blue lines) cells. The data is presented as mean values ± SEM relative to WT control sample that was set to 1.

Yet another key consequence of proteasome inhibition is the cytosolic accumulation of misfolded and damaged proteins and subsequent initiation of the heat shock response (Bush *et* al., 1997; Mitsiades *et al*., 2002). In contrast to acute heat shock, which predominantly activates HSF1, proteasome inhibition activates all members of the HSF family (Kawazoe

et., 1998). Therefore, we also wanted to determine whether the BTZ-induced activation of the heat shock response would be affected in the 2KO cells. We used the normalized expression data to study the inducible expression patterns of all human molecular chaperone genes (Kampinga *et al.*, 2009) and found that the BTZ-inducible chaperone expression profiles between WT and 2KO cells are nearly identical (Figure 2C in IV). Only *DNAJ12* and *DNAJC18* displayed clearly different expression patterns (Figure 2C in IV). A closer examination of selected *HSP* genes (*HSPA1A*, *HSPA6*, *HSPB1*, and *HSP90AA1)*, revealed that the expression of these genes is equal or even higher in the 2KO cells when compared to the WT cells (Figure 2D in IV). HSF2 has been previously shown to negatively regulate the stress-inducible expression of distinct Hsp target genes (Elsing *et al.*, 2014; Östling *et al.*, 2007), which likely explains the higher expression levels of *HSPB1* and *HSP90AA1* observed in here. In addition to Hsps, HSF1 and HSF2 bind also to the promoters of Hsp90 co-chaperones and polyubiquitin genes in response to heat stress (Vihervaara *et al.*, 2013). To study whether HSF2-depletion would impact the expression of such genes upon BTZ-treatment, the expression of Hsp90 co-chaperones *PTGES3* (p23) and *AHSA1* (AHA1), and the polyubiquitin genes *UBB* and *UBC* was analyzed. Similarly to Hsps, we observed no significant differences in the levels of these genes between the BTZ-treated WT and 2KO cells (Figure 2E in IV), and thereby conclude that the heat shock response is not impaired in cells lacking HSF2. Of note, although HSF2 has been previously shown to be dispensable for the transcriptional induction of specific heat shock genes (Mathew *et al.*, 1998, Rossi *et al.*, 2014), this is the first study to demonstrate the HSF2 is not required for the global induction of heat shock response during proteotoxic stress.

Decreased expression of proteasome subunits has been previously proposed as one of the mechanisms causing the decreased survival of HSF2-deficient MEFs during MG132-induced proteasome inhibition (Lecomte *et al.*, 2010). To examine if the proteasome subunits are significantly downregulated in the HSF2-depleted U2OS cells and thereby contribute to the sensitivity of the cells to BTZ, we used the normalized gene expression data to analyze the mRNA expression of all human proteasome subunits in control and BTZ-treated WT and 2KO U2OS cells. In contrast to the results by Lecomte and co-workers (2010), no significant downregulation of proteasome subunits (either 20S or 19S) was detected in the 2KO cells (Figure 18), suggesting that HSF2 disruption does not predispose U2OS cells to BTZ treatment through misregulated expression of proteasome subunits. Interestingly, reduced expression of the 19S particles has been shown to also protect human cancer cells from proteasome inhibiting treatments (Acosta-Alvear *et al*., 2015; Tsvetkov *et al*., 2015). Although the expression of proteasome subunits was modestly decreased in the 2KO cells, it did not provide any survival advantage to the 2KO cells upon exposure to BTZ. Thereby it is unlikely that misregulated expression of proteasome subunits would cause the drastic survival differences detected between the WT and 2KO cells upon BTZ treatment (Figure 1B in IV). Nevertheless, as RNA-seq only examines the expression of specific genes at the mRNA level, it would be beneficial to determine the expression of proteasome subunits also at the protein level or the overall functionality of the proteasome with a proteasome activity assay.

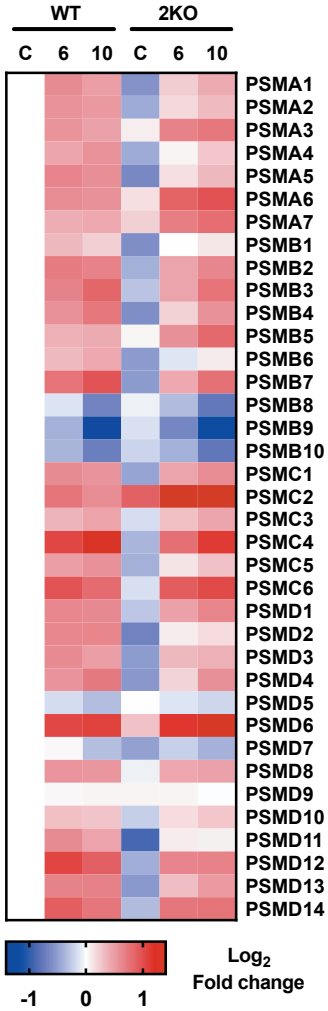

Figure 18. Expression of proteasome subunits is not compromised in HSF2 KO cells. U2OS WT and 2KO cells were treated with 25 nM BTZ for 6 or 10 h. Control cells were treated with DMSO. Gene expression changes were analyzed with RNA-seq. Differentially expressed genes in each comparison pair, were determined with Bioconductor R package Limma (Ritchie *et al.*, 2015) (FC≥3, FDR<0.001). Normalized expression data for all human proteasome subunit genes was used to calculate the fold change of each gene in relation to respective expression in WT control sample. The data is presented as heatmap of $\log_2$ transformed fold changes and it was generated with GraphPad Prism.

Altogether these results propose that the inducible survival programs are not impaired in the 2KO cells and thereby it remains to be established why HSF2 is so prominently upregulated upon BTZ treatment. Since this study mainly relies on analyzing large gene sets that were acquired with stringent cutoff criteria, it fails to unveil the importance of single genes or genes that are more subtly changed in response to BTZ. Moreover, as HSF2 has been shown to act as both an activator and a repressor (Östling *et al.*, 2007) it is possible that HSF2 has a highly complex target gene selection during proteotoxic stress. Since the current literature regarding the HSF2 target genes is fairly scarce, we can only speculate on the importance of distinct genes that were found misregulated in the 2KO cells. However, one gene worth mentioning is *CDKN1A*, which encodes a cyclin-dependent kinase inhibitor 1, p21, a well-established p53 target gene required to arrest cell cycle (Fischer, 2017). Unlike other p53 target genes, which were similarly regulated in BTZ-treated WT and 2KO cells, the 2KO cells failed to induce the expression *CDKN1A* upon exposure to BTZ (Figure 19). This might suggest that the 2KO cells are not able to regulate the progression of mitosis similarly to the WT cells. Considering the notion that HSF2 is downregulated during mitosis (Elsing *et al.*, 2014), it would be interesting to examine, if the pronounced upregulation of HSF2 is required

for stress-induced regulation of mitotic progression and thereby also for cell survival upon such conditions.

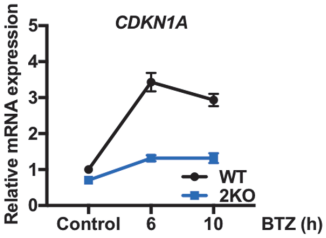

Figure 19. Upregulation of *CDKN1A* is impaired in cells lacking HSF2. Normalized gene expression data was used to analyze the expression kinetics of CDKN1A in control (C) and BTZ-treated (6 or 10 h) WT (black lines) and 2KO (blue lines) cells. The data is presented as mean values ± SEM relative to WT control sample that was set to 1.

4.4 HSF2 affects genes related to cell-cell adhesion

To unravel the differences between the WT and 2KO cells, we next examined the significantly changed genes in 2KO:WT comparison pair at each experimental time point (control, 6 h, and 10 h) (Figure 3A in IV). Interestingly, absence of HSF2 resulted in significant misregulation of 819 genes (272 upregulated, 547 downregulated) already in control conditions, whereas after 6 and 10 h treatments 765 (250 upregulated, 515 downregulated) and 688 (207 upregulated, 481 downregulated) genes, respectively, showed altered expression in 2KO cells (Figure 3B in IV and Table Supplement 1). GO term analysis of the misregulated (both up- and downregulated) genes in each time point revealed specific enrichment of terms related to cell adhesion and included terms such as biological adhesion and cell-cell adhesion *via* plasma membrane adhesion molecules (Figure Supplement 3A in IV). Notably, the terms between all studied comparison pairs were highly similar, strongly suggesting that the lack of HSF2 affects genes relevant to cellular adhesion properties both under normal growth conditions and upon exposure to BTZ-induced proteasome inhibition (Figure Supplement 3A in IV).

To define the genes that are misregulated in 2KO cells in all experimental time points, we generated Venn diagrams of both upregulated and downregulated genes in each comparison pair with the BioVenn web tool (BioVenn, http://www.cmbi.ru.nl/cdd/biovenn/). In total of 114 common genes were found upregulated and 277 common genes downregulated in all comparison pairs (Figure 3B in IV). Analysis of the 114 upregulated genes with DAVID functional cluster annotation tool (Dennis *et al.*, 2003) revealed strong association to cell adhesion and included genes associated with extracellular matrix attachment, such as collagens (*COL16A1*, *COL18A1*), laminins (*LAMB1* and *LAMA5*), and integrins (*ITGB4*) (Figure Supplement 3B in IV). Excitingly, the 277 downregulated genes were linked with GO terms such as cell-cell adhesion, and IPR (protein amino acid and sequence annotation) terms such as cadherin and N-terminal cadherin (Figure 3D in IV), suggesting for involvement of a specific type of cell-cell adhesion molecules, namely the cadherins. The cadherins are essential transmembrane adhesion molecules, which mediate Ca^{2+}-dependent cell-cell adhesion *via* their highly conserved extracellular cadherin domains (reviewed in Hirano & Takeichi, 2012). The human genome encodes 110 cadherin genes, which together form a cadherin superfamily that can be further divided into distinct sub-families. The main role of cadherins is to mediate tissue integrity and they are essential for proper development of multicellular organism (reviewed in Hirano & Takeichi, 2012). Intriguingly, the significantly downregulated genes included members from multiple different cadherin sub-families, such as protocadherins (*e.g. PCDHA1* and *PCDHA7*), desmosomal cadherins

(*DSC2*) and Fat-Dachsous cadherins (*FAT2*), indicating that the cadherin-mediated cell-cell adhesion is misregulated in 2KO cells at multiple levels (Figure 3D in IV). The most prominent sub-family was, however, the protocadherins, as 13 distinct protocadherin genes were found significantly downregulated in all examined comparison pairs (2KO:WT in control, 6 h and 10 h) (Figure 3D in IV).

4.5 Abnormal expression of cadherins in cells lacking HSF2

Due to the relatively stringent cutoff criteria used in this study (FC ≥3, FDR <0.001), the more subtly changed genes were automatically excluded from the analysis. Because cadherins appeared as a completely novel group of HSF2 target genes, we wanted to examine the expression of all the cadherin superfamily genes in WT and 2KO cells in more detail. Normalized gene expression data was used to generate a heat map governing all the currently known cadherin genes in the human genome (reviewed in Hirano & Takeichi, 2012). Surprisingly, by comparing the expression of each gene to the level detected in the WT cells, we observed extensive downregulation of the whole cadherin superfamily, as at least one member from all sub-families was markedly downregulated in 2KO cells (Figure 4A in IV). These included classical cadherins (*CDH2* and *CDH6*), desmosomal cadherins (*DSC2* and *DSG2*), CDH23-PCDH15 cadherins (*CDH12*), Fat-Dachsous cadherins (*FAT2* and *FAT4*), Flamingo cadherins (*CELSR1*), and Calsyntenins (*CLSTN2*) (Figure 4A in IV). The most striking downregulation was detected in the sub-family of clustered α-, β-, and γ-protocadherins, of which 92% were found abnormally expressed in the 2KO cells (Figure 4A in IV). These results indicated that HSF2 is a key regulator of multiple cadherin superfamily genes.

The extensive downregulation of the cadherin genes raises important questions about the mechanism(s) by which HSF2 regulates the cadherin expression. HSF2 is a potent DNA-binding factor and thus it is possible that HSF2 affects cadherin gene expression by directly binding to these genes. Previously, genome-wide HSF2 binding sites have been analyzed with ChIP-seq in human erythroleukemia K562 cells (Vihervaara *et al.*, 2013) and in mouse testis (Korfanty *et al.*, 2014), which are two very different model systems. The non-adherent K562 cells are deficient of endogenously expressed classical cadherins and specific protocadherins (Ozawa & Kemler, 1998) and, perhaps not surprisingly, HSF2 occupies only the *CLSTN* gene in normal growth conditions (Vihervaara *et al.*, 2013). However, upon exposure heat stress, HSF2 binding is observed at members of classical cadherins (*CDH4*), desmogleins (*DSG2)*, Fat-Dachous cadherins (*DCHS2*), Flamingo cadherins (*CELSR2*), and CDH23-PCDH15 cadherins (*CDH23*) (Vihevaara *et al.*, 2013), indicating that multiple cadherin genes are direct HSF2 targets in human cells. Furthermore, in mouse testis, HSF2 occupancy was observed at *CDH15*, *CDH5*, *CDH7-11*, *CDH18*, *CDH20*, *CDH13*, *FAT1*, *FAT4*, *PCDH9*, *PCDH17*, and *PCDHA1* (Korfanty *et al.*, 2014), demonstrating that multiple cadherin superfamily genes are direct HSF2 targets in both murine and human cells. Thereby, it is plausible that the altered cadherin expression observed in 2KO cells in this study, results from lack of HSF2 binding on those genes.

However, as we did not examine the direct HSF2 targets in the U2OS cells, it is also possible that HSF2 affects cadherin gene expression indirectly *via* some other gene regulatory factors. For example, the expression of clustered protocadherins, which were the most extensively misregulated cadherin superfamily members in the 2KO cells, has been previously shown to be regulated by chromatin modifying factors such as CTCF (CCCTC-binding factor)/cohesin-complex (Golan-Mashiach *et al.*, 2012), methyltransferase DNTMB3 (DNA

(cytosine-5-)-methyltransferase 3 beta) (Toyoda *et al.*, 2014), and SETDB1 (SET domain, bifurcated 1) -repressor complex (Jiang *et al.*, 2017). Interestingly, the expression of *CTCF*, cohesin-complex subunits *SMC1A*, *SMC3*, and *RAD21* (Song & Kim, 2017), *DNTMB3,* or *SETDB1* was not altered in the 2KO cells (Figure 20), proposing that the downregulation of clustered protocadherins is not caused by misregulation of these factors in the 2KO cells. Therefore, it would be important to determine (*e.g.* with ChIP-seq) if HSF2 can directly bind to cadherin superfamily genes in U2OS cells and explore the mechanism(s) by which HSF2 regulates the expression of these genes.

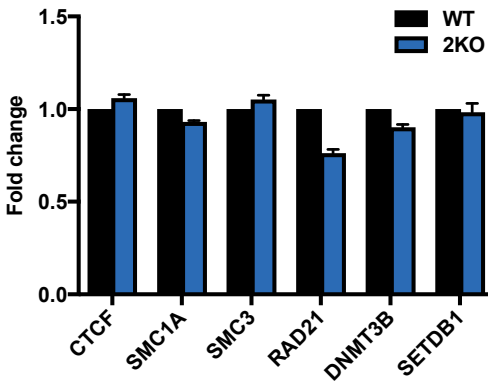

Figure 20. The expression of chromatin modifying factors, previously identified as regulators of protocadherin expression, is not altered in 2KO cells. Gene expression profile of WT and 2KO U2OS cells was analyzed with RNA-seq. Normalized gene expression data for *CTCF*, *SMC1A*, *SMC3*, *RAD21*, *DNMT3B*, and *SETDB1* was used to calculate the fold change of each gene in relation to respective expression in the WT cells. The data is presented as mean values +SEM relative to WT sample that was set to 1.

To determine the biological relevance of the extensive cadherin downregulation, we next analyzed the protein expression levels of γ-protocadherins (Pan-PCDHγA) and N-cadherin by immunoblotting. We observed that both γ-protocadherins and N-cadherin are downregulated also at the protein level in BTZ-treated 2KO cells (Figure 4B in IV). Since cadherins are essential in mediating Ca^{2+}-dependent cell-cell adhesion (Hirano & Takeichi, 2012), their functionality can be assessed with a cell aggregation assay, which measures the cellular ability to adhere to neighboring cells in liquid (Emond *et al.*, 2011). For this, U2OS WT and 2KO cells were suspended in aggregation assay buffer supplemented with either 3 mM $CaCl_2$ or calcium chelator EDTA (ethylenediaminetetraacetic acid) and rotated for 2.5 h at 37°C, 150 rpm. Following that, the cell aggregates were examined with microscopy. Intriguingly, when supplemented with Ca^{2+} the WT cells formed large cell aggregates, which were abolished in the presence of Ca^{2+} -chelating EDTA (Figure 4C in IV). In stark contrast, 2KO cells were completely unable to form cell aggregates even in the presence of Ca^{2+}, clearly demonstrating that the cells are deficient of Ca^{2+}-dependent cell-cell adhesion molecules. Altogether, these results show that HSF2-depleted U2OS cells exhibit cadherin downregulation at both mRNA and protein levels, which results in functional inability to form relevant Ca^{2+}-dependent cell-cell contacts.

Considering the functional impairment of cadherin-mediated cell-cell adhesion in 2KO cells, it is tempting to speculate that the misregulation of cadherins is one of the molecular mechanisms behind the developmental defects, namely disrupted spermatogenesis and corticogenesis, observed in *hsf2*$^{-/-}$ mice (Kallio *et al.*, 2002; Wang *et al.*, 2003, Chang *et al*., 2006). In testis, the seminiferous epithelium lining the lumen of the seminiferous tubules accommodates both the differentiating spermatocytes as well as the Sertoli cells that nurse the differentiating sperm cells. Interestingly, one of the key elements of correct sperm production is the various cell-cell contacts between the Sertoli cells and the differentiating

cells, which not only regulate the polarity of the cells but also direct the cell transport and release from the epithelium (reviewed in Gao & Cheng, 2016). As one of the most prominent spermatogenic defects in the *hsf2*$^{-/-}$ mouse is the accumulation of apoptotic cells, it is intriguing to hypothesize that the lack of appropriate cell-cell contacts leads to misregulation of sperm release and subsequently promotes apoptosis in the developing sperm cells. In addition to spermatogenesis, also corticogenesis is affected in *hsf2*$^{-/-}$ mouse and has been shown to be caused by defects in neuronal migration and positioning (Chang *et al.*, 2006). Though protocadherins are fairly unexplored group of proteins, their role in brain development and neuronal migration has been implicated (Hayaishi & Takeichi, 2015). Since protocadherins were identified in this study as the main cadherin sub-family downregulated in HSF2 KO cells (Figure 4A in IV), it would be interesting to examine, whether the HSF2-dependent misregulation of cell-cell contacts contributes to the defective corticogenesis in the *hsf2*$^{-/-}$ mouse. In support of this, both protocadherin 2A (*PCDH2A*) (Hirano *et al.*, 2002) and HSF2 (Kallio *et al.*, 2002) expression has been detected particularly in the ependymal cell layer forming the lining of lateral ventricles, indicating that the proteins share similar tissue expression patterns in mouse brain. Consequently, it would be extremely interesting to study, if lack of HSF2 results in cadherin downregulation also in *hsf2*$^{-/-}$ MEFs and in *hsf2*$^{-/-}$ mouse tissues and subsequently utilize the *hsf2*$^{-/-}$ mouse to define the physiological relevance of our results.

4.6 Impaired cell-cell contacts predispose cells to chronic proteotoxic stress

Albeit the importance of cadherins has been demonstrated in a variety of developmental systems, their role in the cellular resistance to proteotoxic stress has remained poorly understood. To elucidate if the cadherin-mediated cell-cell adhesions contribute to the cellular resistance against BTZ-induced stress, we re-introduced N-cadherin to the HSF2-depleted cells aiming to restore cell-cell adhesion. As evidenced by figure 5A (in IV), U2OS 2KO cells transfected with the Mock-plasmid displayed lower N-cadherin levels than the Mock-transfected WT cells, whereas in the 2KO cells transfected with the N-cadherin encoding expression plasmids, the levels were modestly increased (Figure 5A in IV). Of note, we were not able to fully rescue the expression to the level observed in the WT cells, which likely was caused by low transfection efficiency and cell survival after transfections (data not shown). Following the transfections, cells were let to recover for 24 h, after which they were counted and re-plated for BTZ treatments. Strikingly, when exposed to BTZ, the N-cadherin transfected 2KO cells accumulated significantly less cleaved PARP-1 than the Mock-transfected cells (Figure 5B in IV), indicating that re-introduction of N-cadherin can increase cell survival during BTZ-induced proteotoxic stress. Since N-cadherin was not the only cadherin downregulated in 2KO cells, we next wanted to study if global destabilization of cadherin-mediated cell-cell contacts can sensitize the U2OS cells to BTZ. For that, U2OS WT and 2KO cells were first treated with 25 nM of BTZ for 20 h in serum free media (SFM) to induce accumulation of misfolded proteins inside the cells. Following day, the cadherin-mediated cell-cell adhesion contacts were destabilized by depleting the extracellular Ca^{2+} with 3 mM EDTA (2 h) and the effect on cell viability was examined with Calcein AM assay (Figure 5C in IV). Intriguingly, Ca^{2+}-depletion was found to enhance the apoptotic effects of BTZ, since the WT cells treated with both BTZ and EDTA exhibited significantly reduced cell survival and more apoptotic phenotype when compared to the EDTA-treated cells (Figure 5C in IV). In 2KO cells the effect of Ca^{2+}-depletion was even more dramatic and resulted in nearly complete abolishment of living cells when combined to BTZ treatment (Figure 5C in IV). Altogether these results indicate, that destabilization of cadherin-mediated cell-cell contacts can predispose U2OS cells to BTZ-induced proteotoxic stress.

It is important to note that Ca^{2+}-depletion does not only disrupt cadherin-mediated cell-cell contacts but affects a variety of cellular features. Thus, we cannot exclude the possibility that the observed results are caused by some other yet unidentified factors and for that it would be beneficial to inhibit the functionality of the cadherins with more specific methods. The challenge is, however, the simultaneous downregulation of multiple cadherin superfamily members in the 2KO cells, which makes *e.g.* siRNA-based downregulation methods unfeasible. Although not really assisting with the challenge of multiple cadherins, one way to specifically inhibit the function of distinct cadherins would be to use subtype specific antibodies to neutralize the cadherin extracellular domains. In principle, such method is highly similar to the Trastuzumab-based inhibition of the HER2 receptor, *i.e.* it utilizes a monoclonal antibody that specifically binds to extracellular domains of transmembrane proteins. In fact, N-cadherin targeting monoclonal antibody GC-4 (manufactured by Sigma-Aldrich), has been successfully used to block N-cadherin-mediated cell-cell adhesion in bone marrow-derived mesenchymal stem cells (Dubon *et al*., 2017) and thus it would be important to examine, whether simultaneous treatment with GC-4 and BTZ also reduces the viability of WT U2OS cells.

4.7 HSF2 protects cells against accumulation of misfolded proteins

In this and other studies, HSF2 has been shown to function as a cellular responder to proteasome inhibition and mediate cell survival during proteasome inhibition induced proteotoxicity (Mathew *et al.*, 2001; Lecomte *et al.*, 2010; Rossi *et al.*, 2014). However, it has remained unclear, if HSF2 activation is caused by proteasome inhibition in particular or if the factor functions as a more general responder to cytosolic protein damage. To study that, we exposed both WT and 2KO cells to amino acid analogue L-canavanine to induce accumulation of damaged proteins without directly affecting the proteasome. U2OS WT and 2KO cells were first starved in L-arginine depleted growth media for 17 h, after which the cells were supplemented with L-canavanine, a structural analogue of arginine. Starvation of the cells for a particular amino acid stalls the translation at the ribosomes, whereas supplementing the cells with an amino acid analogue reinitiates the translation and leads to incorporation of the supplemented amino acid to the growing peptide chain. Incorporation of an incorrect amino acid disturbs the folding of the peptide chain and thus results in accumulation of misfolded proteins in the cytosol. After the treatments, the expression of HSF2 and PARP-1 was examined through immunoblotting and the cells were visualized with microscopy. Excitingly, the WT cells supplemented with L-canavanine displayed higher HSF2 expression than the untreated cells, indicating that the U2OS cells respond also to amino acid analogue-induced stress by upregulating HSF2 (Figure 21A). Moreover, the 2KO cells were found to be clearly more sensitive to L-canavanine, as they accumulated more cleaved PARP-1 and appeared more apoptotic than their WT counterparts (Figure 21A and B). These results show that, similarly to BTZ-induced proteasome inhibition, also amino acid analogue exposure results in upregulation of HSF2 and is more toxic to 2KO cells than to WT cells. As HSF2 DNA-binding activity has been detected during azetidine (a proline homologue) treatment (Sarge *et al.*, 1993), it is tempting to speculate that HSF2 does not specifically respond to proteasome inhibition, but rather functions as a more general surveillance transcription factor monitoring the cytosolic protein folding state.

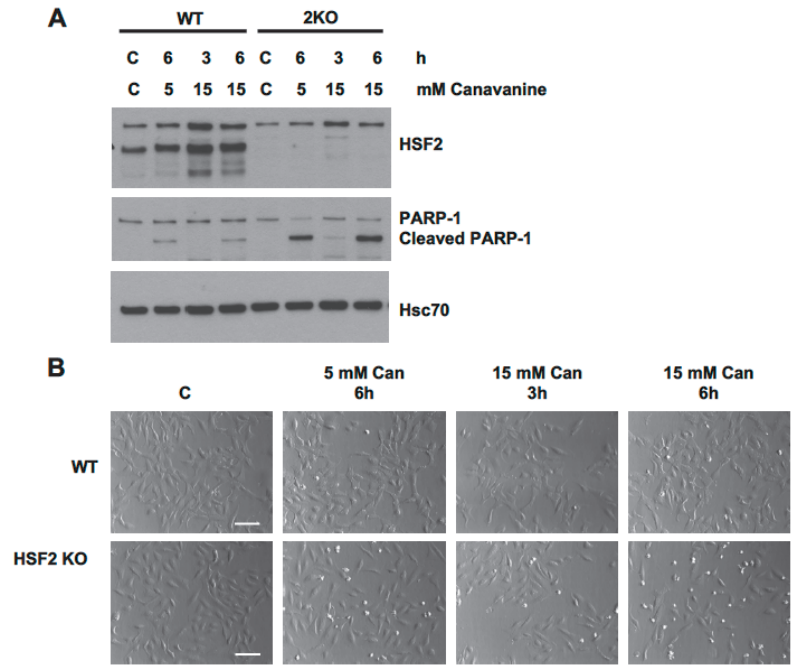

Figure 21. HSF2 is required for cell survival upon amino acid analogue induced proteotoxicity. (A) Cells were starved for 17 h in L-arginine free growth medium (A14431-01, Gibco) supplemented with 10% fetal calf serum and 100 µg/ml penicillin-streptomycin. Following that, L-Canavanine sulfate salt (C9758, Sigma) was applied to cells in indicated concentrations. Cells were treated for 3 or 6 h. Expression of HSF2 and cleaved PARP-1 was analyzed through immunoblotting with indicated antibodies. Hsc70 was used as a loading control. Cells were harvested and visualized with Leica phase contrast microscope. Scale bar 100 µm.

In conclusion, this study revealed that HSF2 is an essential cellular survival factor during prolonged proteotoxic stress. By examining the gene expression profiles of wild-type and HSF2-depleted U2OS treated with BTZ, we found that HSF2 disruption does not impair the induction of classical heat shock response in BTZ-treated cells. Albeit HSF2 has been previously shown to occupy distinct gene loci upon proteasome inhibition, this is the first study to reveal that HSF2 is not required for the global induction of stress-responsive heat shock genes. Excitingly, by comparing the differentially expressed genes between the wild-type and HSF2-depleted cells, we identified cell adhesion as the main biological process disrupted in cells lacking HSF2. Moreover, functional cluster annotation of the HSF2-dependent genes proposed for involvement of a distinct type of cell adhesion molecules, namely the cadherins, which were found extensively downregulated in the HSF2-depleted cells. The most striking downregulation was observed with the clustered protocadherins, of which the great majority was found misregulated in cells lacking HSF2. To the best of our knowledge, this is the first study to identify HSF2 as a key regulator of cadherin gene expression.

By examining the biological impact of cadherin downregulation with a cell aggregation assay, we demonstrated that disruption of cadherin expression results in functional impairment of cell-cell adhesion contacts. This result is particularly important when considering the developmental defects (disrupted corticogenesis and spermatogenesis)

observed with the *hsf2*$^{-/-}$ mouse, which might at least partially be caused by misregulated cell-cell adhesion contacts. Thereby, it would be essential to determine in the future studies, whether HSF2-disruption leads to cadherin downregulation also in other cell types and in mouse tissues. During prenatal exposure to alcohol, HSF2 binding on its target genes is impaired, which leads to brain developmental defects associated with the fetal alcohol spectrum disorder (FASD) (El Fatimy *et al.*, 2014). Recently, Laufer and co-workers examined the FASD specific DNA-methylation patterns and identified clustered protocadherins as one of the main genes affected in FASD diagnosed children (Laufer *et al.*, 2015). Thus, it would be interesting also to examine, whether these two observations are in fact connected and disturbed HSF2 binding is the molecular phenomenon leading to differential methylation pattern of protocadherin genes associated with FASD.

In mammals, HSF2 occupies a great number of target genes (Korfanty *et al.*, 2014; Vihervaara *et al.*, 2013), whose importance in stress resistance has not been characterized. In this study, we re-introduced N-cadherin to HSF2-depleted cells and revealed that cadherin mediated cell-cell contacts are required for cell survival during prolonged proteasome inhibition. To the best of our knowledge, this is the first study to describe the importance of cell-cell adhesion in proteotoxic stress resistance. Moreover, the study demonstrated that HSF2 is not only important during proteasome inhibition but protects cells also against amino acid analogue induced proteotoxicity. Together these results suggested that HSF2 is an essential cellular surveillance factor, which by maintaining the correct cell-cell adhesion contacts, protects the cells against prolonged proteotoxicity. Considering the notion that the cellular inability to maintain proteostasis is a key feature of aging and neurodegenerative diseases (Douglas & Dillin, 2010), it is tempting to speculate that HSF2 might also contribute to the outcome of neuronal proteotoxicity. Intriguingly, by expressing a mutant polyglutamine construct (polyQ81-GFP) in *Hsf2*$^{-/-}$ CEFs (chicken embryonic fibroblasts), Shinkawa and co-workers have demonstrated that lack of HSF2 promotes the accumulation of polyQ-inclusions (Shinkawa *et al.*, 2011). Moreover, in the same study, HSF2-null Huntington´s diseases mice were shown to contain more insoluble polyQ-aggregates in the striatum and have shorter lifespans when compared to the Huntington´s disease mice with wild-type HSF2 expression. These results suggest that HSF2 is required to protect neurons from gradual accumulation of damaged proteins.

Finally, although BTZ was used in this study only as a tool to induce prolonged proteotoxic stress, we cannot ignore the clinical relevance of the molecule and thereby also the possibility that HSF2 might contribute to the outcome of BTZ treatment. Since HSF2 was shown in this study to protect malignant cells from BTZ-induced proteotoxic stress, inhibiting the functions of HSF2 might result in cellular sensitization to the drug. Hence, it would be important to elucidate, if downregulation of HSF2 predisposes also other types of cancer cells to BTZ. BTZ is predominantly used to treat multiple myeloma and mantel cell lymphoma, but it is not similarly effective towards solid tumors (Chen *et al*., 2011). Considering the observations made in this study, one might argue that the solid tumors are more resistant to BTZ because of their proper cell-cell adhesion contacts. Thereby, the possibility that destabilization of cell-cell contacts increases the efficacy of the drug should also be examined. Furthermore, in the third study of this thesis (III), we identified HSF2 as a novel regulator of prostate cancer progression and showed that downregulation of HSF2 is linked to the initiation of invasive behavior. Considering the observations made in this study, it is possible that the downregulation of HSF2 promotes the initiation of invasive behavior by destabilizing the cadherin-mediated cell-cell contacts. Although purely hypothetical, it is also interesting to speculate that if the initiation of invasion requires HSF2 downregulation,

perhaps it is the invasive cells that are the most sensitive to BTZ treatment. Since 90 % of the cancer-associated deaths are caused by metastases (Lambert *et al*., 2017), it would be essential to be able to target specifically the invasive tumor cells that have escaped from the original tumor site to the circulation or lymphatics (*aka.* circulating tumor cells, CTCs). Thereby, the future studies should define whether the invasion-related downregulation of HSF2 sensitizes metastatic cancer cells to BTZ-induced proteotoxic stress.

CONCLUDING REMARKS

Sumoylation is an essential PTM and development of novel unbiased methodologies to study sumoylation is pivotal in our attempts to unravel the complex cellular sumoylome. In the beginning of this thesis work, the methods that were used to identify sumoylated proteins mainly relied on *in vitro* sumoylation reactions and cumbersome identification of putative target peptides by mass spectrometry. Such methods were often challenged by the biological and biochemical properties of sumoylation, which greatly hindered the detection of novel sumoylation substrates. In this thesis, we developed a novel method to study sumoylated proteins *in vivo* in cells. The method is based on engineered human SUMO1 and enables better purification and more accurate identification of both consensus and non-consensus sumoylation substrates. At the time of its publication, the method provided an important advancement to the sumoylation research and many of its biochemical details are still applied in the currently used sumo methodologies.

The second study of this thesis focused on HSF1 phosphorylation, which was the first identified post-translational modification to co-occur with HSF1 activation. For years, hyperphosphorylation was considered as the main modification regulating HSF1 activity and multiple research groups published their studies regarding the importance of single-site phosphorylation in the regulation of HSF1. We aimed at elucidating the importance of HSF1 hyperphosphorylation by generating a phosphorylation deficient HSF1 mutant and investigating its functionality during heat shock response. The study demonstrated that HSF1 phosphorylation is not required for the inducible heat shock response, but functions as a fine-tuning mechanism for the heat-inducible transcription of Hsps. Our observations are elementary to the HSF1-field and provide a conclusion to the reports implicating phosphorylation as a key requirement of HSF1 activity. However, many questions still remain open. For example, we do not understand how phosphorylation mediates the observed effects and whether these findings are applicable also in specific developmental and disease contexts.

Due to its strong ability to enhance cell survival, HSF1 is a potent inducer of carcinogenesis and overexpression of HSF1 has been detected in multiple human cancer types. In contrast, the importance of HSF2 in cancer progression was not known prior to study III of this thesis. We showed that decreased HSF2 mRNA expression correlates with prostate cancer progression and that HSF2 downregulation occurs simultaneously with the initiation of invasive behavior of PC-3 prostate cancer organoids. By modulating HSF2 expression levels, we demonstrated that HSF2 maintains the epithelial phenotype in organoids and suppresses invasion. The study identified key cellular pathways affected by HSF2 in prostate cancer cells and proposed that decreased HSF2 expression in a general characteristic of human malignancies. As the levels of both HSF1 and HSF2 appear to be aberrant in cancer, it would be important to clarify how the expression of these factors is mechanistically regulated. From the clinical perspective, further studies are required to elucidate whether HSF2 could serve as a novel biomarker for prostate cancer progression.

The final study of this thesis aimed at understanding the importance of HSF2 in the cellular response to prolonged proteotoxicity. Prior to this work, HSF2 had been shown to be activated in response to proteasome inhibition induced proteotoxic stress, but the mechanisms by which HSF2 protects cells were unknown. In this work, we utilized genome-wide gene expression profiling (RNA-seq) to examine the differentially expressed genes

between wild-type and HSF2-depleted U2OS cells and observed significant alterations in genes related to cell adhesion. More specifically, we found that the cadherin superfamily genes are the main genes misregulated in cells lacking HSF2 and demonstrated that disruption of proper cadherin-mediated cell-cell contacts predispose human cells to proteasome inhibiting treatments. By describing a previously unidentified class of HSF2 target genes the study proposed a completely novel role for HSF2 in cellular physiology and identified a new determinant of stress resistance. Altogether, this work lays the ground for the future studies examining the molecular details behind HSF2-mediated developmental defects and thus provides essential information regarding the importance of HSF2 in human physiology and disease.

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Regulation of Cellular Stress Proteins in Physiology and Disease

This Ph.D. thesis describes a method to study sumoylated proteins in cells and investigates the role of heat shock transcription factors (HSFs) in stress and disease conditions. Post-translational modifications, such as phosphorylation or sumoylation, mediate rapid signal-responsive alterations in protein function and have multiple target substrates in mammalian cells. In this thesis, a modified human SUMO-1 is used as a basis for a method that can be used to identify novel sumoylation substrates in cells. Phosphorylation, in turn, is examined in the context of HSF1, which is hyperphosphorylated in response to proteotoxic stress. On the contrary to the prevalent view, this thesis reveals that HSF1 hyperphosphorylation is not essential for its activity but rather fine-tunes the HSF1-mediated transcriptional response. In addition to HSF1, the mammalian cells express another HSF-family member, HSF2, whose role in specific biological processes is still partially uncharacterized. In this thesis, HSF2 is identified as an important regulator of prostate tumorigenesis and thereby this work describes a novel role for heat shock factors in human diseases. One of the main findings of this thesis presents HSF2 as a key survival factor upon proteotoxic stress and identifies not only a completely novel group of HSF2 target genes but also a previously unknown determinant of proteotoxic stress-resistance. Altogether the work presented in this thesis elaborates on HSF-mediated cellular survival pathways and lays a ground for future studies regarding HSFs as important regulators of human physiology and disease.

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