

SENSING MECHANISMS AND INDIVIDUALITY OF HEAT STRESS IN MAMMALIAN CELLS

Ph.D. Thesis

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ABBREVIATIONS

ASK1	Apoptosis signal regulating kinase 1
ATT	Acquired thermo tolerance
ATF6	Activating transcription factor 6
ATP	Adenosine triphosphate
AMP	Adenosine monophosphate
AMPK	AMP activated kinase
Cer	Ceramide
CL	Cardiolipin
CSR	Cellular stress response
DAG	Diacylglycerol
DDL1/2	DAF-16(Forkhead box-O transcription factor homologue in <i>C.elegans</i>) dependent longevity 1/2
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
ERK 1/2	Extracellular signal regulated kinase 1/2
FAK1	Focal adhesion kinase 1
GFP	Green fluorescent protein
GlCer	Glucosyl ceramide
GM3	Monosialodihexosylganglioside
GPI	Glycosylphosphatidylinositol
GRP78	Glucose-regulated protein 78
GSK3 β	Glycogen synthase kinase 3- β
GSTM1	Glutathione S-transferase Mu1
GTP	Guanosine triphosphate
HBSS	Hank's buffered salt solution
HSP	Heat shock proteins
HSR	Heat shock response
HSF1	Heat shock factor 1
IGF-1	Insulin-like growth factor 1
IGF1R	Insulin-like growth factor receptor 1
IR	Insulin receptor
IRE1	inositol-requiring transmembrane kinase/endonuclease
IRS	Insulin receptor substrate
JNK	c-Jun N terminal kinase
LPA	Lysophosphatidic acid
LPC	Lysophosphatidylcholine
LPI	Lysophosphatidylinositol
MAPK	Mitogen activated protein kinase

MEK	MAPK/ERK kinase
mTOR	Mechanistic target of rapamycin
PA	Phosphatidic acid
PBS	Phosphate buffer saline
PDK 1/2	Phosphoinositide dependent kinase 1/2
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphoinositol
PS	Phosphatidylserine
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphoinositide 4,5-bisphosphate
PIP3	Phosphoinositide 3,4,5-triphosphate
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
PLD	Phospholipase D
PN	Proteostasis network
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SAPK	Stress activated kinase
SH2	Src homology 2
TRPV1	Transient receptor potential vanilloid 1 ion channel
UPR	Unfolded protein response

1. INTRODUCTION

"Anything that causes stress endangers life, unless it is met by adequate adaptive responses; conversely, anything that endangers life causes stress and adaptive responses. Adaptability and resistance to stress are fundamental prerequisites for life, and every vital organ and function participates in them."
(Selye, 1950)

1.1. Stress

In 1936 a single author letter written by Hans Selye was describing the first observations on stress which initiated a still growing wave of research. He defined stress as a non-specific response of the body to any demand made upon it and according to his notion stress is a part of life that cannot be avoided and would be undesirable to avoid since it is an inducement, however the organism experiencing a stressful episode will pay for its survival by aging or by becoming susceptible to diseases (Selye, 1950).

According to general adaptation syndrome he defined, as organisms are stressed an alarm stage begins which pursues the organism to the fight-or-flight response. For instance, an animal facing a predator will start secreting stress hormones and mobilize energy resources towards muscles so that it can run faster. However organisms that are sessile will activate cellular machineries to minimize the damage caused by the stressors. In the second stage, resistance; the organism is still on an alerted state and continues to reduce the effects of stressors, but energy is consumed to repair the damages originated. Third stage exhaustion is when the stressor persists, so that the organism exhausts resources and becomes susceptible to disease and death (Szabo, Tache, & Somogyi, 2012).

Nevertheless, it is beneficial for the organism to be exposed to a certain level of stress (positive stress/eustress) as it results in increased survival in a subsequent more severe stress. It is important to note another observation Selye made is that the perception of stress and response given to it is highly individualized. A stressor that is mild to an organism can be severe for another one or the extent of defense

may not be the same. Thus both in the multicellular organism and cellular level, the stressor and response must be studied for the better understanding of stress.

1.1. *Cellular Stress Response*

All organisms are challenged by environmental changes such as fluctuations in the temperature, salinity (Krasensky & Jonak, 2012), osmotic pressure (Ho, 2006), high cell density, exposure to heavy metals (Flora, Mittal, & Mehta, 2008), hypoxia/ischemia (Majmundar, Wong, & Simon, 2010) as well as pathological conditions such as infection or inflammation (Muralidharan & Mandrekar, 2013). Cells monitor changes on the macromolecules and cellular stress response (CSR) can be defined as the cells' response towards the damage. Protein aggregation, DNA damage or lipid related changes such as raft disruption or lipid oxidation can trigger cellular stress response (CSR) (Kültz 2005). Depending on the type of the stressor cells can activate different responses which are grouped as heat shock response (HSR), unfolded protein response and DNA damage response. However these responses are interconnected and share common elements (Fulda et al. 2010).

Contingent upon the level of stress and organisms capacity of resources, cells activates mechanisms to minimize the damage and re-establish homeostasis unless damage is severe and cannot be reverted thereby pathways leading to cell death are turned on (Sørensen, Kristensen, & Loeschcke, 2003). The persistence of cells will result in the production of heat shock proteins (HSP), which leads to improved survival for a secondary more severe stressful condition. Interestingly cross-tolerance is possible, the secondary stressor does not essentially be of the same type of stressor (Ely, Lovering, Horowitz, & Minson, 2014; Lindquist, 1986).

1.2. *Stress Sensors*

Cells have several sensors to detect the damages occurred on the macromolecules and these machineries can activate stress signaling to produce stress response.

1.2.1. Protein Unfolding

Proteins as important macromolecules of the cells are involved in vast array of functions that are highly dependent on the configuration and 3-D structure. Therefore it is not a surprise that cells have quality control mechanisms to ensure the presence of functioning proteins and to degrade the misfolded, non-functional proteins. Indeed, there is a constant flux of synthesis and degradation in the cells. Maintenance of the balance between these two states is called proteostasis and it is preserved by proteostasis network (Figure 1.3) (Balch et al. 2008). Translational and transcriptional errors, incorporation of amino acid analogs, mutations and post translational defects are factors affecting the folding of proteins. Other factors such as change in the redox balance, temperature and local variations in ionic strength can directly or indirectly cause a flux of unfolded/aggregated proteins (Gidalevitz, Prahlad, and Morimoto 2011). Unfolded protein response and heat shock response mechanisms can be activated by the increased unfolding/aggregation.

Endoplasmic reticulum (ER) is the major organelle where secretory and endomembrane proteins are targeted to meet their final tertiary and quaternary structure (e.g. with the addition of disulphide bonds) and posttranslational modifications (e.g. glycosylation) (Vitale, Ceriotti, and Denecke 1993). Perturbation of ER function can be monitored in several ways;

In ER, Ca^{2+} is found mainly bound to high Ca^{2+} affinity proteins. Disruption of these molecules result in perturbation of ER-luminal Ca^{2+} which causes the calnexin oligomerization and its interaction with protein disulphide isomerase and calreticulin to initiate UPR signaling (Schröder and Kaufman 2005).

Formation of disulphide bonds requires oxidation and reduction reactions, the disruption of the protein folding machinery, therefore will impair the redox balance (Bulleid 2012).

Disruption in the glycosylation of proteins in one hand causes protein flux towards cytosol for degradation but also results in a transmembrane ER protein activating transcription factor 6 (ATF6) to be underglycosylated which itself serves as a sensor to activate UPR (Carrara, Prischi, and Ali 2013).

Interaction of unfolded proteins with ER resident chaperones themselves also results in dissociation of the chaperones from sensors like IRE1 (inositol-requiring transmembrane kinase/endonuclease) and results in spontaneous dimer formation, phosphorylation and activation of signalling pathways (Hetz et al. 2011).

Due to basic thermodynamic principles the elevation of temperature may also causes denaturation of proteins. In the cytoplasm, misfolded/denatured proteins are refolded by chaperones or eliminated by ubiquitin-proteasome machineries. However, accumulation of denatured proteins activates HSF1 (heat shock factor -1) through a mechanism currently still in debate. Under normal conditions, HSF1 primarily found as monomers bound to HSP70 or HSP90. Protein denaturation causes the dissociation of HSF1 from HSPs and result in formation of active trimers to be translocated to the nucleus to induce heat shock protein genes (Morimoto 1998).

Nucleus is the largest organelle of the cell and is crucial for many activities to occur e.g. transcription, processing of RNA (Bański, Kодиha, and Stochaj 2011). During stress chaperones are transported to the nucleus promptly after temperature elevation. Even though the exact mechanism of the transport and the trigger is unknown, nucleolus is suggested to be an important place where prevention of aggregation of proteins is crucial therefore a possible place for a stress sensor (Shibata and Morimoto 2014).

1.2.2. Depletion of ATP

Proteins that are involved in the stress management use energy to repair the damage. Depending on the level and duration of the stress, adenosine triphosphate (ATP) production by mitochondria may not be sufficient or it may be unfunctional. Cells have active mechanisms to sense the level of adenosine monophosphate (AMP)/ATP ratio. AMPK (AMP activated kinase) orchestrates cells' energy sources and also the stress response based on the AMP/ATP ratio. AMPK is allosterically regulated; binding of AMP activates the complex making it more susceptible for phosphorylation. Phosphorylated AMPK can activate signalling pathways to restore

ATP supplies and limit the ATP consuming biosynthetic processes (Hardie 2011; Hardie, Ross, and Hawley 2012; Chou et al. 2012).

Heat stress, hypoxia and glucose deprivation are stressors that cause ATP depletion. Moreover chaperones that have ATPase activity for the folding proteins are interconnected to ATP depletion mechanisms (Peng et al. 2005).

1.2.3. *Reactive Oxygen Species*

There is a balance of ROS production and antioxidant capacity of the cells. However stresses such as heat, UV radiation and hypoxia are known to impair this balance and reactive oxygen species damage macromolecules such as proteins, lipids and nucleic acids (Belhadj Slimen et al. 2014; Akbarian et al. 2016; Yang et al. 2010). Hydrogen peroxide (H_2O_2), hydroxyl radical (OH^\cdot) and superoxide (O_2^\cdot) are among the molecules which are produced when cells are stressed. Oxygen radicals can be formed with the radiolysis of water with ionizing radiation whereas ROS can be formed endogenously within cellular compartments such as mitochondria and endoplasmic reticulum. ROS induced damage in DNA such as formation of double-strand breaks can result in apoptosis (Takahashi et al. 2008). Lipid autoxidation is initiated by free radicals such as hydroxyl radical and polyunsaturated fatty acid residues mostly in their *cis* configuration are either converted to *trans* configuration or electrophilic aldehydes are formed (Krumova and Cosa 2016).

Like any other stress signals, ROS can transduce signals through protein tyrosine kinases, serine/threonine kinases, phosphatases and calcium (Ben Mahdi, Andrieu, and Pasquier 2000).

1.2.4. *Membranes as Sensors*

Cell membranes are highly dynamic and complex structures formed mainly by lipids and proteins. Being more than a barrier to the external polar environment, they perform wide variety of important cellular functions. The most abundant lipids

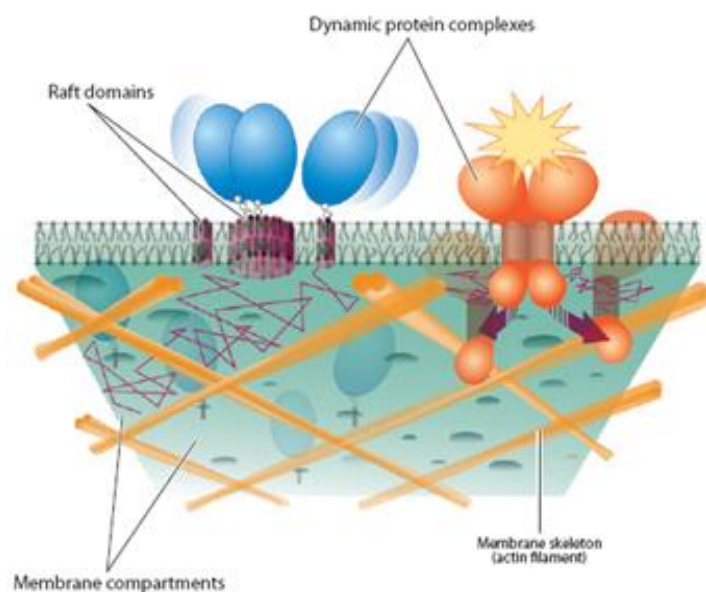


Figure 1.1 Representative model for plasma membrane architecture (adopted from (Kusumi et al. 2012)).

in the membranes are glycerophospholipids, of which more than thousand different species are involved to create the necessary environment to host membrane proteins (Hung and Link 2011). Sphingolipids and cholesterol are tightly packed lipid classes with high melting temperatures and capability of resisting mechanical stress. Their mixtures with glycerophospholipids form membrane rafts/domains that are 10-200nm size, highly dynamic and heterogenous which can be stabilized to form larger platforms through protein-protein interactions. Membranes are therefore highly structured for the necessary functions and their composition is unique to each compartment/organelle. Plasma membrane is highly heterogenous by means of nanoscale organization of lipids, membrane proteins and cortical actin network (Levental, Grzybek, and Simons 2011; Sezgin et al. 2012; Sezgin, Gutmann, et al. 2015)

Another very critical membrane feature is its fluid structure that also contributes to its complexity and dynamic attribute. Fluidity allows molecules to diffuse and rotate freely within the membrane meanwhile the variation of lipid composition in nanoscale compartments permits molecules to interact and influence each other (Bernardino de la Serna et al. 2016; Klotzsch and Schütz 2013; Simons and Sampaio 2011).

Membrane sensor theory is based on these important features. Lipid-phase behaviour is dependent on the temperature hence the fluidity of the membranes and redistribution of the rafts. Since the aforementioned nano-scale domains are influenced by lipid alterations, signalling pathways are either activated or inhibited, new interactions between proteins are formed and cells activate mechanisms to stabilize the highly dynamic organization of their membranes by remodelling lipid composition under stress. (Török et al. 2014; Sezgin, Waithe, et al. 2015). Recently, considerable evidence has been accumulated to the favor of the “Membrane sensor hypothesis” which predicts that, the level of HSPs changes as a result of alterations to the plasma membrane. This is especially pertinent to mild heat shock, a condition present during fever (Török et al. 2014; Nagy et al. 2007). It is conceivable that sensor(s) evolved in parallel to the fever response in vertebrates by detecting the elevation of temperature via membrane thermometers without any recognizable damage to the main cellular functions, thereby supplying the evolutionary basis for the membrane sensor theory.

1.3. *Stress Activated Signalling Pathways*

The orchestration of heat shock protein response is a rapid, highly regulated cellular function, which involves coordinated control of multiple signal transduction pathways. The mitogen activated protein kinases (MAPK) and protein kinase B (Akt/PKB) pathways are the most notable heat activated signalling pathways. Extracellular-signal regulated kinase 1/2 (ERK 1/2), c-Jun N-terminal kinases (JNK) and p38MAPK are Serine/Threonine kinases that are activated upstream of HSF1 phosphorylation and HSP gene transcription. However the direct link with a trigger that activates these pathways is not known.

1.3.1 ERK pathway

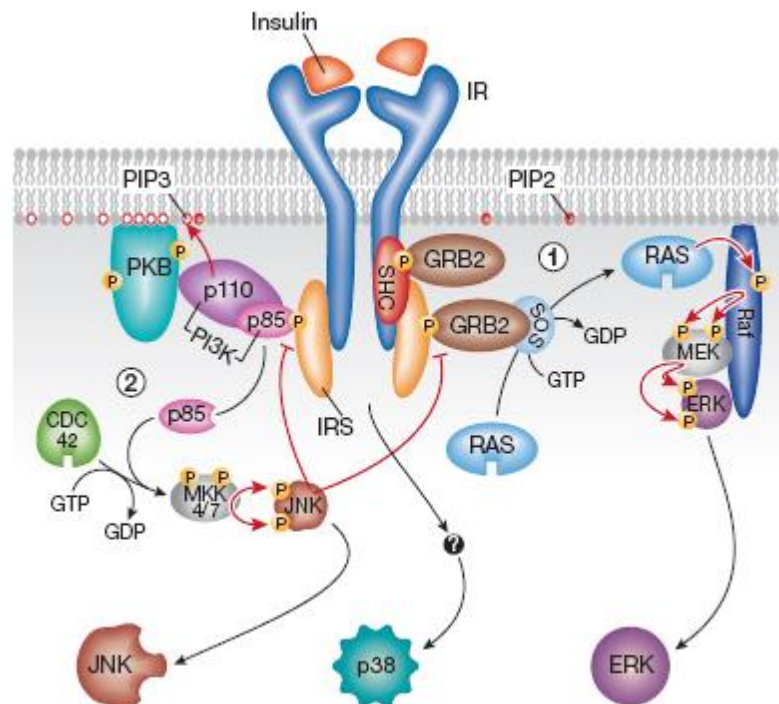


Figure 1.2. Mechanisms of receptor activated MAPK pathway (adopted from Gehart, Kumpf, Ittner, & Ricci, 2010.) CDC42, cell division cycle 42; ERK, extracellular-signal regulated kinase; GRB2, growth factor receptor-bound protein 2; GTP/GDP, guanosine-tri-(di)phosphate; IRS, insulin receptor substrate; JNK, Jun N-terminal kinase; MEK, MAPK/ERK kinase; MAPK, mitogen-activated protein kinase; MKK4/7, MAPK kinase 4/7; PI3K, phosphatidylinositol 3 kinase; PIP3/PIP2, phosphatidylinositol-tri-(di)phosphate; PKB, protein kinase B; SHC, Src homology 2 domain containing; SOS, son of sevenless.

The primary activation of ERK 1/2 (p44 and p42 respectively) are by cell surface receptors such as epidermal growth factor receptors, receptor tyrosine kinases and may result in differentiation or proliferation depending on the duration of the signals (Marshall 1995). Cell surface receptors are dimerized due to external stimuli e.g. ligand binding that results in autophosphorylation of the tyrosine kinase domains and recruitment of small GTPases to plasma membrane. Small GTPases are 21-30kDa monomeric guanine nucleotide binding proteins belonging to Ras superfamily with remarkable diversity of structure and functions, thus diverse effectors (Yang et al.

2010). ERK 1/2 is activated by the Ras-Raf-MEK pathway (Cargnello and Roux 2011). Mechanical stretch, hyperosmolarity, heat and hydrogen peroxide can activate receptor activated Ras-Raf-MEK-ERK 1/2 pathway. In the mechanical stress studies the inhibition of growth factor receptors by suramin blocked the activation of ERK. The dominant mutant Raf-1 has also shown to have inhibitory effects on the same pathway (Richard Z. Lin et al. 1997; Hong et al. 2006).

An alternative pathway for the activation of ERK is receptor independent. If the cells were exposed to a severe stress in which either the duration is longer or the temperature is higher, the activation of MEK decreases. However, the dual phosphatases which controls the level of phosphorylated ERK becomes inactive due to heat induced aggregation/insolubilization causing an increased levels of phosphorylated ERK (Yaglom et al. 2003). It is also interesting that overexpression of HSP70 can stabilize these phosphatases and also by competing with an Raf activating protein all of which abolishes ERK activation by heat (Song, Takeda, and Morimoto 2001; Yaglom et al. 2003).

Nevertheless, ERK activation is generally accepted as a pro-survival signal for the cells. The inhibition of MEK activation or expression of dominant negative mutant decreases the cell survival (Nadeau and Landry 2007).

1.3.2 JNK pathway

JNK pathway can partially be activated by receptor tyrosine kinases through phosphoinositide 3-kinase (PI3K). It converts phosphoinositide (PI) 4,5-bisphosphate (PIP2) to PI 3,4,5-triphosphate (PIP3). PI3K regulatory unit is recruited to the plasma membrane by the adapter protein IRS and phosphorylated receptor tyrosine kinase (Gehart et al. 2010). Suramin which is an inhibitor for growth factor receptors can completely block the activation of JNK by UV and hyperosmotic stress (Dorion and Landry 2002). Other pathway involves a small GTPase Rac1 activation and recruitment to the membrane. Mild stress is shown to recruit Rac1 to the plasma membrane and causes the phosphorylation of JNK. The dominant negative mutant of

Rac1 inhibits JNK activation significantly, however this effect is abolished as the temperature increases (Han et al. 2001).

Main trigger for JNK is thought to be due to inactivation of phosphatases under stressful conditions and it is usually considered as apoptotic signal (Nadeau and Landry 2007). Interestingly HSP70 when overexpressed or induced after stress is shown to protect heat denatured phosphatase, therefore can be responsible for the increased survival and thermotolerance (K. H. Lee et al. 2005; Gabai et al. 1997).

1.3.3 p38 MAPK pathway

p38 MAPK pathway can be activated through variety of stresses including heat stress, UV radiation, hypoxia, ischemia and oxidative stress. In contrast to ERK and JNK pathways, it is not activated through membrane receptors. Activation of p38 is not abolished by suramin or a dominant negative EGFR mutant nor requires the phosphatase inhibition (Nadeau and Landry 2007; Dorion and Landry 2002). In fact, it requires specific canonical MAPK kinases and share common elements with JNK activation. Apoptosis signal regulating kinase 1 (ASK1), a MAPK kinase kinase is shown to activate p38 and JNK when cells are exposed to heat through the titration of thioredoxin and GSTM1 (Dorion, Lambert, and Landry 2002). Oxidative stress causes the thioredoxin dissociation from Ask1 and heat stress causes GSTM1 which creates a distinction on stress sensing pathways and p38 activation. Finally HSP70 has regulatory effects on p38 activation which can be linked to the increased survival of second heat shock or thermotolerance (Gabai et al. 1997).

1.3.4 Akt/PKB pathway

Akt activity can be modulated by several players. Two phosphorylation sites found on Akt are subjected to phosphorylation and dephosphorylation by two different kinases and phosphatases. Thr308 is found on the catalytic domain and Ser473 is found on the hydrophobic domain which is a non-catalytic part (Bayascas and Alessi 2005). Even though it is suggested that maximal Akt activity is obtained when both

residues are phosphorylated, depending on the severity of the signal and interaction of chaperones it can be modulated differently

Activation of Akt/PKB pathway by heat stress results in survival signals as of ERK pathway. Its activation is linked to PI3K by receptor tyrosine kinases. PI3K activation results in accumulation of PIP3 which grounds Akt to interact with the plasma membrane and causes the phosphorylation by PDK 1/2 on threonine 308 (C.-M. Hung et al. 2012). The treatment of the cells with PI3K inhibitors completely blocks the activation of Akt and causes the apoptosis of heat shocked cells (Nadeau and Landry 2007; Shaw, Cohen, and Alessi 1998).

GSK3 β is another kinase which has important roles in the regulation of heat shock response and HSF1 repression (Takaki and Nakai 2016). Akt phosphorylation has an inhibitory effect on it, therefore indirectly stimulates HSF1. It is also shown to inhibit ASK1 activation of p38, therefore attenuates the apoptotic signals (Zhang et al. 2005). However in the case of severe stress inhibition of Akt by PI3K inhibitors did not result in p38 inhibition nor inhibition of p38 did not block the Akt activation (Bijur and Jope 2000).

Phosphorylation of Akt on Serine 473 is catalyzed by mTOR complex and the stimulation abolished in serum deprived cells (Sarbasov et al. 2005; Bayascas and Alessi 2005). Severe ER stress caused an increase in Ser473 phosphorylated Akt and it is regulated by an ER chaperone GRP78 (Yung et al. 2011). HSP90 and HSP27 are among the binding partners of Akt with similar effects where HSP27 promotes the binding of activating kinases and HSP90 prevents dephosphorylation. HSP70 on the other hand causes Akt degradation (Koren et al. 2010; Konishi et al. 1997; S. Sato, Fujita, and Tsuruo 2000).

1.4. Heat Shock Response

Depending on the severity of the stress a decision is made the cells either activate protective mechanisms and induce set of genes that are called heat shock proteins or cell death occurs via activation of apoptotic pathway. Minimal stress proteome

defines cells capacity and includes proteins involved in sensing of stress, redox control, cell cycle control, macromolecular repair and energy metabolism (Kültz 2005). Essential part of the stress proteome is heat shock proteins. They assure the cellular protein homeostasis (proteostasis) by aiding in the proper folding of de novo synthesized proteins or by preventing aggregation therefore assuring a functional proteome (figure 1.3) (Gidalevitz, Prahlad, and Morimoto 2011).

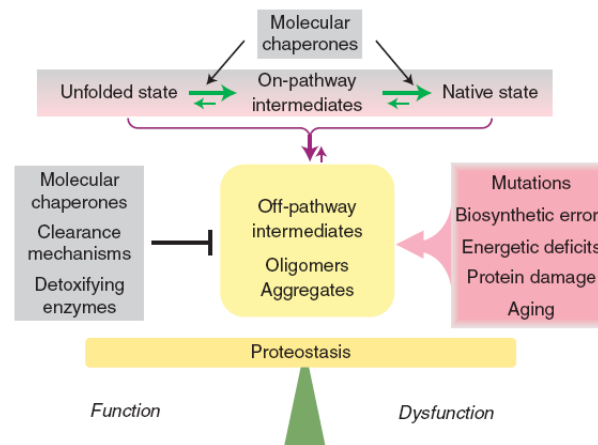


Figure 1.3. Cellular Proteostasis (adopted from Gidalevitz et al., 2011)

1.4.1. Heat Shock Factor

Induction of heat shock proteins are tightly regulated at the transcriptional level by heat shock factors. Stress activates HSFs to bind to heat shock elements which are short 14bp promoter elements found upstream of heat shock genes. Binding of HSF1 requires its transition from the HSF monomer-HSP complex to the trimer state. It is still a debate how this transition occurs, however removal of HSPs from the complex and binding of non-coding-RNAs by heat are possible explanations. Among the HSP families, HSP70 and HSP90 are reported to be binding partners of HSF1. When cells are stressed the repression of HSPs on HSF1 activation is released and the chaperones switch their interaction to unfolded protein and protein complexes (Figure 1.4) (Calderwood 2010; Velichko et al. 2013).

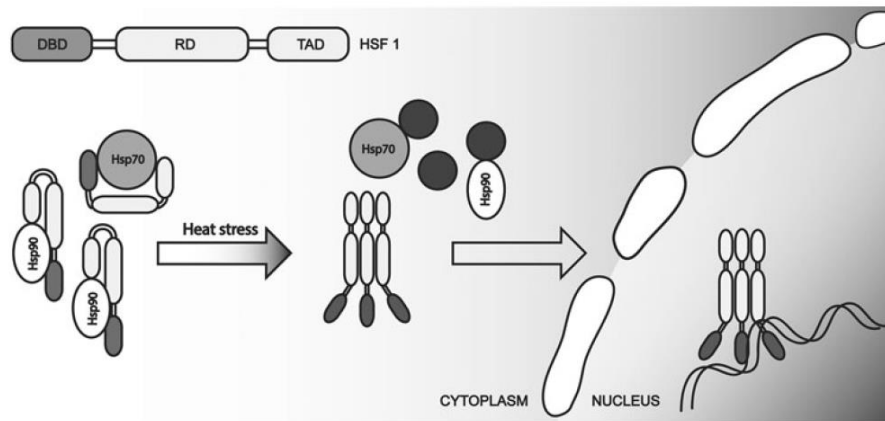


Figure 1.4. HSP interaction with HSF1 upon stress (adopted from Velichko, Markova, Petrova, Razin, & Kantidze, 2013).

HSF1 is composed of highly conserved DNA binding domain, oligomerization domain, nuclear localization signal and regulatory domain (transcriptional activation domain). Nuclear translocation signals are required for the transport of HSF1 and unique trimer structure allows it to bind to DNA with high (Vihervaara and Sistonen 2014). Activation and regulation of HSF1 is rather complex; sumoylation, acetylation, thiol oxidation and phosphorylation are known to be involved in its regulation (Dayalan Naidu and Dinkova-Kostova 2017). While the formation of HSF1 trimer and its binding to DNA is not sufficient for the transcription of genes, the hyperphosphorylation of HSF1 is shown to be required for the transcriptional competent state and for enhanced activity (Guettouche et al. 2005). On figure 1.5 known phosphorylation sites and the enzymes reported to phosphorylate those sites are shown.

Serine 303 and Serine 307 are the constitutively phosphorylated residues required for the repression of transcriptional activity, stability of HSF1 and further sumoylation at Lysine 298. Phosphorylation on Serine 121 is also causing the repression of HSF1 by promoting HSP90 binding. It is also important to note that without the regulatory domain, HSF1 is constitutively transcriptionally competent, therefore this domain may also be regarded as stress sensor (Dayalan Naidu and Dinkova-Kostova 2017).

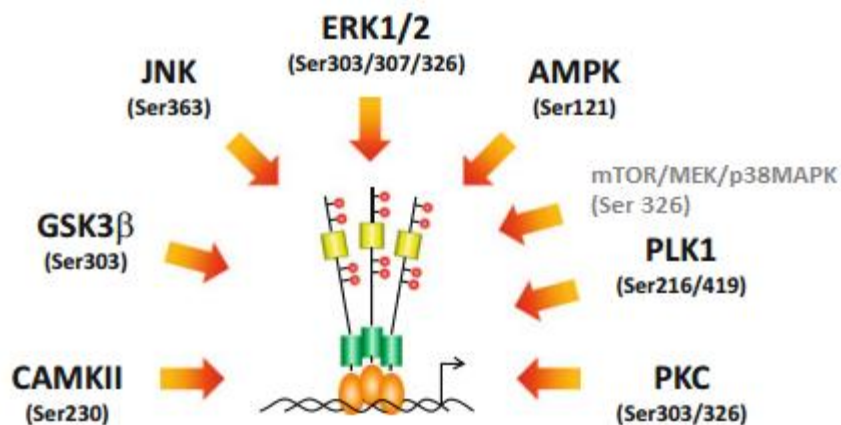


Figure 1.5. Schematic diagram of the structure and post-translational modifications of HSF1 (adopted and modified from Takaki & Nakai, 2016)

The major activating sites on HSF1 were found to be Serine 230 and Serine 326. Mutational studies showed that disrupting the phosphorylation sites on the regulatory domain changes activation thresholds of HSF1 (Holmberg et al. 2001; Budzyński et al. 2015). Phosphorylation on Serine 230 through calcium/calmodulin dependent protein kinase II and it is required for the activation of the trimer (Holmberg et al. 2001; R. E. Wang et al. 2009). Serine 326 is shown to be the hallmark of HSF1 activation leading to HSP gene transcription upon stress. Mutations on this site greatly reduced the heat induction of HSP70 (Guettouche et al. 2005). mTOR has a positive effect on this particular site, as it is required for dissociation of HSP90 and release of this negative regulator. Furthermore HSP70 promoter activity is reduced if mTOR signaling is not active (Chou et al. 2012). Moreover RAS-MEK-ERK pathway has divergent effects on HSF1. MEK is in physical contact with HSF1 to phosphorylate its serine 326 whereas ERK suppresses the activity of MEK (Tang et al. 2015). Another very recent finding is that p38 MAPK kinase is active on the very same site with a highlight on the extent and duration of the stress affecting the extent of phosphorylation (Dayalan Naidu et al. 2016).

1.4.2. Heat Shock Proteins







Heat shock proteins (HSP) are highly conserved family of proteins present in all organisms. Their synthesis is induced at high temperatures, therefore the first roles assigned to HSPs were their protein chaperone functions that is their involvement in the folding and inhibiting the aggregation of proteins. However they are also involved in a variety of other important processes ranging from maintaining homeostasis, transport of proteins through membranes, stabilizing protein complexes, signalling, cell cycle to apoptosis (Tkáčová and Angelovičová 2012). HSPs were classified according to their molecular weight and functions. Cellular localizations and functions of major HSP families are shown in Table 1.

HSP90 is a highly conserved ATP-dependent molecular chaperone with a very dynamic homodimer structure and one of the abundantly found proteins in the cells (Pearl and Prodromou 2006). However its main function is not folding of de novo synthesized proteins. It is involved in late stage folding and activation of specific set of proteins. Therefore it is involved in many events such as signal transduction, cell cycle progression, apoptosis, trafficking of proteins and immunity (Pratt and Toft 2003; Taipale, Jarosz, and Lindquist 2010; Trepel et al. 2010). It is induced by heat stress and has important roles for the survival of the cells.

HSP60 is considered as a mitochondrial chaperone however it is also found in the cytosol. It has important functions in protein folding and signalling pathways (Saibil 2013). HSP60 induction is associated with carcinogenesis and increased survival. Its presence in the extracellular space and peripheral blood makes it a good diagnostic marker for the cancer cells (Cappello et al. 2008).

HSP40 are homodimeric proteins sharing many substrates with HSP70 and involved in processes like protein folding, gene expression, protein degradation and translational initiation (J. C. Young 2010). It regulates the ATPase activity of HSP70 with a direct binding N-terminal ATPase domain but also directs HSP70 towards specific target proteins at precise subcellular locations (Kampinga and Craig 2010).

Table 1. Cellular localizations and proposed functions of HSPs (adopted from (Zorzi and Bonvini 2011))

Family	Organism	Chaperones	Location		Function
Hsp100	E.coli	ClpA, B, C	cytosol	 6- to 7-mer	Role in stress tolerance; it helps the resolubilization of heat-inactivated proteins from insoluble aggregates
	S.cervisiae	Hsp104	cytosol		
Hsp90	E.coli	HtpG	cytosol	 Dimer	Role in signal transduction (e.g., interaction with steroid hormone receptors, tyrosine kinases, serine/threonine kinases); regulation of heat shock response; role in cell cycle and proliferation; maintenance of mitochondrial integrity
	S.cervisiae Humans	Hsp83 Hsp90 GRP94 TRAP1	cytosol, nucleus ER mitochondria		
Hsc/Hsp70	E.coli	DnaK	cytosol	 Monomer	Roles in lambda phage replication; regulation of heat shock response; interaction with nascent chain polypeptides; functions in interorganellar transport; roles in signal transduction; refolding of denatured proteins <i>in vitro</i> ; roles in cell cycle and proliferation; anti-apoptotic activity; potential antigen-presenting molecule in tumour cells
	S.cervisiae Humans	Ssa1-4, Ssb1,2 Kar2, Ssc1 Hsc70, Hsp70 BIP, mHsp70	cytosol ER, mitochondria cytosol, nucleus ER, mitochondria		
Hsp60	E.coli	GroEL/ES	cytosol	 14-mer	Roles in folding and stability of denatured protein <i>in vitro</i> ; cofactor in diverse proteolytic systems; role in the assembly of bacteriophages and plant proteins (Rubisco)
	S.cervisiae Plants Humans	Hsp60 Cpn60 Hsp60	mitochondria chloroplast mitochondria		
Hsp40	E.coli	dnaJ	cytosol	 Monomer	Essential co-chaperone for Hsp70 ATPase activity, substrate binding and release
	S.cervisiae Humans	Ydj1 Hdj1, Hdj2	cytosol nucleus		
Small Hsps	E.coli Humans	IbpA, IpbB Hsp27 crystallin	cytosol cytosol cytosol	 8- to 24-mer	Inhibition of <i>in vitro</i> protein aggregation and heat inactivation in <i>S. cervisiae</i> ; Role in cell thermotolerance through stabilization of actin microfilaments; anti-apoptotic activity

HSP70 was discovered by its high induction after heat stress and found in many organisms from bacteria to humans in many cellular localizations e.g. GRP78 a 70kDa ER chaperone (Lindquist 1986). Its cycling ATPase activity is essential in folding and assembly of newly synthesized proteins. The ATPase activity and the stability of HSP70-substrate complex is controlled by co-chaperones and nucleotide exchange factors. It has very diverse functions within the cell (Mayer 2013). It aids the folding of proteins, prevents aggregation, involved in quality control, disassembly of protein complexes, stabilizing regulatory proteins, cell cycle regulation, aiding signal transduction and apoptosis (Kirkegaard et al. 2010; Koren et al. 2010; J. C. Young 2010). Upon stress it is highly induced and this induction often related with increased survival in a subsequent stress (Carper, Duffy, and Gerner 1987). HSP70 localizes to nucleus during stressful conditions and this event is assisted by special nuclear

transport proteins and binding (Kose and Imamoto 2014; Kose, Furuta, and Imamoto 2012; Velazquez and Lindquist 1984). Besides its chaperoning functions, inside nucleus HSP70 aids formation of an aggregation foci and also protects single-stranded DNA breaks (Kotoglou et al. 2009; Nollen et al. 2001; Shibata and Morimoto 2014).

Small HSPs are a diverse family sharing an α -crystallin domain and with a main function of prevention of aggregation. They are small in size however they can form large dynamic oligomers which changes the size upon stress and stabilize proteins to be re-folded by other ATPase chaperone complexes or degradation pathways (Kostenko and Moens 2009). They are generally considered as cytoprotective since their presence can lower the level of irreversible protein aggregation (Vos et al. 2011). The formation of dimers or monomers requires the phosphorylation of HSPs. P38 MAPK is responsible for this phosphorylation and interaction of monomers with actin causes stabilization (Geum, Son, and Kim 2002). During stress, sHSPs localize to nucleus in some cell types to prevent the aggregation. Translocation mechanism is rather controversial; it was shown to be phosphorylation dependent and once inside the nucleus oligomers form but phosphorylation deficient proteins were also shown to translocate to the nucleus (Bryantsev et al. 2002; Bryantsev et al. 2007; Michaud et al. 2008).

1.4.3. *Acquired Thermotolerance*

An intriguing effect of the heat stress is the cells ability to become temporarily more resistant to the subsequent lethal heat treatments. This phenomenon is called acquired thermotolerance (ATT) (Gerner and Schneider 1975). The basis of this enhanced survival is either due to cells increased capacity to cope with the damages that occur during the heat treatment or to attenuate the formation of the damage. Induction of heat shock proteins was shown to be responsible for thermotolerance in prokaryotes, yeast and Drosophila cells (Carper, Duffy, and Gerner 1987; Parsell et al. 1993). Induction of HSPs stabilizes the proteins and preserves proteostasis during the second heat treatment (Kabakov et al. 2002). Increased survival can also be

attributed to the anti-apoptotic effect of HSPs. Both HSP27 and HSP70 are shown to block apoptotic signalling cascades (Gabai et al. 1997; Mymrikov, Seit-Nebi, and Gusev 2011; Kennedy et al. 2014). HSF1 as the master regulator of heat stress controls the induction of the heat shock proteins and therefore knocking out HSF1 would reduce protein levels as well as survival of the cells in a subsequent stress. (McMillan et al. 1998). Even though no increase in survival was obtained in HSF1-/- cell lines, HSP25 and HSP70 constructs did not restore thermotolerance which suggests the involvement of HSF1 related induction of other genes (Luft et al. 2001). Furthermore calcium increase, an important hallmark of stress is when blocked with chelator molecules inhibits the induction of HSPs but not thermotolerance (Burdon 1987). Nonetheless in a given cell only one of the HSPs may correlate to resistance (G. C. Li 1985).

An additional aspect is the severity and the duration of the first preconditioning treatment. Similar level of thermotolerance can be achieved by long treatment of a low temperature and short treatment of a higher temperature (Burgman, Nussenzweig, and Li 1995). Earlier reports point out a possible membrane involvement in the development of ATT since changing the membrane composition alters the sensitivity of cells to heat (Jóźwiak and Leyko 1992; Konings and Ruifrok 1985). However no direct link has been reported between thermotolerance and plasma membrane (Anderson and Parker 1982; Bowler 2005; Harwood and Vigh 1998).

1.4.4. *Membrane Sensor Theory*

As discussed earlier, plasma membrane is a very unique environment; very dynamic and heterogeneous organization making it to be an important platform for many proteins to interact. The fluid behaviour enables molecules to diffuse freely, to rotate and to re-orient within plasma membrane. Interaction of lipids with different fatty acyl chain length and unsaturation, chemical structure of head groups create physico-chemical heterogeneities enabling optimal environments for different proteins or for protein-protein interactions. In addition to these intrinsic properties,

plasma membrane is in direct contact with the surrounding environment such as extracellular matrix or ligands and with cytoplasm, cortical actin and cytosolic proteins. Due to the temperature controlled lipid phase behaviour and all the interactions that can be linked to it, plasma membrane is an important sensory site for the heat shock. Due to the complex nature of biological membranes it is difficult to capture the spatio-temporal changes in the plasma membrane, however there are numerous experimental evidence for membrane-associated stress sensing (Török et al. 2014).

In recent years, our understanding of the plasma membrane has changed considerably as our knowledge of lipid microdomains has expanded. The level of fluidization induced by the chemical reagents equivalent of fluidity increase seen at 42°C resulted in a similar increase of HSP70 (Balogh et al. 2005). The effect of chemical agents such as benzyl alcohol exerts its effects through the dissolving raft-like cholesterol-sphingomyelin domains, therefore a distinct organization of microdomains may be required for the initiation of stress signals (Nagy et al. 2007; Gombos et al. 2011). The inhibition of stress signal integrator Rac1 has effects both on the plasma membrane microdomain organization and heat shock response as such the average diameter size of the microdomains increases at 37°C but decreases at 43°C with a strong decrease in HSP25 and HSP70 gene expression (Gungor et al. 2014).

Transient receptor potential channels are a large family of cationic channels which contribute to many physiological functions. TRPV1 is shown to be a molecular sensor for environmental stimuli such as chemical reagents (capsaicin, resiniferotoxin), acidity (<6.0) or noxious heat (>43°C) (Szallasi et al. 2007). It is a plasma membrane protein, however it can be found in ER and endocytic vesicles. PKC mediated phosphorylation can regulate the translocation of the TRPV1 from ER to plasma membrane which is triggered by nerve growth factor or insulin growth factor activation (Ho, Ward, and Calkins 2012). Activation of the TRPV1 channels causes Ca^{2+} increase in the cytoplasm which is a hallmark of heat stress. Depletion of raft forming lipids; cholesterol by M β CD, sphingomyelin by sphingomyelinases and ganglioside a complex ceramide by myriocin demolished the activation potential of the channel in

response to capsaicin and resiniferotoxin (Szoke et al. 2010; Sághy et al. 2015). However as for the heat activation of the TRPV1 has not been shown to be inhibited by M β CD even though the data provided supports the capsaicin activation (Jansson et al. 2013). Others reported the PIP₂ regulation of TRPV1 channels and the Ca²⁺ induced PLC δ activity. PIP₂ as mentioned earlier is a minor phospholipid in the plasma membrane and can be converted to signalling molecules inositol phosphate and diacylglycerol by PLC activity to release Ca²⁺ from ER and activate PKC, respectively (Falkenburger, Dickson, and Hille 2013). PI(4,5)P₂, PI(4)P and PI(3,4,5)P₃ were both shown to activate the TRPV1 channels, although the activation is not as high as capsaicin induced activation in excised patches. Moreover purified PLC δ which binds to PI(4,5)P₂ inhibits the channel activity causing a feedback inhibition (Rohacs 2015). Nevertheless, in the intact cells there is a dual effect; in the presence of high concentrations of capsaicin PIP₂ activates TRPV1 channel, in the low capsaicin or moderate heat treatment increased PIP₂ levels inhibited the channel activity whereas a moderate PIP₂ depletion activated (Lukacs et al. 2007). It is possible that the inhibitory effect is indirect since it is not observed in the excised patches via PIP₂ binding protein that is absent in the patches. In a recent study it has been shown that TRPV1 has a direct effect on the HSP expression. Antagonists of TRPV1 inhibited the heat induced HSP70 in HEK-293, MLE-12, HT-29 and MCF-7 cell lines heat whereas the addition of agonist capsaicin activated HSF-1 without heat treatment in HEK-293 cells. Moreover addition of EGTA a calcium chelator also blocked HSP70 induction by (Bromberg et al. 2013; Saidi et al. 2009).

Growth factor receptors are the other candidates of membrane initiated heat shock response. The connection has been made long ago when increase in lifespan was observed in worms and flies when insulin-like growth factor 1 (IGF-1) signalling was disrupted and resulted in upregulation of the stress resistance genes (Akerfelt, Morimoto, and Sistonen 2010). The insulin or insulin-like growth factor activation causes the activation of serine threonine kinases that down regulate the HSF1 and consequently HSP induction (Longo and Fabrizio 2002). Insulin and Insulin-like growth factor receptors (IR/IGFR) are complex structures of disulphide linked homodimers or heterodimers with an extracellular ligand binding domain and intracellular tyrosine

kinase domain which when phosphorylated recruits downstream adaptor proteins and other kinases (Kavran et al. 2014). PI3K/Akt and Ras/MAPK are two principal canonical signalling pathways. Briefly, ligand binding induced autophosphorylation of the tyrosine kinase domain of IR/IGF1R recruits PI3K to the plasma membrane through p85 regulatory domain. PIP₂ to PIP₃ conversion that is catalyzed by PI3K, recruits another kinase PDK which then phosphorylates and activates Akt. Ras/MAPK activation occurs through another adaptor protein which recruits Ras and Raf to the plasma membrane and phosphorylates ERK1/2 through MEK. The activation of PI3K/Akt pathway regulates the glucose uptake whereas Ras/MAPK pathway is involved in the regulation of transcription of several genes. Importantly, ligand/receptor complexes are internalized by endosome/lysosome system leading to the degradation of the ligand, inactivation and recycling of receptors (Siddle 2011). The levels and activation of IR/IGF1R are in control of over 500 genes in a ligand concentration dependent manner (Boucher, Tseng, and Kahn 2010). First level of control on the HSF-1 is through a direct binding of DDL1/2 to hinder HSF-1 in an inactive form (bound to a protein complex). The phosphorylation of DDL1/2 complex is inhibited when the IR/IGF1R is active, therefore attenuation of the insulin-IGF1 signalling causes HSF1 to be released and causes more stress induced HSF-1 to be available (Barna et al. 2012; Chiang et al. 2012). The second level of regulation is through the Akt pathway which negatively regulates HSF1 through indirect phosphorylation on Ser303 (Chu et al. 1996; X. Wang et al. 2003). Membrane link to the IR/IGF1R signalling came when stress factors such as osmotic stress was shown to activate the adaptor proteins of IR/IGF1R. Hyperosmolarity failed to induce autophosphorylation of tyrosine kinase domains in kinase dead knock out mutants of IR suggesting that no other kinases are involved in the activation (Ouwens et al. 2001). However, this did not abolish the activation of ERK1/2, JNK and p38 MAPK by osmolarity suggesting an alternative mechanisms to trigger these pathways. An increased IR/IGF1R phosphorylation has been associated with the use of a hydroximic derivative BGP-15 which is also a co-inducer of HSP. On the other hand BGP-15 was also shown to alter the organization of cholesterol-rich membrane domains and prevents transient disintegration of membrane domains (Gombos et al. 2011). The proposed mechanism for the protective effect on IR/IGF1R signaling shows

similarities. In atrial fibrillation susceptible animal model, depressed IGF1R in atrial samples is correlated with the increase ganglioside levels that reduces membrane fluidity and inhibits phosphorylation of IGF1R. In the presence of BGP-15, membrane fluidity is increased and ganglioside levels are reduced, therefore it is suggested that BGP15 attenuates ganglioside interaction to restore IGF1R signalling (Sapra et al. 2014). Another report is the activation of IGF1R by sheer stress causing the downstream ERK 1/2 activation in an IGF1R dependent manner. Moreover, inhibition of focal adhesion kinases and Rous sarcoma oncogene controls the activation IGF1R (Tahimic et al. 2016).

Focal adhesion kinase 1 is a non-receptor protein tyrosine kinase that has important functions in cell adhesion via providing link between the active cytoskeleton and extracellular matrix proteins, therefore can be a good candidate for the sensing of environment and heat. Growth factors, neuropeptides and lysophosphatidic acid are among those which can cause the phosphorylation of FAK (Schaller, Hildebrand, and Parsons 1999). After its autophosphorylation it is localized to membrane to recruit Grb2 adaptor proteins with SH2 domain and interacts with the actin proteins. It has anti-apoptotic roles during the cell spreading (Ben Mahdi, Andrieu, and Pasquier 2000). Although it is not a membrane protein, it is a regulatory protein of the focal adhesion complexes and it is shown to be responsive to the fluidity changes in the plasma membrane and has regulatory roles in the calcium influxes. Stabilization of the rafts is linked with the Rac activation and FAK-directed localization of a ganglioside (GM1) to the membrane. FAK also recruits PI3K through its p85 regulatory domain and increases the generation of PI(4,5)P₂s (Mitra, Hanson, and Schlaepfer 2005). Shear stress is also shown to cause the phosphorylation of FAK and subsequent activation of ERK 1/2 and JNK signalling cascades in a Grb2 adaptor protein dependent manner (S. Li et al. 1997; S. R. L. Young et al. 2009). In stressed cells, HSP27 phosphorylation by p38 MAPK is linked to focal adhesion organization, detachment of the cells and apoptosis. JNK co-localized to focal adhesions in FAK dependent manner whereas a phosphorylation deficient mutant of HSP27 accelerated the stress-induced changes in focal adhesions and apoptosis (De Graauw et al. 2005). In fact, even under non-stressful conditions phosphorylated HSP27 is

shown to interfere with the migration of the cells (Gerthoffer and Gunst 2001). Finally, HSP72 also interacts with the FAK during the ATP depletion and inhibits its degradation (Mao et al. 2003).

1.4.5. *Lipid Signalling in Heat Stress*

Clues for connecting lipids to the stress response were first provided when thermosensitivity and induction of HSPs were altered due to changes in physical state of the membrane either by chemical agents or by changing the lipid composition of the cells (Konings and Ruifrok 1985; Horváth et al. 1998). It was demonstrated that changing the growth temperature of cyanobacterium *Synechocystis* caused a significant shifts in the threshold temperatures for the HSP induction. When cyanobacteria were grown at 22°C HSP induction started at 38°C, however the value is shifted to 44°C for 36°C grown bacteria. During acclimatization to the heat lipid unsaturation levels has changed suggesting the importance of membrane physical state (Horváth et al. 1998). Moreover addition of fluidizers such as benzyl alcohol and heptanol also caused downshifts of HSP induction thresholds in K562 human lymphoblast cell (Balogh et al. 2005). Therefore the membrane lipid composition is an important determinant of the stress response and it must strictly be regulated. In fact stress itself induces changes in the lipid metabolism to stabilize the heat induced changes in the membranes and also lipids act as mediators for activation of signalling pathways (Balogh et al. 2013).

Sphingolipids are abundant structural elements of the lipid bilayer and their interaction with the membrane domains can modulate the physical state of the lipid bilayers. In addition, they account for regulation of enzyme activities and considered as important mediators for signalling events resulting in apoptosis, differentiation (Breslow and Weissman 2010) (Figure 1.6). The dramatic shift in the sphingolipid metabolism during heat stress was first observed in yeast (Wells, Dickson, and Lester 1998; G M Jenkins et al. 1997). Upon stress ceramide is shown to be accumulated which could be related with cell cycle arrest, activation of pro-survival signalling pathways or apoptosis depending on the severity of the temperature shift. The

targets of ceramide include PKC and ceramide activated protein kinase. It is reported to activate a major stress signalling pathway MAPK through the activation of MEK (Hannun and Luberto 2000) or Raf1 (Woessmann, Meng, and Mivechi 1999).

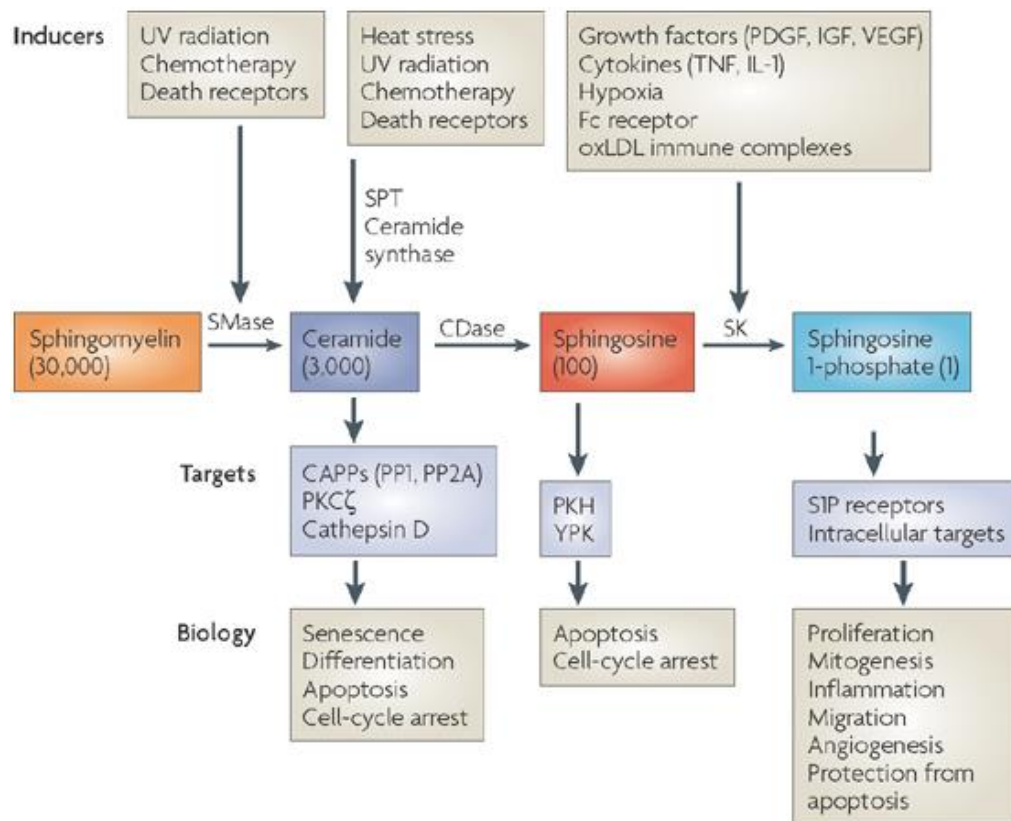


Figure 1.6. Overview of sphingolipid synthesis and sphingolipid dependent signalling (adopted from Hannun & Obeid, 2008). SMase, Sphingomyelinase; SPT, Serine palmitoyl transferase; CDase, ceramidase; SK, sphingosine kinase; CAPP, ceramide-activated Ser–Thr phosphatase; IGF; insulin-like growth factor; IL-1, interleukin-1; oxLDL, oxidized low-density lipoprotein; PDGF; platelet-derived growth factor; PKC, protein kinase C; PKH, PKB homologue; TNF, tumour necrosis factor- ; VEGF, vascular endothelial growth factor; YPK, yeast protein kinase.

It is important to note the impact of ceramide formation on the physical state of the membranes. There are studies pointing out hydrolysis of sphingomyelin to ceramide and activation of sphingomyelinases linked to plasma membrane disruption (Tam et al. 2010). Saturated and long acyl chain ceramides promote formation of gel domains in physiological temperatures therefore has a distinct impact of the membrane lateral organization (Pinto et al. 2011).

Cholesterol is an essential lipid of the plasma membrane. Its hydrophobic interactions with the glycerophospholipids result in the formation of membrane domains. It is a bidirectional regulator of the membrane fluidity as it stabilizes the membranes at high temperatures but prevents stiffening at low temperatures (Silva et al. 2007). It does not act as a signalling mediator however it controls the membrane dependent signalling cascades, trafficking, lipid and protein sorting. Ceramide for example is more soluble in cholesterol rich fluid membranes suggesting the ceramide rich domain formation in cholesterol poor membranes which is a requisite for ceramide induced apoptosis (Castro et al. 2009). Plasma membrane cholesterol can be depleted using methyl- β -cyclodextrin and results in diverse effect on the signalling activities and membrane trafficking (S. B. Sato et al. 2004). Heat shock response is altered by the cholesterol content of the cells; higher HSP expression was observed when cells contain higher cholesterol levels (Gombos et al. 2011; Csont et al. 2002).

Phosphatidylinositols constitute only 1% of the membrane phospholipids however they modulate two important signalling cascades; PI3K pathway and PLC to regulate exocytosis, cytoskeletal organization, plasma membrane recruitment and activation of target protein kinases such as Akt (Czech 2000). Heat stress activates PI3K in a time dependent manner which in turn activates Akt pathway (Richard Z. Lin et al. 1997). PI3K activity therefore controls HSF1 phosphorylation indirectly by inactivation of glycogen synthase kinase3 (Calderwood 2010) and HSP induction (Shinohara et al. 2006).

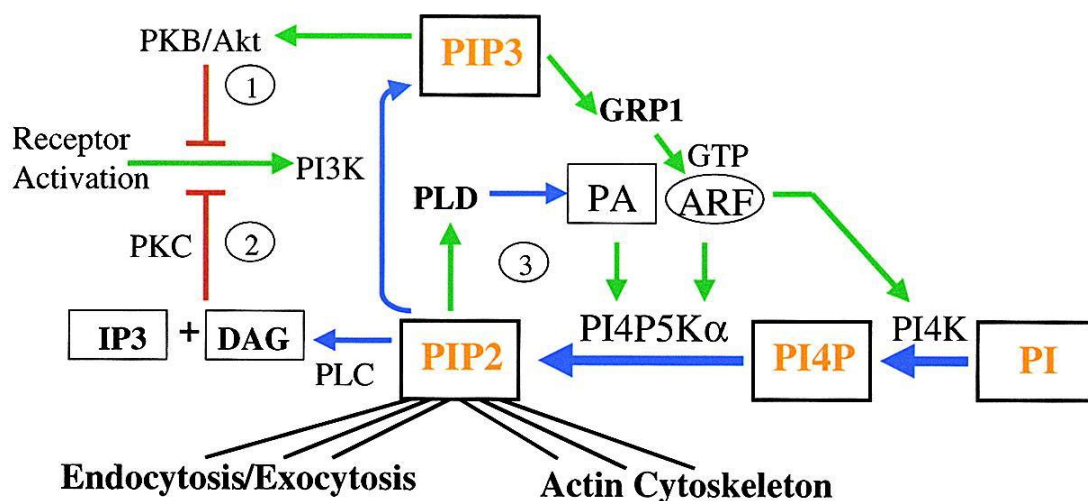


Figure 1.7 Regulation of PIP₂ and PIP₃ Synthesis. Green arrows denote stimulatory effects; blue arrows denote synthetic pathways; red denotes inhibitory effect. Feedback inhibitory loop (1). Cross-talk between receptor signaling pathways (2). Feed-forward loop (3) (adopted from (Czech 2000)).

1.4.6. Dependence of HSR on the severity of stress

As discussed earlier, the damage and the response of the cells are highly dependent on the duration and the severity of the stress. Mild stress is accepted as natural and beneficial to the organism whereas severe stress is mostly destructive. The duration of heat, temperature, cell type, cell cycle phase and environmental conditions can all effect the sensitivity of the cells hence the level of stress. Table 2 describes these factors and regulatory mechanisms based on mild and severe stress.

Mild stress is promoting the cell cycle progression by cyclinD1 through Rac1, ERK 1/2 and PI3K-Akt-GSK3 pathways whereas severe stress causes cell cycle arrest and apoptosis. Rac1 dependent membrane ruffling is similar to growth factor treatment. Moreover mild heat is proposed to activate growth factor receptors which can be linked to pro-survival signals (Park et al. 2005). Transient PI3K activation is observed during heat treatment and it is dose (both temperature and duration) dependent in the absence of apoptotic signals. As a matter of fact, there is interplay between PI3K/Akt and apoptotic pathways which is cell and stressor dependent. Doxorubicin induced apoptosis for example, causes a transient level of Akt phosphorylation which

is then suppressed resulting in ERK1/2 phosphorylation and cell death (E. R. Lee et al. 2006).

Fever, corresponding to mild heat shock could protect the hosts in several ways; that it is cytotoxic to the pathogens meanwhile causing HSP production in the host cells which confers to a better survival or it can also induce the HSPs in the pathogen which in return stimulates the host immune system and defenses and finally it is important since it optimizes the host cell's immune response and increases the chances of survival (Hasday and Singh 2000).

Activation of T-cells involves the reorganization of the plasma membrane where both the actin and membrane proteins are involved. The changes occurred when cells are exposed to ethanol or mild hyperthermia are identical to the T-cell activation (Zynda et al. 2015). Therefore it is a supportive evidence for membranes being the most upstream of the membrane sensing in a stimulating manner during the mild temperatures and being beneficial to the organism (Gombos and Vigh 2015). The beneficial roles of mild stress were also observed when cells experience glucose deprivation or heat shock prior glucose deprivation. Heat shock treatment leading to upregulation of HSP27 switches cell death by the necrosis to apoptosis. In addition to HSP27, activation ERK 1/2 played a role in preventing necrosis and inhibiting PI3K inhibited this effect which also accounts for the protective signalling caused by PI3K (Lim et al. 2010).

Table 2. Comparison of mild and severe stress (adopted from Park, Han, Oh, & Kang, 2005)

	Mild heat stress	Severe heat stress
Denaturation of nascent polypeptides	+	+
pre-existing proteins	–	+
HSF1 activation	+	++
	(Rac1 dependent)	(Rac1 independent)
HSP synthesis	+	++
	(Rac1 dependent)	(Rac1 independent)
Cell cycle arrest p21	–	G1/S and G2/M arrest
Cell proliferation	+/-	–
	(depending on cell types)	
cyclin D1	+	↓
cyclin A	+	–
Differentiation	+/-	–
	(depending on cell types)	
Apoptosis	–	+
Acquisition of thermotolerance	+	+
Signaling pathways		
Ras/Rac 1	+	–
PI3K-AKT	+	–
ERK1/2	+	+
SAPK/JNK	+	+
p38MAPK	+	+
Biological relevance	adaptation of growth conditions	cell death or cell morbidity

1.5. Cell to Cell Variation in Stress Response

Understanding of how cells sense, respond and adapt to environmental changes is of great importance. Knowledge gained by the gene expression of protein analysis is obtained from populations of cells. However, it is a valid question whether all cells in a culture dish suffers the same consequence of stress or whether the response is identical. Treatment of the cells with antibiotics where the persistent cells are shown to regenerate the original population that is a mix of persistent and sensitive cells is a good example for the phenotypic variation observed within the isogenic

populations (Avery 2006). Therefore, one can expect variations within the population and averaging the measure of gene or protein expressions will be misleading. Secondly, analysis of single cells allows a more direct approach to correlate molecular implications and mechanistic insights can be obtained.

The first single cell studies are obtained by the use of flow cytometers where parameters like cell size, cell cycle and a protein of interest can be monitored simultaneously (Crissman and Steinkamp 1973). Since then major establishments in microscopy, microfluidics, flow cytometry, RNA and DNA sequencing allowed researches to do deeper analysis increasing our understanding on how tumor cells can twist their genome or how a single cell can be a decision point to start an electrical impulse, to migrate or to differentiate (Schubert 2011).

Cell cycle and cell division phenotypes are the important and well-studied factors resulting in population heterogeneity. Reports showed that the cell-to-cell variation in heat survival can partly be explained by the cell cycle. G1 cells are shown to be more tolerant compared to G2/M phase whereas cells in the S phase do not develop thermotolerance at all (Park et al., 2005). Single cell studies are, therefore important to reveal the nature of individuality of stress resistance.

Microscopy based assays, on the other hand provide information about the micro-environment, cell-to-cell interactions in a population context. Use of green fluorescent protein (GFP)-tagged protein constructs reveals the dynamics of protein interactions under the microscope. The repair of double stranded breaks by a novel chromatin associated ubiquitin ligase is identified by analyzing the time lapse data obtained from genotoxic stress induced cells (Doil et al. 2009). In another study, a fluorescent construct is generated to measure the activity of MAPK in single cells. It is shown that when the cell wall integrity is compromised, heterogeneity of the MAPK activation occurs independently of the cell cycle phase but low glucose levels caused a lower activity (Durandau, Aymoz, and Pelet 2015). The analysis of the images and the data mining are concurrently developing fields which have a high importance in retrieving data from high throughput studies and making biological conclusions (Conrad and Gerlich 2010).

Studies carried by Snijder et al. shows the population context of the virus infection (2009). They define the heterogeneity of the population as a combination of intrinsic and extrinsic factors. Cells grown on the edge of the colonies have higher GM1 content and phosphorylated focal adhesion kinases, which positively correlate with simian virus 40 infection. Moreover, the crowding actively regulates the transcriptional factors and tunes the membrane lipid composition. Cells in the crowded regions shows higher fluidity compared to more ordered counterparts on the sparse regions. Cholesterol content is also higher in the low crowding regions (Frechin et al. 2015).

1.6. *Aim of the study*

The major aim of this study was to understand how cells sense the heat, how membranes contribute to the heat sensing and cellular response to heat. For this purpose, sequence of events during different level of heat stress and the individuality of stress response were studied. The questions addressed are as follows;

- What is the sequence of early events during the perception of temperature stress?
- How different temperature treatments (heat dose) affect the cellular response in terms of membrane dependent changes, protein induction and activation of stress signaling pathways?
- Does the altered membrane structure and composition relate with the heat resistance?
- How cell cycle affects the stress response and to what extent it contributes to it?
- What are the early events of heat sensing? Is it possible to use these as parameters to analyze the individuality of the stress response?
- Is the population heterogeneity of the heat stress signal linked to the membrane?

2. MATERIALS AND METHODS

2.1. Cell culture

Chinese Hamster Ovary (CHO) cells and CHO cells with glycosylphosphatidylinositol (GPI)-mGFP construct (Brameshuber et al., 2010) was a kind gift of Prof. Dr. Gerhard J. Schütz were cultured in F12-K (Gibco-21127022) medium supplemented with 10% FCS.

Mouse embryonic fibroblasts (MEFs) (a kind gift of Prof. Lea Sistonen) and MCF-7 human breast carcinoma cells (a kind gift of Dr. István Krizbai) were grown in DMEM containing 10 % FCS and 4 mM L-glutamine. Cells were maintained at 37 °C in a humidified 5 % CO₂-atmosphere.

2.2. Treatments

For heat shock treatments, cells were plated with 250cells/mm² density in complete medium. After 24 hours the plates were sealed with parafilm and immersed in a water bath (with a precision of $\pm 0.1^{\circ}\text{C}$) set to the indicated temperature for given temperature. Samples were prepared either right after heat shock treatments or after recovery times as indicated.

For Lyso-PC treatments, CHO cells with GPI-mGFP constructs were grown overnight. HBSS was used to wash the cells twice before 10mM 1-Palmitoyl-2-hydroxysn-glycero-3-phosphocholine (16:0 Lyso-PC, 855675; Avanti Polar Lipids) were added to the at given final concentrations.

2.3. Western blot

Western blot protocol was carried out to determine protein levels. For phospho-proteins and HSF1 cells were harvested right after given treatment, for HSP levels 6 hours recovery time is given unless otherwise stated. Cells were collected in 1x Laemli sample buffer, mixed for 30 minutes at room temperature, incubated at 100°C for 10

minutes and spun down for 5 minutes at 14000rpm (Eppendorf table top centrifuge). Total amount of protein was measured by using Pierce 660 nm Protein Assay (Thermo Scientific) with the addition of ionic detergent compatibility reagent (Thermo Scientific). 20ug of protein samples were loaded on 12% or 10% SDS polyacrylamide gels (depending on the size of the protein of interest). After separating the proteins, they were transferred onto PVDF membranes (0.45µm, Immobilon-P, Millipore). Primary antibodies as listed in Table 3 were used with overnight incubation at 4°C with constant agitation. Secondary antibodies were used in 1:50000 dilutions for 1 hour at room temperature. Equal protein amounts were measured using Pierce 660nm protein assay (22660, Thermo Scientific) and loaded on 8% (for HSF-1) or 10% SDS gels (all other determinations) for western blotting experiments. Enhanced chemiluminescence was detected and analyzed using Alpha Ease FC Software v6 (San Jose, CA, USA). Anti-GAPDH (G9545, Sigma-Aldrich, Saint Louise, MO) antibody was used to control the protein load and for data normalization.

Table 3. Details of the antibodies used in Western Blot experiments

Antibody	Description	Dilution
pSAPK/JNK (thr 183/185)	#9251 -Cell Signalling	1:2000
ERK 1/2	#9102 -Cell Signalling	1:1000
phospho ERK1/2 (pT202/pY204)(pT185/pY187)	#16869 -Abcam	1:5000
phospho p38 MAPK (Thr180/Tyr182)	#9211 -Cell Signalling	1:1000
phospho IGF1Rβ /IRβ(Tyr1162/1163)	#44804 -invitrogen	1:500
HSF1	RT-425-PABX -Thermo Scientific	1:1000
HSF1 phosphoSer326	ADI-SPA-902 -Enzo LifeSciences	1:1000
HSP27 phosphoSer15	SPA-525 -StressGen	1:1000
HSP25 phosphoSer86	#44536 -invitrogen	1:1000
HSP70	ADI-SPA-810 -Enzo LifeSciences	1:1000
HSP90	SMC-107A/B -StressMarq	1:1000
HSP25	ADI-SPA-801 -Enzo Lifesciences	1:1000
HSP60	SPC-105 -StressMarq	1:1000
GRP78	SPC-180 -StressMarq	1:2000
GAPDH	G9545 -Sigma Aldrich	1:10000

2.4. *Colony Formation assay*

For colony formation assay CHO cells were submitted to heat shock twice. The first (priming) HS was conducted at different temperatures (40°C, 42.5°C, and 44°C) for 20 min. After the priming HS, the cells were allowed to recover for 6 h at 37°C, in line with other experiments (western blot and immunolocalizations). The second HS comprised a 20-min exposure to 46°C. Immediately after the second HS, the cells were trypsinized, counted, and serially diluted with media before plating. The plated cells were incubated at 37°C for 3–4 days. To visualize and assess colony formation, the cells were fixed in 4% paraformaldehyde, permeabilized with 2% Triton X-100, and stained with 500nM propidium iodide (Molecular Probes). Imaging of the colonies was done by using Olympus SZX12 stereomicroscope (Olympus optical, Tokyo, Japan), with 530–560 nm excitation filter and 590–650 nm emission filter.

2.5. *Immunofluorescence Labelling and Microscopy Experiments*

Multi-color imaging experiments were carried out on wild-type CHO cells. Cells were fixed in 4% (w/v) paraformaldehyde for 10 min immediately after treatment. Cells were permeabilized with 0.5% Triton-100 for 10 min, and blocked with 1% bovine serum albumin in PBS for 30 min and incubated with HSP25 (ADI-SPA-801, Enzo) and HSP70 (ADI-SPA-810, Enzo) antibodies, Secondary antibodies from AlexaFluor (Thermo Scientific) were selected according to the host species of the primary antibody, and were used at 1:300 dilution, with incubation for 30 min at 37°C. The cells were washed with PBS three times after each treatment. For the detection of both HSP25 and HSP70, 1:50 antibody dilution was used, with Hoechst33342 (Thermo Scientific) as a DNA counterstain. Images were taken using Operetta High Content Imaging system using 40X dry objective (PerkinElmer, Inc. Waltham, MA).

2.6. High Content Microscopy Experiments

Highthroughput assays were imaged using Operetta High-Content Imaging System (Perkin Elmer). Cells grown and labelled in 96-well plates (89626, ibidi, WI,USA) were used to image using 40x dry objective (PerkinElmer). For AlexaFluor 488 detection 475/30nm excitation, 525/50nm emission filter, for AlexaFluor 555 detection 535/30nm excitation, 595/20 emission and for Hoechst 33342 detection 380/40nm excitation, 445/70 emission filter sets were used.

Analysis of the images were carried out using Perkin elmer's Harmony software and MATLAB (Mathworks). Cells were segmented based on the the nuclear intensities and then cytoplasm was detected using Harmony software's own algorithms (Figure 2.1). Hoechst and HSP25 intensities were measured in nucleus and cytoplasm for each single cell. Objects on the border of the image and with very large nucleus were discarded to ensure the quality of the data. For the calculation of HSP25 contrast in the nucleus the following formula was used;

$$\text{Contrast} = (F_{\text{nucleus}} - F_{\text{cytoplasm}}) / (F_{\text{nucleus}} + F_{\text{cytoplasm}})$$

where F represents the mean intensity.

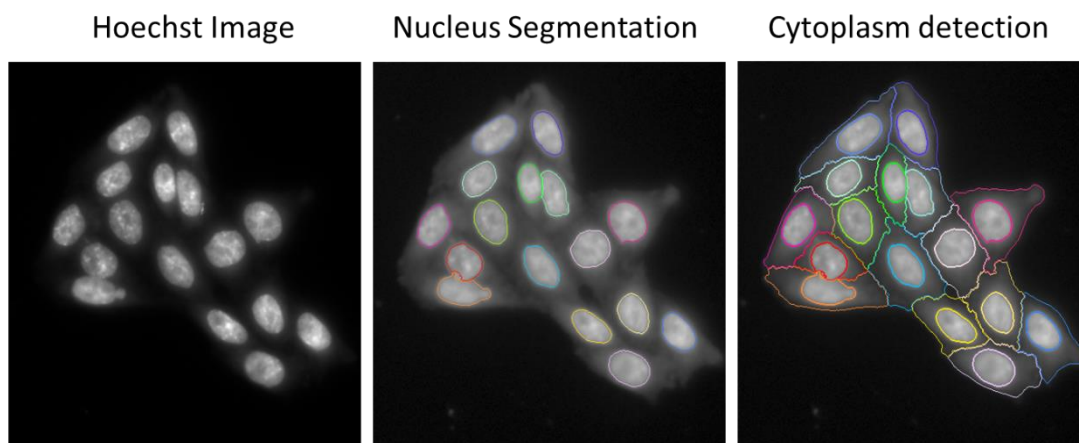


Figure 2.1 Representative images for object analysis in High-throughput assays. Hoechst images were used as a basis of nucleus segmentation which is followed by cytoplasm detection. Detected objects and object borders were drawn by Harmony software algorithms as represented. Data analysis were carried within the determined areas.

2.7. Image based Fluorescent Correlation Spectroscopy (ImFCS)

Labeling and instrumentation

Cells were seeded into glass bottom dishes (MatTek Corporation, MA, USA) two days before experiment. Measurement was performed in culturing media without phenol red at 37°C with CO₂ administration for reducing stress conditions. A POC-R cell cultivation system (Zeiss, Jena, Germany) was attached to the stage of microscope for obtaining the growth conditions. This system includes controlled objective heater and CO₂/Air gas chamber. Objective type Total Internal Reflection illumination was used for achieving the thinnest excited sample volume with a high numerical aperture objective (alpha Plan-FLUAR 100, Zeiss). Excitation wavelength 488 nm from a Spectra-Physics Stabile 2018 (Spectra-Physics, CA, USA) laser as light source was introduced to the microscope (Zeiss Axiovert 200) by two tilting mirrors. The laser beam was focused on the back focal plane of the objective after a 488 nm cleanup filter and aZT488/647/780rpc-UF1 dichroic mirror (Chroma Technology GmbH, Olching, Germany). Sample signal was collected by the objective and filtered by a 535/70 emission filter (Chroma). For acquisition we used a ProEM512 EMCCD camera (Princeton Instruments, NJ, USA) with 3 ms effective exposure time and 20x40 pixel acquisition area per measurement (pixel size 0.16 µm) ImFCS plugin (http://www.dbs.nus.edu.sg/lab/BFL/imfcs_image_j_plugin.html) for ImageJ software was used for data evaluation.

Data analysis

The autocorrelation functions (ACFs) for every pixels were calculated using a multi-tau correlation scheme (Sankaran et al. 2010). An exponential of polynomial bleach correction was used to correct data before fitting. For obtaining the diffusion coefficient (D) for all pixels ACFs were fitted according to the equation below.

$$G(\tau) = \frac{1}{N} + \left[\frac{\text{erf}(p(\tau)) + \frac{(e^{-(p(\tau))^2} - 1)}{\sqrt{\pi p(\tau)}}}{\text{erf}(p(0)) + \frac{(e^{-(p(0))^2} - 1)}{\sqrt{\pi p(0)}}} \right]^2 + G_{\infty} p(\tau) = \frac{a}{\sqrt{4D\tau + \omega_0^2}}$$

Where $G(\tau)$ is the ACF in dependence of the correlation time (τ), N is the number of detected particles, a is the pixel size and ω_0 is the $1/e^2$ radius of the Gaussian approximation of the point spread function. To identify and describe the mode of membrane organization by investigating the size-dependency of diffusion coefficient we used the Imaging FCS type of FCS diffusion law (Ng, Bag, and Wohland 2015). According to that the diffusion time (τ_D) of the fluorescent probe depends on the observation area (A_{eff}) as it is described by

$$\tau_D(A_{eff}) = \tau_0 + \frac{A_{eff}}{D}$$

where A_{eff} means that area of the membrane where the labeled particle travels which is calculated by the convolution of the detection area with the point spread function. τ_0 is the intercept of the diffusion law plot on the y axis of A_{eff}/D against A_{eff} . This parameter provides information about diffusion confinement. Diffusion law can be plotted by using different A_{eff} values which were calculated by post-inquisitional binning of pixels. In the case of free diffusion D is constant regardless of A_{eff} so it shows a straight line passes through the origin ($\pm 0.1s$) in the diffusion law graph. A heterogeneous system where exists membrane domains or meshwork, however allows spatial scale dependent diffusion which results remarkable different diffusion law plot with positive or negative intercepts for domain partitioning or meshwork diffusion, respectively (Ng, Bag, and Wohland 2015).

2.8. Thinning out clusters while conserving stoichiometry of labeling (TOCCSL) experiments

TOCCSL experiments were carried as described by Brameshuber et al.(2010) in Institute of Applied Physics, Technical University of Vienna. A Zeiss Axiovert 200 microscope equipped with a 100× NA=1.46 Plan-Apochromat (Zeiss, Oberkochen, Germany) was used to excite cells in objective-based total internal reflection fluorescence (TIRF) configuration via the epiport using 488 nm light from an optically pumped semiconductor laser (Sapphire; Coherent, Santa Clara, CA). The power was adjusted to 2–10 kW/cm² on the sample. A slit aperture (Thorlabs, Newton, NJ) with

a width of $\sim 7 \mu\text{m}$ in the object plane was used as the field stop. For exact timings, an acousto-optic modulator (Isomet, Springfield, VA) was used. Timing protocols were generated and controlled by an in-house-written program package implemented in LABVIEW (National Instruments, Austin, TX). After the appropriate filtering (dichroic mirror: ZT488/640rpc 2 mm, Chroma, Bellows Falls, VT; emission filter: FF01-538/685-25, Semrock, Rochester, NY), fluorescence images were recorded using a back-illuminated, nitrogen-cooled CCD camera (Roper Scientific Micro Max 1300-PB; PI Acton, Trenton, NJ). The temperature during experiments was maintained at 37°C by means of an in-house-built incubator box equipped with a heating unit and an objective heater (PeCon, Erbach, Germany). Briefly, after recording a pre-bleach image with a power density of $\sim 2 \text{ kW}/\text{cm}^2$ and an illumination time of $t_{\text{ill}} = 2 \text{ ms}$, the sample was bleached for $t_{\text{bleach}} = 400 \text{ ms}$ with a 5-fold higher power density. After an adjustable recovery time, $t_{\text{recover}} = 0.5\text{--}3 \text{ s}$, up to 20 images with a typical delay of 20 ms were recorded with same settings as used for the pre-bleach image. To confirm that bleaching was complete, an optional experiment was carried out with a recovery time of $t_{\text{recover}} = 1 \text{ ms}$. Then the recovery time was increased to allow for the reentry of unbleached GPI-mGFP molecules from the shielded region. The first recorded image after the adjustable recovery time, t_{recover} was used for brightness analysis (TOCCSL image), and the last image was used to determine the single-molecule brightness of mGFP. Due to the low bleach stability of GFP, the probability of observing more than one active GPI-mGFP per diffraction-limited spot in the last image is negligible.

2.9. Measurement of Intracellular Calcium Levels

For calcium measurements MCF-7 cell line was used. Cells were loaded with $10 \mu\text{M}$ Fura-2 AM (F1221, Thermo Scientific) at a final concentration of $10 \mu\text{M}$ for 45 minutes at 37°C followed by a 30 minutes incubation at 37°C without the probe. Cells suspensions with an optical density of 0.15 at 380 nm were measured for change in fluorescence ratio of Ca^{2+} bound and Ca^{2+} free probe as described in manufacturers protocol. Heat treatments were carried in the heated sample holders of fluorimeter.

The fluorescence signal was measured with a PTI spectrofluorometer (Photon Technology International, Inc., South Brunswick, NJ, USA) with emission at 510 nm and dual excitation at 340 and 380 nm (slit width 5 nm). The autofluorescence from the cells not loaded with the dye was subtracted from the Fura-2 signal. Calculations for the calcium concentration was carried as described in Palmer and Tsien (2006). Briefly measurements were carried in 0.05% Triton in HEPES buffered HBSS containing 3 mM EGTA for the completely ion-free indicator and 10 mM CaCl₂ for ion-saturated indicator using the given formula;

$$[Ca^{2+}] = K_d Q \frac{(R - R_{min})}{(R_{max} - R)}$$

where R represents the fluorescence intensity ratio $F_{\lambda_1}/F_{\lambda_2}$, in which λ_1 (~340 nm) and λ_2 (~380 nm) are the fluorescence detection wavelengths for the ion-bound and ion-free indicator, respectively. Ratios corresponding to the titration end points are denoted by the subscripts indicating the minimum and maximum Ca²⁺ concentration. Q is the ratio of F_{min} to F_{max} at λ_2 (~380 nm). K_d is the Ca²⁺ dissociation constant of the indicator described by the manufacturer as 0.14 μ M.

2.10. Lipidomics Analysis by MS-MS

GPI-mGFP-expressing CHO cells were treated at specified temperatures for 20 min, washed twice with cold PBS, collected in Eppendorf tubes (1.4x10⁶ cells per tube), and centrifuged. The pellets were shaken in 1 mL of methanol containing 0.001% butylated hydroxytoluene as an antioxidant, for 10 min, and centrifuged at 10,000xg for 5 min. The supernatant was transferred into a new Eppendorf tube and stored at -20°C (Péter et al. 2017).

Lipid standards were obtained from Avanti Polar Lipids (Alabaster, AL). The solvents used for extraction and for MS analyses were of liquid chromatographic grade (Merck, Darmstadt, Germany) and Optima LCMS grade from Thermo Scientific

(Waltham, MA). All other chemicals were purchased from Sigma-Aldrich (Steinheim, Germany) and were of the best available grade.

MS analyses were performed using an LTQ-Orbitrap Elite instrument (Thermo Scientific, Bremen, Germany) equipped with a robotic nanoflow ion source TriVersa NanoMate (Advion BioSciences, Ithaca, NY). Lipid classes and species were annotated according to the lipid classification systems (Fahy et al. 2009), and the lipids were identified by LipidXplorer software (Herzog et al. 2011). Further measurement details are provided in the Supplementary methods. Lipidomics data are presented as the mean \pm SD; statistical significance was determined by Student's t-test and was accepted for $p < 0.05$. O-PLS-DA and cluster analyses of lipidomics datasets were performed using MetaboAnalyst (Xia and Wishart 2011).

The LTQ-Orbitrap Elite instrument (Thermo Fisher Scientific Bremen, Germany) equipped with a robotic nanoflow ion source TriVersa NanoMate (Advion BioSciences, Ithaca, NY), using chips with 5.5 μ m diameter spraying nozzles. The ion source was controlled by Chipsoft 8.3.1 software (Advion). The ionization voltages were +1.3 kV and -1.9 kV in positive and negative mode, respectively, and the backpressure was set at 1 psi in both modes. The temperature of the ion transfer capillary was 330°C. Data acquisition was performed at the mass resolution $R_m/z400=240,000$.

Phosphatidylcholine (PC, diacyl, and PC-O, alkyl-acyl), lysophosphatidylcholine (LPC), and sphingomyelin (SM) were detected and quantified in the positive ion mode; phosphatidylethanolamine (PE, diacyl, and PE-PI, alkenyl-acyl), lysophosphatidylethanolamine (LPE), phosphatidylinositol (PI), lysophosphatidylinositol (LPI), phosphatidylserine (PS), phosphatidic acid (PA), phosphatidylglycerol (PG), cardiolipin (CL), ceramide (Cer), glucosyl ceramide (GlCer), and ganglioside (GM3) were detected and quantified in the negative ion mode.

For quantification, 10 μ L of the lipid extract was diluted with 140 μ L of the infusion solvent mixture (chloroform:methanol:iso-propanol, 1:2:1, by vol.) containing an internal standard mix (71 pmol PC d31-16:0/18:1, 25 pmol PE d31-16:0/18:1, 11 pmol PI d31-16:0/18:1, 19 pmol PS d31-16:0/18:1, 2.5 pmol PG d31-

16:0/18:1, 1 pmol PA d31-16:0/18:1, 1.5 pmol CL 56:0, 5 pmol SM d18:1/17:0, 2 pmol Cer d18:1/17:0, 3 pmol Glycer d18:1/12:0 and 5 pmol GM3 d3-d18:1/18:0). Next, the mixture was split in two, and 5% dimethylformamide (additive for the negative ion mode) or 3 mM ammonium chloride (additive for the positive ion mode) were added to the sample halves. Each quantified lipid species accounted for more than 0.5% of the respective lipid class. The mass tolerance was 3 ppm. Data files generated by LipidXplorer queries were further processed by in-house Excel macros.

2.11. Plasma Membrane Isolation

Plasma membrane was isolated from CHO-GPI-mGFP cells right after given heat shock treatment to investigate the changes in the total lipid composition. This isolation method was carried out as described in Maeda *et al.* (1983). Briefly, 5 x 10 cm plates were seeded with 250 cells/mm² density and grown in complete medium for 24 hours. After given heat treatments cells were washed 3 times with ice cold PBS, scraped and collected in 1 ml ice cold TNM buffer (10 mM NaCl, 1.5 mM MgCl₂, 10 mM Tris-HCl pH 7.4). Cell lysis carried out using glass beads. After inspecting lysate under the microscope, it was spun at 3000 rpm for 5 minutes at 4°C. Supernatant was collected and centrifuged at 8800 rpm, 4°C for 15 min in F28/13 rotor in a Sorvall super-speed centrifuge (RC28C). Supernatant was discarded and pellet was re-suspended gently in 8 mL of TNM buffer. Parallel to this 36 % sucrose in TNM buffer was prepared and 4 ml filled in ultra-centrifuge tubes on top of which re-suspended pellet was delicately layered. The tubes were centrifuged at 27000 rpm, 4°C for 3 h in SW41 rotor (sorvall WX Ultra Series, Thermo Fischer Scientific). Interphase of each sample was collected into TNM buffer and centrifuged again at 27000 rpm, 4°C for 1 h in SW41 rotor. Supernatant was removed and the pellet (PM fraction) was dissolved in 10uL ultrapure water for further analysis.

2.12. Plasma membrane sheet preparation

Cells were grown on poly-L-lysine treated coverslips over night. Cells were rinsed with ice-cold PBS and rinsed with cold hypotonic buffer (23mM KCl, 10mM HEPES pH 7.5, 2 mM MgCl₂, 1mM EGTA) three times which causes cells to swell are then placed into sonication buffer (70 mM KCl, 30 mM HEPES pH 7.5, 5 mM MgCl₂, 3 mM EGTA) containing beaker. Sonication was carried out with 19mm-tip fitted Branson sonifier cell disruptor B-30 with optimized operation settings (power 1.5, duty cycle 80%) for 3 times. Finally sonicated coverslip was rinsed with sonication buffer to be further used for the microscopy experiments.

2.13. Cell sorting

Sorting experiments were carried on using BD FACSJazz instruments. CHO cells expressing GPI-mGFP construst grown in normal growth conditions for 24h were stained with 20ug/mL Hoechst 33342 for 30 minutes and trypsinized. 300.000 cells were sorted based on the cell cycle stages according to Hoechst histogram using 450/50nm filter set excited by 405nm laser. To remove doublets and triplets from sorting, hoechst intensity versus signal duration graph was used as discussed in Wersto *et al.* (2001) (Figure 2.2) and only the Unsorted and unstained samples were also analyzed to confirm experimental artifacts.

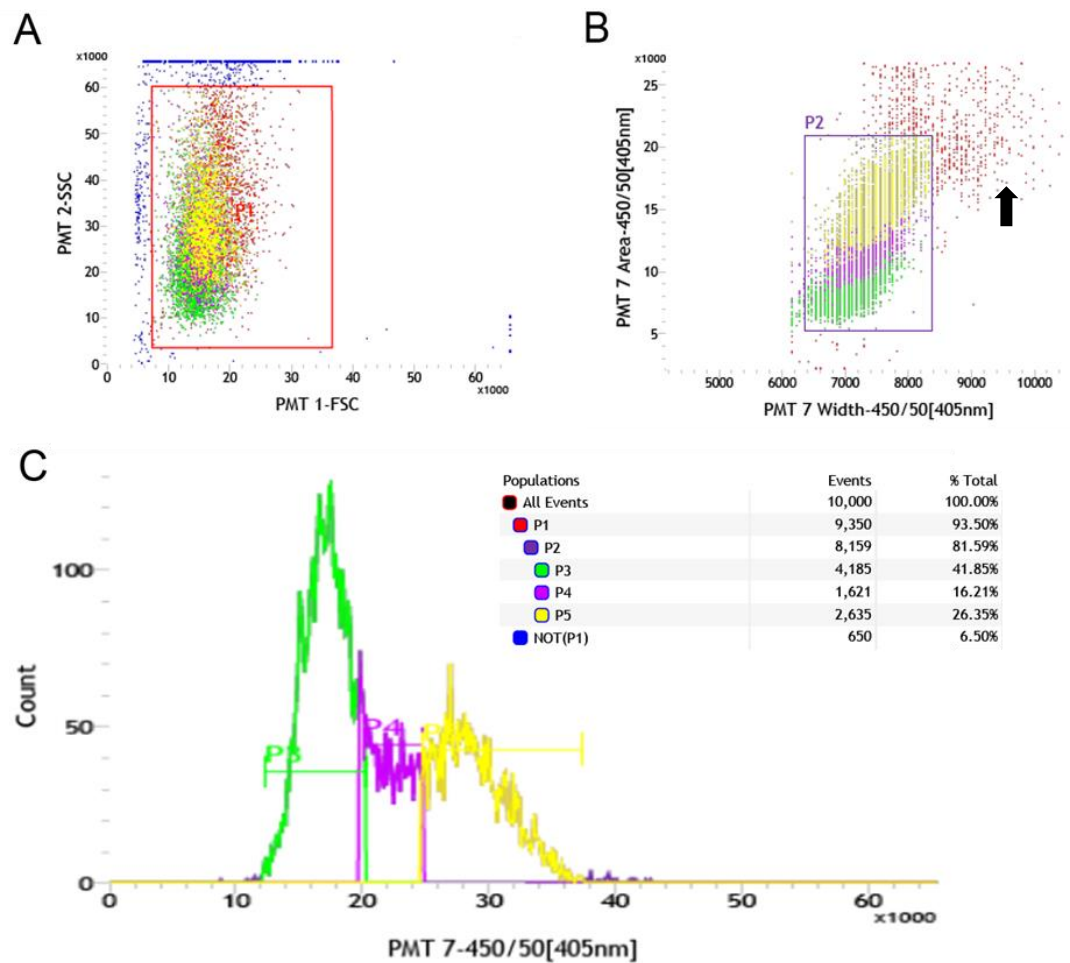


Figure 2.2 Representative data for fluorescence activated cell sorting. CHO cells expressing GPI-mGFP construct were sorted based on the given selection. For separating cells from cellular debris forward scatter (FSC) and side scatter (SSC) plots were drawn (A) and 93.5% (P1) of the objects were selected for the subsequent cell cycle analysis. As doublets and G2 phase cells have the same amount of DNA, area (Hoechst intensity) and width (time) of the detected intensity were plotted (B) to separate the doublets (marked with arrow) on P2 (81.5%). G1 (P3 -41.85%), S (P4-16.21%) and G2 (P5-26.35%) were selected based on the Hoechst intensity histogram (C).

3. RESULTS AND DISCUSSION

3.1. HEAT-STRESS SENSING

In order to create a better understanding on how cells sense and respond to stress, the effect of heat on HSP induction was analyzed in parallel with the changes in the membrane structure, acquired thermotolerance, lipid composition and stress signalling pathways.

3.1.1. *Heat shock proteins are induced differentially during heat stress*

Heat stress induces the HSPs depending on the level and the duration of the treatment that is the dose of heat. How the signals are generated during heat treatment and initiation of the induction of proteins are very much dependent on the dose. We therefore conducted systematic experiments to reveal the protein induction patterns of different doses of treatments using CHO cells with GPI-mGFP reporter construct. GPI linked proteins are preferentially localized in the ordered microdomains of plasma membrane, therefore they are useful tools to study the changes in the organization of the plasma membrane. For that reason, CHO line expressing GPI-mGFP construct was used in all experiments unless it is stated otherwise.

Westerblot analysis experiments revealed a distinct profile of HSP induction (Figure 3.1). 40°C did not increase the level of HSPs whereas 42.5°C which is indicated as moderate stress resulted in increase in both HSP25 and HSP70 to a different extent. 10 minutes treatment was sufficient to raise HSP25 and HSP70 levels for CHO cells. The minimal heating time was app. 20 minutes for B16 F10 cell type (data not shown) for the HSP induction suggesting cell type dependence. For HSP70, there was a biphasic behaviour. The initial increase started after 10 minutes however a major ramp was observed after 40 minutes (Figure 3.1). These findings suggest a differential regulation for HSP25 and HSP70 expressions. Minimum recovery time for HSP25 and HSP70 to be synthesized were measured to be 3 hours and 5 hours respectively after 1 hour heat treatment at 42.5°C (data not shown), therefore 6 hours recovery time is sufficient for the protein synthesis pointing a regulational difference at the

transcriptional level. Moreover intron free HSP70 mRNA by-passes splicing machineries which is repressed during heat stress on the post-transcriptional level and also cap-dependent translation on the pre-translational level (Silver and Noble 2012). HSF1 binding and initiation of transcription however can be altered as reported by Trinklein et al. (2004). Chromatin co-immunoprecipitation experiments revealed a very different and specific pattern of HSF1 and HSF2 binding to several HSP promoter regions depending on the heat treatment or differentiation all of which could be possible regulatory mechanisms. Therefore further experiments are needed for a better understanding of the phenomenon. It is also important to note that a similar biphasic behaviour was observed in temperature dependent inductions of HSP70 (Figure 3.2); initial increase started at 42.5°C but the major increase was observed at 44°C after 20 minutes of treatment. Other HSPs, HSP60 and HSP90 showed minor but significant increase at 42.5°C and 44°C. GRP78 an endoplasmic reticulum chaperone was induced only at 44°C that can be linked to protein unfolding/denaturation dependent trigger (Fulda et al. 2010).

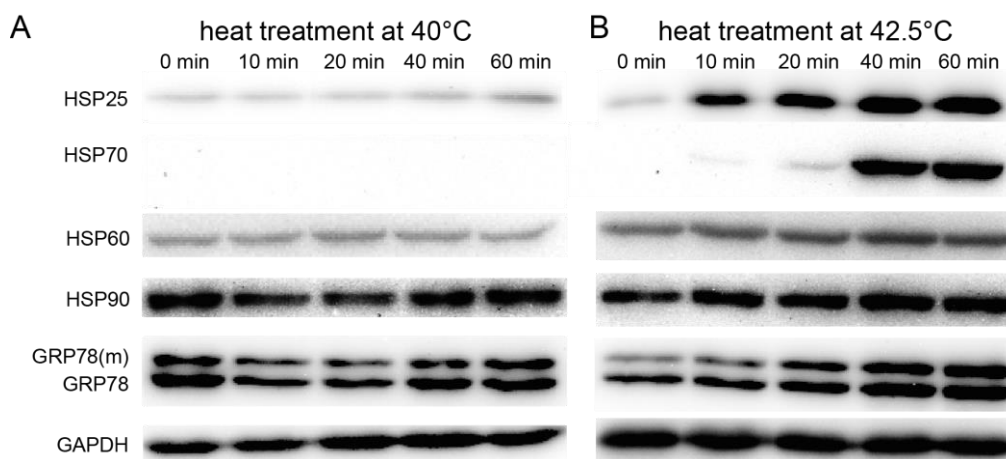


Figure 3.1 The effect of heat shock duration on HSP induction. A GPI-mGFP expressing CHO cell line was subjected to 40°C (A) and 42.5°C (B) heat for the specified time periods, and samples were prepared for western blotting after 6 h of recovery at 37 °C.

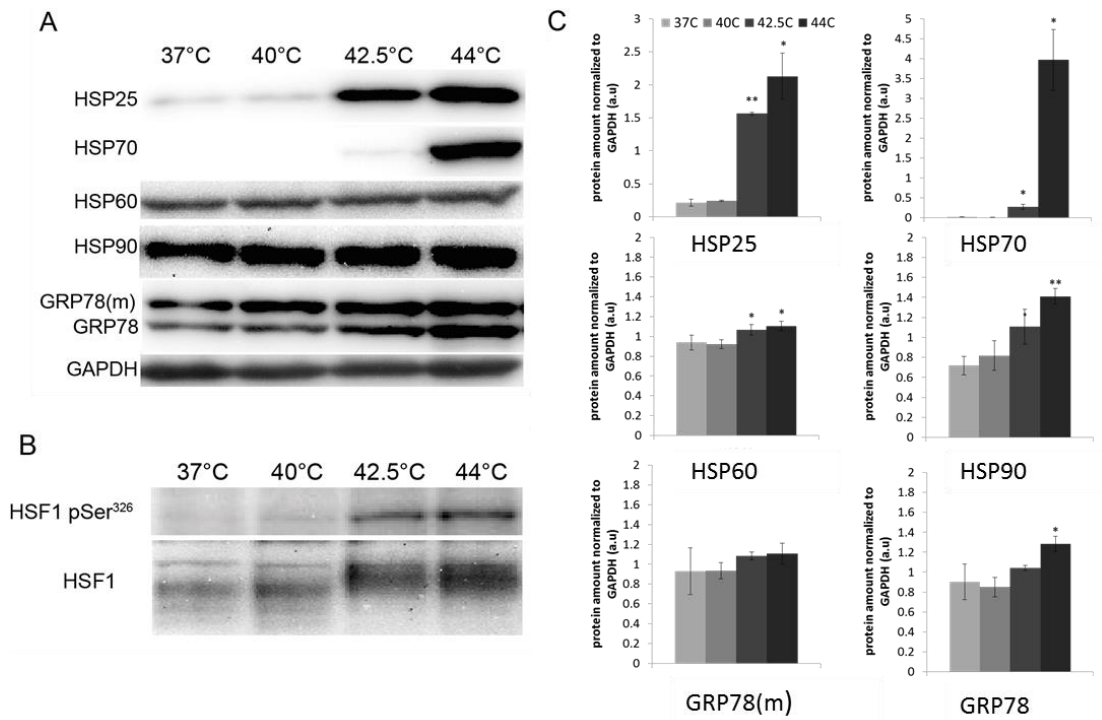


Figure 3.2 The effect of temperature on HSP induction and HSF1 activation. (A) A GPI-mGFP-expressing CHO cell line was subjected to a 20-min of incubation at the specified temperatures; the samples were prepared for western blotting after 6 h of recovery at 37 °C. (B) HSF1 phosphorylation was analyzed in samples after 20 min of heat treatment without recovery. Data are represented as Mean \pm SEM, N=3, * $p < 0.05$ and ** $p < 0.01$ compared to 37°C.

HSF1, the major orchestrator of the HSP synthesis is modified before it interacts with heat shock elements and then starts the transcription of heat shock proteins. Not all the modifications result in HSP transcription, but a specific phosphorylation site on Serine 326 is shown to be correlated with HSP induction (Guettouche et al. 2005) Therefore we investigated both the total HSF1 modifications as bandshift and phosphorylation on Ser326. In these experiments, sample collection was done right after 20 minutes of heat treatment. The observed phosphorylation and increase in the molecular weight of HSF1 was observed at 42.5° and 44°C seemingly in a comparable extent. No change was observed at 40°C in HSF-1 post translational modifications in line with absence of HSP induction (Figure 3.2 (B)).

3.1.2. Heat shock protein redistribution is dependent on the level of heat stress

One of the consequence of HSR is the redistribution of HSPs within the cellular compartments to prevent and protect the protein aggregation (Velazquez and Lindquist 1984). In order to investigate redistribution patterns of the most heat inducible HSPs; HSP25 and HSP70 were monitored after 20 minutes heat treatment which was found to be sufficient to cause an increase in protein levels at 42.5 °C (Fig 3.1). Both HSP25 and HSP70 were found to be transiently localized to nucleus in a dose dependent manner (Figure 3.3).

HSP70 localization in the nucleus was reported to protect thermo labile proteins while forming a large insoluble loci for aggregated proteins. Its translocation is aided and specific through binding partners and carrier proteins (Kose and Imamoto 2014; Velazquez and Lindquist 1984). At 40°C in the majority of the cells HSP70 showed nuclear accumulation which was enhanced further at 42.5°C with the appearance of nuclear spots. At 44°C HSP70 was localized in the particular spots in the perinuclear area.

Heat induced HSP25 relocalization was in a similar fashion; increased nuclear localization at 40°C and 42.5°C but a perinuclear appearance at 44°C. Interestingly, HSP25 was more heterogeneous at 40°C and less responsive compared to HSP70 in terms of nuclear enrichment. This effect was also not in line with protein induction experiments where HSP25 was shown to be more responsive to heat with a significant induction at a lower stress dose. HSP25 slows down the heat induced aggregation in vitro and binding of heat denatured nucleosolic proteins may serve for the subsequent chaperoning or degradation of the bound proteins (Bryantsev et al. 2007; Hayes et al. 2009). Although some reports suggest a phosphorylation dependent translocation, both the phosphorylation mutant version and treatment with kinase inhibitors are failed to inhibit accumulation in the nucleus in support of our results (Figure 3.4) where major phosphorylation was observed at 44°C in two possible residues Serine 15 and Serine 86.

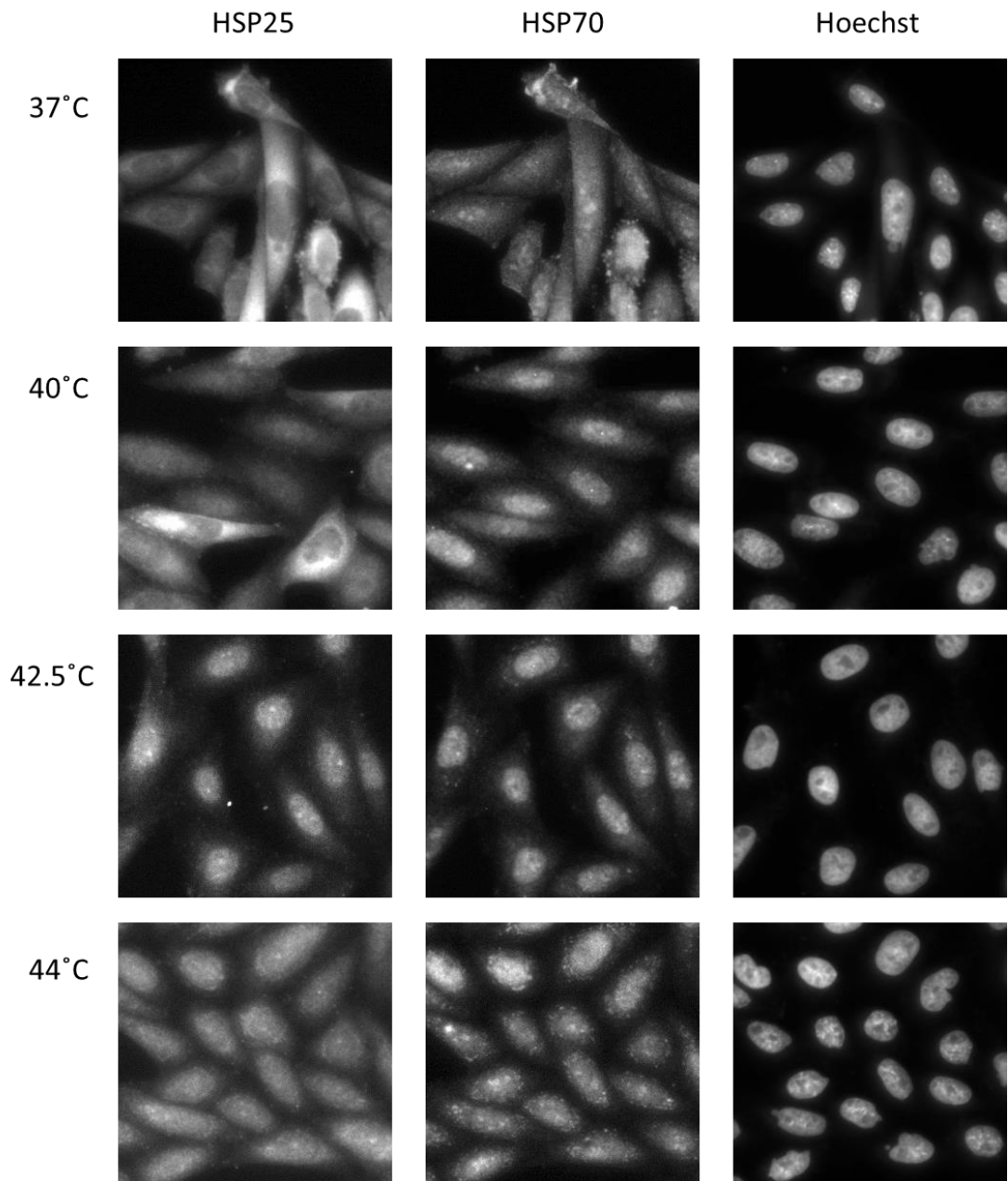


Figure 3.3 Redistribution of HSPs is dose dependent. Representative images ($n=40$) of CHO cells after a 20-min heat treatment at the indicated temperatures are shown.

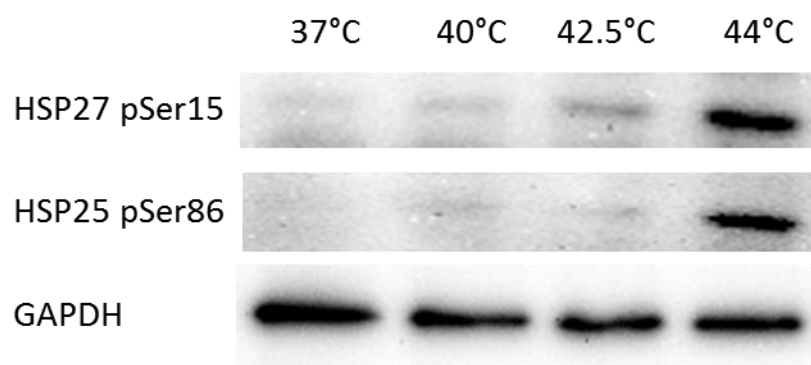


Figure 3.4 The effect of heat treatment on the phosphorylation status of HSP25. CHO cells harboring the GPI-mGFP construct were treated at a given temperature for 20 min before sample preparation for western blotting.

3.1.3. Acquired thermotolerance is highly dependent on stress dose

Ability to withstand a subsequent stress is observed in the heat shocked cells. Acquired thermotolerance is often related with the induction of HSPs and their antiapoptotic functions (Yokota et al. 2003; Kennedy et al. 2014). HSF-1 knock-out cell lines are shown not to gain thermotolerance, and transfecting HSP25 and HSP70 constructs to HSF-1 knock out cell line does not restore ATT (Luft et al. 2001). In order to test the effect of heat dose on the increased survival ATT assay was carried out. CHO cells were treated with 40°C, 42.5°C and 44°C for 20 minutes. After recovery period at 37°C for 6 hours, a lethal heat treatment was performed at 46°C for 20 minutes. Cells which were not heat shocked before showed very poor survival after (Fig 3.5A). As expected at 42.5°C and 44°C where HSP induction was observed, survival was above 50%. However, 40°C treatment resulted in significant ATT (Figure 3.5A) suggesting the involvement of other factors which can contribute to better survival. Moreover at 42.5°C treatment for 20 minutes resulted in an increase of HSP25 (Figure 3.1C), whereas at 44°C both HSP25 and HSP70 were induced (Figure 3.1C). Therefore it is not possible to link the survival to either HSPs alone.

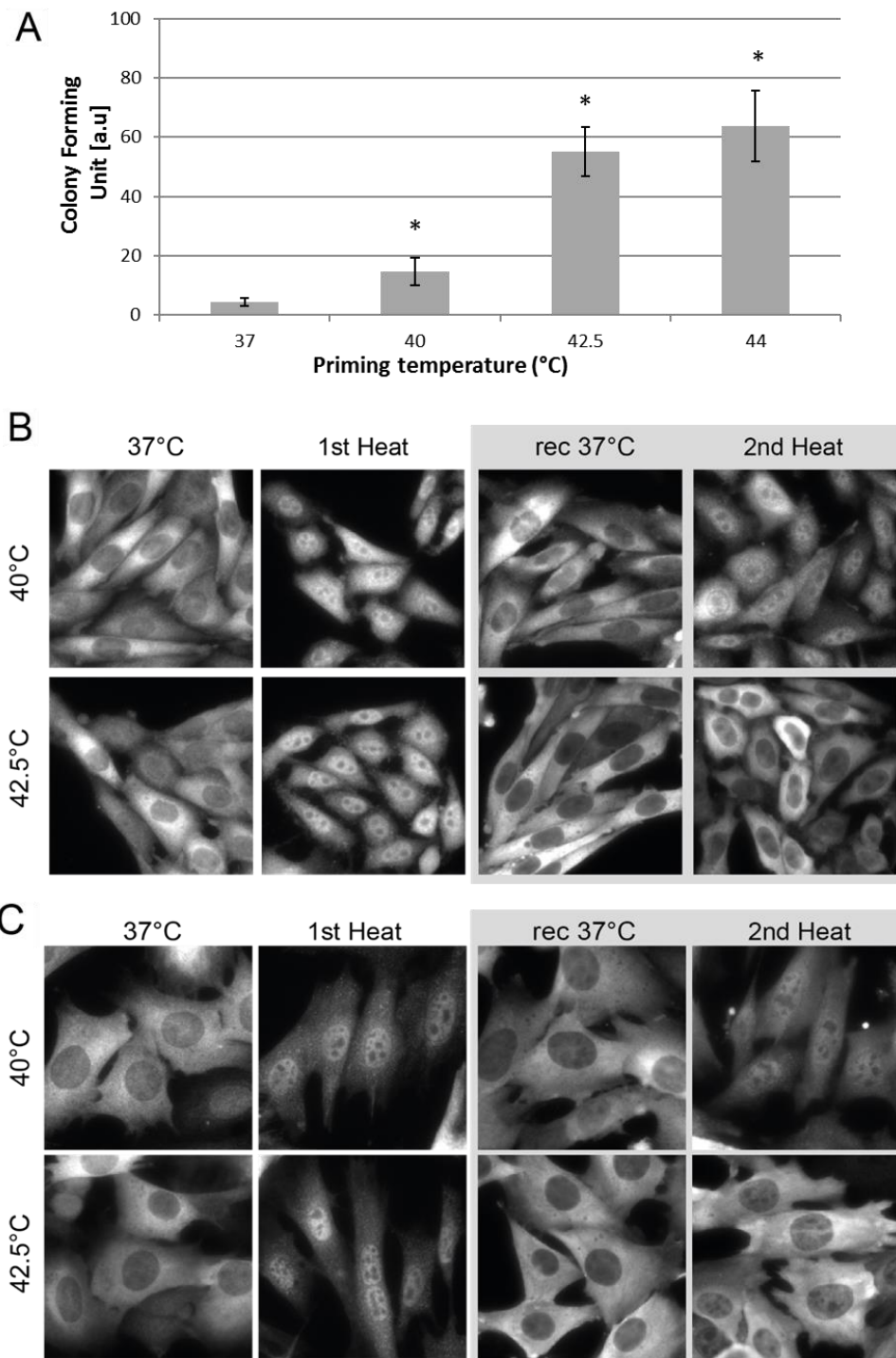


Figure 3.5 Different heat treatments affect ATT in a distinct manner. CHO cells were treated at given temperatures for 20 minutes followed by a recovery period of 6 hours at 37°C and a subsequent heat treatment at 46°C in order to test the survival (A). Cellular localizations of HSP25 were tested after two subsequent heat treatments separated with 6 hours of recovery at 37°C in CHO (B) and MEF (C) cells. All treatments were carried out for 20 minutes at given temperatures.

Next, the effect of thermotolerance was tested on HSP25 translocation to the nucleus in a modified thermotolerance assay. CHO cells were treated with 40°C and 42.5°C for 20 minutes and returned back to 37°C for recovery period of 6 hours in order to allow HSP25 synthesis to occur. Then a second heating cycle was carried out with the same temperatures. As expected translocation to nucleus occurred at 40°C in a more heterogeneous context whereas at 42.5°C it occurred more uniformly (Figure 3.5B). After the recovery period, at both 40°C and 42.5°C treatments, nuclear HSP25 was lowered and cytoplasmic signal was observed. Moreover, at 42.5°C HSP25 signals increased supporting Western blot experiments (Figure 3.1C). After the second heating cycle, the effect of 40°C was similar to the first heating cycle. As for 42.5°C, nuclear enhancement did not occur despite the fact that the nuclear HSP25 signals were found to be comparable (data not shown).

3.1.4. Cells promptly respond to heat by remodelling plasma membrane

Heat stress is shown to induce the alterations in the physical properties of plasma membrane such as fluidity and microdomain organization (Török et al. 2014). In order to reveal the temperature dependent changes at mild and moderate stress camera based type of Fluorescence Correlation Spectroscopy (FCS) was used to monitor lateral diffusion and confinement properties of fluorescent probes in relatively high temporal resolution. Total internal reflection fluorescence microscopy allows to carry the measurements with a depth of 100 nm into the sample, therefore restricting the observations to the plasma membrane region. After post-analysis of the images, diffusion coefficients (D) and diffusion times (τ_0) were calculated which describe the lateral diffusion of the probe and the membrane organization, respectively. In the membrane there are three diffusion modes; hop diffusion ($\tau_0 < 0$), free diffusion ($\tau_0 = 0$) and confined diffusion ($\tau_0 > 0$) (Ng, Bag, and Wohland 2015).

In CHO cells imaging GPI-mGFP revealed a significantly increased diffusion coefficient at 42.5°C whereas unexpectedly at 40°C it remained unchanged (Figure 3.6A). A possible explanation for sustained fluidity is the cells' ability to maintain the membranes by lipid modelling which was supported by the elevation of long chain

sphingolipids and removal of lysophospholipids (Figure 3.9 and 3.10). In order to test this hypothesis, plasma membrane sheets were prepared by removing all the cellular structures with the use of sonication except the plasma membrane attached to the bottom of the coverslip. This methods allows the measurement of fluidity and lateral organization without any maintenance provided and it resulted in constant temperature dependent increase in diffusion coefficients including 40°C (Figure 3.6 C).

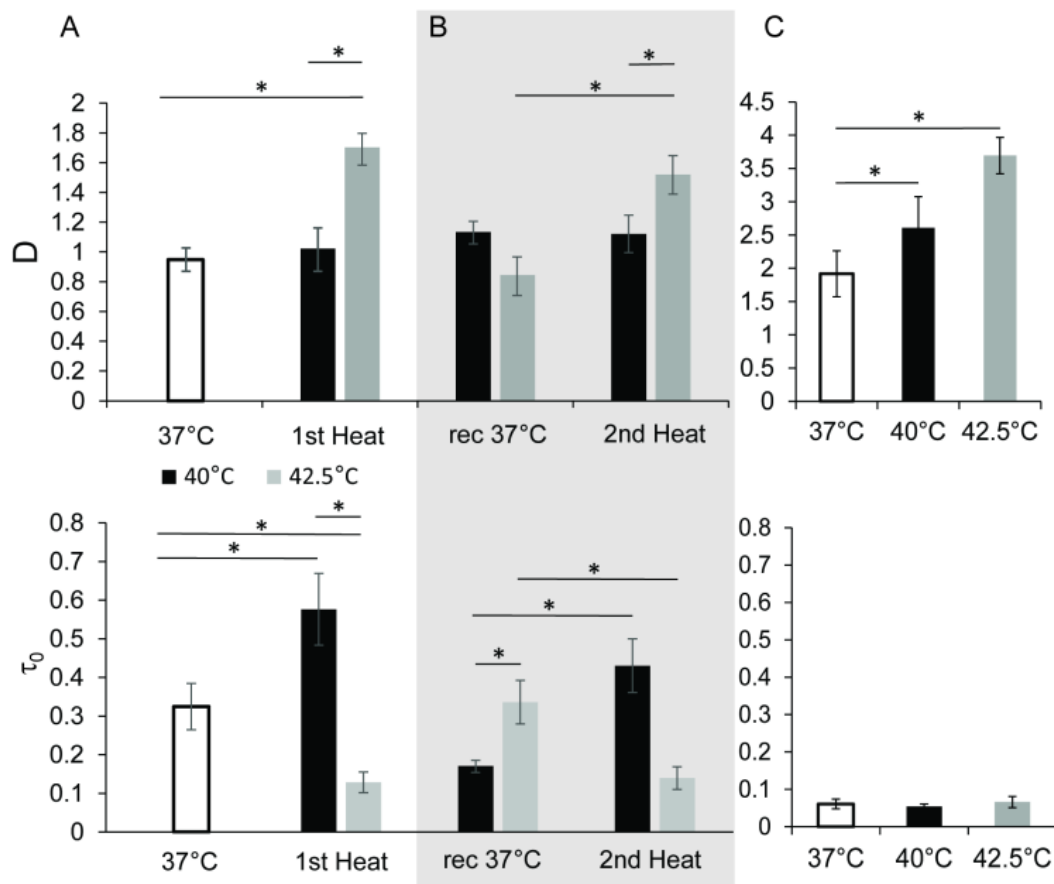


Figure 3.6 Lateral diffusion of GPI-mGFP behaves differently upon mild and moderate heat in live cells. Diffusion coefficient (D) and diffusion time (τ_D) of GPI-mGFP on the surface of CHO cells at 37°C (white bars), 40°C (black bars) and 42.5°C (grey bars) after given heat treatments (A), second heat cycle after 6 hours recovery period (B) and on CHO plasma membrane sheets (C). Error bars represents the standard error of mean (SEM) ($n=8$, $p<0.05$).

Positive diffusion time hence confined diffusion was observed at all temperatures, but under the effect of heat an increase confinement was observed at 40°C which was below the normal at 42.5°C (Figure 3.10A). Approximately 20-40% of GPI-anchored proteins on the membrane are present as clusters and the rest are monomers which are in a dynamic movement towards in and out of the relatively immobile nanoclusters (Sharma et al. 2004; Sengupta et al. 2011). Therefore the measured confinement is possibly due to these interactions with relatively immobile platforms. Increase confinement at 40°C can be due to cellular regulation and nucleation of GPI-mGFP clusters. Moreover, this effect was lost when the plasma membrane sheets were exposed to elevated temperature.

In order to understand the effect of second heating cycle on the membrane biophysics, experiment was repeated on the samples recovered for 6 hours after the first heating cycle with the same temperature regime. The heat induced changes were very similar to the first heating cycle for both D and τ_D (Fig. 3.10B). On the other hand, in case of second 40°C treatment the GPI-mGFP diffusion was significantly less confined then before the first heating cycle (smaller τ_D).

3.1.5. Lipidomics reveals distinct stress lipidomes

In order to assess lipid changes occurring during heat shock comprehensively, electrospray ionization mass spectrometry based lipidomics analysis was carried out using total cellular membrane lipid extracts. Approximately 160 molecular species were identified and analyzed using this high-sensitivity and high-resolution shotgun lipidomics approach. Sparse partial least squares discriminant analysis revealed a clear separation of 4 temperature treatments; 37°C, 40°C, 42.5°C and 44°C. Hierarchical clustering analysis was illustrated on the heat map depicts the changes occurred in different temperatures (Figure 3.7). Mild (40°C) and moderate (42.5°C) stress resulted in 30% of the lipidome to be altered whereas severe (44°C) stress altered 50% of the lipidome.

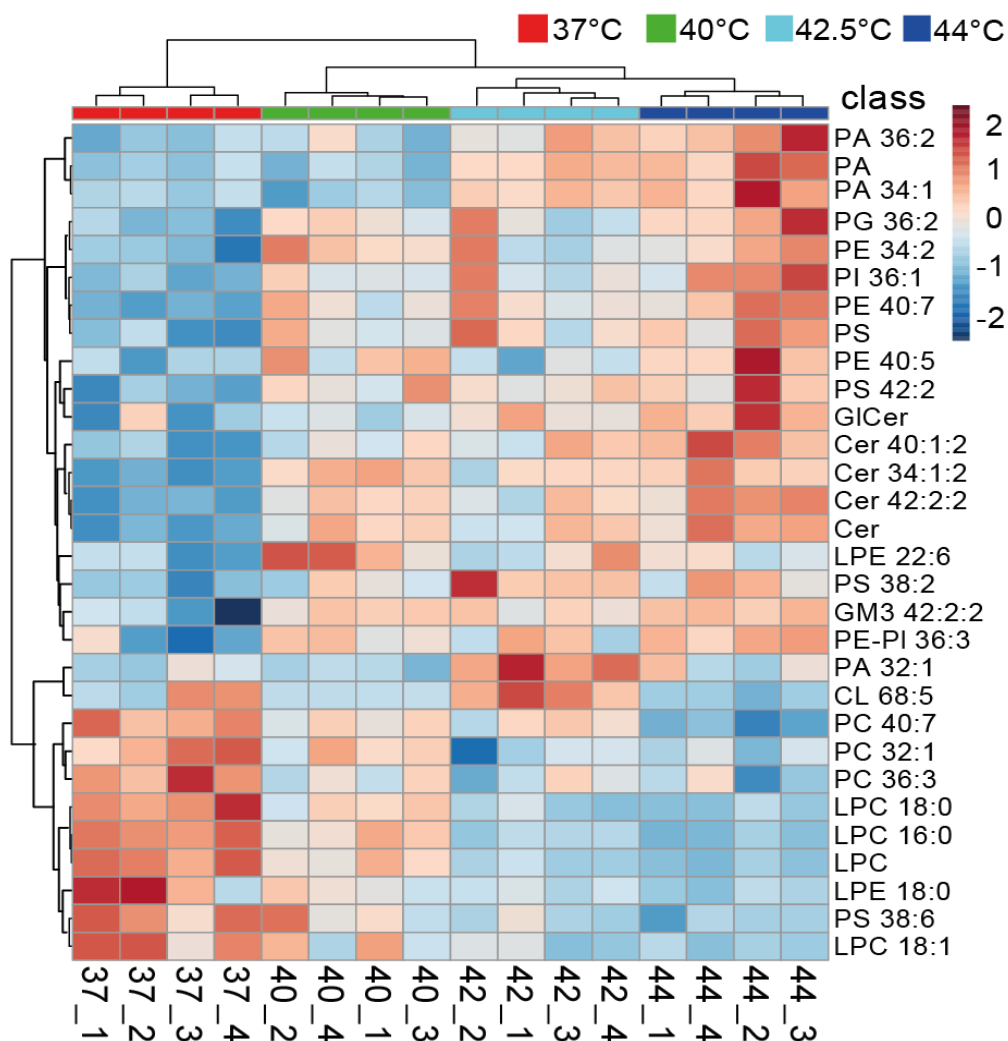


Figure 3.7 Characterization of lipidomes of GPI-mGFP-expressing CHO cells exposed to different levels of HS. Heatmap representation of hierarchical clustering of lipid profiles at 37°C, 40°C, 42.5°C and 44°C. The top 30 most significant species were selected using ANOVA, Euclidean distance, and clustering algorithm Ward. The heat color-code represents normalized values (z-scores), ranging between [-2] (darkest shade of blue) and [+2] (darkest shade of red). Data from four independent experiments with control and HS-treated cells are shown.

All mild, moderate and severe stress treatments resulted in distinct alterations in the lipid compositions. PA is a low abundant lipid of cellular membranes which can act as a second messenger and precursor for other lipids. Increase of intracellular PA is found to be linked to phosphorylation of Akt and mTOR-dependent signalling (Bromberg and Weiss 2016). Our results showed an increase in PA at moderate stress with further gradual increase as the temperature increases (Figure 3.8). Increase in PA is often related with the activation of PLD which hydrolyzes PC and PLC (Y. Liu, Su, and Wang 2013).

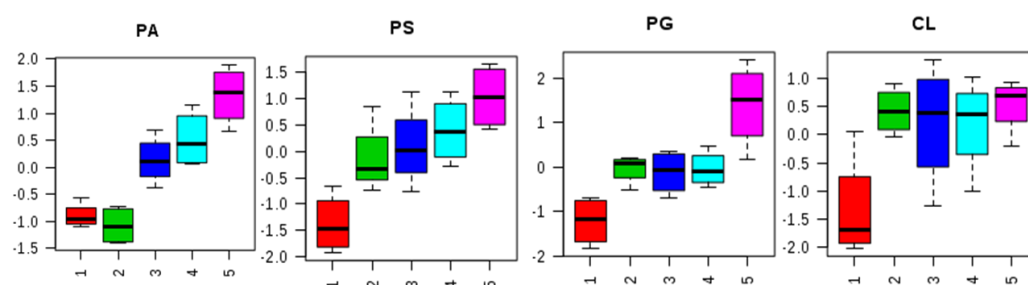


Figure 3.8 Key features of HS-associated lipid remodeling of CHO lipidomes. CHO cells with GPI-mGFP constructs were either unstressed (37°C (1)) or stressed at 40°C (2), 42.5°C (3), 44°C (4) and 46°C (5) for 20 min. Phosphatidic acid (PA), phosphatidyl serine (PS), phosphatidyl glycerol (PG) and cardiolipin (CL) levels were shown in arbitrary units normalized by mean-centering and dividing by the standard deviation of each variable.

At mild temperature 40°C, a significant increase was observed in PS, PG and CL (Figure 3.8). PS is a negatively charged phospholipid mainly found on the inner leaflet of the plasma membrane unless the cell enters the apoptotic pathway in which outer leaflet localization of PS is the hallmark. Increase of PS on the plasma membrane can have two outcomes; first it can attract membrane domains that can recognize the head group stereospecifically, secondly its negative charge can attract number of signalling molecules such as small GTPases (Kay and Grinstein 2013). CL which is a unique lipid mainly found in the mitochondrial membranes and has fundamental functions in maintaining functional integrity and mitochondrial dynamics. Alterations

in the CL has very important consequences in the cell such as triggering apoptosis and causing an increase in ROS (X.-X. Li et al. 2015). In eukaryotes, the synthesis of CL is catalyzed from PG. Mutation of the enzyme responsible for this reaction causes the perturbation of mitochondrial metabolism, increased mitophagy and vacuolar defects under stress conditions (de Paepe, Lemaire, and Danon 2014). During mild stress, the accumulation of CL and its biosynthetic precursor PG is related to temperature induced mitochondrial biogenesis, aiding increased cellular respiration to supply the elevated energy demand of the cells during stressful conditions (C.-T. Liu and Brooks 2012).

Ceramide is a well documented lipid which are not only the constituents of the cell membranes but also second messengers. They can trigger specific responses that can reprogram the gene expressions causing the cell to proliferate, differentiate or undergo apoptosis. In addition sphingolipids are found to be one of the early responders to the stress (Chen et al. 2015).

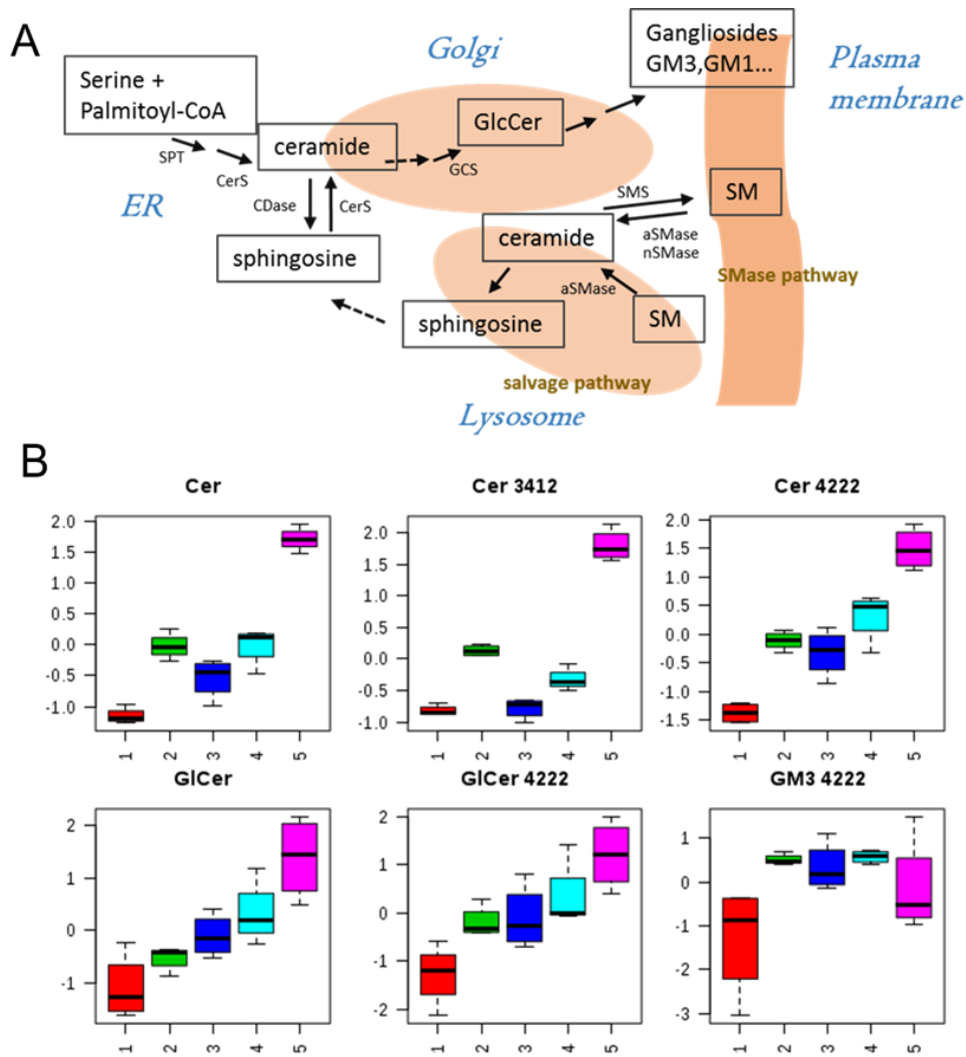


Figure 3.9 Heat induced changes in the ceramide metabolism. Biosynthetic pathways for ceramide synthesis are shown based on cellular compartments (A). Heat induced alterations after 20 minutes of treatment at 37°C (1), 40°C (2), 42.5°C (3), 44°C (4) and 46°C (5) in CHO cells with GPI-mGFP construct. Ceramide (Cer), Ceramide 34:1:2 (Cer 3412), Ceramide 42:2:2 (Cer 4222), Glucosylceramide (GlCer), Glucosylceramide 42:2:2 (GlCer 4222) and Ganglioside GM3 42:2:2 (GM3 4222) levels were shown in arbitrary units normalized by mean-centering and dividing by the standard deviation of each variable. (Serine palmitoyltransferase (SPT), ceramide synthase (CerS), neutral (n) or acid (a) sphingomyelinase (SMase), ceramidase (CDase), glucosylceramide synthase (GCS), sphingomyelin synthase (SMS)).

Our results showed a significant increase in ceramide levels (Figure 3.9B). Specially in the case of mild heat treatment at 40°C, two ceramide species 34:1:2 and 42:2:2 were significantly elevated as well as the total ceramide. At 42.5°C there was no further increase observed except Cer 34:1:2 where no change was measured. At 46°C, the increase was much higher, however this treatment was carried to measure the change in a lethal, apoptosis inducing heat shock. Moreover complex sphingolipids were also shown to be elevated. Glucosyl ceramide and ganglioside GM3 elevation was measured to start at 40°C and increase further with increasing temperature (Figure 3.9B). A simplified illustration of the very complex sphingolipid metabolism is shown on Figure 3.9A. Briefly *de novo* ceramide synthesis occurs in the ER and then transported to Golgi apparatus either by vesicles or ceramide transfer proteins. Ceramide can be converted to glycosphingolipids either in ER or in golgi apparatus whereas sphingomyelin synthesis occurs either in golgi apparatus or in plasma membrane. However, there is evidence on ceramide synthesis in the plasma membrane from sphingosine through $\text{Ca}^{2+}/\text{Mg}^{2+}$ dependent ceramide kinases (Gault, Obeid, and Hannun 2010). Another pathway for ceramide synthesis in plasma membrane is the conversion of sphingomyelin to ceramide via sphingomyelinases. ASMase is predominantly a lysosomal enzyme, after its secretion into the extracellular space it can metabolize outer leaflet SM on the plasma membrane. An important regulation on this mechanism is reported to be Ca^{2+} directed endosomal trafficking to a specific area in the plasma membrane (Tam et al. 2010). nSMase can be found either in golgi or in plasma membrane and has preference for C24:0 to C24:1 species. Due to the complexity of the sphingomyelin synthesis, it is not possible to reveal the cellular compartment which is responsible for ceramide increase. Inhibitor based studies yet reports *de novo* synthesis pathway to be responsible (Gary M. Jenkins et al. 2002). Nevertheless independent of its origin, ceramide is capable of triggering signal transduction pathways which is highly important in lipid controlled heat shock response. JNK is a pro-apoptotic signalling pathway, PKC ζ and Ras can be activated with increased ceramide levels whereas PKC α and Akt can be inhibited (Ruvolo 2001). The activation of the signal transduction pathways can arise from the impact of ceramide in the biophysical features of the membranes since it has capability of promoting lateral segregation of lipid domains. It was shown that very long chain

unsaturated ceramides (C24:1) can drive the formation of gel domains (Castro, Prieto, and Silva 2014) which is supported in our results as 42:2:2 ceramide species shown to be increased both at 40°C and further within 10 minutes of treatment (Figure 3.9).

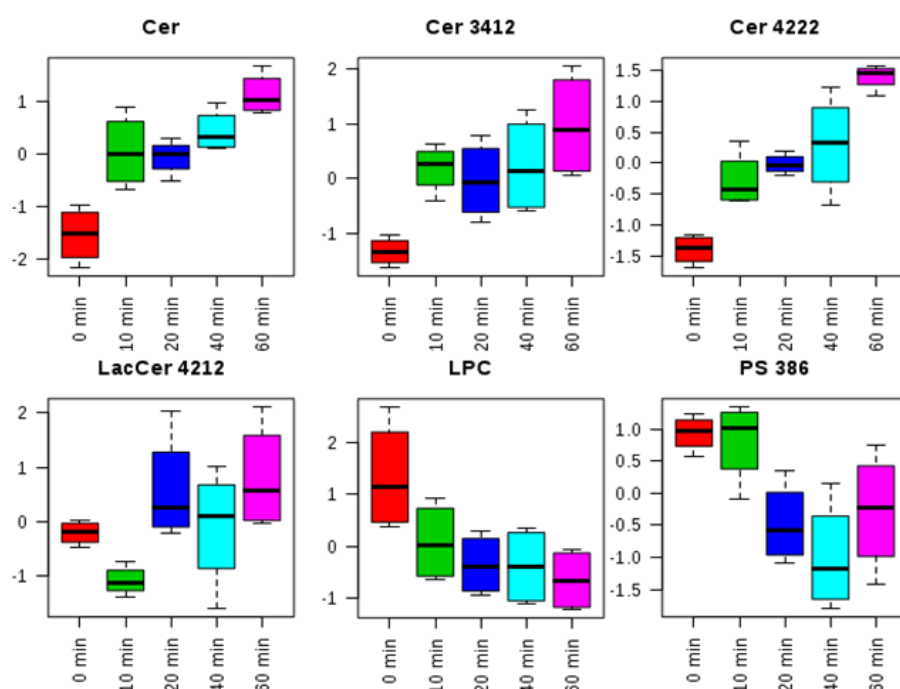


Figure 3.10 Time dependent key lipid changes in CHO cells with GPI-mGFP construct. Heat induced changes were measured at 42.5°C at 0, 10, 20, 40 and 60 minutes. Ceramide (Cer), Ceramide 34:1:2 (Cer 3412), Ceramide 42:2:2 (Cer 4222), Lactosylceramide 42:1:2 (LacCer 4212), Lysophosphatidylcholine (LPC), Phosphatidylserine 38:6 (PS 386) levels were shown in arbitrary units normalized by mean-centering and dividing by the standard deviation of each variable.

In order to investigate the time based differences, lipidomics analysis was performed at 42.5°C at different time points. As early as 10 minutes, changes in sphingolipid metabolism was observed (Figure 3.10). Ceramide species were elevated as the duration of stress increased. The prompt decrease in LacCer can be explained by catalysis of it for the formation of Cer, however further analysis are needed to prove the direct link and effect. Rapid elevation of sphingolipid species has been proposed to trigger changes in several cellular processes, including transcription, translation, growth, and actin organization (Meier et al. 2005). Prompt increase in

long FA containing complex sphingolipid (Figure 3.9 and 3.10) may support the reorganization of plasma membrane domains (Chen et al. 2015).

Another significant change was observed in lysoPC which was gradually decreased with time (Figure 3.10) and with the increased heat treatment (Figure 3.7). Due to its inverted cone shape, LysoPC is a strong curvature inducing and destabilizing lipid class. It is formed by the hydrolysis of PC by phospholipases. Therefore, these results suggest the activation of phospholipases by heat. Due to its curvature inducing properties, change in the plasma membrane structure can also be expected and in order to stabilize the heat induced fluidity, decreasing LysoPC species can be a good strategy of the cell. Therefore, the effect of exogenous addition of LysoPC on plasma membrane organization was tested. Although the increase of LysoPC was analyzed and confirmed by lipidomics analysis (Figure 3.11B), no effect on the integrity of GPI-mGFP nanoplateforms were observed (Figure 3.11A) (Brameshuber et al. 2016). There are reports on the effects of lysolipid induced phosphorylation of focal adhesion kinase (Schaller, Hildebrand, and Parsons 1999) and a possible transient receptor potential channel modulation (Taberner et al. 2015). Further experiments are needed in order to understand the background of heat induced changes in lysophospholipids.

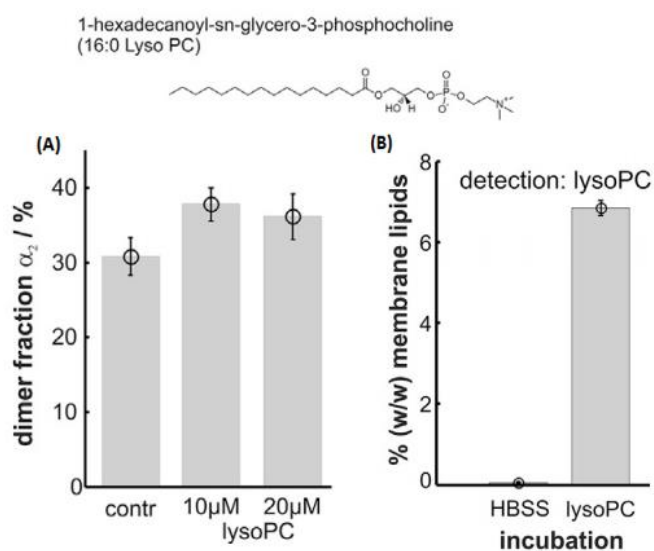


Figure 3.11 Effect of Lyso-PC addition on plasma membrane of CHO cells with GPI-mGFP construct. After 20 minutes of exposure to 10 μ M Lyso-PC, GPI-mGFP dimer fraction was measured (A) and MS analysis was carried to measure the incorporation of Lyso-PC (B).

3.1.6. Stress signalling is linked to plasma membrane remodelling

The observations on the heat induced changes on the plasma membrane biophysical properties and in the cellular lipidome supports the “membrane sensor hypothesis” which suggests that the alterations in the plasma membrane can affect the level of HSPs. Plasma membrane localized calcium channels and insulin receptors are good candidates for possible membrane sensors which can get activated due to surrounding lipid changes (Török et al. 2014; Nagy et al. 2007).

Calcium signalling

TRPV channels are studied intensively for their involvement in the heat shock response. Not only their presence on the plasma membrane and mechano-sensitive properties but also the stress induced Ca^{2+} increase makes TRPV channels a possible membrane sensor of heat (Bromberg and Weiss 2016). In support of this hypothesis blocking Ca^{2+} entry to the cytosol by using molecular chelators was shown to have negative effect on the plant heat shock response (Saidi et al. 2009) and

acclimatization to higher temperatures results in lowered Ca^{2+} peaks (Saidi et al. 2010).

Therefore, we monitored temperature dependent increases in cytosolic calcium by measuring Fura-2 fluorescence which is a ratiometric indicator. It's excitation shifts in the presence of Ca^{2+} allowing a concentration independent measurement. MCF-7 cells were used instead of CHO cells, because the multidrug resistance transporters extrude the fluorescent probe from CHO cells (Homolya et al. 1993). By applying a general phosphatase inhibitor sodium orthovanadate, the effect was reversed and CHO cells were loaded efficiently (data not shown), however due to possible intervention with the heat shock signalling pathways experiments were carried out using MCF-7 cells.

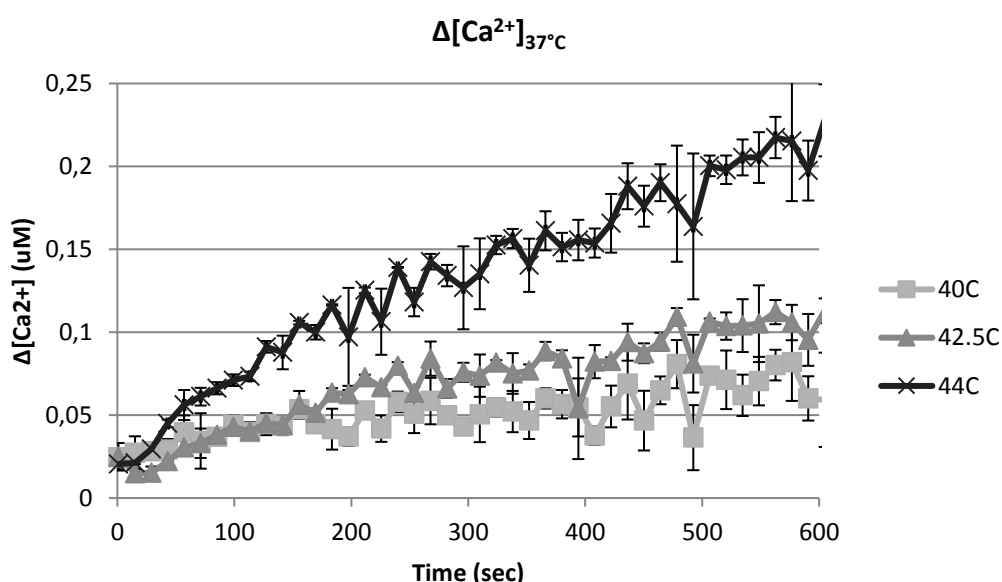


Figure 3.12 Heat dose dependent Ca^{2+} increase in MCF-7 cells. Cells were loaded with Fura-2 AM and the intracellular calcium increase was measured in a heated fluorimeter. $\Delta[\text{Ca}^{2+}]_{37^\circ\text{C}}$ was calculated by subtracting the basal levels at 37°C ($N=6$ and error bars represents standard deviation).

Intracellular Ca^{2+} concentration was $0.11 \pm 0.02 \mu\text{M}$ in MCF7 cells at 37°C . Figure 3.12 shows the change of intracellular Ca^{2+} as a function of time at different temperatures. Elevation to 40°C or 42.5°C resulted in comparable change of cytosolic

Ca²⁺ concentrations while in case of severe 44 °C stress intracellular Ca²⁺ increase was more pronounced. These findings lend support to a possible sensing of environmental changes by the calcium channels. Further analysis of the agonist and antagonists to modify the channel activity however did not result in any effect on the HSP25 translocation (data not shown). Moreover at 40°C observed Ca²⁺ increase does not cause the HSP induction in MCF-7 cells (data not shown). However, the effect of Ca²⁺ sensitive/dependent changes such as activation of phospholipases and subsequent release of lipid mediators can be linked to the perturbation of the plasma membrane (Balogh et al. 2005). Another important finding was the difference in the level of Ca²⁺ with mild/moderate and severe stress. The source of cytoplasmic Ca²⁺ increase can be external through plasma membrane and/or the internal stores of ER and mitochondria. The consequences of the depletion of internal stores has been linked to induction of apoptotic pathways (Kaufman and Malhotra 2014; Görlach et al. 2015) and therefore in order to understand if the higher increase in Ca²⁺ with higher temperature is linked to depletion of internal stores, further experiments are needed.

IGF1R signalling

One of the most probable candidates for membrane sensors under these conditions are receptors or receptor networks, since specific receptors are inconsistent with the lack of stress response specificity for the stressor itself. The structural changes in the members of this putative stress sensing receptor network could provide both a rapid signal and an immediate feedback to control the effectiveness of the cellular stress response. For instance the nonspecific clustering of growth factor receptor tyrosine kinases and cytokine receptors during osmotic, UV-radiation and oxidative stress can activate these receptors and downstream stress signalling in mammalian cells (Rosette and Karin 1996; Ouwens et al. 2001; Handayaningsih et al. 2011; Tahimic et al. 2016). We chose IGF1R/IR receptors as models since these receptors were implicated many times in stress signalling in *C.elegans* (Barna et al. 2012), hyperosmotic stress in CHO (Ouwens et al. 2001).

We observed that IGF1R/IR receptor was significantly activated upon mild heat treatment (Figure 3.13). At a moderate heat (42.5°C) the phosphorylation of this receptor shows a transient pattern having an increased activity for a few minutes followed by a downregulation in parallel with ERK1/2 activation (Figure 3.13 and Figure 3.14). It is well established that glucose deprivation induces HSP synthesis through the downregulation of IGF1R/IR pathway (Chiang et al. 2012; Barna et al. 2012; Hsu, Murphy, and Kenyon 2003; Hooper and Hooper 2005). This effect is completely antagonized by dexamethasone, the synthetic glucocorticoid which is a well know membrane fluidizing agent with the ability to remodel cellular lipid composition (Kasambalides and Lanks 1985). A more general role for insulin in the heat shock response has already been provided by the finding that cell surface insulin binding is reduced by heat shock in HA-1 CHO cells. It has been suggested that the fate of insulin receptor mirrors intimately the ultimate destiny of the cells after heat treatment (Calderwood and Hahn 1983). Alteration in the microenvironment of the receptor might cause allosteric effects and lead to changes in its equilibrium binding properties. However, the affinity constants of thermotolerant cells were similar, thereby it was proposed that heat induced receptor depolimerization could be responsible for the observed effect. Their explanation was based on an earlier observation on the lipid composition and membrane fluidity dependent dimerization of the insulin receptor (Ginsberg, Jabour, and Spector 1982). Receptor tyrosine kinases were suggested to be connected to HSP signalling pathways in NIH3T3 fibroblasts by Lin et al (R. Z. Lin et al. 1997). Their results indicated that the signal pathways used by heat shock to activate MAPK and p70 S6 kinase resemble those used by growth factors, and heat shock activation of MAPK is blocked by suramin, a tyrosine kinase receptor inhibitor. Protein perturbation caused by hyperthermia may in some way stimulate cell surface receptor tyrosine kinases to initiate the downstream signaling events. Indeed, inhibition of heat shock activation MAPK by tryphostin AG1478 and a dominant negative EGFR mutant strongly suggests that EGFR may also be involved in the heat shock response. Integrin signaling also regulates activation of IGF1R and downstream targets in response to both IGF1 and a mechanical stimulus (Tahimic et al. 2016).

Further supporting that insulin signalling is involved in membrane sensing of heat the protection of a small molecule, BGP-15 against heart failure and atrial fibrillation was associated with an increased phosphorylation of the insulin-like growth factor 1 receptor (IGF1R) (Sapra et al. 2014). Since BGP-15 was also shown to be an HSP co-inducer and its ability to activates stress signal transduction pathways by remodeling plasma membrane rafts (Gombos et al. 2011).

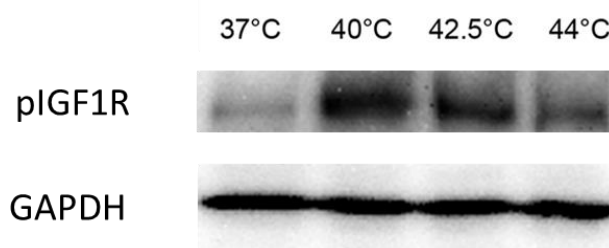


Figure 3.13 Mild stress activates the IGF1R. CHO cells with GPI-mGFP construct were treated with heat for 20 minutes at given temperatures. (N=3)

MAPK signalling

Cells activate signalling pathways in order to transmit the signals from external stimuli and to make critical decisions on poliferation, migration etc. MAPK pathway is three layer signalling cascade which involves several tyrosine kinase domains and regulatory tyrosines. Growth factor receptors and stress are known activators for these pathways (Cargnello and Roux 2011). ERK 1/2, JNK and p38 MAPK are three major branches which are shown to be activated through different signals. ERK1/2 and partially JNK can be activated due to activation of growth factor receptors that is also a possible stress sensor in mild temperatures (Katz, Amit, and Yarden 2007). Our results showed a low level of activation at 40°C of ERK1/2 and JNK due to phosphorylation (Figure 3.14). With increasing temperature, the phosphorylation levels were elevated with a strikingly high phosphorylation at 44°C (Figure 3.14). For ERK 1/2 and JNK the unstabilized phosphatases were shown to be responsible for the phosphorylation at protein denaturing temperatures (Nadeau and Landry 2007).

At mild temperatures, ERK1/2 activation at 40°C is possible through the receptor activation due to change in plasma membrane physical properties (confinement and fluidity) (Figure 1.2 and 3.6). It has been shown recently by Zhou and coworkers that PS maintains the lateral segregation of Ras nanoclustering and therefore a potential mediator for a lipid mediated signaling (Zhou et al. 2014; Zhou and Hancock 2015). In addition to that, treating human fibroblasts by PS resulted in an increased ERK 1/2 signaling which is known to be activated through Ras (Donyo et al. 2016). On the otherhand JNK can be activated through the activation of small GTPases with increasing PS on the plasma membrane as well (Figure 3.8) (Han et al. 2001; Kay and Grinstein 2013). However, in the mild temperature range (39°C), a small GTPase Rac1 was shown to inhibit the activation of SAPK/JNK in a Rac1 dominant negative mutant containing Rat-2 fibroblast cell line, whereas temperatures above that (41-43°C) preserved the activation of SAPK/JNK which suggests another level of regulation. Another supporting evidence for that is inhibition of SAPK/JNK activity in the first 10 min treatment of 41°C, however due to the inhibition after 20 min SAPK/JNK activation was observed (Han et al. 2001). Correspondingly the induction of HSPs were affected negatively in a similar manner without any effect on heat induced HSF1 phosphorylation (Gungor et al. 2014; Han et al. 2001).

Activation of p38MAPK by stress is distinct from both ERK and JNK. EGFR activation, inhibition by the specific inhibitors does not effect the p38 MAPK and inactivation of phosphatase is not involved (Nadeau and Landry 2007). Under oxidative stress, endoplasmic reticulum stress, infection and calcium influx specific set of kinases are involved in the activation of p38MAPK, namely ASK-1 (Shiizaki, Naguro, and Ichijo 2013; Matsukawa et al. 2004). Upon increase in sphingosine and ceramide during heat stress, ASK1 is titrated from its repressor glutathione S-transferase and activates the p38MAPK pathway (Nadeau and Landry 2007). Moreover p38MAPK is a known upstream effector of HSP25 phosphorylation (Vertii et al. 2006). Therefore, we investigated the level of p38MAPK in CHO cells expressing GPI-mGFP construct in a dose dependent manner. Westernblot analysis was carried out after 20 minutes of given heat treatment and no recovery time was given. Our results showed a highly enhanced p38MAPK phosphorylation at 44°C whereas a

minimal activation was also observed at 42.5°C (Figure 3.14). Furthermore increase in p38MAPK phosphorylation correlated well with the HSP25 phosphorylation levels at 44°C (Figure 3.4).

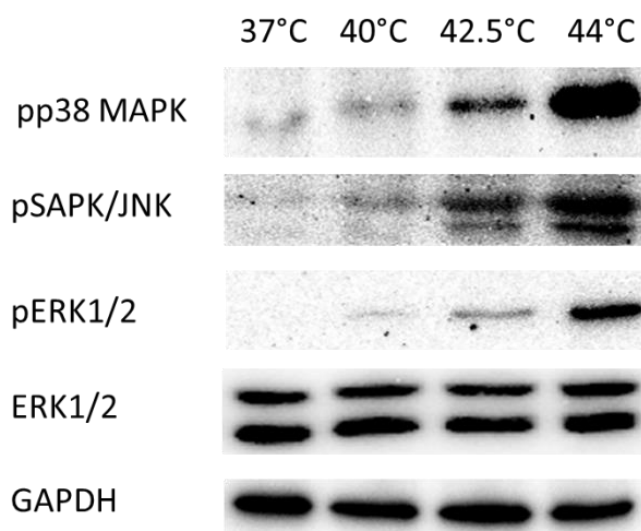


Figure 3.14 Dose dependent activation of stress signaling pathways. CHO cells expressing GPI-mGFP construct were treated at given temperatures for 20 minutes and collected for western blot experiments (N=3).

3.2. CELL TO CELL VARIATION OF HEAT SHOCK RESPONSE

3.2.1. Stress response of individual cells

It is a fundamental question whether all cells in a populations sense the imposed stress on the same level and whether they respond in the same manner. Majority of our knowledge on the cellular responses such as gene and protein expression are derived from the averages of a population of cells. The appearance of two sub-populations with different expression characteristics can result in the same mean of the big population, however the information is lost during the averaging process. Therefore single cell studies has the power of preventing information loss in that way. Furthermore, the correlation between the measured effect and the source of

heterogeneity e.g. gene expression profile and plasma membrane structure allows a better understanding of a biological phenomenon. In order to investigate the cell-to-cell variations and population context based differences in stress sensing and the stress response, single cell experiments were carried out following different approaches.

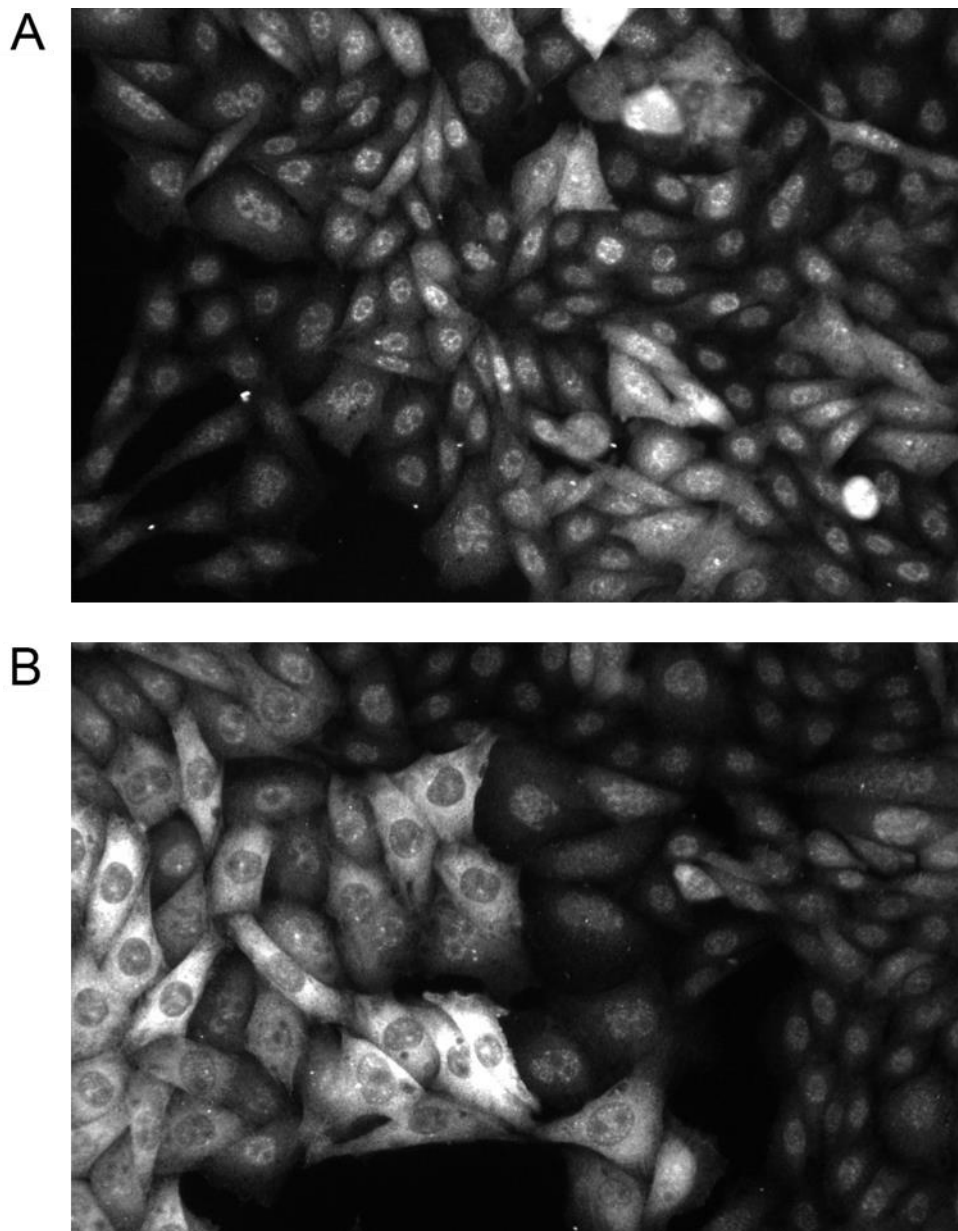


Figure 3.15 *Cell to cell variation is observed in cellular heat shock response. Immunolocalization of HSP25 after 20 minutes heat treatment at 40°C in CHO (A) and MEF (B) cells was represented.*

Fluorescent imaging is a powerful tool to investigate cell-to-cell variability. It allows the visualization of protein localization, intensity based differences of the probes in use and gives information about the surroundings of the cells such as local cell crowding. Therefore we applied fluorescence microscopy to analyze the individuality of heat-shock response in two cell lines by following the redistribution of HSPs into the nucleus as described in figure 3.2. Our results showed a pronounced heterogeneity in HSP25 redistribution (Figure 3.15). Since cell cycle phase is considered as a source of heterogeneity, cell cycle dependence of heat induced HSP25 redistribution was further investigated. Lipidomics of cell populations in different cell cycle phases were also analysed after isolating them using fluorescence activated cell sorter.

3.2.2. HSP25 translocation reveals the heterogeneity within the population

As described in stress models, the probability of a genotype/phenotype increases in the population with the highest fitness. However, if the stress levels are permitted to rise too far, the stress threshold may be reached. This causes the mass die-off, sterility and possible a local extinction in studies carried using fruitflies (Krebs and Loeschcke 1994; Clark and Fucito 1998; Jackson, Nielsen, and Singh 2015). In order to understand how population distribution is affected with heat dose, stress sensitivity was analyzed with HSP25 translocation to nucleus as a function of HSP25 intensity contrast in nucleus. The measurement of contrast allows an unbiased comparison of nuclear and total intensity obtained from HSP25 immunolocalization and the normalization of different samples. The heat dose dependent HSP25 translocation (contrast) indeed showed a greater variance when cells were treated with mild 40°C (Figure 3.16). As the temperature increased and cells reached to the stress threshold the variance dropped drastically (44°C). In accordance to these observations, the single cell experiments were carried out at moderate stress level (42.5°C) to be able to detect both low and high threshold of stress. An additional observation obtained was the continuous profiles of the population distribution. Subpopulations could not

be dissected at this resolution, however categories could be obtained by further image analysis of multiple parameters.

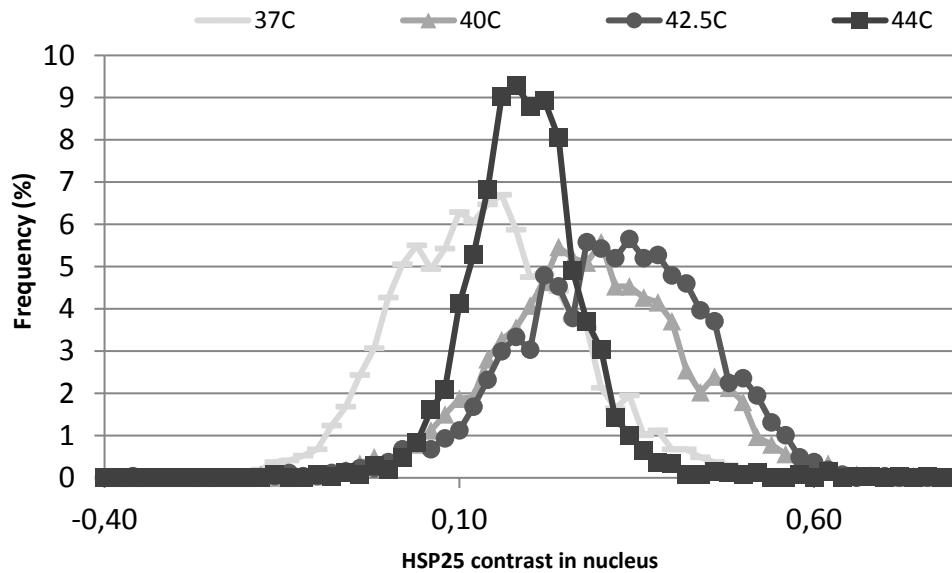


Figure 3.16 Stress dose controls the individual variation. CHO cells with mGFP-GPI construct were treated at given temperatures for 20 minutes and then immunofluorescence images of HSP25 were analyzed for contrast in nucleus ($N \sim 2000$).

3.2.3. Lipidomics reveals cell cycle dependent lipidomes

Heterogeneity of the “seemingly identical” isogenic cell populations are always present. One of the major contributor for this cell-to-cell variation is considered to be the cell cycle. Even after mitosis, the protein, DNA and mitochondria content of the two daughter cells are unequal and the cellular programs differ through the stages of cell cycle (Darzynkiewicz et al. 1982).

In order to understand the effect of cell cycle in the lipid profile of the cells and the stress response, CHO cells with mGFP-GPI construct were sorted based on the DNA (Hoechst intensity) content and lipidomics analysis were carried out. As membrane lipid compositions are sensitive to changing environments, first the effect of sorting and staining of DNA was tested. These procedures did not altered the

3.2.4. A microscopy-based method to analyze cell cycle phases in non-synchronized mammalian cells to dissect the effect of cell cycle on heterogenous HSR

Nutritional and metabolic status of a cell must be assessed to ensure the progression of cell cycle and succession of the cell division. Therefore external stimuli and conditions are important factors that manipulate cell cycle span. The challenge to investigate the effects of cell cycle in stress sensing or response is the counter-interaction of both; stress causes delays in the cell cycle and cell cycle states have different metabolisms that in turn may result in different responses. When cells experience stress, the usual outcome is the induction of HSPs which occur after a period of time that is required to initiate transcriptional and translational processes making it difficult to link the level of induction with the initial cell cycle state. Methods like starvation and chemical treatments to synchronize cells, on the other hand are stressors for the cells and can influence the cellular response. For these reasons, unsynchronized cells were analyzed based on their DNA content simultaneously with the early stress response of HSP25 translocation to the nucleus (Figure 3.2).

Microscopy-based analysis of cell cycle we developed is based on the analysis carried out in flow cytometry where the histogram of a ratiometric DNA stain is used to determine whether cells are in G1, S and G2 phase (Dolbeare et al. 1983). The DNA histograms were plotted after image analysis described under "Materials and Methods" (Figure 3.15). The first peak which corresponds to the highest percentage of the cells is G1 phase that takes longest in the cycle. The second peak has almost double the intensity of the first peak hence the DNA amount corresponds to G2 phase. In between is the S phase where Hoechst intensity varies. This classification of cells were used in subsequent analysis of cell cycle dependent HSR (HSP25 translocation to the nucleus).

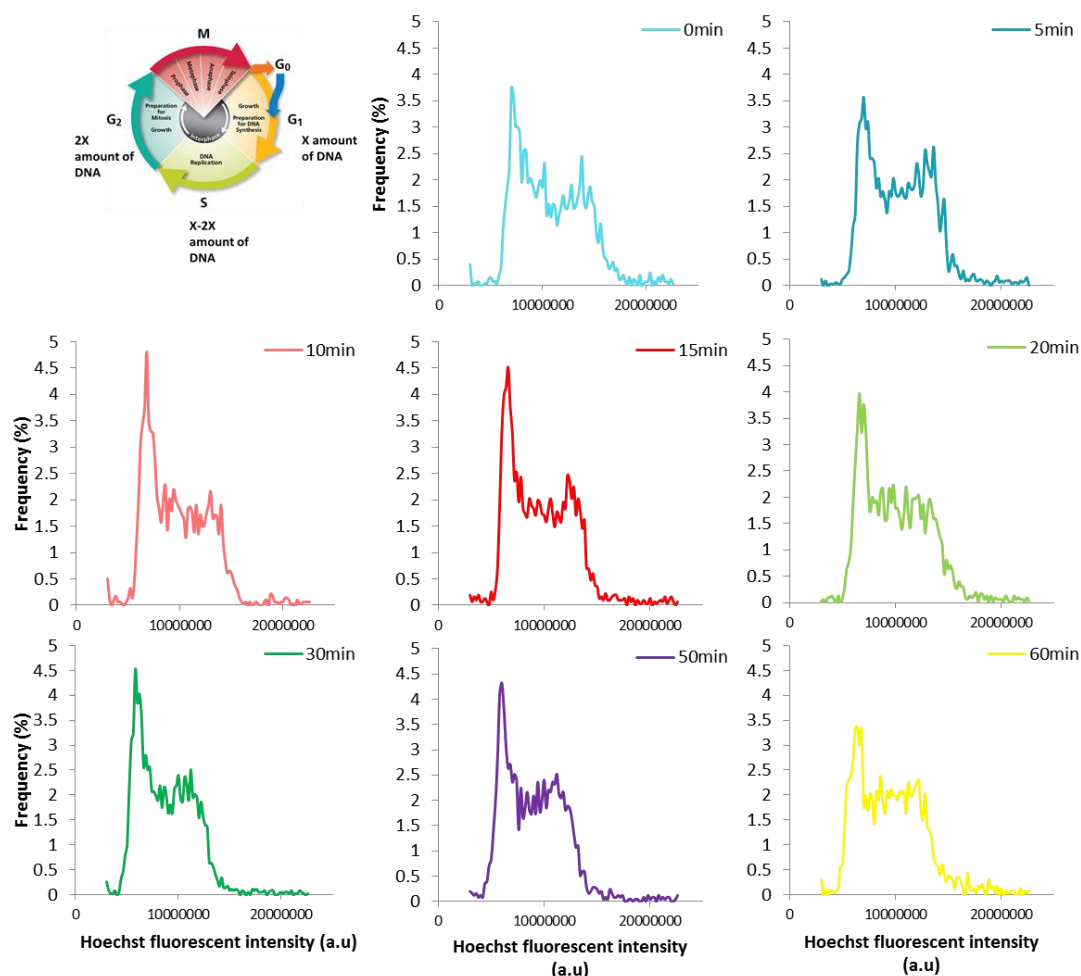


Figure 3.18 Image analysis of DNA content reveals the cell cycle phases. DNA histogram was plotted to analyze the cell cycle phases from the microscopic analysis of CHO cells with GPI-mGFP construct. Cells were stained with Hoechst 33342 and fluorescent intensity was measured from single cells ($N \sim 2000$).

Transient localization of HSP25 from cytosol to nucleus is a protective mechanism for the cells to prevent heat induced irreversible aggregation of nuclear proteins (Bryantsev et al. 2007). Figure 3.2 shows a heterogeneous profile of the translocation whereas HSP70 translocation was more uniform throughout the population. Therefore, the time lapse analysis was carried out to investigate when the translocation starts and how the single cell differences were organized within the population (Figure 3.15A). During the first 10 minutes a bell shaped distribution curve was observed with a lower HSP25 localization in the nucleus. A striking increase of translocation is detected at 15 minutes followed by a gradual increase reaching a

maximum level at 30 min. The most heterogeneous response was observed 15 minutes suggesting that at this heat dose there are still resistant cells present in the population that may not require the protection of HSP25 in the nucleus.

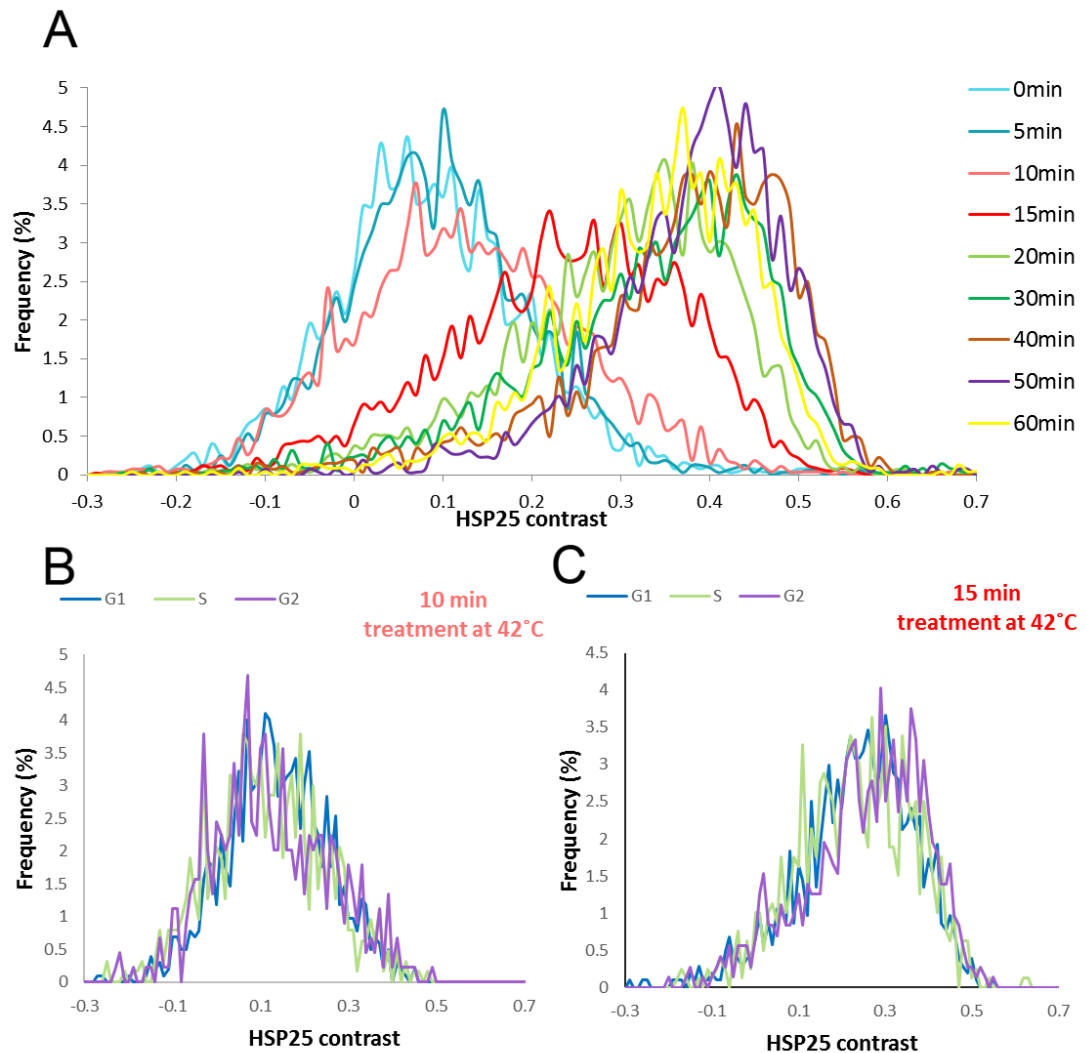


Figure 3.19 Variability of stress response is dependent on the heat dose but not cell cycle phase. CHO cells expressing GPI-mGFP construct were heat shocked at 42°C. Immunofluorescence labelling was carried out for HSP25 and images were analyzed to measure the HSP25 contrast at given time points (A). Cell cycle stages were analyzed using the Hoechst 33342 intensity of the same images by plotting the DNA histogram at 10 minutes (B) and 15 minutes (C). (N~2000)

Next analysis of the cell cycle was carried out at 10 and 15 minutes where the shift towards higher nuclear signal started. Cells were analyzed for their DNA histogram (Figure 3.15) and HSP25 contrast distributions were plotted based on the cell cycle states G1, S or G2 (Figure 3.16 B and C). Cell cycle phase did not influence HSP25 translocation and its heterogeneity. However, it is still possible that the effect of cell cycle can be observed in the extent of protein induction or gene expression. Along with the HSP25 translocation, HSF1 and kinases involved in MAPK signalling (p38MAPK, JNK, ERK 1/2) were tested by high content microscopy. Neither the localization nor the intensity was found to be changed during the treatments (data not shown). The use of other antibodies or assays, however, can be a promising tool to define the source of heterogeneity in the populations.

3.2.5. Single-cell analysis of the calcium peaks of heat shocked CHO cells reveals the heterogeneity of heat shock sensing/response

TRPV1 channels are proposed plasma membrane sensors of heat and their activation results in the increase of intracellular Ca^{2+} (Bromberg et al. 2013). Therefore, we analyzed the increase of Ca^{2+} on the single cell level. CHO cells expressing GPI-mGFP construct was labelled with an Ca^{2+} indicator dye Fura-2AM. Cells were heat shocked under the microscope using a temperature controller. Our results showed a high degree of individuality on the Ca^{2+} response of the cells (Figure 3.20). The intracellular Ca^{2+} signals can be classified into slowly rising signal (Figure 3.20 A (red and blue lines) and B (yellow and purple lines)), transient sustained peak (Figure 3.20A (green line)) and spiking Ca^{2+} signals (Figure 3.20 C). Understanding of these different signal characteristics are not trivial and requires further analysis. It can be important to link these behaviours to the plasma membrane re-organization and heat induced signalling pathways. A recent study addressed the source of variability in Ca^{2+} transients in epithelial tissue damage by using an automated algorithm to examine in detail (Appleby et al. 2015), therefore can be a great base for further analysis.

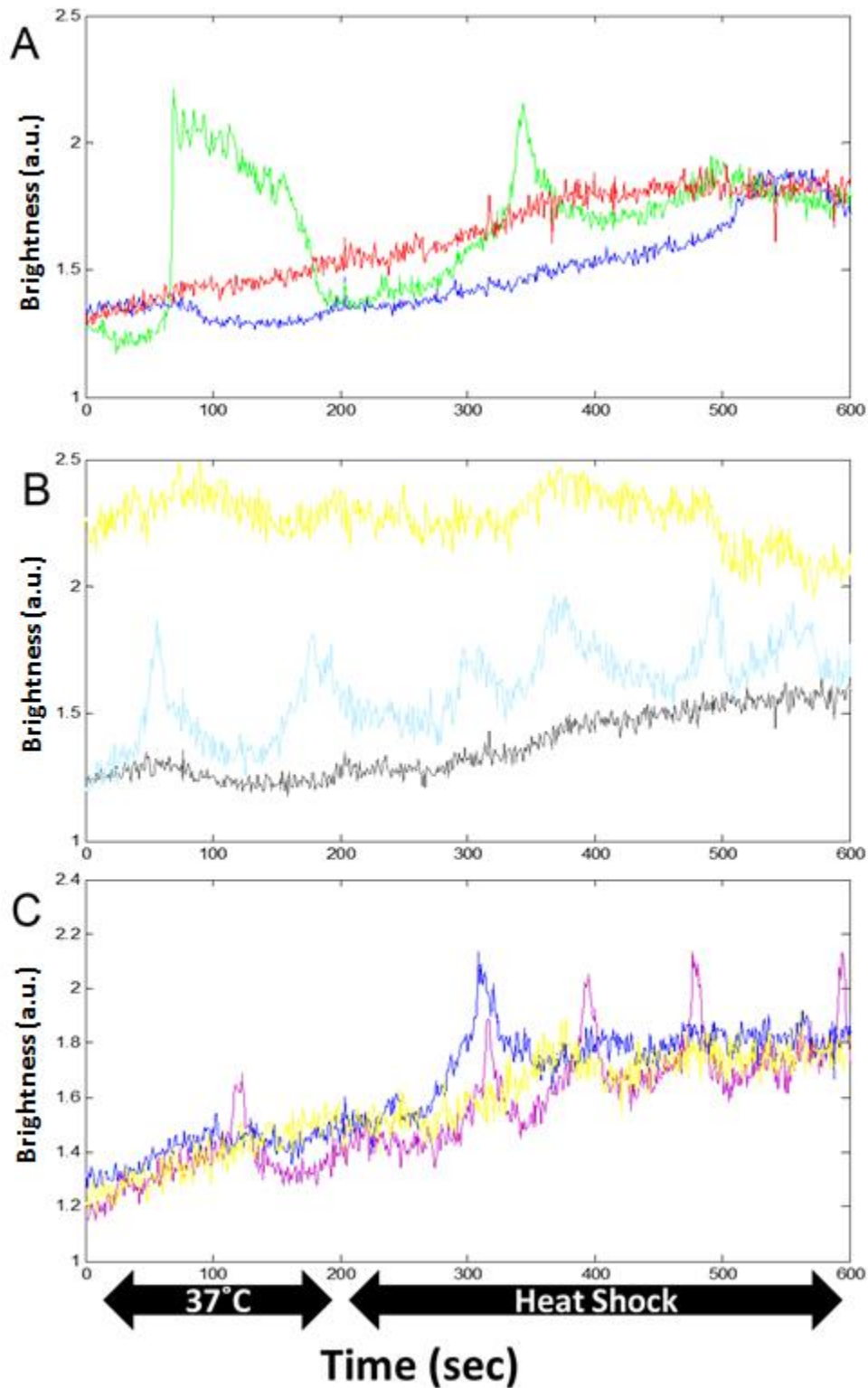


Figure 3.20 Heat induced calcium peaks shows individuality. CHO cells expressing GPI-mGFP construct were labeled with 10 μ M Fura-2AM and calcium signals were measured on the fluorescent microscope heated to 42.5°C. Each plot represents an individual cell.

3.2.6. Heat shock response is dependent on cell density

Previous work carried out by our lab revealed a cell density dependent HSR. Cultured cells of B16 melanoma cell line showed a decreased proportions of polyunsaturated fatty acids, decreasing cholesterol content on the plasma membrane microdomains and decrease in HSR measured by mRNA synthesis of HSP genes with increasing cell density (Péter et al. 2012). There is also evidence on the cell density dependent promoter activation of HSP70 genes where cells are less inducible at high cell densities (Noonan et al. 2007). The authors suggested that the source of this altered responses can be the nutritional media itself. Noonan and coworkers analyzed the effect of the conditioned media from low cell density cultures on the high cell density cultures and observed an enhanced promoter activation. Péter and coworkers analyzed the fatty acid *de novo* synthesis and fatty acid incorporation from the media and observed that the fatty acid synthesis is dependent on the cell density. As a result, from the population analysis of cell number dependence of heat shock response it is not straightforward to assess information on the cellular state and to reveal a direct link to the stress response. A recent study investigating the effect of local cell density on viral infection using single cell analysis is a pioneer work to reveal a media independent differences. In other words, intrinsic differences are obtained since cells are grown on the same plate with no differences on the media input. Their results suggest that simian virus 40 infection is increased in the cells experiencing low local crowding. Moreover, crowding affects the GM1 levels and the focal adhesion kinase activation in a negative direction (Snijder et al. 2009). A follow-up work carried by Frechin et al. identified that in cells experiencing low crowding a more active PI3K and Akt signalling is observed whereas cells in high crowding areas have increased ABC transporter A1 activity (2015). Importantly, lipid droplet levels are dropped as the cell density increased and the fluidity of the total membranes increased due to crowding. Therefore it is important to analyze populations based on the crowding.

As a preliminary effort, the effect of cell density on IGF1R/IR, ERK signalling and HSF1 phosphorylation on Ser326 was analyzed in parallel with HSP induction (Figure 3.21). CHO cells expressing GPI-mGFP construct were seeded in low cell density (LCD)

at 250 cells/mm² and high cell density at 750 cells/mm² (HCD). In order to adapt the cells to these density conditions and to prevent depletion of nutrients each day cells were splitted into same cell number. After 3 days of adaptation, each stock of cells are splitted to LCD and HCD cultures to carry out westernblot analysis after given heat treatments for 20 minutes. For the analysis of phosphorylation cells were collected right after the heat shock and for the analysis of HSP levels sample collection was carried out after an additional recovery time of 6 hours .

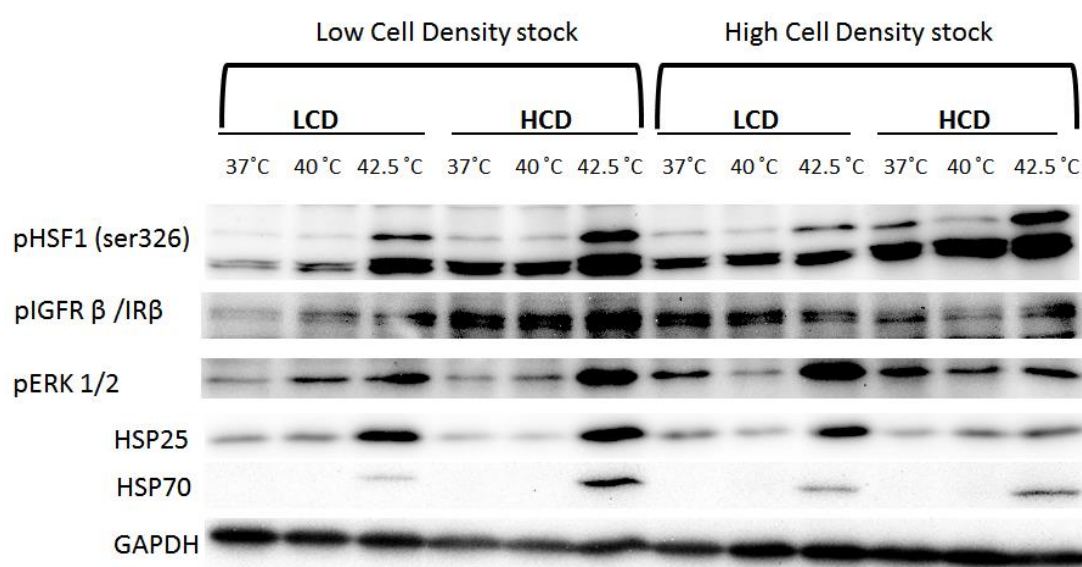


Figure 3.21 Cell density regulates the HSR. CHO cells expressing GPI-mGFP construct were seeded with 250 cells/mm² (Low cell density(LCD) stock) and 750 cells/mm² (High cell density (HCD) stock) for 3 days keeping the cell density constant transferred to subsequent LCD and HCD cultures. pHSF1, pIGF1Rβ/IRβ experiments were carried out 20 minutes after the heat treatment, HSP25 and HSP70 levels were detected after 6 hours of recovery.(N=2)

Our results showed a density dependent HSP induction. HSP25 and HSP70 level increase was more pronounced on HCD sample obtained from a LCD stock. The lowest induction of HSPs were observed when samples were on HCD all the time. HSF1 phosphorylation did not correlated with the induction of HSP25 and HSP70 opposing our previous observations shown on figure 3.2. Further experiments are needed to be carried in order to understand the HSF1 phosphorylation on Ser326 and

its effect on the promoter regions of HSP25 and HSP70. The activation of IGF1R/IR and ERK 1/2 showed a cell density dependence as well. On HCD stock, the phosphorylation of IGF1R/IR was low and unaffected by temperature elevation. Further analysis on the signalling pathways described in this work (Figure 3.14) and HSP levels on population context can be a promising approach to understand the cell crowding based lipid and plasma membrane differences, hence the signalling events and ultimately the cellular response to heat.

4. CONCLUSIONS

The most important conclusions that can be derived from this thesis work can be listed as follows;

- Early events during the perception of temperature stress involve the remodeling of the plasma membrane and cellular lipidome, hyperphosphorylation of HSF1, activation of MAPK signalling pathways and redistribution of HSPs that is followed by an increase in HSP protein levels and gain of acquired thermotolerance.
- Cellular response of CHO cells to heat is both temperature and duration dependent.
- Distinct heat stress levels, namely mild (40°C), moderate (42.5°C) and severe (44°C) can be classified based on distinct cellular lipidomes, plasma membrane organizations, HSP response and signalling pathways.
- Mild stress induces ERK1/2 signalling whereas JNK and p38 MAPK pathways are more active at higher temperatures
- Cells promptly respond to heat by remodeling their plasma membrane. Heat exposure significantly increases the lateral diffusion of fluorescent membrane probes in the plasma membrane of CHO cells at moderate, but not mild temperatures. While the effect of mild heat is successfully balanced by an active microdomain rearrangement (i.e., increased domain confinement), moderate heat resulted in both, an increased diffusion and reduced confinement of GPI-mGFP.
- Cells instantly respond to heat by remodeling their lipidome. PA, PS, PG, CL, LPC and Ceramide changes has been identified as key features while PA is distinctive for moderate and severe stress
- Re-distribution of HSP25 and HSP70 into nuclear and perinuclear compartments is an acute effect of heat and reflects dose dependent stress response.
- HSP25 and HSP70 protein levels are controlled with a different threshold or possibly through different mechanisms that is not connected to HSF1 phosphorylation on Ser326.

- Our results delineated the molecular details of a novel mild type of cellular eustress, when the mammalian cells adapt to fever-type mild heat by maintaining membrane homeostasis, activating lipid remodeling, and redistributing chaperone proteins, leading to acquired thermotolerance; strikingly, these processes occur in the complete absence of the induction of HSPs. At higher temperatures, additional defense mechanisms are activated, including elevated expression of molecular chaperones, contributing to an extended stress memory and acquired thermotolerance.
- At mild temperatures, heat treatment alters lipid composition and plasma membrane structure and it results in a significant increase of acquired thermotolerance therefore pointing out a possible interrelationship of the two.
- Individual variation of the cellular heat shock response is observed in translocation of HSPs to the nucleus (early response) and in induction of HSPs (late response).
- Cell-to-cell variation of heat induced HSP25 redistribution is a cell cycle independent event whereas distinct lipid profiles of G1, S and G2 phases are observed.
- Single cell analysis of heat induced Ca^{2+} signalling indicates that the population heterogeneity of the heat stress signalling can be linked to membranes, but further studies are required in order to better understand this phenomenon.

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SUMMARY

Stress response (SR) is a vitally important biological process that protects organisms against various environmental and pathophysiological conditions. Based on macromolecular damage, cells monitor stress irrespective of its type causing such damage. Therefore, many aspects of the cellular stress response are not stressor specific. One of the most studied stress is heat that results in deleterious damages, i.e. leakage of the plasma membrane or severe protein denaturation, only at relatively high temperatures. However, heat shock response could also be activated under mild pathophysiological conditions, such as in fever, that is unlikely to cause proteotoxicity. Fever appears to be an evolutionarily conserved response to bacterial or viral infection, which implies that it may have survival benefits. Recently, considerable evidence has been accumulated in favor of the "Membrane sensor hypothesis" which predicts that, the level of heat shock proteins (HSPs, also called stress proteins) changes as a result of alterations to the plasma membrane. Stressful conditions activate numerous membrane-related sensors at the top of the signaling pathways which are interconnected by the parameters of the membrane chemical and physical state. Phospholipids, sphingolipids and cholesterol are all involved in the generation of stress-induced second messengers.

The study of cellular stress response is of great importance to our understanding of how cells respond and adapt to various changes in their environments. The current research on cellular heat stress management focuses on the roles HSPs and the proteostasis network under severe stress conditions. The mild, fever-type stress and the maintenance of membrane homeostasis are less well understood. Herein, we characterized the acute effect of mild heat shock on membrane organization, and HSP synthesis and localization in mammalian cells, to delineate the role of membranes in the sensing and adaptation to heat.

In the first part of this work, the sequence of heat induced cellular events was investigated as a function of exposure time and temperature. HSP synthesis, HSP redistribution, re-modelling of plasma membrane structure and cellular lipidome and possible signal transduction pathways were analyzed. Multidisciplinary approach

combining ultrasensitive fluorescence microscopy and lipidomics revealed the molecular details of distinct cellular stress profiles. Chinese hamster ovary (CHO) cells, at mild stress temperature (40°C), adapt to environmental changes by activating lipid remodelling, maintaining membrane homeostasis and redistributing chaperone proteins whereas further increase in the temperature into moderate stress zone (42.5°C) induces plasma membrane reorganization and HSP synthesis in addition to redistribution of chaperones. Under severe stress temperature (44°C), additional defense mechanisms are activated, including elevated expression of molecular chaperones, contributing to an extended stress memory and acquired thermotolerance. Our results revealed the molecular details of a novel mild type of cellular eustress, when the mammalian cells adapt to fever-type mild heat and acquire thermotolerance in the complete absence of the induction of HSPs.

The current work addresses another fundamental question whether all cells in a culture (or in tissue) suffer the same consequence of an imposed stress and whether they respond in the same manner. Knowledge on gene expression and cellular responses in cells is derived from analyses of populations consisting of millions of cells. Although this approach provides useful insights into average population responses, they do not provide information on individual cells or subpopulations within cell cultures. The impact of heterogeneity is significant in many aspects: first, sample heterogeneity is critical for the functionality of cellular networks. Second, cell ensemble data can easily be misinterpreted when ignoring heterogeneity; a specific parameter – e.g. an expression profile, the molecular clustering or mobility – determined by measuring the average ensemble value need not to be preserved in the individual subclasses of cells. Third, correlation analysis at the single cell level allows for identifying molecular implications in a systemic approach; in this aspect, sample heterogeneity is utilized for obtaining mechanistic insights into cellular processes.

In the present project we demonstrated the individual variability in the stress response of genetically homogeneous mammalian cell population by combining state of the art lipidomics and ultrasensitive high content imaging. Cell cycle phase as a first cause of heterogeneity is linked to differential stress responses, however methods

such as serum starvation and chemical treatments to block the cell cycle have collateral effects on the lipid composition of the cells. Therefore, results obtained with such methods can be misleading. The combination of microscopy and fluorescence activated cell sorting applied in this study bypasses the chemical treatments to analyze the cell cycle phases and allows the analysis of individuality of stress response. Our experiments revealed distinct, cell cycle dependent cellular lipidomes, however heat induced redistribution of chaperones was independent of the cell cycle phase. Therefore we extended our single cell approach to the individuality of the stress signaling. Calcium increase which is a hallmark of stress activates many kinases in the cells and hence coordinates the response. In single cells, heat treatment induced unsynchronized, distinct calcium peaks during the course of heat. Further efforts are needed to elucidate the importance of plasma membrane properties in the heterogeneity of calcium signalling and cellular stress response.

ÖSSZEFOGLALÓ

A stresszválasz létfontosságú biológiai folyamat, amely megvédi az organizmusokat a különféle környezeti és patofiziológias körülmények mellett. A sejtek minden stresszt (típustól függetlenül) nyomon követnek, amelyek makromolekuláris károsodást okoznak. Következésképpen a celluláris stresszválasz több szempontból sem stresszor specifikus. Az egyik leginkább tanulmányozott stressz-típus a hő, amely magas hőmérsékleteken komoly károkat eredményez (plazmamembrán integritásának csökkenése vagy súlyos fehérje denaturáció). A hősokk válasz azonban kiváltható enyhe patofiziológias körülmények között is, pl. a láz esetén, amelynél proteotoxicitás nem valószínű. A láz a bakteriális és vírusos fertőzésekre adott evolúciósan konzervált válasz, amely feltételezhetően előnyt jelent a túlélés szempontjából. Az utóbbi időkben számottevő bizonyíték támasztotta alá az ún. „Membrán szenzor hipotézis”-t, amely azt feltételezi, hogy a plazmamembránban történt változások módosíthatják a hősokk fehérjék (a HSP-k vagy stressz fehérjék) szintjét. A stressz számos membránhoz kötött szenzoron keresztül indíthat el különböző jelátviteli útvonalakat. Ezek a membrán kémiai és fizikai állapotában bekövetkezett változásokkal hozhatók összefüggésbe. A stressz-indukált másodlagos hírvivő molekulák képződésében a koleszterin, a foszfolipidek és szfingolipidek játszhatnak elsődleges szerepet.

A sejt stresszválasz vizsgálata rendkívül fontos ahhoz, hogy megértsük, hogyan reagálnak és adaptálódnak a sejtek, ha a környezetükben különféle változások következnek be. A celluláris hőstressz kezeléssel kapcsolatos jelenlegi kutatások a HSP-k szerepére és a proteosztázis hálózatra fókuszálnak súlyos stressz körülmények mellett. Az enyhe, láz-szerű stresszről és a membrán homeosztázis fenntartásáról azonban kevés ismerettel rendelkezünk. Jelen értekezésben az enyhe hősokk által a membrán szerveződésre valamint a HSP szintézisre és lokalizációra gyakorolt akut hatásokat jellemezzük különös tekintettel a membránok hőérzékelésben és az alkalmazkodásban betöltött szerepére.

Kísérleteinkben először a hő-indukálta celluláris események sorozatát vizsgáltuk meg az expozíciós idő és hőmérséklet függvényében. Tanulmányoztuk a HSP szintézist, a

HSP redisztribúciót, a plazmamembrán szerkezet és a celluláris lipidom átrendeződését, valamint a lehetséges jelátviteli utakat. Multidisziplináris megközelítéssel ultraszenzitív fluoreszcencia mikroszkópiát és lipidomikát kombinálva feltártuk a különböző sejtes stresszprofilok molekuláris részleteit.

Kínai hörcsög ovárium (CHO) sejtek a környezeti változásokhoz alkalmazkodva enyhe stressz-hőmérsékleten (40°C) átrendezik a lipidjeiket, fenntartják a membrán homeosztázist és a sejten belül átszervezik a dajka fehérjék (chaperon HSPk) lokalizációját. Ha a hőmérséklet tovább emelkedik a mérsékelt tartományba (42,5°C), a molekuláris dajka fehérjék sejten belüli újra elosztása mellett a sejtek elindítják a plazmamembrán újra szerveződését és a HSP szintézist. Súlyos stressz hőmérsékleten (44°C) további védelmi mechanizmusok aktiválódnak, ide értve a molekuláris dajkafehérjék emelkedett expresszióját, ami hozzájárul a stresszmemória kiterjesztéséhez és a szerzett termotoleranciához. Eredményeink rámutattak egy sejtszintű eustressz tartomány molekuláris részleteire, amelyben az emlős sejtek adaptálódnak a láz-szerű enyhe hőmérséklethez, és kialakul a termotoleranciájuk a HSPk indukciójának teljes hiányában.

A jelen munka során egy másik alapvető kérdést is feltettünk arról, vajon a stressz hatása ugyanolyan következményekkel jár-e a sejt kultúra (vagy szövet) minden sejtjében, és azok azonos módon reagálnak-e a stresszre. A génexpresszióval és a celluláris válaszokkal kapcsolatos ismereteinket több millió sejtet tartalmazó populációk vizsgálatával nyerjük. Ez a megközelítés hasznos információt nyújt a populáció átlagos válaszáról, a kultúrában lévő egyedi sejtek vagy szubpopulációk válaszáról azonban nem. E heterogenitás hatása több szempontól is jelentős: Egyrészt a sejtek individuális variabilitásának ismerete elengedhetetlen a celluláris hálózatok működésének megértéséhez.; Másrészt, a sejtekből együttesen nyert adatokat könnyen félreértelmezhetjük, ha figyelmen kívül hagyjuk a heterogenitást. A jellemző paraméterek (pl. expressziós profil, molekuláris klasztereződés vagy mobilitás) együttesen nyert átlagos értékei nem feltétlenül egyeznek meg az egyedi sejtek értékeivel. Harmadrészt, az egyedi sejtszintű korreláció analízis lehetővé teszi, hogy szisztematikus megközelítéssel molekuláris eseményeket azonosítsunk. Ebben a

vonatkozásban a sejtek heterogenitását arra használjuk fel, hogy mechanisztikus betekintést nyerjünk a celluláris folyamatokba.

Jelen projekt során korszerű lipidomika és ultraszenzitív nagy információtartalmú képalkotó technikák kombinációjával bemutattuk, hogy a genetikailag homogén emlős sejtpopuláció egyedeinek stresszválasza nagy mértékben eltérő. A különböző stresszválaszokban megmutatkozó heterogenitás egyik fő oka lehet a sejtek eltérő sejtciklusa. Az olyan módszerek, mint pl. a széruméheztetés vagy a kémiai kezelések, amelyek célja a sejtciklus megállítása, egyúttal hatást gyakorolnak a sejtek lipid-összetételére is. Ebből adódóan az ilyen módszerekkel nyert adatok félrevezetők lehetnek. A vizsgálataink során alkalmazott mikroszkópia és fluoreszcencia-aktivált sejtválogatás (FACS) kombinációjával kémiai kezelések nélkül elemezhetjük a sejtciklust és az egyedi stresszválasz kapcsolatát. Kísérleteink alapján az eltérő sejtciklusokhoz különböző celluláris lipidomok tartoztak, a hő indukálta dajkafehérje lokalizáció a sejtciklus fázisától azonban nem függött. Ezért az egyedi sejt szintű megközelítésünket stressz-jelátvitel individualitásának vizsgálatával egészítettük ki. A stressz egyik fő jellemzője kalcium szint emelkedése, ami számos kináz aktiválásán keresztül szabályozhatja a választ. Kimutattuk, hogy a hőkezelés az egyes sejtekben eltérő, nem szinkronizált kalcium tranzienseket eredményez. További vizsgálatok szükségesek annak megértéséhez, hogy az egyes sejtek plazmamembránjának fizikai-kémiai tulajdonságai milyen szereppel bírnak a kalcium jelátvitel ill. a stresszválasz heterogenitásában.

7. APPENDICES

7.1. *Publication List*

MTMT number : 10052550

Publications that are related to the PhD thesis :

Peksel, Begüm, Imre Gombos, Mária Péter, László Vigh, Jr., Ádám Tiszlavicz, Mario Brameshuber, Gábor Balogh, Gerhard Schütz, Ibolya Horváth, László Vígh, Zsolt Török. "Mild heat induces a distinct "eustress" response in Chinese Hamster Ovary cells that does not require heat shock protein synthesis". (2017) Scientific Reports (in press)

I.F.: 4.259

Torok, Zsolt, Tim Crul, Bruno Maresca, Gerhard J Schutz, Felix Viana, Laura Dindia, Stefano Piotto, Mario Brameshuber, Gabor Balogh, Maria Peter, Amalia Porta, Alfonso Trapani, Imre Gombos, Attila Glatz, Burcin Gungor, **Begum Peksel**, Laszlo Vigh Jr, Balint Csoboz, Ibolya Horvath, Mathilakath M. Vijayan, Phillip L. Hooper, John L. Harwood, Laszlo Vigh. "Plasma Membranes as Heat Stress Sensors: From Lipid-Controlled Molecular Switches to Therapeutic Applications." *Biochimica et Biophysica Acta* 1838, no. 6 (June 2014): 1594–1618. doi:10.1016/j.bbamem.2013.12.015.

I.F.: 3.498

Brameshuber, Mario, Eva Sevcsik, Benedikt K. Rossboth, Christina Manner, Hans-Peter Peter Digner, **Begüm Peksel**, Mária Péter, Zsolt Török, Albin Hermetter, Gerhard J. Schütz. "Oxidized Phospholipids Inhibit the Formation of Cholesterol-Dependent Plasma Membrane Nanoplatfoms." *Biophysical Journal* 110, no. 1 (January 5, 2016): 205–13. doi:10.1016/j.bpj.2015.11.018.

I.F.: 3.656

Publications that are not directly related to the PhD thesis:

Cadenas, Cristina, Sonja Vosbeck, Eva Maria Hein, Birte Hellwig, Alice Langer, Heiko Hayen, Dennis Franckenstein Bettina Büttner, Seddik Hammad, Rosemarie Marchan, Matthias Hermes, Silvia Selinski, Jörg Rahnenführer, **Begüm Peksel**, Zsolt Török, László Vígh, Jan G. Hengstler. "Glycerophospholipid Profile in Oncogene-Induced Senescence." *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* 1821, no. 9 (September 2012): 1256–68. doi:10.1016/j.bbalip.2011.11.008.

I.F.: 5.547

Molnár, Eszter, Soujanya Kuntam, Pradeep Kumar Reddy Cingaram, **Begüm Peksel**, Bhavyashree Suresh, Gabriella Fábián, Liliána Z. Fehér, Attila Bokros, Agnes Medgyesi, Ferhan Ayaydin, Laszlo G. Puskas. "Combination of Small Molecule Microarray and Confocal Microscopy Techniques for Live Cell Staining Fluorescent Dye Discovery." *Molecules* 18, no. 8 (August 2013): 9999–10013.

I.F.: 2.465

Koos, Krisztian, **Begüm Peksel**, Lóránd Kelemen. "Phase Measurement Using DIC Microscopy" *Acta Cybernetica* 00 (0000) 1–15.

I.F.: 0.28

Other publications:

Kavas, Musa, Oya Ercan Akça, Ufuk Celikkol Akçay, **Begüm Peksel**, Seçkin Eroğlu, Hüseyin Avni Öktem, and Meral Yücel. "Antioxidant Responses of Peanut (*Arachis hypogaea* L.) Seedlings to Prolonged Salt-Induced Stress." *Archives of Biological Sciences* 67, no. 4 (2015): 1303–12.

I.F.: 0.352

Özgür, Ebru, and **Begüm Peksel**. "Biohydrogen Production from Barley Straw Hydrolysate through Sequential Dark and Photofermentation." *Journal of Cleaner Production* 52 (2013): 14–20.

I.F.: 4.959

Öztürk, Yavuz, Abdulmecit Gökçe, **Begüm Peksel**, Muazzez Gürkan, Ebru Özgür, Ufuk Gündüz, Nci Eroğlu, and Meral Yücel. "Hydrogen Production Properties of Rhodobacter Capsulatus with Genetically Modified Redox Balancing Pathways." International Journal of Hydrogen Energy 37, no. 2 (January 2012): 2014–20. doi:10.1016/j.ijhydene.2011.06.078.

I.F.: 3.582

Özgür, Ebru, Astrid E. Mars, **Begüm Peksel**, Annemarie Louwerse, Meral Yücel, Ufuk Gündüz, Pieter A M Claassen, and Inci Eroğlu. "Biohydrogen Production from Beet Molasses by Sequential Dark and Photofermentation." International Journal of Hydrogen Energy 35, no. 2 (January 2010): 511–17. doi:10.1016/j.ijhydene.2009.10.094.

I.F.: 3.582

Presentations in meetings and conferences:

Begüm Peksel, Imre Gombos, László Vígh Jr, Péter Horváth, Mária Péter, Gábor Balogh, Ibolya Horváth, László Vígh, Zolt Török. Cell-To-Cell Variation Of Stress Response Is Linked To Membrane Structure. EMBO Practical Course Single-cell Gene Expression Analysis. 17-22 April 2015, Heidelberg, Germany.

Begüm Peksel, Imre Gombos, László Vígh Jr, Péter Horváth, Mária Péter, Gábor Balogh, Ibolya Horváth, László Vígh, Zolt Török. Cell-To-Cell Variation Of Stress Response Is Linked To Membrane Structure. EMBO/EMBL Symposium: Cellular Heterogeneity. 15-18 April 2015, Heidelberg, Germany.

Begüm Peksel, Imre Gombos, Peter Horvath, Akos Hunya, Anna-Maria Pilbat, Maria Peter, Gabor Balogh, Laszlo Vigh Jr., Kamilla Oglecka, Ibolya Horvath, Laszlo Vigh, Zolt Torok. Individual variability in the stress response of genetically homogeneous mammalian cell population is linked to membrane rearrangement. FEBS workshop on Lipids. 25-30 August 2014, Spetses, Greece.

Begüm Peksel, Imre Gombos, László Vígh Jr, Péter Horváth, Mária Péter, Gábor Balogh, Ibolya Horváth, László Vígh, Zolt Török. Single cell heat stress response: cell profiling and lipidomics. Straub Days – 28-29 May 2014 (oral presentation), Szeged, Hungary.

Begüm Peksel, Imre Gombos, László Vigh Jr., Ibolya Horváth, Mária Péter, Gábor Balogh, László Vígh, Zolt Török. Individual variability in the stress response of

genetically homogeneous mammalian cell population is linked to membrane rearrangement. Quantitative Single Cell Biology in Stem Cell Research, 10-11 October 2013, München, Germany.

Peksel B., Vigh L., Ayaydin F. Increased SUMOylation in response to membrane fluidization, ITC Alumni Meeting, (1-3 September 2011), (oral presentation) Szeged, Hungary.

DECLARATION

Hereby, as corresponding author and supervisor I declare the role of Begüm Peksel Şahin in the following publications;

1. **Peksel, Begüm**, Imre Gombos, Mária Péter, László Vigh, Jr., Ádám Tiszlavicz, Mario Brameshuber, Gábor Balogh, Gerhard Schütz, Ibolya Horváth, László Vigh, Zsolt Török. "Mild heat induces a distinct "eustress" response in Chinese Hamster Ovary cells that does not require heat shock protein synthesis". (2017) Scientific Reports (in press)

The majority of the experiments were designed and carried out by Begüm Peksel Şahin in this article and it is the basis of the thesis. The results of Figure 1, Figure 2, Figure 4, Supplementary Figure S1, Supplementary Figure S2 and Supplementary Figure S3 were done by her. She was also involved in the treatments and sample preparations for the Figure 3, 5 and 6 and in writing the manuscript.

2. Török, Zsolt, Tim Crul, Bruno Maresca, Gerhard J Schütz, Felix Viana, Laura Dindia, Stefano Piotto, Mario Brameshuber, Gabor Balogh, Maria Peter, Amalia Porta, Alfonso Trapani, Imre Gombos, Attila Glatz, Burçin Güngör, **Begüm Peksel**, Laszlo Jr Vigh, Balint Csoboz, Ibolya Horvath, Mathilakath M. Vijayan, Phillip L. Hooper, John L. Harwood, Laszlo Vigh. "Plasma Membranes as Heat Stress Sensors: From Lipid-Controlled Molecular Switches to Therapeutic Applications." *Biochimica et Biophysica Acta* 1838, no. 6 (June 2014): 1594–1618. doi:10.1016/j.bbamem.2013.12.015.

This is a review article including several authors. Begüm Peksel Şahin was actively taken part in collecting literature and writing chapter 6. : Cell profiling and lipidomics: novel tools to explore the primary membrane-associated events critical in the triggering of stress signals.

I also declare that those parts in the above mentioned articles were not or will not be used in the past or in the future, respectively for the purpose of acquiring an academic degree or title.

Dr. Zsolt TÖRÖK

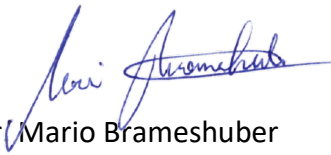
Szeged, June 2, 2017.

Hereby, as corresponding author I declare the role of Begüm Peksel Şahin in the following publication;

3. Brameshuber Mario, Eva Sevcsik, Benedikt K. Rossboth, Christina Manner, Hans-Peter Peter Deigner, **Begüm Peksel**, Mária Péter, Zsolt Török, Albin Hermetter, Gerhard J. Schütz. "Oxidized Phospholipids Inhibit the Formation of Cholesterol-Dependent Plasma Membrane Nanoplatfroms." Biophysical Journal 110, no. 1 (January 5, 2016): 205–13. doi:10.1016/j.bpj.2015.11.018.

Begüm Peksel Şahin performed MS experiments and analyzed data for Figure 3.

I also declare that the part in the above mentioned article was not or will not be used in the past or in the future, respectively for the purpose of acquiring an academic degree or title.



Dr. Mario Brameshuber

Szeged, June 2, 2017.