The role of small GTPase Rac1 in stress signaling

Ph.D. Thesis

Burçin Güngör

Supervisor: Dr. Ibolya Horváth

Doctoral School of Biology

University of Szeged

Institute of Biochemistry

Biological Research Center of the Hungarian Academy of Science

Szeged 2014

List of Content

LIST OF ABBREVIATIONS 5

1. INTRODUCTION 7

1.1. Cellular Stress 7

1.1.1. Heat stress at cellular level 8

1.1.2. Mild versus severe heat stress 9

1.2. Cell shape and Cytoskeletal changes during heat stress 11

1.3. The heat shock response 14

1.4. Heat shock proteins: their roles and functions 15

1.5. Transcriptional regulation of HSPs: the small GTPase Rac1 is an upstream element of HSF1 regulation 18

1.6. Heat stress sensing 21

1.6.1. Micro-domain organization of membranes and membrane initiated stress signaling 24

1.6.2. Small GTPase Rac1 and its role in heat stress signaling 33

2. THE AIMS OF THE THESIS 41

3. MATERIALS AND METHODS 42

3.1. Cell culturing and transfections 42

3.2. Treatments 42

3.3. Crude membrane isolation 43

3.4. Plasma membrane isolation 43

3.5. Immune localization of Rac1 using confocal microscopy 44

3.6. Membrane micro-domain size analysis 45

3.7. Determination of morphological changes by Scanning Electron Microscopy 45

3.8. Determination of F-actin alterations in MEF cells by confocal microscopy 46

3.9. Determination of mRNA levels of inducible *hsp25* and *hsp70* genes 46

3.10. Western blot analysis 47

3.11. F-actin determination by Flow Cytometric Analysis 48

4. RESULTS AND DISCUSSIONS 49

4.1. Rac1 is a membrane-binding protein 49

4.2. Heat stress stimulates Rac1 translocation to membranes in B16F10 melanoma cells 52

4.3. Rac1 palmitoylation affects the PM micro-domain organizations upon heat stress 56

4.4. Rac1 is involved in heat induced changes in cell morphology 59

4.5. Rac1 is involved in the disorganization of actin filaments upon severe heat stress treatment 63

4.6. Rac1 affects the mRNA expression levels of *hsp25* and *hsp70* under heat shock conditions 65

4.7. Rac1 takes part in the HSR at protein level 68

4.8. Rac1 inhibition does not affect the level of HSF1 phosphorylation 71

4.9. p38MAP Kinase is at the downstream of the Rac1-dependent stress signaling pathway 73

5. SUMMARY OF FINDINGS 76

6. REFERENCES 77

7. SUMMARY 92

8. ÖSSZEFOGLALÓ 95

9. ACKNOWLEDGEMENT 98

List of Figures

Figure 1. Effects of heat shock on the organization of the eukaryotic cell. 8

Figure 2. Lamellipodial extension in NIH 3T3 cells. 12

Figure 3. Representation of stress fibers types in cultured animal cells. 13

Figure 4. Stressors that induce HSR. 14

Figure 5. HSF1 activation. 19

Figure 6. Signaling kinase cascades activated by heat shock and their downstream targets. 20

Figure 7. Stress sensors of the HSR in mammalian cells. 22

Figure 8. Regulation of the HSR and the HSF1 cycle. 23

Figure 9. Three-tiered hierarchical mesoscale-domain architecture of the PM. 26

Figure 10. Lipid raft micro-domains. 29

Figure 11. Potential stress sensing mechanisms that are based on lipid membrane perturbation/ rearrangements. 32

Figure 12. Phylogenetic tree of the Rho family GTPases and representatives of other Ras-superfamily GTPases. 34

Figure 13. Structure of Rac1 35

Figure 14. Structure of NSC23766 36

Figure 15. The Rac1 acylation cycle. 38

Figure 16. Scheme of working hypothesis for the cascade of possible heat-stress signal generation and transduction events linking PM to hsp during HSP response induced by mild heat stress. 39

Figure 17. Rac1 PM localization after serum starvation. 49

Figure 18. Localizations of Gfp tagged Rac1 protein in NIH3T3, MEF and B16F10 cell lines. 51

Figure 19. Localization of Rac1 to the crude membrane fraction in response to heat shock treatment. 53

Figure 20. Visualization of the association of Rac1 to the PM. 54

Figure 21. The effect of Rac1 inhibitor and palmitoylation inhibitor administration on the Rac1-membrane binding under heat stress conditions. 55

Figure 22. Change in size of PM micro-domains as functions of temperature and 2-Brp treatment. 58

Figure 23. F-actin changes in B16F10 cells under heat shock conditions. 60

Figure 24. Cell morphology changes in B16F10 cells under heat shock conditions.....61

Figure 25. Effect of Rac1 inhibitors on the actin filaments of heat and inhibitor treated MEF cells. 64

Figure 26. Effect of Rac1 inhibitors 2-Brp and NSC on hsp25 and hsp70 gene expression levels under heat shock conditions. 67

Figure 27. Effect of NSC on HSP25 and HSP70 protein levels under heat shock conditions. 69

Figure 28. Representative western blotting showing the effect of 2-Brp on HSP25 and HSP70 protein levels under heat shock conditions. 70

Figure 29. The effect of NSC on the expression levels of HSP60, HSP90 and HSP110. 71

Figure 30. Effects of Rac1 inhibition on phosphorylation level of HSF1. 72

Figure 31. Effect of Rac1 inhibition by NSC on phosphorylation of p38MAPK under heat shock conditions. 74

Table 1. Criteria for distinguishing between mild and severe heat stress 10

Table 2. Topology of Polypeptide Binding and Action of HSP Families. 16

LIST OF ABBREVIATIONS

AA: Arachidonic Acid

AKT: Protein Kinase B

BA: Benzyl Alcohol

Chol: Cholesterol

Cer: Ceramide

DNA: Deoxyribonucleic Acid

ECM: Extracellular Matrix

ERK: Extracellular Regulated Kinase

FAK: Focal Adhesion Kinase

GAP: GTPase Activating Protein

GDI: Guanine nucleotide Dissociation Inhibitor

GDP: Guanosine Diphosphate

GEF: Guanine nucleotide Exchange Factor

Gfp: Green Fluorescent Protein

GPI: Glycosyl Phophatidyl Inositol

GSK3: Glycogen Synthase Kinase 3

GTP: Guanosine Triphosphate

HSF: Heat Shock Factor

HSE: Heat Shock Element

HSP: Heat Shock Protein

HSR: Heat Shock Response

JNK: c-Jun N Terminal Kinase

L_o: Liquid Ordered

L_d: Liquid Disordered

MAPK: Mitogen Activated Kinase

MEF: Mouse Embryonic Fibroblast

MK2: MAPK-activated protein Kinase 2

NADPH: Nicotinamide Adenine Dinucleotide Phosphate

NSC: NSC23766

Pak: p21-activated Kinase

PBS: Phosphate Buffered Saline

PIP_2: Phosphatidylinositol bisphosphate

PIP_3: Phosphatidylinositol trisphosphate

PI3K: Phosphoinositide 3 Kinase

PKA: Protein Kinase A

Rac1: Ras related C3 botulinum toxin substrate1

PM: Plasma Membrane

RNA: Ribonucleic Acid

SAPK: Stress Activated Protein Kinase

SE Microscopy: Scanning Electron Microscopy

sHSP: Small Heat Shock Protein

SM: Sphingomyelin

TIRF: Total Internal Reflection Microscopy

2-Brp: 2-Bromo palmitate

1. INTRODUCTION

1.1. Cellular Stress

Living organisms are continuously subjected to the environmental challenges. In order to survive, they have to cope with a variety of unfavorable circumstances. Therefore, a necessary condition for survival is to adapt to their environment. First, Hans Selye (1950) defined the "stress" as a physiological perturbation that can be associated with various abnormalities and requires resistance or adaptation (Selye 1950). Later on, subsequent studies revealed that stress can be considered as understanding the relation between living organisms and their dynamic environment.

Stress at cellular level can cause the damage on the structure and the function of macromolecules (Zhang & Andersen 2007; Gupta et al. 2010). The response of the cell to the stress stimuli is to defend against and recover from the harmful effect. However, if cell is not able to handle the stress, then the cell death programs such as apoptosis, necrosis or autophagy are activated to eliminate these damaged cells from the organism (Fulda et al. 2010). The cellular stress response is a universal reaction of extraordinary physiological/pathophysiological changes (Kultz 2003). Depending on the severity and duration of stress encountered, cells either reconstitute cellular homeostasis to the former physiological state or adopt an altered state in the new environment. Responsiveness to diverse stresses at cellular level is mainly non-stressor specific since the impact of disturbance damages the membranes and the macromolecules such as proteins, DNA and lipids (Kültz 2005).

Any sort of abnormal changes in the environment can be a stress factor for cells. Thus, heat or cold, pH alterations, hyper- or hypo-osmolarity result in cellular stress responses. In addition, cellular stress can contribute to, or even trigger many diseases and has an important effect also on aging. Several data show, the presence of stress phenotypes in cancer. Aberrant expressions of microRNAs and heat shock proteins with their chaperon activities are implicated in cell differentiation, metastasis and tumor cell resistance (Ciocca & Calderwood 2005; Leung & Sharp 2011; Jego et al. 2013; Niforou et al. 2014). Studies on neurodegenerative diseases such as Alzheimer and Parkinson etc. point out the substantial and growing evidence for the activation of stress responses (Petrozzi et al. 2007; Brignull et al. 2007; Hetz et al. 2013). Cell stress have a profound effect in triggering/developing the

cardiovascular diseases, type 2 diabetes and aging (Shamaei-Tousi et al. 2007; Haigis & Yankner 2011; Q. Xu et al. 2012; Hooper et al. 2014).

1.1.1. Heat stress at cellular level

Living organisms sense the temperature of their milieu and develop adaptations to growth temperatures in the range of freezing point of the water and circa 100 °C. However, temperature changes within few degrees under or above the optimum growth temperature cause threat for the cells to survive (Richter et al. 2010).

Sustainability of the optimal growth temperature is necessary for the cell functions. Including the extremophiles, a small elevation in temperature can damage cell components and therefore internal organization of the cell (Szalay et al. 2007; Ambily Nath et al. 2011). In eukaryotes, one of the deleterious impacts of heat stress at cellular level is damaging the cytoskeleton. Depending upon the severity of stress, reorganization or fragmentation of actin filaments, stress fiber formation and aggregation of vimentin can be monitored within heat stressed cells (Welch & Suhan 1985; Richter et al. 2010). Along with the disintegration of the cytoskeleton, organelles lose their correct localization (Figure1).

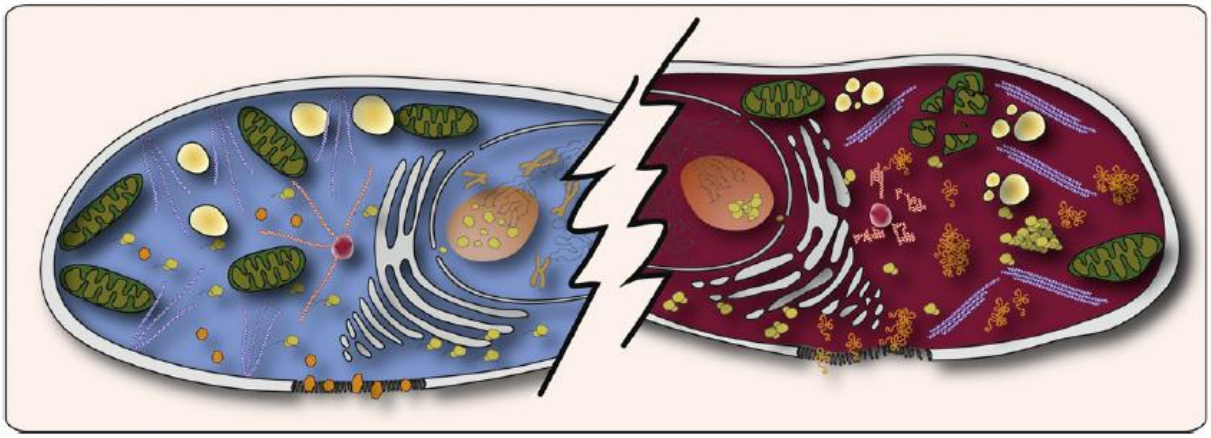


Figure 1. Effects of heat shock on the organization of the eukaryotic cell. Representative cartoon of an unstressed eukaryotic cell (left) is compared to a cell under heat stress (right). The effect of heat stress on the compartments of the cell is shown in different colors. Cytoskeleton components: actin filaments in blue, microtubules in red. Organelles: Golgi and the endoplasmic reticulum in white, mitochondria in green, lysosomes in yellow-white gradient, ribosomal assembly sites and stress granules in yellow, protein aggregates in orange with hexagonal versus spaghetti style (Richter et al. 2010).

After heat-shock treatment of various mammalian cells, a fragmentation and/or disappearance of Golgi complex and endoplasmic reticulum can be observed (Welch & Suhan 1985; Richter et al. 2010). Heat treatment decreases the number and the integrity of mitochondria thus, impairs oxidative phosphorylation and disrupts the mitochondrial energy production (White et al. 2012). The nuclei of the heat-treated cells contain unusual rod-like inclusion bodies that are packed with thin parallel filaments. The nucleoli look swollen and stress granules become visible in the cytosol (Welch & Suhan 1985).

One of the major events occurring within the nucleus is the elevated transcription of genes encoding the stress proteins and decreased transcription and/or processing of transcripts that were active before the stress (Welch & Suhan 1985; Richter et al. 2010). Besides, heat shock remarkably affects cellular membranes, seriously influencing its fluidity, phase state and micro-domain organization. This topic will be discussed in details (see below) (Vigh et al. 1998; Vigh et al. 2005; Balogh et al. 2013; Török et al. 2014).

1.1.2. Mild versus severe heat stress

Heat stress induces orchestrated and multi-component signaling pathways to respond for adaptation and survival. Based on the severity and duration of stress, classification can be made for the heat expositions; mild heat and severe heat. It is very difficult to define the terms ‘mild’ and ‘severe’, since they are determined by both the temperature and the exposure time. For mammalian cells mild stress is typically in the temperature range of 38-42°C for duration of 15 - 20 minutes, and the severe stress is for the same duration interval, in the temperature range of 43 - 45°C (Cates et al. 2011).

Moreover, heat stress sensitivity differs depending on cell or tissue types and developmental stage. Acute exposure of cells to severe heat stress can lead to a transient arrest of cell cycle at the G1/S and G2/M checkpoints. Furthermore, severe heat can direct cells to apoptosis. Mild stress can induce the degeneration of only newly synthesized polypeptides, resulting in partial transcriptional activation of heat shock proteins, whilst under severe stress pre-existing proteins can undergo unfolding and nascent polypeptides can be subjected to misfolding which leads to complete activation of heat shock transcription factor (See Table 1). Mild heat stress is presumed to positively regulate cell cycle progression and differentiation and mild hyperthermia *in vivo* leads to an almost selective destruction of certain solid tumors (Issels 2008). Besides, mild heat stress may regulate cell survival

signaling through triggering a complex cascade of events including Ras, Rac1, mitogen-activated protein kinases (MAPKs) such as, extracellular-regulated kinase ½ (ERK1/2), phosphatidylinositol-3 kinase (PI3K)-protein kinase B (AKT), p38MAPK, stress activated protein kinase (SAPK)\c-Jun N terminal kinase (JNK) (Table 1). The mild heat effect on Rac1 regulates the downstream signaling pathways in the same manner as treatment with epidermal growth factor (EGF) does. Therefore cells perceive the mild heat as an external stimulation rather than an insult (Han et al. 2001; Park et al. 2005) and respond accordingly.

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

Table 1. Criteria for distinguishing between mild and severe heat stress (Modified figure of Park et al. 2005).

The main difference between the mild and severe heat stress is, that while mild stress can trigger cellular activities including protein expressions, severe heat can cause cell death or morbidity (Parks et al 2005). It is noted, that whereas most frequently the toxic effects of severe heat stress-induced cell cycle arrest and apoptosis are on the focus of the studies, in a

physiological sense the cellular response to mild heat stress is more important and relevant. Clearly, because during febrile diseases, the body temperature increases only by 1 -3 °C (Park et al. 2005; Cates et al. 2011).

1.2. Cell shape and Cytoskeletal changes during heat stress

Cell shape comprises the interaction of several fundamental components namely, the cytoskeleton, the cell membrane and cell adhesions. The cytoskeleton is a highly dynamic network of filamentous proteins that link all regions and components of the cell (Keren et al. 2008). All cells, from prokaryotes to mammalians have a cytoskeleton in different forms. The eukaryotic cytoskeleton has three main components; actin filaments, microtubules and intermediate filaments. Assemblies and various combinations of these dynamic proteins construct an internal architecture of a cell through building linkages between plasma membrane (PM) and organelles. Adhesion to extracellular matrix and cell-cell interactions modifies cytoskeleton structures (Clainche & Carlier 2008). Cell adhesion and motility involve the extension of the PM. The dynamic structure of cytoskeleton doesn't only define the shape of the cells but also enables them to move and to react to the extracellular environment and mediate communication across the entire cell. Cytoskeleton can sense the chemo-attractant and other stimuli such as growth factor targeted movement, cell- cell interaction and other stress conditions and respond accordingly. Therefore cytoskeletal changes have tremendous impact on cellular functions (Pollard & Cooper 2009).

As one of the main component of cytoskeleton, actin is the most abundant protein in cells which contributes to cell shape. Actin can be at either filamentous (F-actin) or globular (G-actin) forms (Dominguez & Holmes 2011). Assembly of these structures creates cell protrusions which involve either thin actin rich veils named lamellipodia or finger like projections called filopodia. Both types of cell extensions emerge with the cooperation of actin polymerization and displacement of PM for cell attachment and cell motility (Raucher & Sheetz 2000) (Figure 2).

Stress fibers are contractile bundles of actin and myosin associated with focal adhesions. Stress fibers diverse in their morphology and association with focal adhesions. Therefore they are grouped in four classes: (1) ventral stress fibers that are located at the ventral cell surface and associated with focal adhesions at both ends, (2) dorsal stress fibers that are anchored to focal adhesions at one end, (3) transverse arcs curved actin bundles and

that are not directly attached to focal adhesions and, (4) the peri-nuclear actin cap that positioned above the nucleus. Stress fibers contain antiparallel actin filaments, myosin II, and several actin filament binding proteins, such as talin α-actinin (Tojkander et al. 2012; Vallenius 2013) (Figure 3).

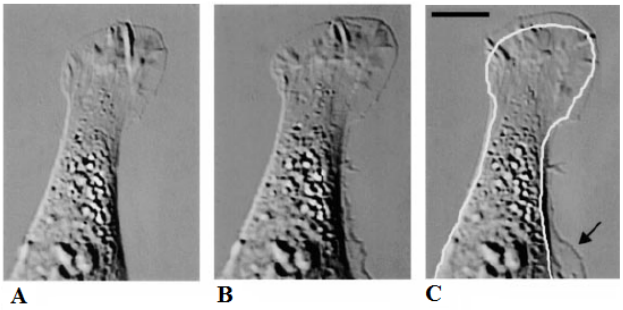


Figure 2. Lamellipodial extension in NIH 3T3 cells. (A) Initial formation of a lamellipodial protrusion in an NIH 3T3 cell. (B) After 1 min exposure of deoxycholic acid to the same cell (C) After 2 min exposure of deoxycholic acid. For comparison, the outline represents the initial cell contour from a. Scale bar, 10 mm (Modified figure of Raucher &Sheetz 2000).

Focal adhesions are the large protein complexes of the integrin family. They provide physical link between actin cytoskeleton and extracellular matrix and play crucial roles in cell-matrix sensing. The cell surface localized transmembrane integrin receptors are heterodimer proteins that bind to other proteins of the matrix such as fibronectin, collagen, vitronectin (Wolfenson et al. 2010; Haynie 2014). Integrin-ligand interactions have role in events such as cell spreading and migration and regulate cell proliferation, cell survival, and gene expression (Margadant et al. 2011).

When cell extends its lamellipodium to the extracellular matrix, integrins come into contact with extracellular matrix (ECM) ligands and cluster in the cell membrane interacting with the focal adhesion kinase (FAK), a-actinin and talin. Focal complex assembly requires Rac1 contribution (Parri & Chiarugi 2010). In focal complexes, lamellipodium extension at the leading edge involves actin polymerization which is controlled in the long run by Rac1 (Clainche & Carlier 2008; Pollard & Cooper 2009; Parri & Chiarugi 2010). As one of the central regulators of actin dynamics Rac1 coordinates stress fiber assemblies (Kovac et al. 2013; Tojkander et al. 2012). Stress fiber components are linked to cellular signaling pathways, resulting in a variety of intracellular responses including the phosphorylation dependent recruitment of signaling proteins (Clainche & Carlier 2008).

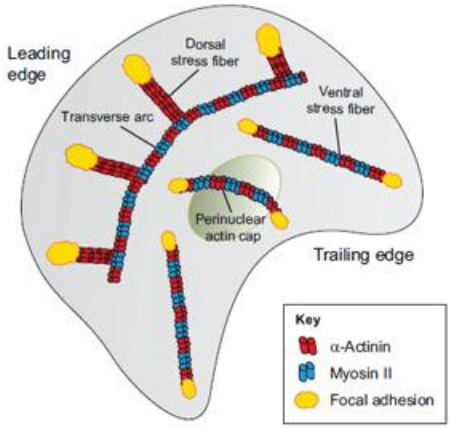


Figure 3. Representation of stress fibers types in cultured animal cells. Motile cells contain four discrete categories of stress fibers; (i) dorsal stress fibers, (ii) transverse arcs, (iii) ventral stress fibers and (iv) peri-nuclear actin cap bundle (Modified figure of Tojkander et al. 2012).

Small heat shock proteins (sHSPs) are known to interact with actin bundles as capping proteins to stabilize the actin filaments. Cytoskeletal disorganizations and phosphorylation of the sHSPs are the earliest events induced by a stress. It has been reported that overexpression of sHSPs prevent actin cytoskeleton disruption by stress induced signals (Mounier & Arrigo 2002). Among the sHSPs, HSP25/27 has been extensively studied to elucidate the actin dynamics. Non-phosphorylated, monomeric HSP25/27 can inhibit actin polymerization (Wettstein et al. 2012). Phosphorylation of HSP25/27 stimulates the actin filament organizations following heat stress in order to prevent the aggregation of denatured actin (Pivovarova et al. 2005; Doshi et al. 2009; Clarke & Mearow 2013).

The complicated multi-component structure of cytoskeleton therefore, can respond rapidly to their environmental changes such as heat stress, especially in mammalian cells (Hildebrandt et al. 2002; Vallenius 2013; Gagat et al. 2013). The response of the cytoskeletal system to hyperthermia differs depending on the cell type and heat intensity. Hyperthermia is reported to disrupt microtubules and form collapsed vimentin filaments (Huang et al. 1999; Pawlik et al. 2013). A rapid loss of stress fibers in Chinese hamster ovary cells has been reported after heat exposure of cell to 45 ºC (Glass et al. 1985). Cell rounding associated with disintegration of F-actin filaments have also been documented upon heat shock treatment (Clarke & Mearow 2013).

1.3. The heat shock response

One of the main pro-survival mechanisms of living organisms, the heat shock response (HSR), is defined as the biochemical response of cells to heat stress (Lindquist 1986; Fulda et al. 2010). In 1962, Italian scientist Ritossa discovered the appearance of expanded chromosomal puffs in Drosophila salivary glands after heat shock indicating the onset of locally enhanced transcription (Ritossa 1962). This discovery was followed by the identification of the transcribed genes and corresponding proteins. Respected studies were carried out in prokaryotes and other eukaryotes. Results underline that the HSR is one of the most ancient and conserved mechanisms found in nature (Horváth et al. 2008; Dai et al. 2007). HSR can govern the cell faith towards cell survival or cell death according to the severity of heat stress (Samali & Orrenius 1998; Powers & Workman 2007).

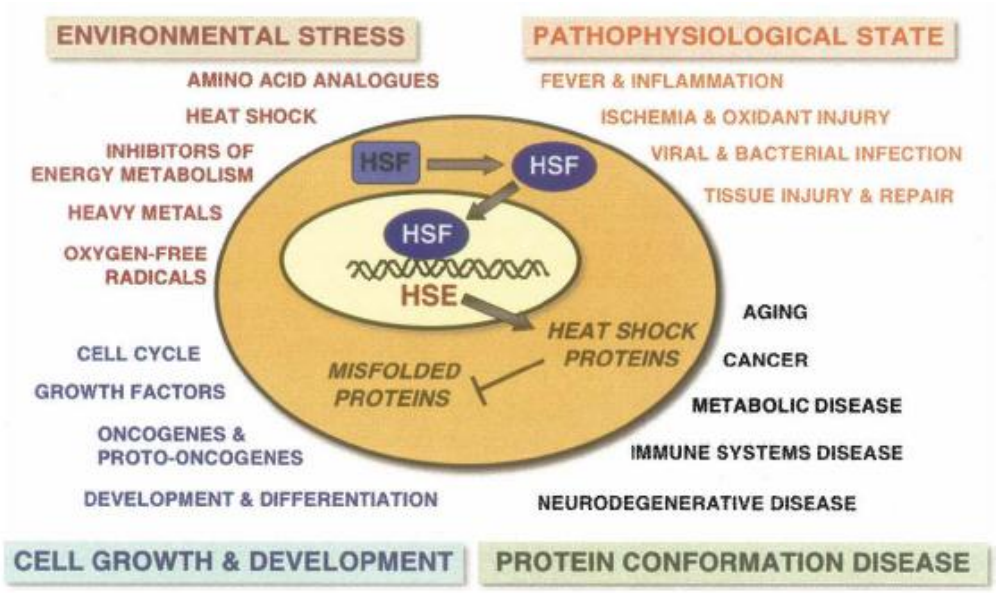


Figure 4. Stressors that induce HSR. The regulatory conditions are represented by environmental and physiological stress and non-stressful conditions, including cell growth and development and pathophysiological states. HSP expression is represented here by the activation of HSF and binding to DNA (Morimoto 2008).

Although increment of the temperature is the classic inducer of HSR, it has been recognized that this response can be activated by many other stresses including pesticides, heavy metals, reactive oxygen species, solvents as well as pathophysiological states and non-stress conditions including cell cycle, growth factors (Morimoto 1998; Gupta et al. 2010). According to a classic view, the major outcome of these stresses is protein inactivation, caused by the unfolding and/or aggregation of proteins. HSR leads to cellular resistance that is

known as “thermotolerance”. HSR arrests the general protein transcription and translation to ease the amount of damaged proteins. However, the development of thermotolerance inevitably involves the coordinated synthesis of heat shock proteins (HSPs) via a group of transcription factors, termed as heat shock factors (HSFs) that enhance the expression of *hsp* genes (Calderwood et al. 2010; Fulda et al. 2010) (Figure 4).

On the other hand, isothermal membrane perturbations induced by membrane stressors (like chemical fluidizers), are reported to initiate the HSR (Horváth et al. 2012; Balogh et al. 2013; Török et al. 2014; Vigh et al. 1998). Detailed information will be given in section 1.6.1.1 “Membrane initiated stress signaling”.

1.4. Heat shock proteins: their roles and functions

The most well characterized HSR is the accumulation of a highly conserved set of proteins called heat shock proteins (HSPs) (Park et al. 2005) or stress proteins. Although the term “heat shock protein” is commonly used synonymously with “chaperone,” distinctions must be made, as not all HSPs are molecular chaperones, and not all chaperones are induced by heat shock (Verghese et al. 2012). Chaperones guide the conformation of proteins, aid in the folding of nascent proteins, modulate assembly of proteins and degradation of misfolded proteins, while HSPs involve in protein homeostasis, in order to alleviate the stress caused unfavorable changes (Kampinga et al. 2009; Kampinga & Craig 2011).

HSPs thus promote the cellular recovery and the development of thermotolerance (Calderwood et al. 2010; Finka et al. 2011). HSP molecular chaperones play several important roles in protein homeostasis through the following processes:

- Folding nascent proteins
- Directing the translocation of proteins to cellular organelles
- Assembling of protein complexes
- Protecting against protein aggregation
- Change the activity of proteins
- Stabilizing inactive, structurally unstable proteins
- Refolding proteins that have inadequate conformation
- Leading the separation and degradation of damaged proteins

"Moonlighting" HSPs have multiple and vital functions in the cell. They reside not only in the cytosol but also in the cellular organelles and can associate with membranes through their lipid interactions (Horváth et al. 2008; Horváth & Vígh 2010).

The diverse HSPs can be classified in five major families according to their molecular weight, amino acid sequence homologies and functions:

- HSP100 family,
- HSP90 family,
- HSP70 family,
- HSP60 family
- Small HSP (sHSP) family (Horváth et al. 2008; Niforou et al. 2014).

Major HSPs and their actions can be seen in Table 2.

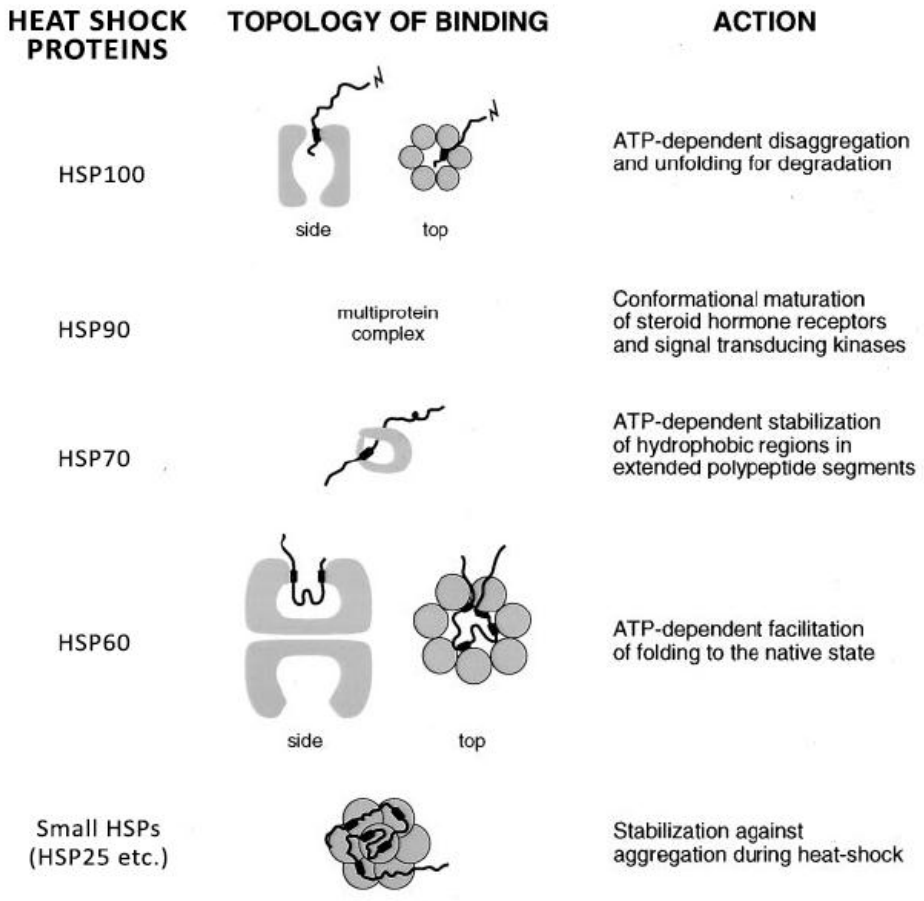


Table 2. Topology of Polypeptide Binding and Action of HSP Families. Bold lines signify polypeptides, and the thickened segments denote sites that become directly associated with chaperone, typically hydrophobic in character. Structures are not drawn to scale (Modified figure of Bukau & Horwich 1998).

HSP100 family: ATP dependent chaperones, the *HSP100 family* belongs to AAA+ (ATPases Associated with diverse cellular Activities) superfamily and members of the family function as unfoldases and disaggregases (Saibil 2013). It has several different homologous such as bacterial ClpB, ClpA, ClpX and eukaryotic HSP104, HSP110. In eukaryotes, HSP100 acts as HSP70 nucleotide exchange factor (Dragovic et al. 2006; Mogk et al. 2008) and cooperates with HSP70 for disaggregation, thus avoiding the toxic effects of aggregation (Saibil 2013).

HSP90 family: HSP90 is a highly conserved family which can be found in both prokaryotes and eukaryotes, abundantly. Besides having common molecular chaperone function, HSP90 is also very essential for cell viability and cell growth. HSP90α and HSP90β are two members which are expressed by two different genes (Jego et al. 2013). It has more than 200 client proteins which covers almost all the cell processes such as transmembrane tyrosine kinases (Her-2, EGFR), metastable signaling proteins (AKT, Raf-1 and IKK), cell cycle regulators (Cdk4, Cdk6), and steroid receptors (androgen, estrogen, and progesterone receptors). Similar to HSP70, HSP90 also functions in ATP dependent manner (Li et al. 2010; Li & Buchner 2012).

HSP70 family: HSP70 family of chaperones is the most conserved proteins in evolution, they are found in all organisms from prokaryotes to eukaryotes. Every eukaryotic cell encodes more than one HSP70. The human Hsp70 family consists of eight highly homologous members that have different expression profile and intracellular localization. Among those, HSP70-1a and HSP70-1b (HSP70, HSP72) are stress inducible HSP70 members. In physiological conditions their expression is at low, nearly undetectable levels in cell type and cell cycle dependent manner (Rohde et al. 2005; Daugaard et al. 2007). HSP70 family members are ATP dependent chaperones that take role in folding of newly synthesized polypeptides, the assembly of multi-protein complexes and transportation of proteins between cellular compartments (Bukau & Horwich 1998). Majority of HSP70 proteins function together with several cofactors and J-proteins (also called HSP40). In organisms, the members of J-protein family are found in large numbers according to their domain composition and cellular functions (Mayer & Bukau 2005; Young 2010; Clare & Saibil 2013).

HSP60 family: HSP60 family, called also as *chaperonins* (GroEL/GroES in bacteria), form oligomeric, high molecular weight complexes of ~1 MDa (Bukau & Horwich 1998; Hartl 2011). GroEL is a double-ring 14mer and GroES is a single-ring heptamer that binds to GroEL in the presence of ATP. In the eukaryotic cytosol the more poorly characterized TRiC

(TCP-1 Ring Complex) the eukaryotic chaperonin, is composed of two rings of eight different though related subunits. TRiC was originally thought to fold only the cytoskeletal proteins actin and tubulin but is now known to fold dozens of substrates. Eukaryotic chaperonins are not thought to utilize a GroES-type cofactor to fold their substrates.

sHSP family: sHSPs are ATP-independent members of the heat shock protein family with molecular weights in the range of 15–30 kDa. sHSPs have a conserved α-crystallin domain that binds misfolded polypeptide chains. sHSPs prevent the aggregation of partially misfolded proteins and support the thermotolerance of the cells. Many sHSPs are not constitutively functioning; they are specifically activated upon the introduction of stress conditions such as elevated temperatures. sHSP complexes are dynamic structures and exchange subunits constantly to form hetero-oligomeric assemblies with other sHSP species present in the same compartment (Sun & MacRae 2005). sHSPs require cooperation with ATP-dependent chaperone systems to release the refolded substrates from the transient sHSP complexes. HSP25/27 and α-crystallin target damaged or mutated proteins for degradation. Moreover, autophagy-mediated degradation of protein aggregates is induced by HSP22 (Horváth et al. 2008; Finka et al. 2011; Niforou et al. 2014). sHsps are also reported to be involved in several apparently unrelated cellular processes, such as modulation of the actin cytoskeleton and the intermediate filaments, cell growth, differentiation, apoptosis, tumorigenesis, and signal transduction (Mounier & Arrigo 2002).

1.5. Transcriptional regulation of HSPs: the small GTPase Rac1 is an upstream element of HSF1 regulation

HSP expression is a transcriptionally controlled process. In *E. coli*, heat shock genes are controlled by a specific transcription factor, σ32, which directs the core RNA polymerase to HSP promoters. Among eukaryotes, in invertebrates such as yeasts, nematodes, and fruit flies, the transcriptional activation of *hsp* genes is regulated by a single heat shock factor (HSF).

There are four different HSFs and one of which, HSF1 is the main transcriptional regulator of *hsp* genes in mammalian cells (Fujimoto & Nakai 2010; Anckar & Sistonen 2011). In mammalian cells, HSF1 is dispensable under physiological conditions but deletion of *hsf1* completely abolishes the transactivation of *hsp* genes in response to a variety of stresses (Dai et al. 2007; Akerfelt et al. 2010). In B16F10 cell line, administration of benzyl

alcohol (BA) as a membrane fluidizer can also stimulate HSF1 activation (Nagy et al. 2007). Moreover HSF1 can't be compensated by other HSFs in mammals (Anckar & Sistonen 2011).

HSF1 is constitutively present in most tissues and cell types in the cytosol where it is kept in inactive form if there is no proteotoxic stress. When cells experience diverse environmental or physiological stress stimuli, HSF1 undergoes multi-step processes; (1)- cytosolic HSF1 dissociates from HSP90, HSP70 or HSP40 which is in a complex with HSF1 during non-stress conditions, (2)- translocates to the nucleus and trimerizes via intramolecular interactions, (3)- binds to extended repeats of the sequence nGAAn, called heat shock elements (HSEs) of *hsp* genes in promoter site of DNA (Voellmy 2005; Shamovsky & Nudler 2008; Tóth et al. 2013) (Figure 5).

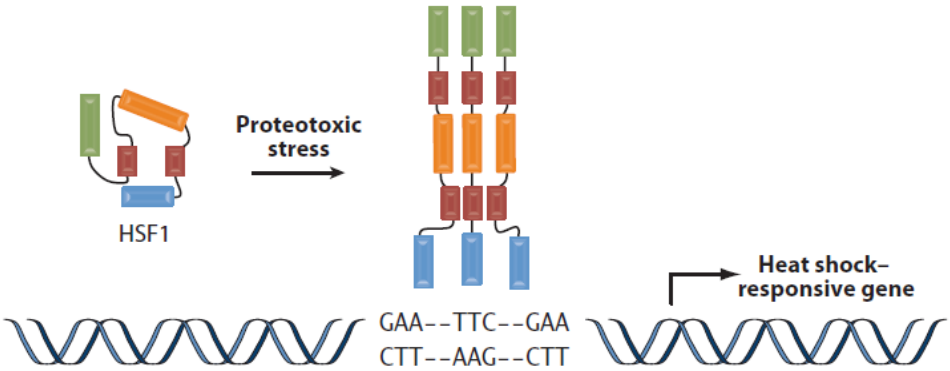


Figure 5. HSF1 activation. HSF1 is activated by a multitude of protein-damaging stresses. Upon activation, HSF1 trimerizes, accumulates in the nucleus, undergoes extensive posttranslational modifications (especially phosphorylation) and binds to HSEs, which comprise inverted repeats of the nGAAn pentamer in the promoters of *hsp* and other target genes (Anckar & Sistonen 2011).

Post translational modifications play important roles in HSF1 activation. HSFs can be modified by phosphorylation and sumoylation on many serine and threonine residues and by acetylation. Phosphorylation is one of the prominent modifications of HSF1; the effect of phosphorylation can be activatory or inhibitory according to phosphorylation site of the trimerized HSF1. Sumoylation represses the trans-activating capacity of HSF1. HSF1 can also be regulated by acetylation on lysine residues that release DNA-bound HSF1 thus, causes inactivation of transactivation potential of HSF1. HSF1 acetylation is controlled by the deacetylase sirtuin1 (SIRT1) (Morimoto 2008; Anckar & Sistonen 2011; Y. Xu et al. 2012).

Lately, it has been reported that a number of kinases are activated by heat stress and can modulate the HSR positively or negatively. Those signaling cascades can have both pro-survival and pro-death functions. This plasticity depends on the diversity of many factors such as environment, intensity and mode of stimulus, cell conditions (cell type, cell-cycle).

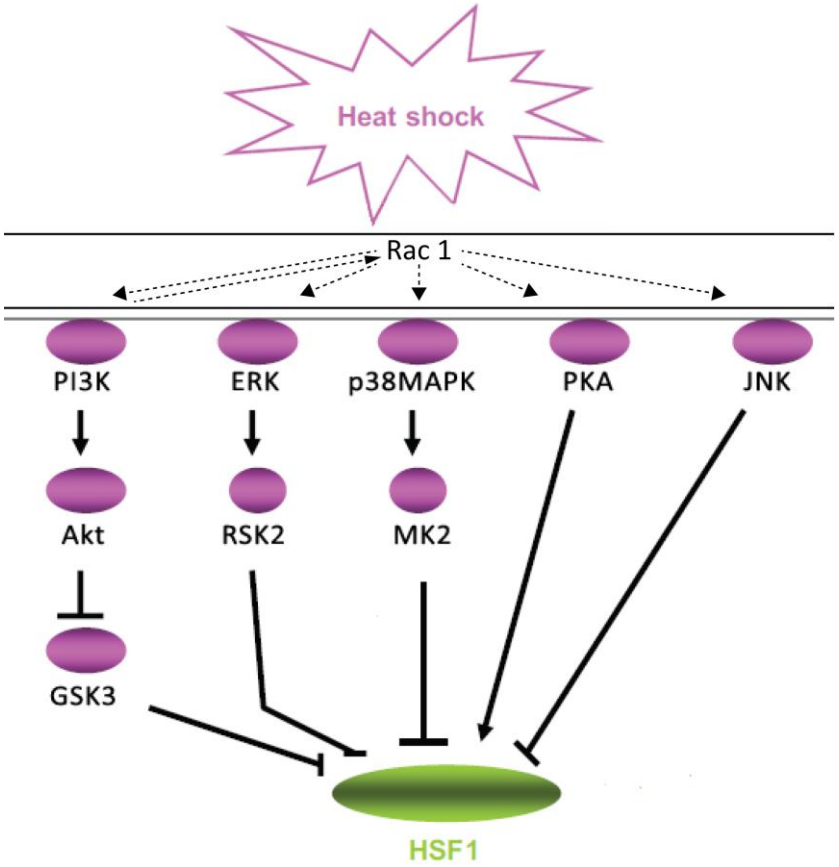


Figure 6. Signaling kinase cascades activated by heat shock and their downstream targets. p38MAPK, p38 mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; ERK, extracellular signal-regulated kinase; PKA, protein kinase A; JNK, c-Jun N-terminal kinase; MK2, MAPK-activated protein kinase 2; AKT, protein kinase B; GSK3, glycogen synthase kinase-3; HSF1, heat shock factor 1 (Modified figure of Calderwood et al 2010).

The most notable heat stress induced signaling pathways consist of three MAPK pathways, namely the extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK) and p38MAPK. Heat stimulation of ERK1/2 has dual effect: it phosphorylates HSF1 on serine 307 residue and causes transcriptional inhibiton as well as induces RSK (ribosomal s6 kinase) to repress HSF1. Induction of JNK mediates apoptosis through inactivating HSF1 by phosphorylating HSF1 at an unknown side under stress stimuli. p38MAPK activation induces its downstream effector MK2 kinase which then phosphorylates

HSF1 on serine 121, inhibiting the activation of HSF1. AKT, a serine/threonine-specific protein kinase (also known as protein kinase B) is another signaling modulator during heat stress. It acts together with PI3K. Rapid activation of PI3K generates phosphatidylinositol 3,4,5-triphosphate which directs AKT membrane transloction. PI3K/AKT cascade can trigger the HSF1 activation indirectly via inhibiting glycogen synthase kinase 3 (GSK3) which inhibits HSF1 activation by phosphorylating the serine 303 residue. Besides, HSF1 is activated by protein kinase A (PKA) (Figure 6) (Calderwood et al. 2010; Nadeau & Landry 2007).

Small GTPase Rac1 is believed to be another upstream element of HSF1 regulation. Han et al. (2001) showed the inhibitory effect of dominant negative mutant of Rac1 (Rac1N17) on HSF1 activity under mild heat shock conditions while upon severe heat exposure, Rac1N17 caused no inhibition of HSF1 activity (Han et al. 2001). Besides, Ozaki et al. (2000) tested Rac1 contribution on HSF1 activation under hypoxia/reoxygenation stress and sodium arsenite administration (Ozaki et al. 2000). Consistent with Han et al.'s finding Rac1N17 inhibits the HSF1 activation and HSP expression under those conditions. However, constitutively active Rac1V12 does not induce HSF1 activation suggesting that Rac1 may be necessary but insufficient for HSF1 activation (Ozaki et al. 2000; Han et al. 2001).

Effect of Rac1 on HSF1 regulation happens through its upstream lipid intermediates such as phosphatidylinositols (PtdIns) and downstream signaling elements such as MAPKs (Park et al. 2005; Wang et al. 2006) (Figure 6).

1.6. Heat stress sensing

In order to resist life threatening insults, ranging from environmental changes to unfavorable metabolic abnormalities, cells respond producing HSPs to preserve their integrity. Understanding the stress sensing and signaling is a target of researcher's interest (Vígh et al. 2007).

Cells can sense the stress in different ways. Stress signal then results in alteration of HSPs through the association of HSF1 with HSE in the promoter region of *hsp* genes. Potential stress sensors are shown on Figure 7.

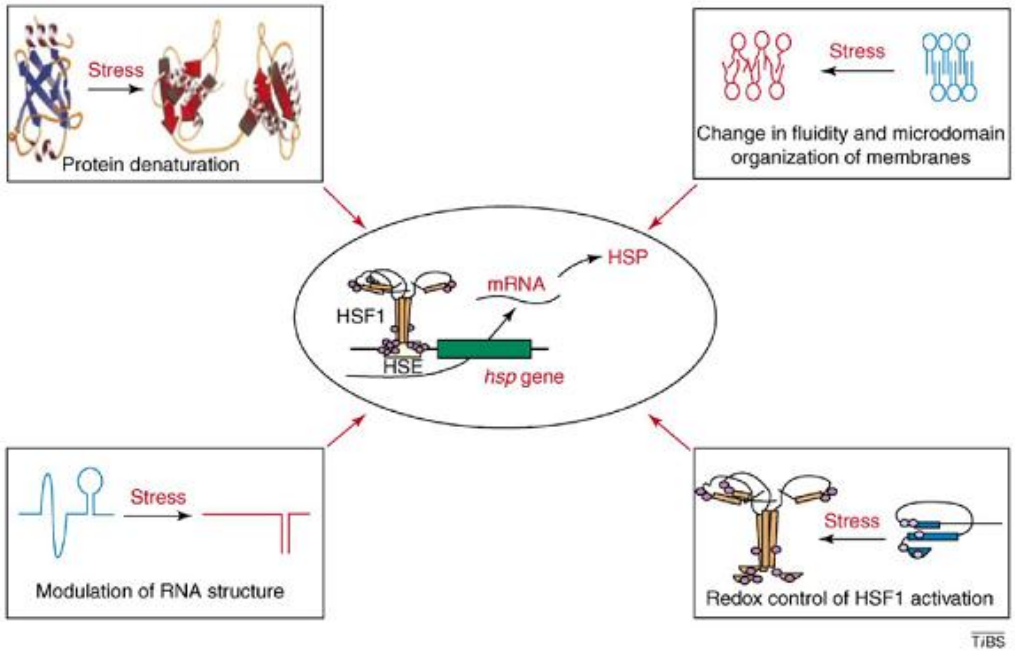


Figure 7. Stress sensors of the HSR in mammalian cells. Protein denaturation, membrane fluidity or micro-domain organization, RNA structure and redox control are the potential stress sensors (Vígh et al. 2007).

Denatured protein sensor model hypothesizes that various stress conditions can cause the accumulation of damaged proteins in the cell. Denaturation of proteins by heat stress may serve as a signal for the induction of HSPs via activation of HSF1. According to this hypothesis, under non-stress stimuli, HSF1 monomers are in a complex with HSP90 or HSP70 in cytoplasm and this state is considered as *inactive state*. Under stress stimuli, the presence of the elevated amounts of denatured proteins liberates HSP90 and HSP70 from HSF1 to fulfill their chaperone function and allows HSF1 trimerization. The *active state* of HSF1 binds to HSE of DNA. Increasing quantities of expressed HSPs (HSP90 or HSP70, HDJ-1) attenuates the transactivation of HSF1 (Figure 8) (Morimoto 1998).

However this hypothesis is not applicable in many circumstances. Many diseases and aging alter the expression of HSPs without increased levels of denatured proteins and also insulin stimulated HSP expression cannot be explained by denatured protein model. Moreover, HSP expression has also been reported to be induced by mild, fever range temperatures which don't create any protein denaturation (Horváth et al. 1998; Vigh et al. 2007).

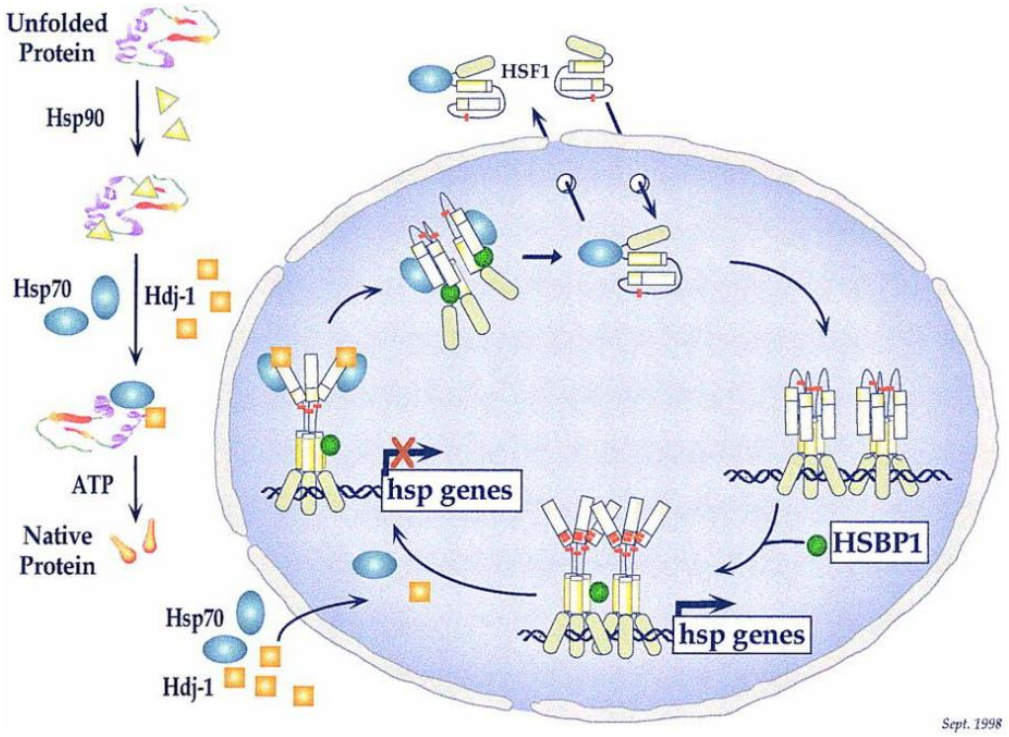


Figure 8. Regulation of the HSR and the HSF1 cycle. Presence of unfolded proteins activates the HSF1. Activated HSF1 locates to nucleus, trimerize and binds to promoter of *hsp* gene. HSF1 activation is repressed by direct binding of Hsp70 and Hdj-1. HSF binding protein 1 (HSBP1), which binds to both HSF1 and HSP70, negatively regulates HSF1 trimers. Then HSF1 trimers dissociate (Morimoto 1998).

HSF1 can directly detect changes in the **redox state** via assembling into a homo-trimer in a reversible and redox-regulated manner. Two cysteine residues of HSF1 DNA binding domain are required to form redox-sensitive disulfide bonds. These cysteine residues (C35 and C105) are necessary for HSF1 transactivation (Ahn & Thiele 2003).

According to **RNA thermo-sensor** model, transactivation of HSF1 by trimerization is induced by a ribonucleoprotein complex consisting of a translation elongation factor, eEF1A, and a constitutively expressed noncoding RNA called HSR-1 (heat shock RNA-1) during heat stress in mammalian cells (Shamovsky et al. 2006). Although regulation of HSR-1 and contribution to HSF1 activation remains to be elucidated, it is possible that the RNA molecule per se might act as a thermo-sensor via a heat-induced change in its conformation (Kugel & Goodrich 2006; Shamovsky & Nudler 2008). Also, as a result of heat stress, disintegration of cytoskeleton releases its binding protein eEF1A which then enables to interact with HSR1 and HSF1 to activate HSR (Shamovsky & Nudler 2008).

The alternative cellular temperature-sensing mechanism is intimately associated with the composition and physical state of membranes; even subtle temperature changes can be sensed by membranes. Membranes are known to be the main targets of temperature adaptation, they respond to various environmental perturbations by changing their composition and micro-domain organization. Besides, changes in the physical state of membranes of *Synechocystis, Escherichia coli* or yeast have been demonstrated to affect profoundly the temperature-induced expression of *hsp* genes (Horváth et al. 1998; Balogh et al. 2013; Horváth et al. 2012). Various membrane fluidizers are reported to modulate HSP expression without inducing cell proteotoxicity (Balogh et al. 2005; Nagy et al. 2007; Balogh et al. 2010). More, different disease states cause changes in the fluidity and micro-domain structure of membranes (Crul et al. 2013; Török et al. 2014). Based on the findings mentioned above we proposed the **membrane sensor model** which postulates that the membrane's physical properties and micro-domain organization play an initiating role in the HSR (Horváth et al. 1998; Vigh et al. 2007).

Alterations in lipid membranes can affect signaling pathways either through the changes on the physical state of the membranes or via specific lipid-protein interactions and distinct membrane domains can recruit amphitropic proteins to transduce the membrane initiated signals. **Membrane sensor** hypothesis underlines the importance of membrane structure, micro-domain organization and their lipids in HSR (Vigh et al. 2005; Balogh et al. 2013; Török et al. 2014).

1.6.1. Micro-domain organization of membranes and membrane initiated stress signaling

One of the universal features of all cells is an outer limiting membrane called the PM. The PM is a multifaceted entity which provides a separation of the extracellular and intracellular milieu (Head et al 2014). Detailed investigations have explored its multiply functions including compartmentalizing the membrane binding compounds, hosting receptor proteins, perceiving the external stimuli, having roles in differentiation, cellular uptake and cell motility, representing the cell-cell, cell-tissue adhesion zones and enabling the control of influx and efflux of specific ions (Vereb et al. 2003; Patra 2008; Klotzsch & Schütz 2013). PM composed of proteins and lipids regulates cell signaling and many other cellular processes through its unique "dynamic, yet structured" feature (Vereb et al. 2003; Escribá et al. 2008; Kusumi et al. 2012; Török et al. 2014).

Possessing the amphipathic nature, membrane lipid molecules create highly hydrophobic core in the intramembrane milieu and their hydrophilic portion gets in contact with aqueous environment which forms the physical basis of the PM bilayer. Eukaryotic PMs consist of glycerophospholipids, sphingolipids, and sterols (particularly cholesterol in mammalian cells). The major structural lipids in eukaryotic membranes, the glycerophospholipids include phosphatidylcholine (PtdCho), phosphatidylethanolamine (PtdEtn), phosphatidylserine (PtdSer), phosphatidylinositol (PtdIns) and phosphatidic acid (PA). Due to different geometry and the structure of the glycerophospholipids, they are asymmetrically distributed between two bilayer leaflets. The hydrophobic backbone of sphingolipids is ceramide (Cer). They vary in many different carbohydrate structures in the head groups. The main sphingolipids in mammalian cells are sphingomyelin (SM) and the glycosphingolipids (GSLs). Besides, gangliosides (such as GM1) are GSLs with terminal sialic acids. Sphingolipids adopt the solid gel phase and can be fluidized by sterols. Cholesterol (Chol) is the major non-polar lipid of the membranes in mammalian cells. Chol thickens the lipid bilayer leaflets. Not all the eukaryotic cell lipids have cylindrical shape (lipid species with "non-bilayer propensity"), thus they can't provide the spontaneous formation of a lamellar phase. Contribution of bilayer-type lipids and specific proteins, however, ultimately results in functional macromolecular assemblies and formation of asymmetric membrane bilayers (Vereb et al. 2003; van Meer et al. 2008; Head et al. 2014). Breakdown products of membrane lipids such as arachidonic acid (AA) released by signaling-induced hydrolysis, can serve as secondary lipid messengers. Phoshoinositides are phosphorylated PtdIns intermediate molecules that recruit cytosolic signaling proteins (Head et al. 2014).

According to the fluid mosaic model proposed by Singer and Nicolson in 1972, the PM has a two dimensional phospholipid bilayer, hosting membrane proteins which are dispersed randomly and homogenously within the lipid bilayer. Free partitioning of proteins and lipids occurs via undergoing thermal diffusion (Singer & Nicolson 1972). However, further investigations showed that the PM houses numerous proteins and thousands of lipid species which compose complex, heterogenic and asymmetrical biological membranes with differences in lipid and protein compositions between the inner and the outer leaflet of the bilayer (Vereb et al. 2003; Escribá et al. 2008; Truong-Quang & Lenne 2014). In 1990s studies showed that protein diffusion undergoes in a more complex manner and is restricted;

lipid domains were proposed to solve the problem of sorting and trafficking lipids and lipid binding proteins (Edidin 2003; Vereb et al. 2003).

Kusumi et al. proposed new membrane architecture by hierarchical three-tiered mesoscale-domain architecture of the PM (Figure 9). In their research they underlined the importance of actin based membrane-skeleton-induced compartments. By removing the actin membrane skeleton increased the diffusion coefficient of phospholipids by a factor of ~20. They reported that the actin based membrane skeleton as "fence" and transmembrane proteins as "pickets" hinder the diffusion rate of membrane molecules (Kusumi et al. 2012).

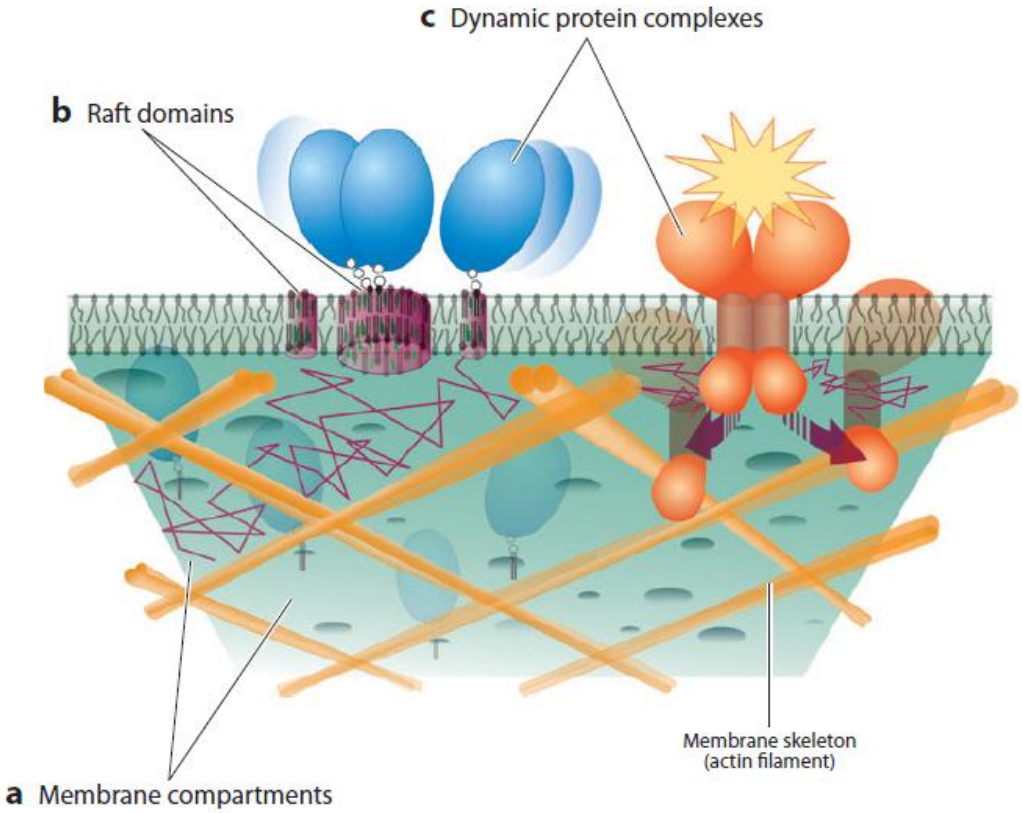


Figure 9. Three-tiered hierarchical mesoscale-domain architecture of the PM. (*a*) Membrane compartments, generated by the partitioning of the entire PM, by the actin cytoskeleton (fence) and transmembrane proteins anchored to the membrane skeleton fence. (*b*) Raft domains enriched in Chol, glycosphingolipids, and glycosylphosphatidylinositol (GPI)-anchored proteins. (*c*) Dynamic protein complex domain consists of dimers and greater oligomers of integral membrane proteins (Kusumi et al. 2012) .

Bilayer heterogeneity can be explained by introducing the term of membrane micro-domains (also called rafts). Different lipid constituents can segregate into functionalized micro-domains (Simons & Ikonen 1997). These lipid clusters are existed in a liquid ordered (L_o) state within the generally liquid disordered (L_d) lipid membrane environment (Kusumi et

al. 2011; Truong-Quang & Lenne 2014). Lipid domains are initially described as heterogeneous, highly dynamic, Chol and sphingolipid enriched micro-domains which compartmentalize cellular processes and function as scaffolds for assembling the specific signaling molecules. Under some conditions small lipid rafts can coalesce and form larger and more stable platforms through protein-protein, protein-lipid interactions (Pike 2006). Owing to their dynamic structures, reported size and lifetime distributions of rafts vary greatly (Kusumi et al. 2011). Lipid micro-domains influence the membrane protein and receptor trafficking (Pike 2009, Kusumi et al 2011). Biochemically, lipid rafts are detergent resistant membrane fractions, therefore they can be separated from high density cytoskeletal proteins. The high amounts of detergents used for raft isolation processes can change the thermodynamic properties of lipids, thus may affect the observed domains (Klotzsch & Schütz 2013). Membrane micro-domains have characteristic lipid compositions. Chol is essential component of rafts, it is found in double amount in rafts compared with PMs (Pike 2009). It serves as cofactor for signaling molecules and as precursor for steroid hormones (Anchisi et al. 2012). Chol is required for forming L_o phases in model membranes (Goni et al. 2009). Also, depletion of Chol by using methyl-β-cyclodextrin results in lipid raft dispersion causing hindered lateral mobility of membrane proteins. The key regulatory phospholipid mediator, phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) is concentrated in Chol-dependent domains and Chol extraction results in a decreased amount of PtdIns(4,5)P_2 on the PM (Kwik et al. 2003). SM levels are also increased by 50% in lipid rafts compared to PMs. While SM level is elevated, PC levels are decreased so that choline-containing lipid levels remain similar in rafts and PMs (Pike 2009). Gangliosides (especially GM1 and GM3 in mammalians) are another constituent of lipid rafts and their presence results in the coalescence of small rafts into micrometer-sized domains and the reorganization of known raft proteins in the micro-domains (Pike 2009; Head et al. 2014). Model lipid rafts can demonstrate different features than PMs. Various studies showed that L_o-L_d phase separation (Lingwood & Simons 2010) and diffusion properties (Kusumi et al. 2012) differ between model membranes (composed of lipids) and native PM. The reason can be well explained on the base of Kusumi and coworker's recent membranes models (Head et al. 2014). On the other hand, model-membrane work emphasizes the fact that certain lipids exhibit preferential association with one another and provides a framework for understanding how heterogeneity in cell membranes may arise (Lingwood & Simons 2010).

One of the most important properties of lipid rafts is that they can either long term or temporarily host several proteins ("residents" and "visitors") (Escribá et al. 2008). Among those; transmembrane receptors such as integrins, G-coupled protein receptors (GCPRs) and receptor tyrosine kinases (RTKs) including epidermal growth factor receptor (EGFR) are typically involved in detecting environmental signals (Cordwell & Thingholm 2010; Truong-Quang & Lenne 2014). Moreover, numerous studies suggest that lipid modifications such as GPI anchors, palmitoylation, or myristoylation can target proteins to lipid rafts (Pike 2009). Lipid modified caveolins, flotillins, Src-family kinases, small GTPases including H-Ras and Rac1 are detergent resistant raft binding proteins and are involved in various cellular functions including stability and signaling (Simons & Toomre 2000; Echarri et al. 2007; Patra 2008).

It has been reported that palmitoylation increases the affinity of signaling proteins for raft localization. However, it is important to point out that palmitoylation is not the only modification directing proteins to raft association. There are many reported palmitoylated proteins which are not raft associated. Indeed C-terminus polybasic region of palmitoylated proteins is also necessary for the affinity of proteins to localize to the raft regions or PMs (Jack et al. 2008; Greaves et al. 2009; Simons & Sampaio 2011). As cytoskeleton and PM are connected, when isolated, the rafts retain some of their associated cytoskeletal proteins (Pike 2009).

Recently del Pozo and co-workers have shown that Rac1 can incorporate palmitate at cysteine 178 and indeed, this specific post-translational modification targets Rac1 for stabilization at actin cytoskeleton-linked ordered membrane regions (Navarro-Lérida et al. 2012). Moreover, it was documented, that palmitoylation of Rac1 requires its prior prenylation. It was also evidenced, that non-palmitoylated Rac1 displays decreased GTP loading and reduced association with detergent-resistant rafts. In fact, mammalian cells expressing a palmitoylation-deficient mutant have an increased content of disordered membrane domains. Taken together, these data firstly identified palmitoylation as a mechanism for Rac1 function in actin cytoskeleton remodeling by controlling its membrane partitioning, which in turn regulates membrane organization (Navarro-Lérida et al. 2012). (Further details will be discussed later).

There are two common types of lipid rafts: caveolin containing lipid rafts called as caveolae (little caves) planar lipid rafts (also referred to as non-caveolar, rafts) (Figure 10). Formation and maintenance of caveolae is primarily due to the protein caveolin (Allen et al.

2007; Head et al. 2014). Cell detachment or phosphorylation of caveolin triggers caveolae internalization. It has been reported that changes in lipid composition strongly affect caveolae dynamics such that, caveolar endocytosis can be elevated by exogenous glycosphingolipids (GSLs) and by increased endogenous amounts of GM1 or Chol (Echarri et al. 2007). Pharmacological inhibition of phosphatases also induces caveolae internalization, and similar effects are achieved by insults such as oxidative stress, heat stress and hyperosmotic shock (Echarri et al. 2007).

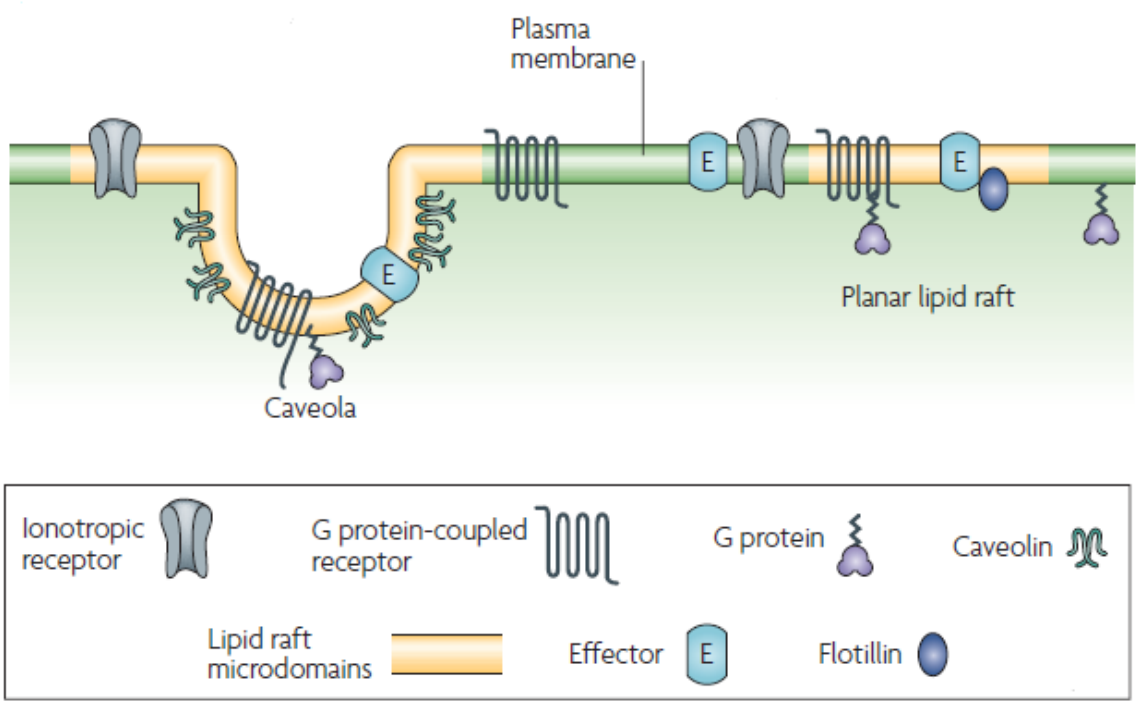


Figure 10. Lipid raft micro-domains. Planar lipid rafts and caveolae can be seen in the figure. Planar rafts don't contain distinguishing morphological features. On the other hand caveolae is flask-shaped membrane invaginations that contain caveolins. Both have similar lipid composition (Modified figure of Allen et al. 2007).

Caveolins and flotillin can recruit signaling molecules into lipid rafts. As rafts are signaling platforms therefore many signaling effectors such as second-messenger generating enzymes are found in lipid rafts (Echarri et al. 2007; Allen et al. 2007). Also, cytoskeletal components interact with lipid rafts for communication to the ECM via integrins in adherent cells (Head et al. 2014). Activation and/or ligand binding induces integrin clustering. Activated integrins then recruit the signaling molecules and actin filaments to lipid raft platforms (Echarri et al. 2007; Allen et al. 2007). Integrin mediated activation of signaling intermediates, including ERK, PI3K, FAK, Src family tyrosine kinase and small GTPases are

also regulated by caveolin (Del Pozo & Schwartz 2007). Furthermore, integrin signaling is an important regulator for the caveolae internalization such that sudden loss of cell adhesion induces striking effects in caveolae internalization. Besides, caveolae internalization causes disappearance of Rac and other signaling proteins from their original sites resulting in the blocking of signal transduction (Parton & del Pozo 2013). The recruitment of signaling molecules and cytoskeletal components in raft regions stimulates multiple signaling cascades that can result in changes in cell polarity, cell migration, cell cycle progression, gene expression and survival (Echarri et al. 2007; Del Pozo & Schwartz 2007).

Due to their wide range of cellular functions, small GTPase Rac1 and PI3K signaling pathways overlap. Several studies showed that PI3K activation via production of lipid second messenger PtdIns (3,4,5)P_3 (or PIP_3) leads to the activation of guanine-nucleotide exchange factors (GEFs) that activate Rac1. Vice versa, Rac1 may also induce PI3K activation through contributing the generation of PtdIns (4,5)P_2 (or PIP_2) (Welch et al. 2003; Kwiatkowska 2010).

Rafts are enriched in lamellipodia of PM and required for cell spreading. Rac1 localizes to rafts in lamellipodial regions (Balasubramanian et al. 2007), acts in membrane ruffling (Itoh et al. 2008; Schwarz et al. 2012) and its targeting to lipid rafts dictated by integrins. Raft located Rac1 can then bind its effectors and trigger downstream signaling (Echarri et al. 2007; Del Pozo & Schwartz 2007). Rac1 activation can be stimulated not only by growth factors but also exposure to heat, hypoxia and arsenite (Park et al. 2005). As Rac1 activation is coupled with its translocation to PM, Rac1 can be one of the major stress sensing elements of PM structures.

As discussed before exposure of cells to hyperthermia stress disturbs the pre-existing physical state and architecture of membranes: it generates membrane hyperfluidization and rearranges micro-domains structures (Gombos et al. 2011). Besides their roles in the structural organization of PMs, different membrane lipids can be metabolized and give rise to signaling molecules in response to stimuli and thus alter the gene expression of HSPs (Vigh et al. 2007; Balogh et al. 2013; Török et al. 2014). According to lipid analysis results, heat stress stimulates the accumulation of Chol and Cer levels while SM levels are slightly reduced. The level of AA, a known potential HSP modulator, is elevated under heat shock conditions (Balogh et al. 2010). Various membrane fluidizers or compounds which can interact with certain membrane lipids, have the ability to modulate the HSP expression without creating any proteotoxic effect. Benzyl alcohol (BA), a known membrane fluidizer weakens the van

der Waals interactions between the lipid acyl chains (Török et al. 2014) and generates such alterations in Chol, Cer and AA levels that show similar trends to the effect of heat shock (Balogh et al. 2010). Microscopy experiments revealed that BA treatment results in a characteristic rearrangement of the Chol-rich micro-domains which can be also observed after heat shock treatment. Membrane perturbing effect of BA is coupled with altered HSP levels as BA can induce HSP expression in a dose dependent manner and in a HSF1 dependent way (Nagy et al. 2007; Balogh et al. 2013). Another membrane interacting compounds, the non-proteotoxic hydroximic acid (HA) derivatives are intercalating in PM lipid raft. Among them BGP15 is a well-established HSP co-inducer which amplifies the expression of HSPs induced by mild heat or pathophysiological stresses rather than showing direct inducing effect on HSP response. As a multi target drug BGP15 has proven clinical effects in animal models of muscular dystrophy, atrial fibrillation, and type-2 diabetes (Gombos et al. 2011; Crul et al. 2013; Török et al. 2014). BGP15 fluidizes but also stabilizes the membranes and remodels their lipid rafts (Crul et al. 2013). Although the precise molecular mode of action of BGP15 is not yet completely clear, it has recently been shown that BGP15 is able to increase the stability of Chol/SM complexes in an *in vitro* monolayer experiments. It has been shown in B16F10 cells that BGP15 can preserve the integrity of these rafts when challenged by thermal stress (Gombos et al. 2011). According to HSF1 acetylation analysis on human embryonic kidney (HEK293) cells, BGP15 pretreatment prior to heat shock can attenuate HSF1-acetylation leading to prolonged activation of HSF1 therefore enhanced HSP expression (Gombos et al. 2011; Crul et al. 2013; Török et al. 2014).

Membrane perturbations by the physical effects of environmental stresses, such as oxidative stress and mild heat stress are associated with altered permeability, membrane protein rearrangement, alteration in transmembrane potential, formation of lipid peroxides together with lipid compositional changes (Kültz 2005). Membrane thermosensor model emphasizes that subtle changes in the lipid phase of surface membranes caused by even mild heat stress can initiate stress signals which recruit lipases, receptors, receptor-like molecules, PI3K and other signal transducing proteins such as Rac1 to the PM and alter eventually HSP expression (Vigh et al. 2005).

During the stress, clustering of nonspecific growth factor receptors stimulates PI3K. The rapid induction of PI3K after the onset of stress catalyzes the conversion of PIP_2 to PIP_3. The generation of PIP_3 activates the small GTP-binding protein Rac1, which, in turn, stimulates NADPH oxidase. NADPH oxidase produces hydrogen peroxide (H_2O_2) (Figure

11). Stress stimulated H_2O_2 generation can be also originated from lipid peroxidation which products induce multiple signaling pathways, including MAPK pathways. During stress integral membrane proteins liberate active signaling molecules. For example, phospholipase A_2 (PLA_2) activity depends on membrane integrity and is elevated during stress. PLA_2 catalyzes the hydrolysis of membrane glycerophospholipids, resulting in release of AA (Figure 11), an important signaling molecule in cells (Balogh et al. 2010; Head et al. 2014; Török et al. 2014). Another change in membrane permeability and the activity of ion channels during stress evoke Ca^{2+} influx into the cytosol and accumulation of Ca^{2+} induces HSP synthesis in mammalian and plant cells (Balogh et al. 2005; Saidi et al. 2010). A Ca^{2+}-transporting ATPase may be required to restore cytosolic calcium levels after the initial stress signal has been perceived (Figure 11) (Kültz 2005).

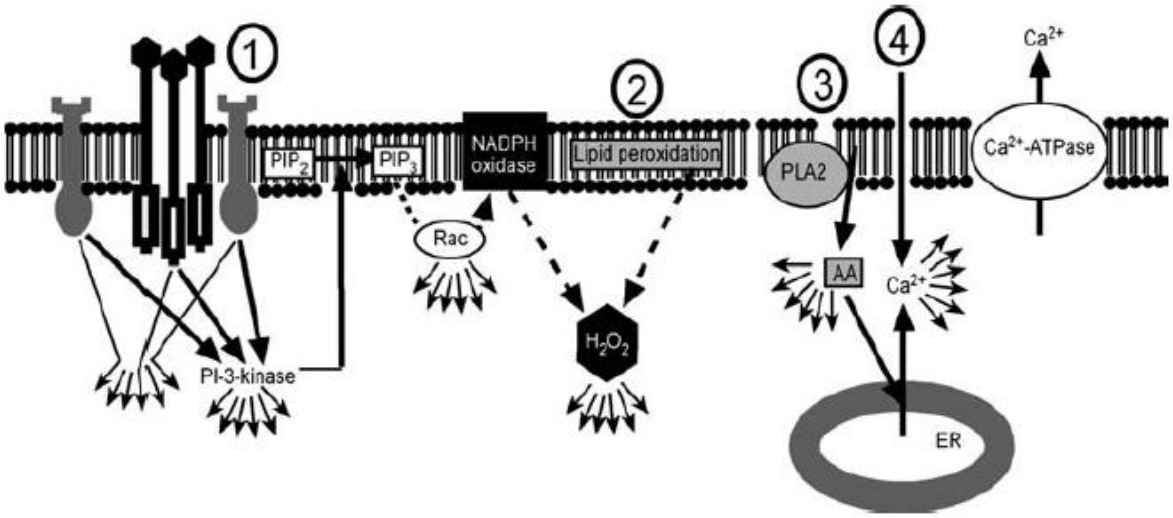


Figure 11. Potential stress sensing mechanisms that are based on lipid membrane perturbation/ rearrangements. (1) Membrane rearrangements stimulate nonspecific clustering of growth factor and cytokine receptors. (2) receptor activation caused NADPH oxidase activation (1) and lipid auto-oxidation generate H_2O_2 that serves second messenger. (3) Alterations in membrane tension or lipid rearrangement result in PLA_2 activation liberates AA from membranes. (4) Changes in membrane permeability induce calcium influx into the cytosol. Multiple arrows from several elements illustrate possibilities for further signaling cascades (Kültz 2005).

Although these mechanisms have been still under investigations in order to gain a universal applicability to a broad spectrum of cells and stresses, they represent potential sensing and signaling pathways for membrane lipid perturbation/ rearrangements.

In this thesis we focus on the Rac1 signaling pathway which is believed to be one of the key elements of HSR signaling. Under the following title we give more information about Rac1 structure, functions and more importantly its role upon heat stress conditions.

1.6.2. Small GTPase Rac1 and its role in heat stress signaling

Rac1 was firstly identified as Ras-related C3 botulinum toxin substrate. It is a ~21 kDa protein in a subfamily of the Rho family of GTPases (Didsbury et al. 1989). Members of this family in humans are divided into 6 classes: Rho (RhoA, RhoB and RhoC), Rac (Rac1, Rac2, Rac3 and RhoG), Cdc42 (Cdc42, Tc10, TCL, Chp/Wrch-2 and Wrch-1), RhoBTB, Rnd and Miro proteins (Figure 12). The most studied members are RhoA, Rac1 and Cdc42. Rac1 share 83% identity with Rac2 and 77% identity with Rac3 in primary sequence and all three isoforms have a high homology of ~89% at the amino acid level. Rac1 and Rac3 proteins are widely expressed in different tissues whereas Rac2 is observed only in hematopoietic cells (Wennerberg & Der 2004; Pai et al. 2010; Bustelo et al. 2012; Wertheimer et al. 2012). Rac1b, an alternative splice form of Rac1, has been previously shown to be upregulated in colon and breast cancer cells. It possesses an increased intrinsic GTP loading rate and exhibits impaired GTPase activity, thus it has an enhanced association with the PM (Wennerberg & Der 2004).

Cells have evolved a series of regulatory factors responsible for controlling signaling events mediated by the Rho GTPases. Guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) catalyze activation via GDP/GTP exchange and inactivation via GTP hydrolysis, respectively (Bishop & Hall 2000; Henneman et al. 2010). Guanine nucleotide dissociation inhibitors (GDIs) represent a third class of regulatory proteins that are critical to the control of signaling events mediated by the Rho GTPases. The GDIs are unique among regulatory proteins in that they exhibit multiple effects on their Rho-family substrates, controlling both the nucleotide state of the GTP-binding proteins as well as their cellular location.

Rac1 has numerous roles in normal physiology and disease state of cell functionality such as cell cycle regulation (Michaelson et al. 2008), lamellipodia formation, membrane ruffling (Steffen et al. 2013), regulation of NADPH oxidase activity in NADPH complex (Flinder et al. 2011), cellular adhesion (Lawson & Burridge 2014), proliferation (Woodcock et al. 2010), survival, differentiation and malignant transformation (Wertheimer et al. 2012),

cell migration, actin polymerization, spreading (Kovac et al. 2013). Rac1 is also important in various oncogenesis pathways including initiation, progression, invasion and metastasis (Davis et al. 2013) and stress sensing (Han et al. 2001).

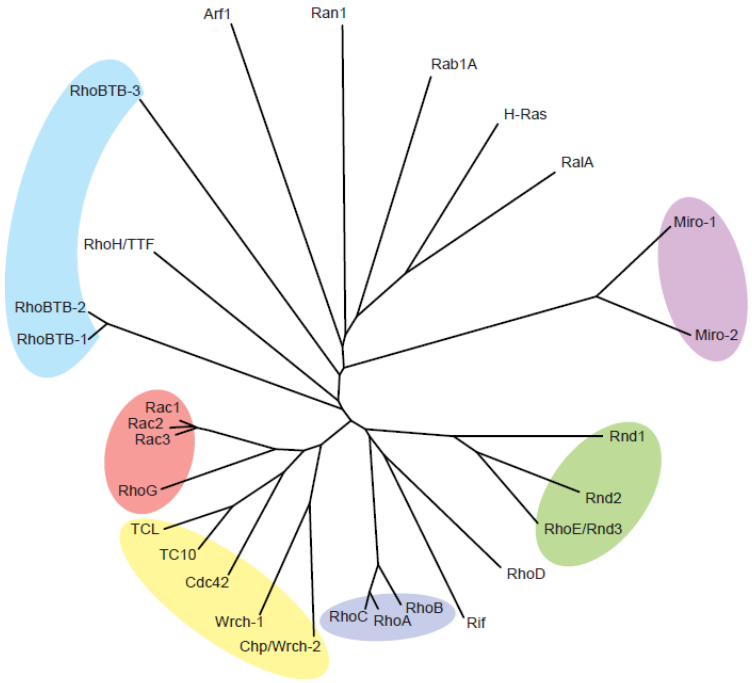


Figure 12. Phylogenetic tree of the Rho family GTPases and representatives of other Ras-superfamily GTPases. The Rho family can be roughly classified into six major branches: RhoA-related, Rac-related, Cdc42- related, Rnd proteins, RhoBTB proteins and Miro proteins (Wennerberg & Der 2004).

Rac1 protein possesses three functional regions that include Switch I, Switch II and the Insert region (Figure 13). Switch regions are responsible for the molecular interactions of Rac1, except those that deal with membrane interactions. Switch I primarily interacts with downstream effectors and proteins in the NADPH complex. Therefore it is called as "effector region". Switch II interacts with guanine nucleotide exchange factors (GEFs). The Switch II region is the site where Rac1 becomes activated in its GTP-bound state (Krauthammer et al. 2012; Kumar et al. 2013). The Insert Region (12 amino acids, not present in Ras) exists only in the Rho subfamily of GTPases and therefore is an identification element of Rho GTPases. The Insert region is essential for mitogenesis and apoptosis. It also has a significant role in the regulation of interactions with its downstream effectors, specifically in the NADPH complex (Kumar et al. 2013).

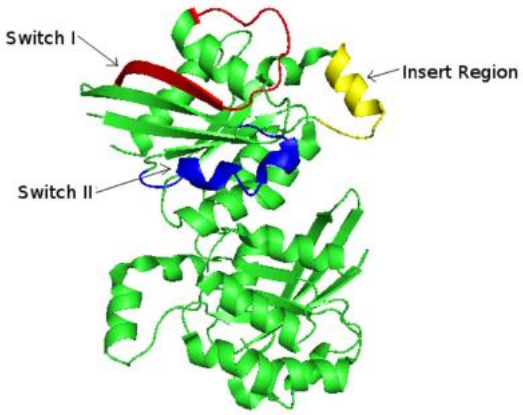


Figure 13. Structure of Rac1. Switch I, Switch II and Insert region of Rac1 can be followed with the color codes as Switch I is red, Switch II is blue and Insert region is yellow (Kumar et al. 2013).

The C-terminus of Rac1 participates in the binding to the membranes. Rac1 undergoes posttranslational modifications such as prenylation and reversible palmitoylation in the C-terminal CAAX (where C represents cysteine, A is an aliphatic amino acid, and X is a terminal amino acid) and polybasic region which is upstream of CAAX tetrapeptide motif. These posttranslational modifications direct Rac1 to PM association for further signaling events (Grizot et al. 2001; Roberts et al. 2008; Navarro-Lérida et al. 2012).

Like other small GTPases, Rac1 acts like molecular switch by cycling between an inactive GDP bound state and an active GTP bound state. GTP bound, active form of Rac1 typically induced by GEFs that are activated by receptor dependent kinases. GAPs remove the γ-phosphate and return the active GTPase to a GDP bound form of the protein which turns Rac1 inactive (Pai et al. 2010). Rac1 has similar affinities to both nucleotides and GEF binding doesn't effect on choosing one to another. The increase in GTP-bound over GDP-bound form in Rac is rather due to the higher cellular concentrations of GTP relative to GDP. Trio, GEF-H1, and Tiam1 are a subset of the Dbl family of GEFs that are responsible for catalyzing the GDP–GTP exchange reaction specifically of Rac1 but not the closely related Cdc42. Rac1 GEFs have recently been reported to have important roles in cancer (Wertheimer et al. 2012; Davis et al. 2013). It has been shown that Trp56, which is present between the two switch regions, is the necessary and critical amino acid of Rac1; hence targeting the Trp56 site by an interfering compound can affect the Rac1 activity (Gao et al. 2001).

In resting cells Rho GTPases are located in the cytosol and are associated with a Rho-GDI. Rho-GDI interacts with Switch I and Switch II regions but not with insert region of

Rac1 (Grizot et al. 2001). In the Rac1-Rho-GDI structure, Rac1 is in a GDP conformation. Rho-GDI binds to Rac1 of 1:1 ratio together with one Mg^{2+} (Kumar et al. 2013). Rho-GDI shields the geranyl-geranyl moiety of Rac1 and keeps Rac1 soluble in cytosol. Palmitoylation of Rho GTPases is reported to block Rho-GDI binding (Michaelson et al. 2001). The multiple regulatory factors of Rac1 such as GAPs, GDIs, and GEFs have been shown to undergo overexpression, downregulation, or mutation in different types of cancer (Ellenbroek & Collard 2007). As one can imagine, once an upstream signal is changed, the activity of its targets downstream, i.e. the Rho proteins, will change in activity.

First generation small-molecule NSC23766 (NSC) (Figure 14) is a highly water soluble and cell permeable inhibitor of Rac1 (Wertheimer et al. 2012), which fits into the surface groove, centering on Trp56 of Rac1. According to the suggested docking model of NSC by Gao and co-workers (Gao et al. 2004), the binding site for NSC in Rac1 is a surface cleft formed by the residues Lys5, Val7, Asp38, Asn39, Trp56, Asp57, Thr58, Leu70, and Ser71. The nitrogen atoms of NSC form three hydrogen bonds with residues from the backbone of Rac1, and the stacking effect of the middle pyrimidine ring of NSC with the indole ring of Trp56 governs the binding specificity of NSC. It is possible that, NSC not only interferes with the interaction between the insert region of Rac1 and Tiam1 but it also inhibits the interaction of the switch regions with Tiam1. Therefore NSC is able to efficiently inhibit the activation of Rac1 by Tiam1. Gao and coworkers reported that NSC inhibits Rac1 in a dose dependent manner and is ineffective on Cdc42 or RhoA. Furthermore, inhibition by NSC does not change the p21-activated kinase (Pak) binding activity (Gao et al. 2004).

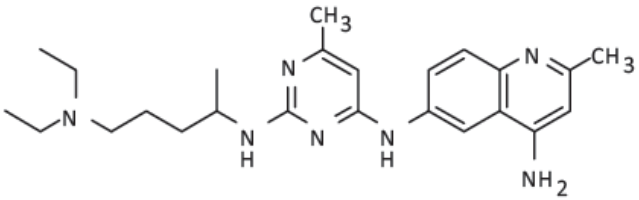


Figure 14. Structure of NSC23766 (Hernandez et al. 2010).

Besides the above cited work of Gao et al, subsequent *in vivo* studies where Rac1 activity was inhibited by NSC have emphasized the role of Rac1 in various cellular functions, such as growth factor mediated cell migration, actin cytoskeletal regulation, formation of

focal complex structures and lamellipodia/membrane ruffles, cell cycle, cell signaling as a response to an external stimuli including UV irradiation, cancer progression, metastasis, and tumorigenesis in several cell lines such as breast cancer types, osteosarcoma, medulloblastoma and lung adenocarcinoma (Mukherjee et al. 2009; Dokmanovic et al. 2009; Yuan et al. 2010; Akunuru et al. 2011; Yan et al. 2012).

A key basis for the functional diversity of Rho GTPases is their association with distinct subcellular compartments, which is dictated in part by three posttranslational modifications signaled by their carboxyl-terminal tetrapeptide motifs. CAAX motifs are substrates for the prenyltransferase-catalyzed addition of either farnesyl or geranyl-geranyl iso-prenoid lipids (Roberts et al. 2008). In Rac1, the CAAX motif is modified with geranyl-geranylation via a thioether, catalyzed by a geranyl-geranyl transferase. The last three amino acids are then released, and the C-terminal cysteine residue is carboxy methylated (Grizot et al. 2001). Upstream of CAAX motif Rac1 has a polybasic region (KKRKRK) with a net charge of +6 and the cysteine residue which undergoes palmitoylation, resides upstream of KKRKRK sequence. Palmitoylation occurs by covalent linking of the 16-carbon fatty acid palmitate, mostly via a thioester bond. Palmitoylation of Rac1 requires its prior prenylation and the intact C-terminal polybasic region and is regulated by the triproline-rich motif. Non-palmitoylated Rac1 shows decreased GTP loading and lower association with detergent-resistant (liquid-ordered) membranes. Therefore, palmitoylation considered to be the key signal which leads Rac1 to lipid rafts within PM (Figure 15) (Navarro-Lérida et al. 2012; Tsai & Philips 2012). Interestingly, unlike the two other types of lipid modification that also tether proteins to cytosolic membrane surfaces, namely prenylation and myristoylation, which remain attached to the protein throughout its lifetime, a distinguishing feature of palmitoylation is its reversibility, thus one aspect of palmitoylation is the dynamic regulation of membrane association of proteins through the regulated addition and removal of palmitoyl modifications.

Localization of Rac1 to Lo domains leads to raft coalescence and initiates F-actin polymerization via interacting with Arp2/3 (Arp2/3 complex plays a major role in the regulation of the actin cytoskeleton). Initiation of Rac1-mediated actin polymerization recruits coronin 1A/ArhGEF7 complex which associates with Pak1 protein, a serine/threonine p21-activated kinase which serves as target for Rac1 and has been implicated in a wide range of biological activities such as lamellipodia formation, actin polymerization and stress signaling (Bishop & Hall 2000; Han et al. 2001). This association then further interacts with Rho-GDI

bound Rac1, representing a positive feedback loop (Castro-Castro et al. 2011; Symons 2011). Recently, Navarro et al proposed that Rac1 can act as a membrane organizer through a mechanism dependent on the actin cytoskeleton (Navarro-Lérida et al. 2012; Wertheimer et al. 2012).

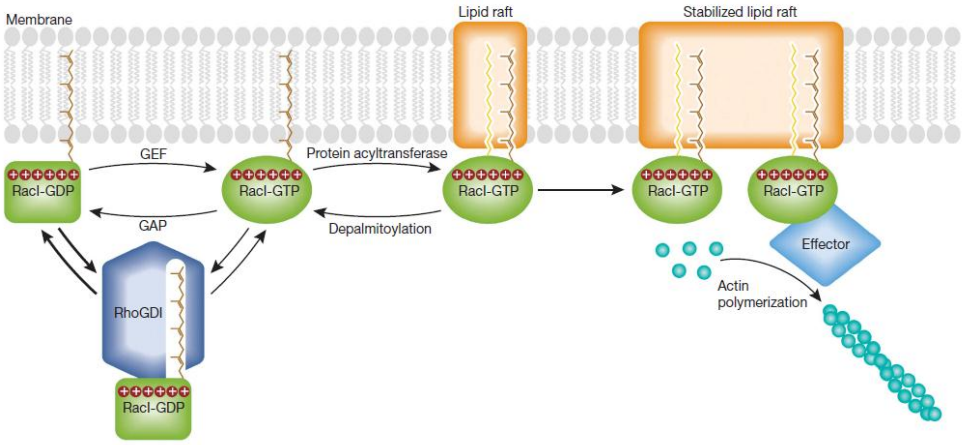


Figure 15. The Rac1 acylation cycle. Rac1 associates to the PM by its geranyl-geranyl modification (shown in brown) and its +6 polybasic region (shown in red). When Rac1 is released from Rho-GDI, it is activated by a GEF and undergoes palmitoylation (palmitate is shown in yellow). Palmitoylated Rac1 segregates to cholesterol-rich, L_o domains (lipid rafts) in the PM and stabilizes these micro-domains in a process associated with actin polymerization (Tsai & Philips 2012).

One of the attractive properties of Rac1 is that it is stimulated under fever range mild temperatures. This acts in a similar fashion to growth factor stimulated conditions where stress doesn't generate proteotoxicity on cells and cells can receive this external signal as stimulation for its favorable growth adaptation. Rac1 has been reported to be affecting HSF1 activation therefore HSP expression (Ozaki et al. 2000; Han et al. 2001). Accordingly stimulations by mild heat, hypoxia/reoxygenation and sodium arsenite treatments on HSF1 activities can be inhibited by dominant negative Rac1, Rac1N17 (where 17^{th} amino acid serine is substituted to asparagine). Therefore, decreased levels of HSP70 are attained while upon severe heat exposure, Rac1N17 is causing no inhibition in HSF1 activity. However, constitutively active Rac1V12 (where 12^{th} amino acid glycine is changed to valine), does not induce HSF1 activation (Han et al. 2001; Park et al. 2005) suggesting that Rac1 may be necessary but insufficient for HSF1 activation. These results also suggest that Rac1 signaling pathway may specifically respond to newly synthetized, misfolded nascent polypeptide under

mild stress conditions, but not of formerly folded aggregated proteins to activate HSF1 (Park et al. 2005).

As mentioned previously in section 1.6.1. Micro-domain organization of membranes and membrane initiated stress signaling,, there are several different signaling platforms originating from PM to sense the stress and activate their downstream cascades. Signaling elements fulfill their tasks most likely with collaborations and crosstalk between them.

In favor of "membrane stress sensor" model, our working hypothesis is that Rac1 pathway is involved in stress signaling through the effect of heat stress on membrane micro-domain organization (Vigh et al. 2005) (Figure 16).

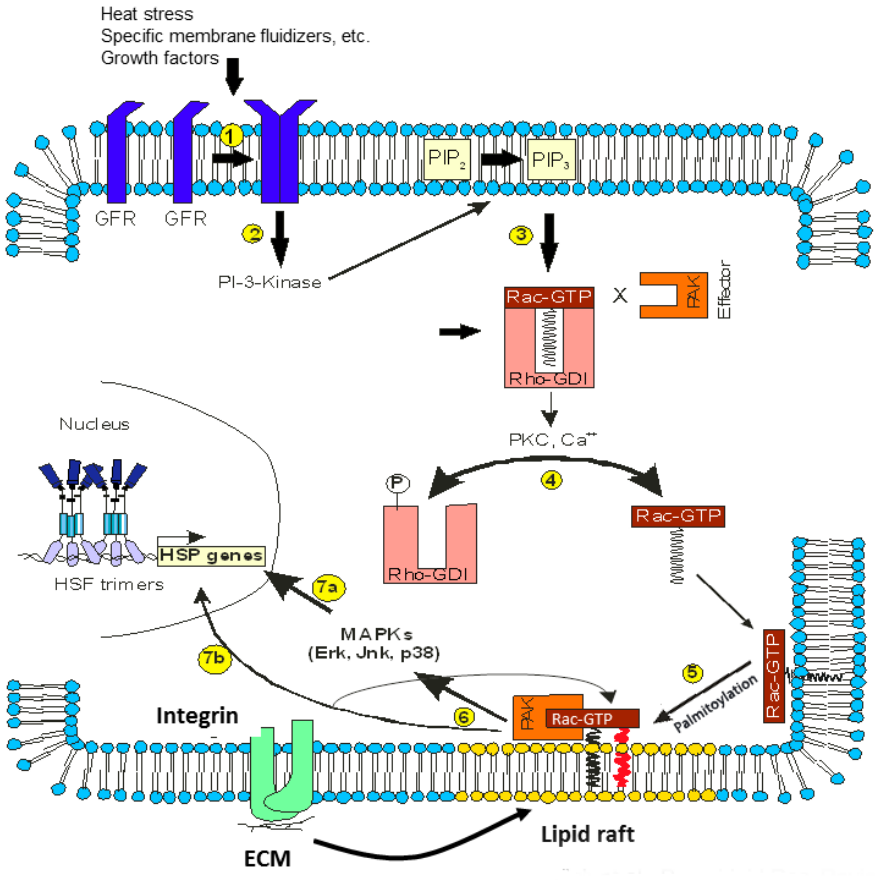


Figure 16. Scheme of working hypothesis for the cascade of possible heat-stress signal generation and transduction events linking PM to hsp during HSP response induced by mild heat stress. (for details, see the text) (Modified figure of Vigh et al 2005).

We assumed that membrane rearrangement and/or membrane hyperfluidization by mild heat stress may activate growth factor receptor tyrosine kinases by causing their non-specific clustering (1). Activation of such cell surface receptors has other potential

consequences including the activation of PI3K (2), which in turn activates the Rac1 (3). In the absence of stress a Rho-GDI protein keeps Rac1 solubilized by shielding its geranyl-geranyl group from the solvent in the cytosol. Ca^{2+} and protein kinase C (PKC)-dependent phosphorylation of Rho-GDI promote the release of bound Rac1 (4) and Rac1 undergoes reversible palmitoylation, then subsequent translocation to the Chol rich domain of PM (5). Membrane localization of Rac1 is required for activation of its effector, Pak, which mediates also a down-stream signaling cascade to MAPKs such as p38MAPK (6), affecting HSP expression (7/a) (Vigh et al. 2005). Translocation of Rac1 to the Chol-SM enriched PM domains through its palmitoylated feature (see Figure 15) is essential for activating downstream effectors and requires integrin-mediated adhesion of cells to ECM (del Pozo et al. 2004; Navarro-Lérida et al. 2012; Tsai & Philips 2012). Activation of Pak may also lead to a direct activation of HSF1 (7/b) (Vigh et al. 2005) (Figure 16).

2. THE AIMS OF THE THESIS

The main goal of this thesis is to better understand the changes within the cell under heat shock conditions and to explore the participation of Rac1 in stress signaling. Specifically our studies are aimed at gaining insights into functions of the PM localization of Rac1 through its palmitoyl moieties under heat stress conditions and also heat shock induced alterations starting on membrane structures and resulting in HSP expressions and cytoskeletal changes. Therefore, we have used 2 different inhibitors targeting Rac1; a general palmitoylation inhibiting compound 2-bromopalmitate and Rac1 specific inhibitor NSC in order to check if the inhibition of activity and/or localization of Rac1 has any consequences on any possible downstream pathways leading to HSP expression.

Thus, the aim of the present work was:

- to test the Rac1 PM membrane localization upon heat stress and monitor the PM micro-domain alterations in connection with Rac1 localization and activity.
- to demonstrate the effect of Rac1 on cytoskeletal changes upon heat stress.
- to figure out the involvement of Rac1 in HSP expression and,
- to check whether Rac1 contributes to the pathway(s) of HSP induction by membrane interacting HSP co-inducer drug candidates.

3. MATERIALS AND METHODS

3.1. Cell culturing and transfections

B16F10 mouse melanoma cells (CRL-6475) were purchased from ATCC and were cultured in RPMI 1640 medium supplemented with 10% FCS and 4 mM L-glutamine. Mouse embryonic fibroblasts (MEFs) (a kind gift of Prof. Lea Sistonen) and NIH3T3 cells (ATCC, CRL-1658) were grown in DMEM containing 10 % FCS and 4 mM L-glutamine. Cells were maintained at 37 °C in a humidified 5 % CO2-atmosphere.

We used expression vectors encoding wild type and mutant forms of Rac1; pcDNA3GfpRac1WT, pcDNA3GfpRac1V12 and pcDNA3GfpRac1N17 which were kind gifts of the group of Gauthier-Rouviere (CNRS, France). The plasmids were transiently transfected with Turbofect transfection reagent (Thermo Scientific) according to manufacturer instructions for visualization of the localization of Rac1 proteins in B16F10 and other fibroblast cell cultures.

3.2.Treatments

For heat shock treatments, cells were plated with $2.5x10^4/cm^2$ density in complete medium. After 24 hours the plates were sealed with parafilm and immersed in a water bath (with a precision of ±0.1 ºC) set to the indicated temperature for 1 h. Samples were prepared right after heat shock treatments in order to check the localizations of Rac1 and F-actin proteins, mRNA levels of *hsp25* and *hsp70* genes, membrane Chol-SM containing micro-domain alterations and HSF1 and p38MAPK phosphorylation levels.

Inhibitors were administrated in concentrations and at time points as follows; 100µM of Rac1 specific inhibitor NSC (Santa Cruz Biotechnology, USA) (dissolved in water) for 2 h, 25 µM of palmitoylation inhibiting compound 2-Bromopalmitate (2-Brp) (Sigma Aldrich) (dissolved in dimethyl sulfoxide (DMSO)) for 30 min and 10 µM of HSP co-inducer BGP15 (N-Gene) right before heat shock treatments at 41.5 (mild) and 43 ºC (severe). HSPs at protein level were investigated by western blotting after an overnight recovery of treated cells in fresh complete medium without any added, compound.

When serum starvation was applied, cells plated with $2.5x10^4/cm^2$ density were grown in complete medium for 24 hours. Then medium was removed and plates washed three times

with phosphate buffered saline (PBS) thereafter the cells were incubated in serum free medium for 24 h. Serum re-addition was performed by exchanging serum free medium for serum containing medium.

3.3. Crude membrane isolation

Crude membrane was isolated from B16F10 cells right after 1h heat shock exposure to investigate Rac1 protein localization to the total membrane fraction (which encompass PM and all the organelle membranes) at indicated temperatures. For this, $2x10^7$cells were used for each sample preparation. Cells, heat shocked at indicated temperatures were washed twice with ice cold PBS, harvested by scraping on ice and re-suspended in 1 ml of ice cold SCA Buffer (20 mM HEPES-KOH pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose and protease inhibitor cocktail (Sigma)). After 30 min incubation on ice, cells were lysed by 30-40 strokes in Dounce homogenizer and centrifuged at 750 g for 5 min. Supernatant was transferred into another tube and centrifuged again at 750 g for 5 min. Pellet was discarded and supernatant was supplemented to 12 ml with SCA buffer and further centrifuged at 37000 rpm by using SW41 rotor for 1h at 4 ºC. The pellet was mixed with 1:3 volumes of 3X Laemmli Sample Buffer and after protein determination by Pierce 660 nm Protein Assay (Thermo Scientific). Equal amounts of protein samples were subjected to western blot analysis which is explained in detail in section 3.10 Western Blot Analysis.

3.4. Plasma membrane isolation

B16F10 cells were used for plasma membrane (PM) isolation. This isolation method was carried out as described in Maeda et al. (1980). Briefly, 5 x 10 cm plates were seeded with $2x10^6$ (which is equivalent to $2.5x\ 10^4/cm^2$) cells and grown in complete medium for 24 hours when the cell number reached approx. $8x10^6$/plate. Then cells were kept in serum free media for 24 h. After serum administrations for the indicated time intervals, cells were washed 3 times with ice cold PBS, scrapped and collected in 1 ml ice cold TNM buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 7.4) and kept on ice for 20 min. After that, cells were homogenized by passing through 30 times a 26 G bore size needle attached to 5 ml syringe. Lysates were spun down at 3000 rpm, 4 ºC for 5 min. Supernatant was collected and

centrifuged at 8800 rpm, 4 ºC for 15 min in F28/13 rotor in a Sorwall super-speed centrifuge. 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 ºC for 3 h in SW41 rotor. Interphase of each sample was collected into TNM buffer and centrifuged again at 27000 rpm, 4 ºC for 1 h in SW41 rotor. Supernatant was removed and the pellet (PM fraction) was dissolved in 2 X Laemmli Buffer (130 mM Tris-HCl pH 6.8, 10 % β-mercaptoethanol, 4 % SDS, 20 % glycerin, 0.01 % bromophenol blue). Equal amounts of protein samples were run on SDS-PAGE and subjected to western blot analysis which is explained in detail in section 3.10 Western Blot Analysis.

3.5. Immune localization of Rac1 using confocal microscopy

Appropriately pretreated B16F10 cells were washed with PBS twice and fixed with 4% paraformaldehyde (PFA) (Sigma Aldrich) for 8 min at room temperature, then permeabilized with 0.1% TritonX100 (Reanal) for 15 min at room temperature. After washing with PBS, blocking was done by 1 % bovine serum albumin (BSA) solution in PBS for 30 min at room temperature. Monoclonal anti-Rac1 (Millipore) antibody (1:100) was used for labeling for 1h, followed by anti-mouse Alexa488 (A21202, Life technologies) antibody (1:300) for 1h at 37 °C. After several washing steps, images were taken with Leica SP5 AOBS confocal microscope using the 488 argon laser line for excitation and 500-530 spectral filters for emission detection. Images were analyzed with ImageJ software: whole cells and plasma membranes were drawn around on at least 15 cell-images for each treatment. The average intensity of the pixels representing the plasma membrane was divided by the average intensity of the whole cell in an equatorial focal plane. Statistical analysis was done by student's t-test calculations. Experiments were repeated 3 times; the results demonstrated the same tendency. A linear region of interest was chosen for every representative image. The fluorescence intensity of the pixels within this region was plotted.

3.6. Membrane micro-domain size analysis

B16F10 cells (seeded with $0.5x10^6$ initial cell number) were grown for 24 h in glass-bottom dishes and treated by different ways as described in section 3.2 Treatments. After treatments, cells were washed with PBS and labeled with 1 µM of BODIPY FL C12-SM (Avanti Polar Lipids) diluted in PBS for 8 min. This probe was used for labeling the cholesterol enriched raft micro-domains (Gudheti et al. 2007). After washing and taking up the cells with RPMI without phenol-red, images were taken by custom made high content fluorescent microscope applying objective-based total internal reflection (TIR) configuration using 488 nm light for excitation and 520±20nm emission filter. Images were analyzed with CellProfiler (www.CellProfiler.org) and ImageJ software (http://rsbweb.nih.gov/ij/). The size (theoretical diameter) of each single detected micro-domain was calculated as $2X * (Npix/\pi)-2$, where X is the size of a pixel in nm and Npix is the number of pixels covering the actual domain. The domains found were sorted into classes according to theoretical diameters. Analysis of the images was done on at least 35 cells for each sample. The data shown are mean values ± SEM. Each experiment was repeated 3 times. For statistical analysis of the domain size distributions Kolmogorov-Smirnov test was performed to verify the equality of distributions.

3.7. Determination of morphological changes by Scanning Electron Microscopy

B16F10 cells were maintained on sterile 5 mm cover slips placed in 24 well plates. After appropriate treatments, cells on cover slips were washed with PBS and fixed with 2.5 % glutaraldehyde (Sigma) solution containing 4.5 % glucose buffered with 75 mM sodium cacodylate buffer (Sigma) (pH7.2) for 3 h at room temperature followed by 3 washes with 100 mM cacodylate buffer. Secondary fixation was done by addition of 1% osmium tetroxide (Sigma) buffered with 50 mM cacodylate buffer for 3 h at room temperature. Subsequently, the cells on cover slips were washed with distilled water, dehydrated in an ethanol series (25 %, 50 %, 75 %, 90 % and 100 %) then immersed in a 1:1 mixture of ethanol/acetone and then kept in pure acetone. Finally, critical point drying was performed with CO_2 in E 3000 critical point drying apparatus (Quorum Technologies). The specimens were mounted on adhesive carbon discs, sputter coated with gold in a SC7620 Sputter Coater (Quorum Technologies) and images were taken with an EVO40 scanning electron microscope (Carl Zeiss GmbH) at

20.0 kV (at the Institute of Material and Environmental Chemistry, Research Center for Natural Sciences, Budapest, by the help of László Szabó). Quantitation of images was done by using ImageJ software. Cell borders were drawn and pixel numbers within the borders were obtained and converted to μm^2 units. For each sample, 30 cells were measured. Statistical analysis was performed by Student's t-test. A level of $p < 0.05$ was considered to be significant.

3.8. Determination of F-actin alterations in MEF cells by confocal microscopy

MEF cells were grown on glass bottom plates. When cells on the plate reached 50-60 % confluency they were exposed to either NSC or 2-Brp pretreatments for 2 h or 30 min, respectively as indicated in 3.2 "Treatments" section and then cells were subjected/or not to mild (41.5 °C) and/or severe (43 °C) heat shock for 1 h. After treatments cells were washed with PBS and then fixed with 4% PFA and labeled by using 200 nM of Alexa 647-conjugated Phalloidin (A22287, Life technologies) for 30 min. DAPI (Life technologies) staining (100 µg/ml) was followed by washing steps for 5 min and then Fluoromount-G (SouthernBiotech) was added and coverslips were mounted onto plates. Confocal laser scanning microscopy was performed using a Leica SP5 AOBS confocal laser scanning microscope (Leica, Germany). Microscope configuration was the following: objective lenses: HC PL APO 20x (NA:0.7); HCX PL APO lambda blue 63x OIL (N.A: 1.40); sampling speed: 200-400Hz; line averaging: 1-2x; pinhole: 1 airy unit; scanning mode: sequential unidirectional; excitation: 405nm (DAPI), 633nm (Alexa 647-phalloidin); spectral emission detectors: 417-525 nm (DAPI), 643-746nm (Alexa 647-phalloidin). DAPI and Alexa 647-phalloidin images were pseudo colored blue and red, respectively. Experiments were done 3 times.

3.9. Determination of mRNA levels of inducible *hsp25* and *hsp70* genes

Right after heat shock or compound administrations, B16F10 cells were washed with PBS and harvested in lysis buffer for total RNA isolation which was performed by using NucleoSpin RNA II kit (Macherey-Nagel). 1 µg of RNA was reverse-transcribed through use of the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas). *hsp70*

(Mm01159846_s1), hsp25 (Mm00834384_g1) and *gapdh (Mm99999915_g1)* primers with TaqMan probes (Applied Biosystems) and TaqMan Universal PCR Master Mix (Applied Biosystems) were used to prepare the reaction mixtures according to manufacturer instructions. Reaction steps: 50 ºC 2 min, 95 ºC 10 min and 40 cycles of 95 ºC 15 sec, 60 ºC 1 min. The PCR runs were performed in a BioRad-CFX96 instrument and BioRad-CFX Manager 2.1 software was used for quantitation. ΔΔ Ct method was performed to calculate the results obtained by qRT-PCR. Relative quantities of mRNAs were normalized to *gapdh*. Reported data are the means of the results of three independent experiments.

3.10. Western blot analysis

To analyze the expression levels of HSP25, HSP70, HSP60, HSP90 and HSP110 stress proteins cells were heat shocked at designated temperatures and harvested after an overnight recovery at 37 °C. For the determination of changes in Rac1 levels, phosphorylation rate of HSF1 and p38MAPK phosphorylation cells were collected instantly after heat shock or other treatment. After indicated treatments cells were collected and lysed by mixing for 1h in Laemmli sample buffer. Total amount of protein was measured by using Pierce 660 nm Protein Assay (Thermo Scientific). After incubation in 100°C water bath for 5 min, samples were spin for 1 min at 14000 rpm (Eppendorf table top centrifuge) and then equal amounts of proteins (20µg) were loaded on 15% or 10% SDS polyacrylamide gels (depending on the size of the protein of interest). . After separating the proteins, they were transferred onto PVDF membranes (0.45 µm, Thermo Scientific). The membrane was probed with monoclonal (mAb) anti-Rac1 (Millipore) (1:1000), anti-Flotillin (F1180, Sigma Aldrich) (1:1000), anti-Caveolin-1 (Sigma Aldrich) (1:1000), anti-HSP25 (SPA 801, Stressgen) (1:1000), anti-HSP70 (SPA 810, Stressgen) (1:2000), anti-HSP60 (SPA 806, Stressgen) (1:10000), anti-HSP90 (ab13492, Abcam) (1:1000), anti-HSP110 (610510, BD Transduction Laboratories) (1:1000), anti-phospho-p38MAPK (Thr80/Tyr182) (Cell Signaling Technology) (1:1000), monoclonal anti-HSF1 (Clones 4B4+10H4+10H8, Thermo Scientific) (1:1000) and anti-Gapdh (Sigma-Aldrich) (1:20000) antibodies as indicated in the figures legends. Rac1 PM association was normalized by using anti-Flotillin antibody on the same membrane. Normalization of Rac1-crude membrane localization was assayed by using anti-Caveolin re-probing on the same membrane. HSP25, HSP70, HSP60, HSP90, HSP110, HSF1 and p-p38MAPK normalizations were done against Gapdh levels of the same membranes by re-probing with anti-Gapdh

antibody. Expression levels were visualized with Enhanced Chemiluminescence (Amersham, USA). Band intensities were measured with Alpha View Software v.1.3.0.7. (Alpha Innotech, USA). Reported data are the means of the results of three independent experiments.

3.11. F-actin determination by Flow Cytometric Analysis

Following heat treatments B16F10 cells were immediately washed with PBS, collected by trypsin treatment, then fixed with 4% PFA and permeabilized with 0.1% TritonX100. Samples were labeled by Alexa Flour 647 Phalloidin (200nM) for 30 min at room temperature. Measurements were performed with BD Accuri C 6 flow cytometer (BD Bioscineces) with 648 laser line for excitation and FL4 channel for detection. Gating was done based on FSC/SSC and FL4A signals. Quantification of the data was analyzed by the CFLow Plus 1.0.227.2. Three independent experiments were performed.

4. RESULTS AND DISCUSSIONS

4.1. Rac1 is a membrane-binding protein

Heat shock induces heat shock protein (HSP) expression through activation of the heat shock factors (HSFs), but the multiply signaling pathways generated by temperature stress are not clearly understood. One of the stress stimulated signaling protein, the small GTPase Rac1, has a well-established role in the production of reactive oxygen species (ROS) (Ozaki et al. 2000; Park et al. 2005). Rac proteins are essential for the assembly of the plasma membrane NADPH oxidase that is responsible for the transfer of electrons to molecular oxygen leading to the production of superoxide anions. Rac1 regulates cell growth, migration, and cellular transformation by controlling the intracellular production of ROS. Furthermore, Rac1-regulated ROS can lead to the activation of redox-sensitive transcription factors such as heat shock factors and thereby participate in the stress-stimulated heat shock response (Ozaki et al. 2000). Rac is required for normal cell proliferation and migration and plays a key role also in cancer progression (Sahai & Marshall 2002; del Pozo et al. 2004; Papaharalambus et al. 2005; Moissoglu et al. 2006).

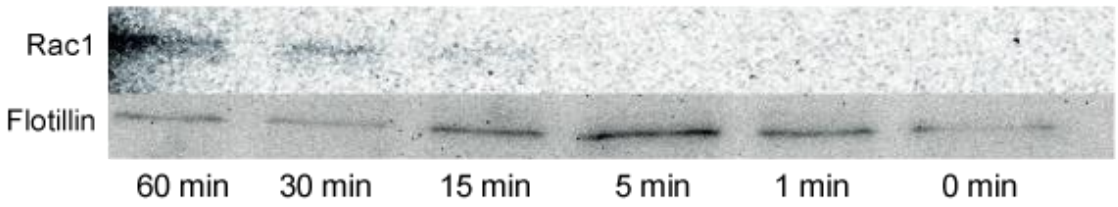


Figure 17. Rac1 PM localization after serum starvation. Serum was added for the indicated time intervals to B16F10 cells serum-starved for 24 h. Right after, plasma membrane isolation was carried out and the plasma membrane fraction was collected in Laemmli Buffer. Equal amounts of proteins were run for western blotting. Rac1 probing was performed for the membrane and flotillin was used as loading control.

In resting cells Rac1 is in inactive (GDP-bound) conformation, and resides in cytosol in complex with Rho-GDI which traps the Rac1 iso-prenyl group and GTP binding domain. Environmental stimulations can activate its target effectors and exert its biological functions; Rac1 dissociates from its Rho-GDI complex and consequently, becomes active by the contribution of GEFs and after posttranslational modification translocate to the plasma

membrane (Bustelo et al. 2012). Since cellular localization of is a key factor of Rac1 function, in our experimental cell line we first investigated serum induced plasma membrane localization of Rac1 after serum starvation conditions (Figure 17).

Upon 24 h serum starvation Rac1 was absent in the plasma membrane fraction. However, after 15 min of serum administration, PM associated Rac1 was observed in a time dependent manner (Figure 17). PM association of Rac1 initiates downstream signaling pathways such as integrin signaling for membrane ruffling and ERK signaling for focal adhesion (Zheng et al. 2010; Flinder et al. 2011). Consistent with previous studies documented for human hepatocyte cell line HepG2 (Ozaki et al. 2000) our present study confirmed that as a response of growth factor stimulation, endogenous Rac1 translocates to the PM in melanoma cells (Figure 17) and presumably induces its downstream signaling pathway which can thus stimulates heat shock factor activation and HSP70 expression. Similarly, growth factor receptor coupled, cyclic strain stress-mediated Rac1 signaling pathway has been also shown to trigger HSF1-induced HSP70 expression in smooth muscle cells (Xu et al. 2000). Moreover, growth factor stimulation of Rac1 has been reported to regulate its downstream signaling pathways similar to mild heat effect (Han et al. 2001; Park et al. 2005). Although the detailed mechanisms of HSF1 and *hsp* gene activation are not fully characterized, Rac1 PM association can mediate signaling pathways for HSP activation.

Next we wanted to study whether Rac1 localization to the membrane is in direct relationship to its activity that is whether mutations affecting its activity can determine PM localization. For these experiments we used three transiently overexpressed Gfp tagged Rac1 variants: wild type (GfpRac1WT), constitutively active (GfpRac1V12) and dominant negative (GfpRac1N17) in NIH3T3, MEF and B16F10 cell lines under normal growth conditions (Figure 18). Constitutively active Rac1 is found always GTP loaded state and dominant negative Rac1 is in the GDP bound inactive form. Both mutations have no any known effect on post translational modifications of the protein.

In the cell lines tested, all three Rac1-variants showed plasma membrane localizations in different extents although a cytoplasmic Rac1 pool was notable. In the GfpRac1V12 overexpressing cells, a more pronounced lamellipodia formation was visible and Gfp signal was accumulated more at around the plasma membrane in all cell lines tested. Similar to GfpRac1WT and GfpRac1V12 overexpressing cells, plasma membrane localization was also observable in GfpRac1N17 transfected cell lines. Interestingly, dominant negative mutation did not influence PM location. In all three transfected NIH3T3 cells, Rac1 localization was

also observed around the large vesicles with an unknown nature. Earlier, Ridley et al. (1992) reported that in Rat2 fibroblasts constitutively active Rac1 was detected not only at the plasma membrane but also in vesicles. Our data also demonstrated large vesicle accumulation (lipid droplet?) in the three different Rac1 overexpressing NIH3T3 cells obviously independently on Rac1 active or inactive form.

.

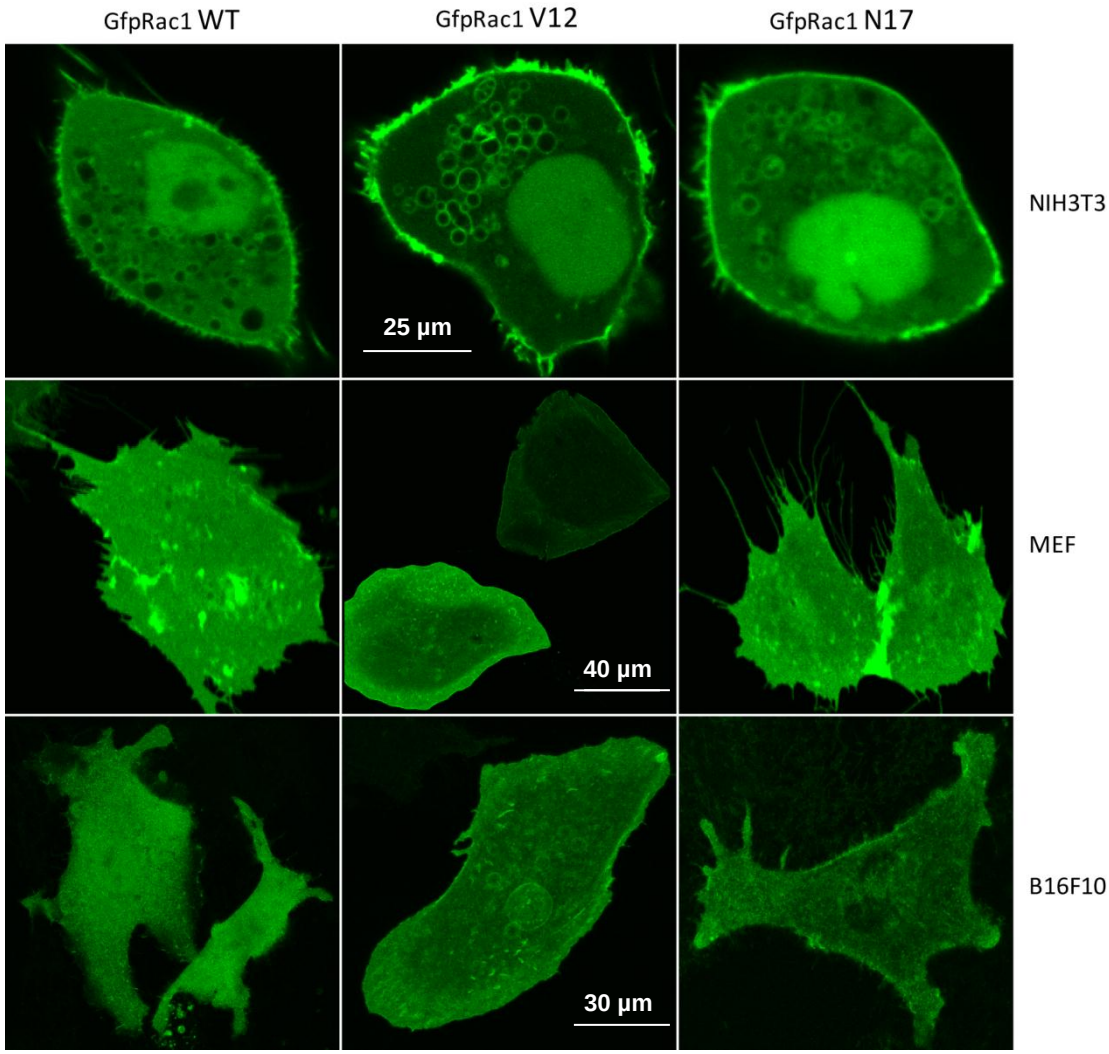


Figure 18. Localizations of Gfp tagged Rac1 protein in NIH3T3, MEF and B16F10 cell lines. Cells were transfected with Gfp tagged Rac1- WT, V12 and N17 constructs. Next day, samples were fixed and photos were taken with confocal microscope.

We expected that mainly overexpressed GfpRac1V12 is in the PM bound form. Surprisingly, GfpRac1N17 expressing cells also displayed Rac1 PM localization, showing

that association of Rac1 with the PM is not sufficient to its activity. Small GTPase Rac1 has been suggested to be regulated by GTP-GDP cycle to initiate the downstream signaling pathways (Chae et al. 2008). Accordingly, inactive form of GDP loaded Rac1 is localized in the cytosol while active, GTP bound Rac1 associates with PM (Moissoglu et al. 2006). When we transfected cells with dominant negative and constitutively active forms of Rac1 constructs, we expected to see the distinct localizations of the overexpressed variants. However, both of the mutant as well as WT overexpressing cells demonstrated cytoplasmic and membrane localizations in all cell lines monitored. Our results clearly suggest that there must be another and more complex regulation of membrane translocation besides the GDP-GTP loading cycle.

Rac1 undergoes two different posttranslational modifications, namely iso-prenylation and palmitoylation. Iso-prenylation occurs in the CAAX motif of C-terminus of Rac1. Also polybasic region of Rac1 at the C-terminus is another decisive region and is required for targeting Rac1 to the cell membranes (Choy et al. 1999; Jack et al. 2008; Higashi et al. 2010). As second post translational modification, palmitoylation regulates Rac1 association to PM (Tsai & Philips 2012). Besides this, it is also known that Rac1 can bind to its main effector Pak1 when it is in GTP-loaded, active form (Higashi et al. 2010). Additionally, Navarro-Lérida et al. (2012) has shown that Rac1 palmitoylation promotes activation of Pak at the PM and thus downstream signaling (Navarro-Lérida et al. 2012).

Testing Rac1-PM association either under serum starvation condition or by overexpression of the mutant Rac1-variants underlines the complexity of the Rac1 pathway.

4.2. Heat stress stimulates Rac1 translocation to membranes in B16F10 melanoma cells

Early findings suggest that Rac1 may have an important role in the initiation of stress response (Han et al. 2001; Vigh et al. 2005). PM translocation of Rac1 is supposed to be the key point of Rac1-mediated stress signaling (del Pozo et al. 2004; Kültz 2005). Therefore, we tested the heat stress dependent Rac1 localization to membrane fractions in B16F10 cells.

The presence of Rac1 in the crude membrane fractions exhibited increased amounts under heat shock conditions (Figure 19). The calculated relative band intensities of western blots (37 ^{o}C=100; 41.5 ^{o}C=132; 42 ^{o}C=310; 43.0 ^{o}C=255) indicated that the exposure of cells to 41.5 ^{o}C did not noticeably influence Rac1 membrane association (Figure 19). By contrary, heat exposures at 42 or 43 ^{o}C caused highly elevated association of Rac1 with cell

membranes. Quantified results shown above suggest that Rac1 association to the cell membranes is involved (or coupled to) in stress sensing and/or signaling pathway.

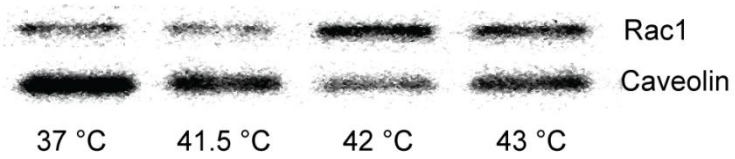


Figure 19. Localization of Rac1 to the crude membrane fraction in response to heat shock treatment. B16F10 cells were subjected to heat shock at the indicated temperatures for 1 h. Immediately after this, the crude membrane fraction was isolated and solubilized in Laemmli buffer. Equal amounts of proteins were run for western blotting. Rac1 probing was performed for the membrane fraction, and Caveolin immunostaining was used for normalization. Changes in normalized Rac1 band intensities (au) are as follow: 37 °C=100, 41.5 °C=132, 42 °C=310, 43 °C=255 .

Based on these findings, heat shock at 41.5 °C as a mild treatment and 43 °C as a severe treatment were selected for the additional experiments. Going one step further, we monitored the Rac1 localization as a result of heat stress with confocal microscope. Rac1 is also localized on endomembranes, such as mitochondria**,** where it is a binding partner of Bcl-2 and stabilizes its anti-apoptotic activity (Velaithan et al. 2011), or on early endosomes where it can generate the enlargement of early endosomes for the raft trafficking (Balasubramanian et al. 2007; Palamidessi et al. 2008; Madhivanan et al. 2012; Wiedmann et al. 2012).

Images showed that both mild and severe heat shock treatments directed endogenous Rac1 to the PM associations in B16F10 cells. Mild heat treated samples (41.5 °C) displayed noticeable Rac1 PM association while cytoplasmic Rac1 signal could also be seen. Under severe heat conditions (43 °C) we obtained very remarkable membrane binding to the PM (Figure 20). Quantification of the confocal images verified the translocation of Rac1 at both mild and severe temperature treatments (Figure 21).

It has been reported that an important step for the Rac1 PM association upon extracellular stimuli such as heat exposure is dissociation of Rac1 from its GDI complex, which shields the iso-prenyl moiety of Rac1 and keeps it in the cytosol in resting cells. When Rac1 gets released from GDI partner then can be loaded with GTP to possess the active form. After that Rac1 undergoes palmitoylation and binds to PM together with its effector Pak1 (Vigh et al. 2005; Bustelo et al. 2012).

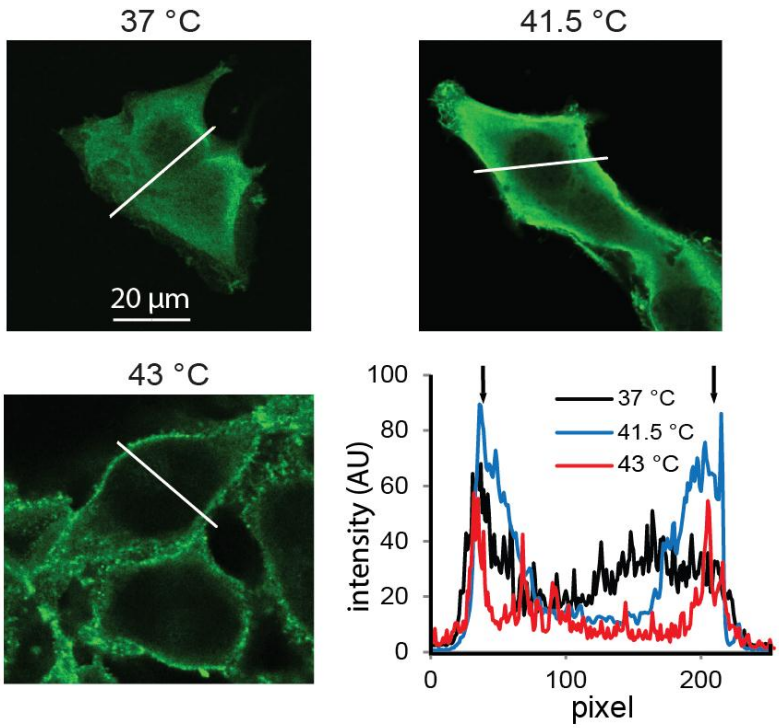


Figure 20. Visualization of the association of Rac1 to the PM. B16F10 cells were treated at the indicated temperatures for 1 h. Cells were then fixed, permeabilized and immunoreacted with Rac1 mAb, and probed with Alexa488-labeled secondary antibody by confocal microscopy. Intensity profiles of regions of interest on confocal images (indicated with white lines) are shown. Black arrows indicate PM location. The red curve refers to 43 °C, the blue curve to 41.5 °C and the black curve to 37 °C.

Receiving membrane initiated stress signaling, Rac1 PM translocation becomes of special importance for its downstream signaling pathway. In our experiments confocal images displayed a significant Rac1 pool in PM region at as mild temperature as 41.5 °C ($p<0.001$) (Figure 20 and Figure 21). This suggests that B16F10 cells could sense the heat stimuli at the clinically relevant range of temperature, initiating Rac1 plasma membrane localization of Rac1 (Figure 20), giving the green light for the activation of the downstream signaling pathways.

Moreover, the ratio of PM localized versus total Rac1 signal intensity was nearly identical under severe heat and mild heat conditions (Figure 21). This can be interpreted as sensing even the non-proteotoxic mild-heat condition at the PM, at a significant level.

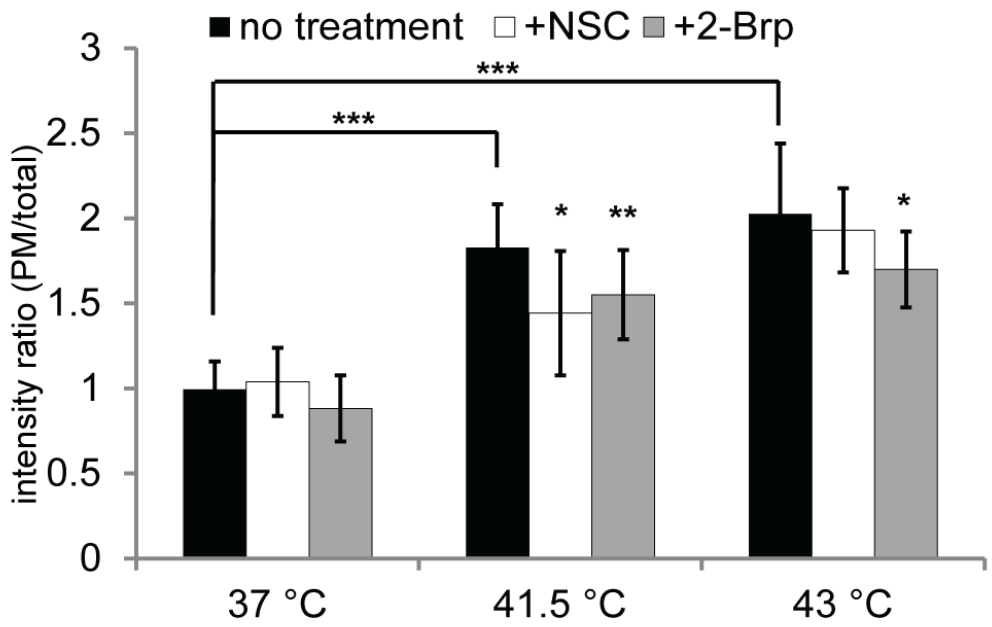


Figure 21. The effect of Rac1 inhibitor and palmitoylation inhibitor administration on the Rac1-membrane binding under heat stress conditions. Cells were treated/or not with the Rac1 inhibitor NSC or 2-Brp, heat-shocked/or not, and then immunostained as Figure 20. Confocal images were taken and 15 cells/3 views for each treatment were quantified by ImageJ. The bars represent the fluorescence intensity of the PM versus the fluorescence intensity of the whole cell. Black bars relate to cells not treated with inhibitor, white bars to NSC-treated and gray bars to 2-Brp-treated cells. Data are means ± SEM, n =3, Student's t-test was used for statistical analysis. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

The importance of Rho family GTPases in disease state (hypertension, cancer, or neurodegenerative diseases) as well as in physiological conditions make them attractive targets for development of inhibitory drugs (Bouquier et al. 2009). Among them, NSC is the most frequently used cell-permeable, reversible inhibitor of Rac1 activation which does not interfere with Cdc42 and RhoA. It specifically binds to the interaction region of Rac1-specific GEFs TrioN and Tiam1 on the surface groove of Rac1 and keeps Rac1 in the inactive form (Gao et al. 2004)..

NSC has been used to demonstrate the importance of Rac1 activity in cancer cell proliferation, metastasis, invasion, migration and therapy resistance in many different cell types. The compound has been administrated in a variety of concentration range and time point in different studies (Dokmanovic et al. 2009; Mukherjee et al. 2009; Yuan et al. 2010; Akunuru et al. 2011; Yan et al. 2012). In our system, 100 µM of NSC application for 2 h along with 1 h heat stress generated significant inhibitory effect on Rac1 PM localization

under mild heat stress at 41.5 °C ($p<0.05$) (Figure 21). Inhibition at severe stress was still present yet, not as strong as under mild heat conditions (Figure 21). Rac1 is known to undergo palmitoylation as a post translational modification besides containing prenyl moiety (Tsai & Philips 2012). Recently, the importance of Rac1 palmitoylation was shown in governing its membrane association and signaling activity (Navarro-Lérida et al. 2012). In order to assess the effect of palmitoylation upon heat shock treatments, B16F10 cells were pretreated with an effective and irreversible inhibitor of protein S-palmitoylation *in vivo* called 2-Brp (Webb et al. 2000; Davda et al. 2013). 2-Brp administration was performed as described by Navarro-Lérida et al.; 25 µM of 2-Brp was applied for 30 min (Navarro-Lérida et al. 2012) before heat shock treatments. This concentration was sufficient to block Rac1 palmitoylation whilst, some other membrane binding proteins such as K-Ras, H-Ras, Fyn etc., need several hours in order to undergo palmitoylation inhibition by 2-Brp (Webb et al. 2000; Goodwin et al. 2005; Rocks et al. 2005). This difference might be the consequence of different palmitate turnover rates (Navarro-Lérida et al. 2012). Consistent with the study obtained using MEF and Cos7 cells mentioned above by Navarro-Lérida et al., we have documented that 2-Brp administration caused a slight reduction in Rac1 PM binding without any heat stress (Figure 21). This may mean that the turnover of the Rac1 palmitoylation is rather slow but palmitoylation inhibition has an interfering effect on PM localizataion.in B16F10 cells. Moreover, inhibition of Rac1 palmitoylation at both mild ($p<0.01$) and severe heat ($p<0.05$) caused a higher degree loss of PM localizations of Rac1 implying an increased turnover of Rac PM location/activity at higher temperatures.

Taken together, the above studies clearly showed that heat stress stimulated the PM localization of Rac1. The localization was more dependent on GTP loaded form under mild condition than under more severe circumstance and that the turnover rate on PM is higher during heat stress than under normal growth condition.

4.3. Rac1 palmitoylation affects the PM micro-domain organizations upon heat stress

Previously, lipid rafts were identified as membrane binding sites for active Rac1 which is shown to localize at lamellipodial edges of PM in MEF, NIH3T3, Swiss 3T3, Cos7 cells (del Pozo et al. 2004; Golub & Caroni 2005; Vasanji et al. 2004). Further studies demonstrated that PM micro-domain reorganizations were accomplished by the relocalization of palmitoylated active Rac1 (Navarro-Lérida et al. 2012; Tsai & Philips 2012). The

"membrane sensor hypothesis" postulates that, not only protein denaturation but also changes in the physical states and micro-domain organization of membranes can activate membrane initiated stress signaling for *hsp* gene expression (Horváth et al. 1998; Vigh et al. 2007). Based on these findings, we investigated the role of palmitoylation inhibition on micro-domain reorganizations in B16F10 cells under heat stress conditions. As raft domains are known to be enriched in SM, we used BODIPY FL C12 fluorescent SM label to detect lipid rafts. BODIPY FL C12 SM labeled, 2-Brp treated/or not, heat shocked/or not B16F10 cells were analyzed with fluorescent microscopy in TIR mode where the PM could be visualized (without the disturbing signal from the cell interior). PM domain size analysis was performed using ImageJ and CellProfiler software and SM labeled domains were categorized in five classes on the basis of their calculated diameters. Heat shock, both moderate and severe, brought about an increase in average PM micro-domain size (Figure 22A, black bars) and a characteristic change in the size-distribution of membrane micro-domains. Namely, in cells subjected to heat stress the number of smaller domains (456-465) decreased whilst the larger domains (929-1136 nm and 1137< nm) were present with higher frequency (Figure 22B).

2-Brp treated control cells exhibited a PM with a highly increased SM labeled average domain size (Figure 22A, gray bars). But when 2-Brp pre-treated cells were exposed to mild heat at 41.5 °C, the level of average domain size didn't change compared to 41.5 °C non-treated cells and was similar to 37 °C 2-Brp treated cells. Very interestingly, treatment of cells at 43 °C resulted in a pronounced and significant ($p<0.001$) increase in average diameter of SM labeled micro-domains. This robust increase was inhibited ($p<0.05$) by 2-Brp pre-administration and these cells showed domain sizes similar to that of 2-Brp treated control and mild heat treated cells. Interestingly, the effect of 2-Brp treatment was opposite when 37 °C cells were compared to 43 °C treated cells. It seemed that 2-Brp treatment adjust a temperature independent PM average micro-domain size (Figure 22).

Additionally we found that 10% of the examined PM area consisted of SM labeled micro-domains. Navarro-Lérida et al. (2012) have shown that Cos7 cells expressing palmitoylation-deficient Rac1 mutant or conditionally Rac1 knock out MEF cells have an increased content of disordered membrane domains (Navarro-Lérida et al. 2012). On the contrary, in our study we didn't observe any changes in the SM labeled L_o micro-domain area over against the total PM surface area tested as a result of 2-Brp treatment in B16F10 cells.

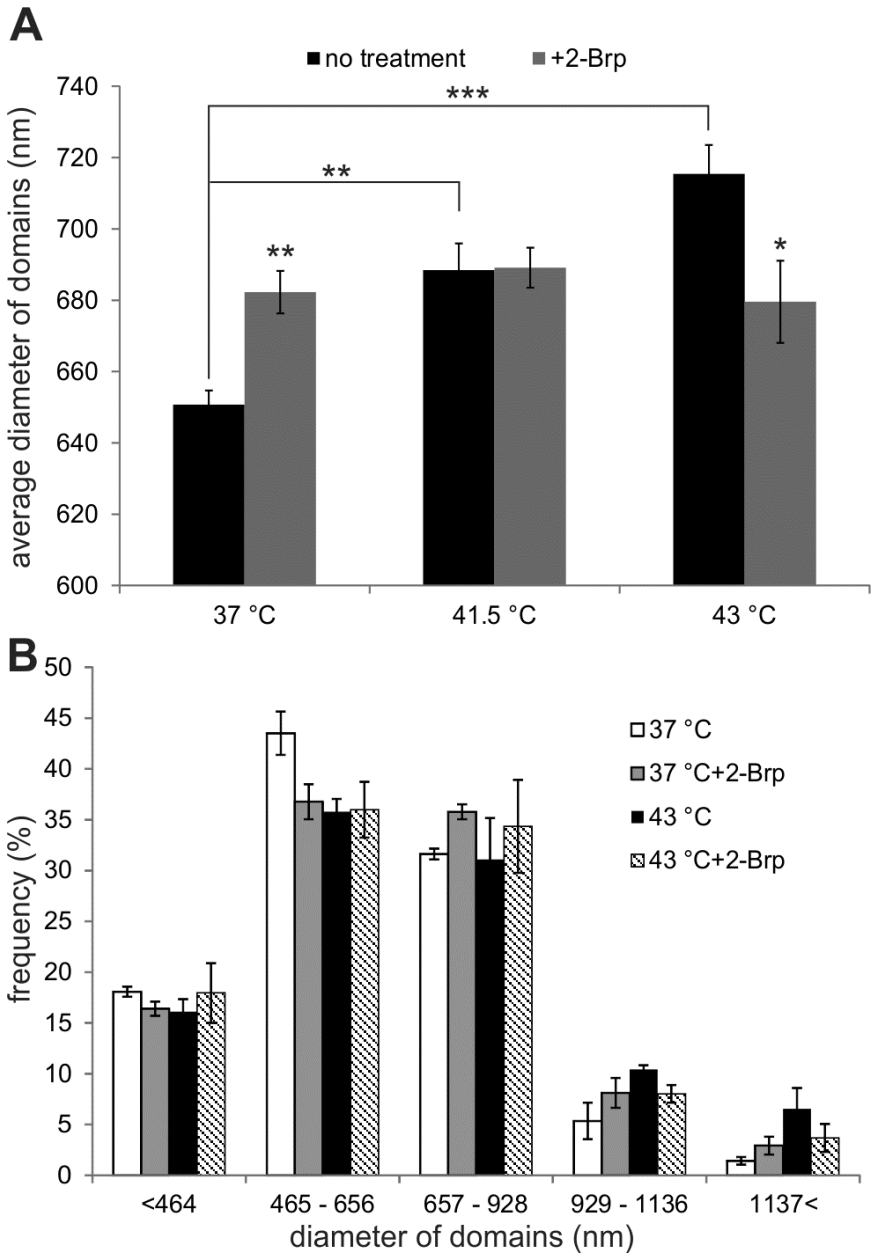


Figure 22. Change in size of PM micro-domains as functions of temperature and 2-Brp treatment. (A) 2-Brp was added/or not to samples for 30 min, and they were then heat-treated at 41.5 °C or 43 °C or kept at 37 °C for 1 h. After BODIPY FL C12-sphingomyelin labeling and TIRF microscopy, the domain distribution was analyzed with ImageJ and CellProfiler and the average domain sizes are shown. (**B**) From the experiment shown in (**A**), the PM micro-domains were separated into five classes, depending on their sizes. The data shown are means ± SEM, n = 3. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

Rafts are dynamic structures whose properties are strongly influenced by their protein and lipid content (Balasubramanian et al. 2007) and the hypothetic stress signal related proteins are known to bind to raft regions of the PM (Echarri et al. 2007; Kusumi et al. 2012).

As one of the posttranslational modifications, palmitoylation of the proteins recruited in stress signaling pathways are regulated by different palmitate turnovers which has not been elucidated in details yet (Greaves et al. 2009). We assume, that inhibition of raft binding protein palmitoylation by 2-Brp, therefore may result is in an aberrant protein turnover. 2-Brp as a general palmitoylation inhibitor may effect different pathways and palmitoylated proteins in the complex *in vivo* systems. We don't have information about the de/re-palmitoylation turnovers of not only Rac1 but also of other potentially affected proteins which may be in charge on HSR pathways.

To complete the elucidation of the temperature independent effect of 2-Brp on lipid raft size and distribution and to judge more specifically the contribution exclusively of Rac1 palmitoylation itself to this effect, Rac1 palmitoylation-incompetent mutant B16F10 cell line is under preparation (in Lajos Mátés's lab) through the use of the combined "Recombinase Mediated Cassette Exchange" (RMCE) and "Sleeping Beauty" (SB) gene transfer methods (Mátés et al. 2009).

4.4. Rac1 is involved in heat induced changes in cell morphology

The stress response of mammalian cells is classified as sequential and discontinuous heat-induced processes (de Gannes et al. 1998; Coakley 1987; Beuthan et al. 2004). While the cell morphology, adhesion behavior and integrity of surface membranes of MX1 breast cancer cells have been shown to be unaffected by mild heat stress (40-41 ºC), the surface roughness increased as a consequence of the destabilization of the actin cytoskeleton (Dressler et al. 2013). Moreover, actin monomers are denatured (and are no longer polymerizable) at temperatures a few degrees above the growth temperature, although actin in the filament form requires much higher denaturation temperatures (Coakley 1987).

In order to monitor the influence of heat stress on F-actin cytoskeletons, B16F10 cells were labeled with Alexa Fluor-647 conjugated Phalloidin which binds specifically to F-actin. Then labeled cells were analyzed by flow cytometry and fluorescent intensity of 10 000 cells which were either heat treated at indicated temperatures or kept at 37 $^{\circ}$C as control. As presented on Figure 23, under the increasing heat stress conditions (41.5 and 43 $^{\circ}$C) B16F10 cells demonstrated gradual reduction of F-actin levels.

Actin is one of the main components of cytoskeleton and has a special importance at membrane ruffling and cell shape (Kusumi et al. 2012; Gagat et al. 2013). Structure of

filamentous actin is reported be effected by heat stress conditions (Clarke & Mearow 2013; Pawlik et al. 2013). The stress fibers consist of bundles of 5-nm actin filaments, and their formation appears to be regulated through PM interactions (Glass et al. 1985). Wiegant et al. (1987) showed disorganized stress fibers upon increased heat stress in H35 Hepatoma cells (Wiegant et al. 1987). Our data set is supporting the previous findings in which Glass et al. (1985) demonstrated a remarkable decrease in the percentage of F-actin under heat stress at 45 °C within 5 min in CHO cells. With an agreement with our findings, another recent study related to actin changes under stress conditions has demonstrated that exposure to heat shock of rat H1299 cells resulted in disintegration of F-actin (Pawlik et al. 2013).

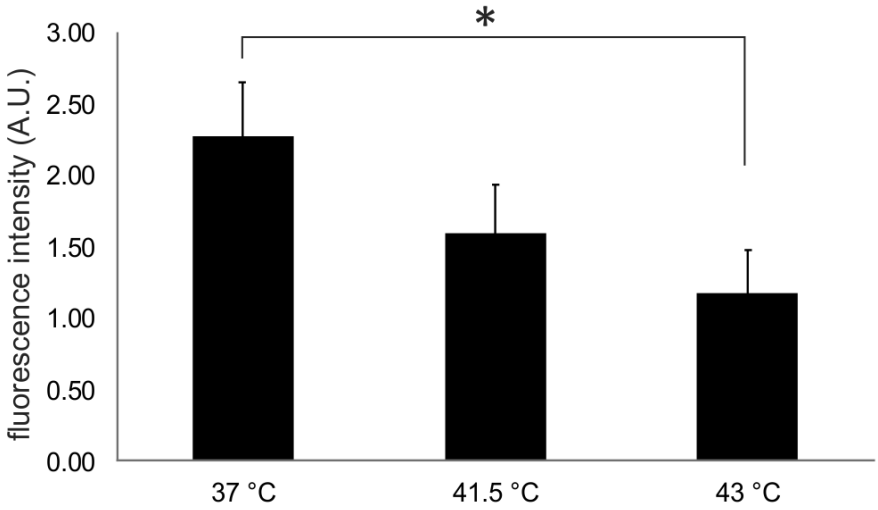


Figure 23. F-actin changes in B16F10 cells under heat shock conditions. Measurement of heat stress-triggered F-actin alterations by using flow cytometer. After heat stress, cells were collected by trypsinization, fixed and labeled with Alexa 647 phalloidin. Flow cytometry measurements were made with a BD Accuri C 6 cytometer and CFLow Plus 1.0.227.2. was used for data analysis. The data from three independent experiments are shown, along with the S.D. Student's t-test was used for statistical analysis. *: $p<0.05$.

Cytoskeleton is the scaffold of the cells the shape of which is determined by the network of cytoskeletal filamentous proteins including microtubules and actin (Frixione 2000). Earlier studies demonstrated that Rho GTPases mediate actin rearrangements involved in cell shape changes induced by stress and extracellular signals (Wojciak-Stothard & Ridley 2003; Civelekoglu-Scholey et al. 2005; Parri & Chiarugi 2010). Cytoskeleton can generate a prompt response to extracellular stimulation such as heat stress (Hildebrandt et al. 2002; Gagat et al. 2013). The response of the cells to thermal stress can vary depending on the mode

and duration of the applied heat stress as well as the biological factors such as the cell type (Garcia et al. 2012; Pawlik et al. 2013). In fact, rearrangements of the actin filaments accompanied by striking changes in cell shapes in MX1 human breast cancer cells were reported under comparatively mild heat stress conditions (Dressler et al. 2013). Also, it was reported that 9L rat brain tumor cells had contracted cell shape after a 15 min 45 ºC heat stress (Wang et al. 1998).

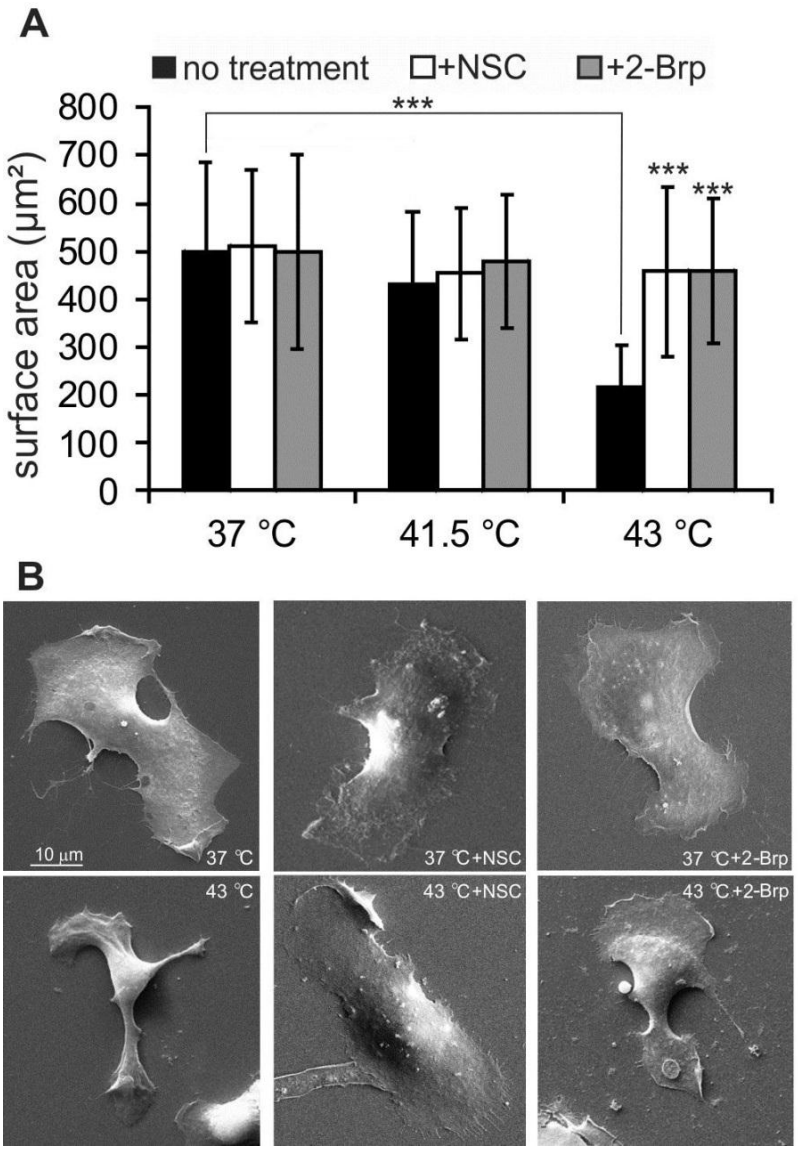


Figure 24. Cell morphology changes in B16F10 cells under heat shock conditions. (A) Surface area changes of B16F10 cells in response to heat shock and Rac1 inhibitor administration. Cells were subjected to stress conditions at 41.5 °C or 43 °C or kept at 37 °C with or without inhibitor administration as indicated, and samples were fixed for SE Microscopy imaging. From the SE Microscopy images, 30 cells were chosen for the calculation of surface area with ImageJ. Black bars relate to the lack of inhibitor treatment, while white and gray bars relate to NSC and 2-Brp administration, respectively. The data from three independent experiments are

shown, along with the S.D. Student's t-test was used for statistical analysis. ***: p<0.001. (**B)** Representative SE Microscopy photos of 37 °C, 37 °C+NSC, 37 °C+2-Brp, 43 °C, 43 °C+NSC and 43 °C+2-Brp-treated cells .

Next we applied SE Microscopy investigations to obtain an overview of morphological responses of B16F10 cells to heat treatments. Control cells at 37 °C exhibited well spread morphology on the glass substrate. Cells in the 41.5 °C stress group were very slightly altered, cell morphology did not display noticeable differences compare to control group. Calculated cell surface areas showed no changes at mild heat stressed cells compared to their control. Again, as a result of calculated cell surface area data, neither NSC nor 2-Brp pretreatments caused surface area changes without heat stress. Furthermore, when mild thermal stress was applied, with or without Rac1 inhibitor NSC and 2-Brp, the cells displayed no significant alterations (Figure 24A). When stressing was performed at 43 °C, the cells became much smaller and more spheroidal shaped compared to their controls. Severe heat also led to a significant reduction in the calculated cell surface area. Treatment with either NSC or 2-Brp however, ceased the heat stress (43 °C) induced cell surface shrinkage (Figure 24).

It has been reported that hyperthermia favors changes in human cancer cell shapes through the integrin modifications (Pawlik et al. 2013). Besides this, both microtubules and actin filaments are known to be affected by heat stress in a variety of organisms (Wang et al. 1998) and Rac1 has been shown to play important roles in cytoskeletal organization (B. Xu et al. 2012). Indeed, flow cytometer and SE Microscopy results showed that at mild stress conditions of 41.5 °C, filamentous actin cytoskeleton and cell shape were not affected in B16F10 cells whereas, exposure of stress temperature of 43 °C caused striking changes of cell shapes and rearrangements of actin filaments. Besides this, both the Rac1 inhibitor NSC and the palmitoylation inhibitor 2-Brp prevented the severe heat stress-induced changes in cell shape and cytoskeleton. The effect of both NSC and\or 2-Brp inhibitors on the active reorganization of plasma membranes (described in section 4.2) and cell shapes alterations at 43 °C suggest a key role of Rac1 under heat stress conditions (Figure 23 and Figure 24).

4.5. Rac1 is involved in the disorganization of actin filaments upon severe heat stress treatment

As explained before, heat stress can govern the actin filament degradation and cell shape changes to create shrunk, rounded morphology and Rac1 inhibitors can save the structure of cell shape under normal growth conditions (see Figure 23 and Figure 24). Having a fundamental role in wide variety of cellular processes such as migration, spreading and morphogenesis, an understanding of the roles of the actin cytoskeleton and stress fibers are getting important (Tojkander et al. 2012; Kovac et al. 2013; Burridge & Wittchen 2013). The process of stress fiber dynamics is currently being rigorously studied and is not yet well understood. In order for contraction to generate forces the fibers must be anchored on something. Stress fibers can anchor to the cell membrane, and the sites where this anchoring occurs are also connected to structures outside the cell. These connection sites are called focal adhesions hence are involved in those cellular processes mentioned above (Burridge & Wittchen 2013).

In order to assess the actin depolymerization under heat shock conditions and the potential role of Rac1 on structural changes of F-actin, we tested the effect of both Rac1 specific inhibitor NSC and palmitoylation inhibitor 2-Brp, upon heat shock conditions. For these experiments MEF cells which are flat and well spread fibroblasts and as such are better amenable for visualizing the cytoskeleton by confocal laser microscopy were chosen. MEF cells were subjected to either 41.5 °C as mild or 43 °C as severe heat stress with or without inhibitor pretreatments. Control cells were kept at 37 °C along with or without inhibitor administrations. Confocal microscopy results demonstrated that, mild stress at 41.5 °C caused no remarkable reorganization of actin filaments in the presence or absence of Rac1 inhibitor. As a consequence of mild heat treatment, actin rich cell protrusions, filopodia and lamellipodia were detected. Besides the fine stress fibers, also some decomposed F-actin structures could be seen. On the contrary, severe heat treated cells at 43 °C revealed considerable F-actin fragmentations. However, NSC treatment prior to severe stress prevented this effect and could save the fine actin structure against heat. Therefore, long and intact actin fibers were still detectable resembling that of the control cells at 37 °C (Figure 25).

When we used palmitoylation inhibitor 2-Brp to test the actin filament morphology of MEF cells following mild and severe heat stress treatment, actin fibers could be still detected. 2-Brp pre-treatment followed by mild heat at 41.5 °C didn’t make any visible changes on filamentous actin structures. Conversely, 2-Brp pretreated cells at 43 °C displayed better-

preserved, intact actin fibers as well as actin rich protrusions whilst 43 °C without inhibitor pretreatment, caused decomposed F-actin structures (Figure 25).

Previously, van Bergen et al. (1985), Glass et al. (1985) and Wiegant et al. (1987) had pointed out the degradation of F-actin and stress fiber upon severe heat stress conditions in different cell lines (van Bergen en Henegouwen et al. 1985; Glass et al. 1985; Wiegant et al. 1987). Recently, Clarke and Maerow (2013) documented that heat shock treatment resulted in rounding of the cells and collapse of the actin filaments in PC12 cells (Clarke & Mearow 2013).

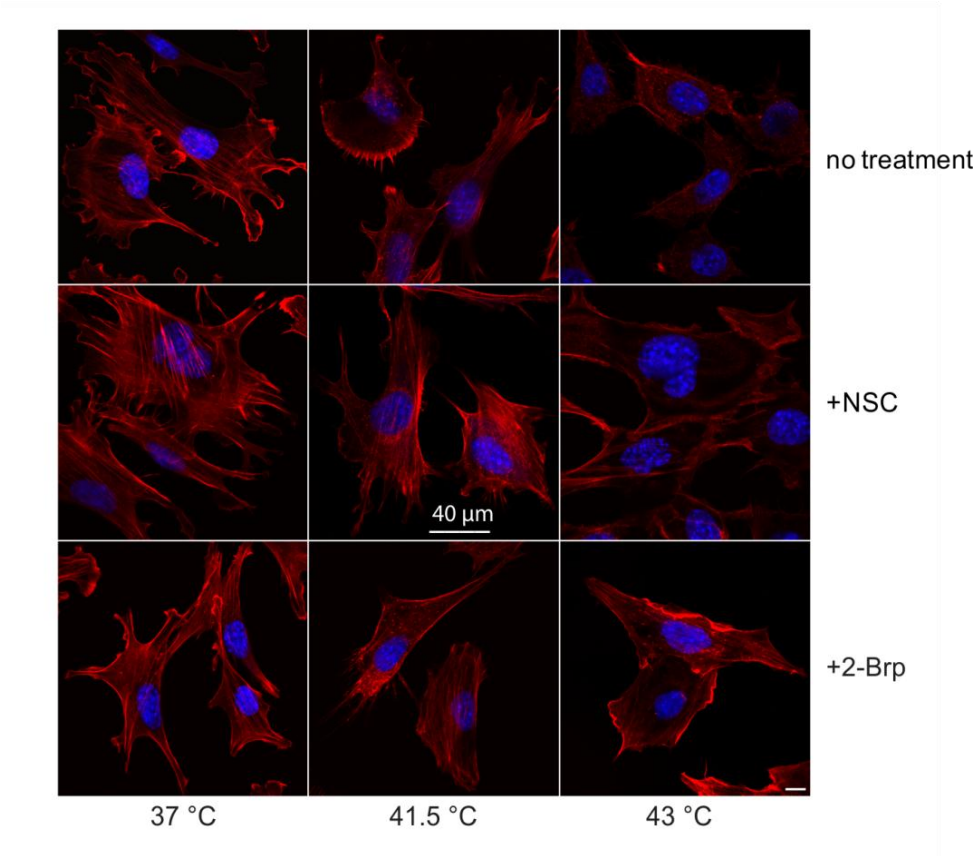


Figure 25. Effect of Rac1 inhibitors on the actin filaments of heat and inhibitor treated MEF cells. Cells were subjected to temperature treatment with or without NSC or 2-Brp treatment. After fixation and permeabilization, cells were labeled with Alexa 647-phalloidin (red, actin) and DAPI (blue, nuclei) and confocal microscopy was used for immune localization. Scale bar: 40 µm.

Coinciding with the previous studies mentioned we demonstrated that severe heat shock caused cell shrinkage and rounding through decomposition of F-actin in MEF cells. Notably, this effect at 43 °C could be prevented by the administration of both Rac1 specific inhibitor NSC and palmitoylation inhibitor 2-Brp prior to heat stress applied.

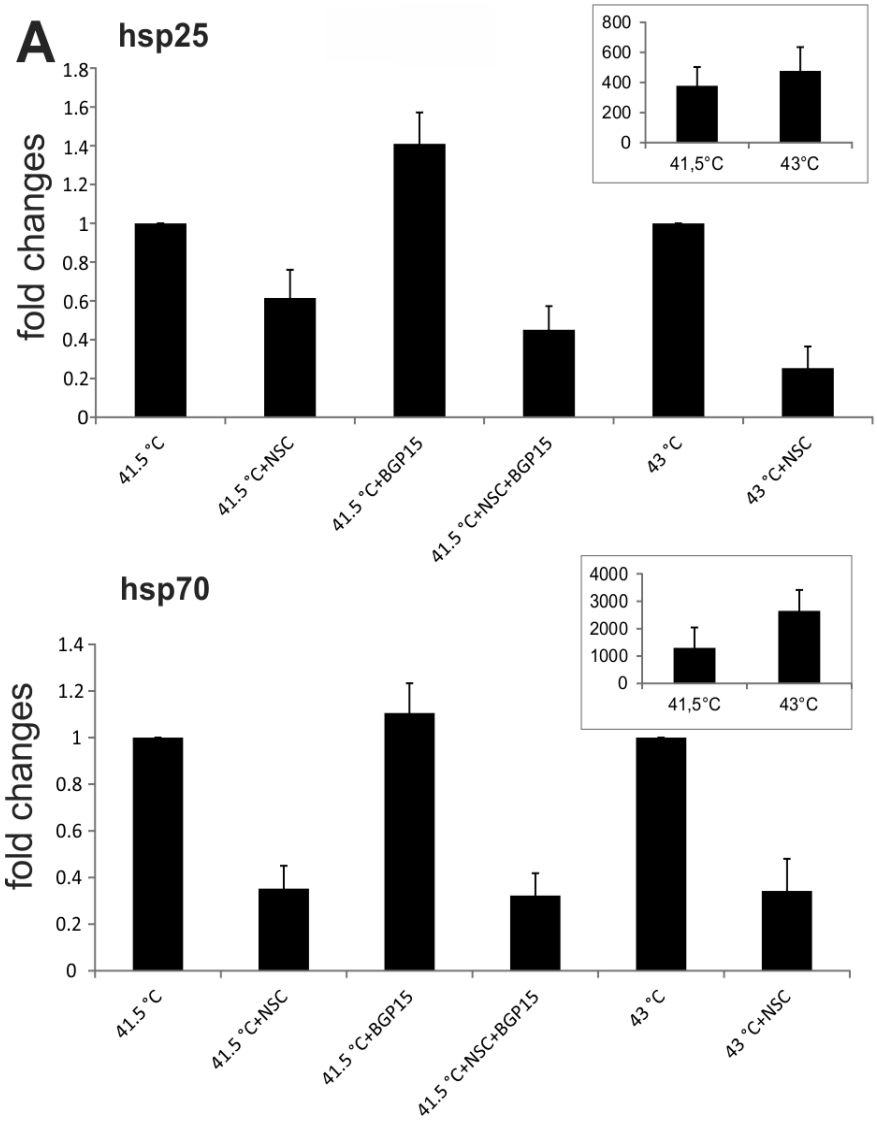
These results presented here highlight the importance of Rac1 on reorganization of actin filaments under heat stress conditions.

4.6. Rac1 affects the mRNA expression levels of *hsp25* and *hsp70* under heat shock conditions

Based on the "Membrane sensor hypothesis", membrane hyperfluidization as well as cholesterol and SM-rich micro-domain (raft) reorganizations are involved in the generation of stress signaling and activation of *hsp* genes in B16F10 cells (Vigh et al. 1998; Vígh et al. 2007).

Taken together, the informations regarding Rac1 PM associations and micro-domain rearrangements (where Rac1 inhibitors could modulate these effects) as a result of heat treatment, we next investigated the possible influence of Rac1 inhibitor NSC and 2-Brp on *hsp25* and *hsp70* inducibility under mild (along with HSP co-inducer, BGP15) and severe heat stress conditions by performing quantitative RT-PCR. For this aim, B16F10 cells with $5x10^5$/6 cm plate initial cell number, were used. Several former studies indicated that the cell density of the culture influences the heat shock response (Noonan et al. 2007; Gombos et al. 2011; Péter et al. 2012). Indeed, Peter et al. (2012) revealed that high cell number plated cultures of B16F10 cells showed 2 fold less HSR to HS compared to low cell number counterparts (Péter et al. 2012). Therefore, we carried out our experiments very precisely in regard to the actual cell number and selected low cell density condition to investigate the effect of Rac1 inhibition on the activity of these two genes under heat shock condition. B16F10 cells were subjected to Rac1 inhibitors prior to heat treatments as described in section "3.2 Treatments". B16F10 cells were also treated with a well-established chaperone co-inducer hydroximic acid derivative BGP15 (Chung et al. 2008; Literáti-Nagy et al. 2012; Crul et al. 2013). BGP15 administration was carried out only under mild heat conditions as we discussed earlier in Gombos et al. (2011). As can be seen in the small charts of Figure 26, *hsp25* and *hsp70* mRNA levels increased several hundred times upon both mild and severe heat shock exposures. As also expected, BGP15 administration further elevated the levels of both inducible *hsp* mRNA under mild heat condition. This effect was more obvious on *hsp25* expression, while *hsp70* mRNA level increased only slightly. The administration of NSC diminished the mRNA levels of both *hsp25* and *hsp70* under mild (41.5 °C) heat shock (Figure 26A). Moreover, as we published in Gombos et al. (2011) and reviewed in Crul et al.

(2013), elevated levels of *hsp25* resulted from HSP co-inducing capability of BGP15 at 41.5 °C was also inhibited by NSC (Gombos et al. 2011; Crul et al. 2013). Interestingly administration of NSC on the expression levels of *hsp70* displayed the same inhibitory effect. NSC pretreatment resulted in drastic inhibitions of both *hsp25* and *hsp70* mRNA levels under severe heat shock conditions at 43 °C (Figure 26A).



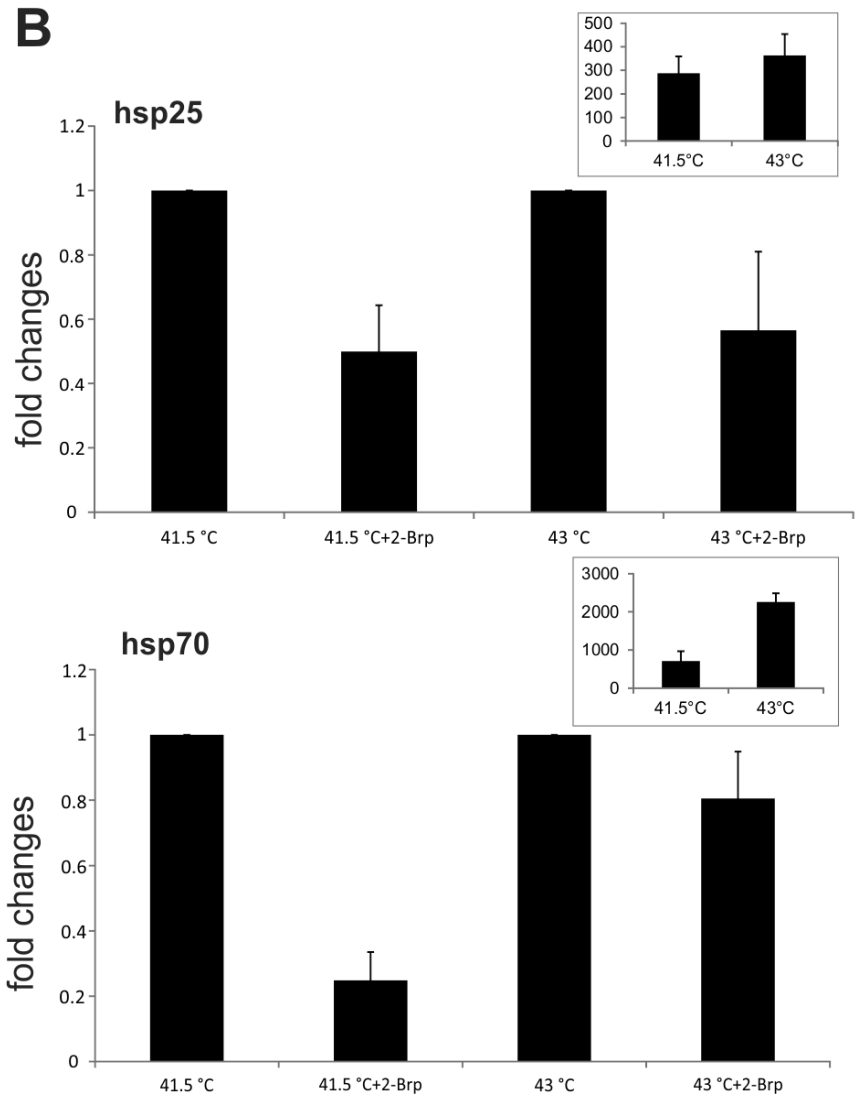


Figure 26. Effect of Rac1 inhibitors 2-Brp and NSC on hsp25 and hsp70 gene expression levels under heat shock conditions. B16F10 cells were either treated/or not with NSC (A) or 2-Brp (B) for 2 h or 30 min, respectively, and then exposed/or not to the indicated heat shock temperatures for 1 h. Total RNA was isolated immediately after the heat shock, and the expression levels of *hsp70* and *hsp25* mRNAs were measured by quantitative RT-PCR and were normalized to *Gapdh*. If BGP15 was used, it was administered at 10 μM during heat shock. Data shown are fold changes compared to the mRNA levels measured in inhibitor untreated, heat shocked samples. Small, inserted graphs represent the fold changes compared to the mRNA levels measured in 37 °C samples. Data are means ± SEM, n=3.

In the case of 2-Brp effect on B16F10 cells heat-treated at 41.5 °C, the compound combined with the heat treatment resulted in an inhibitory effect on the induction of both *hsp25* and *hsp70* mRNA levels and this inhibition was more pronounced for *hsp70* at 41.5 °C.

However, the 2-Brp inhibitory effect was much stronger on *hsp25* mRNA levels compared to *hsp70* mRNA inducibility under severe heat stress (43 °C) (Figure 26B). In contrast to NSC, 2-Brp remained apparently ineffective under severe heat stress conditions.

As an evidence of "membrane sensor hypothesis", in B16F10 cells elevated expression levels of *hsp25* and *hsp70* genes correlated with membrane micro-domain reorganizations under stress conditions (Nagy et al. 2007; Balogh et al. 2010; Péter et al. 2012). Also, Rac1 has been reported to play a role in stress response processes for several stress conditions such as hypoxia, heat stress and oxidative stress (Bornfeldt 2000; Ozaki et al. 2000; Han et al. 2001; Nègre-Aminou et al. 2002; Misra et al. 2012).

del Pozo et al. (2004) documented that active Rac1 binds preferentially to Chol-rich membranes (rafts) and this binding step is specifically determined by the composition and physical state of the membrane lipids (del Pozo et al. 2004). Overall, our data suggest that, reorganization of membrane micro-domains can be coupled with a modulation of the activities of prominent *hsp* genes and Rac1 activity and post- translational modifications have key roles in the operation of stress signaling pathways.

4.7. Rac1 takes part in the HSR at protein level

When cells encounter challenging fluctuation in their environment, they react by increasing the expression level of HSPs. The *hsp* encoded proteins are essential to the cells' ability to survive environmental insults (Baler et al. 1992). Since its discovery in 1962 by Ritossa, the HSR has been extensively studied in different organisms and cell lines by several research groups (Balogh et al. 2005; Nagy et al. 2007; Brameshuber et al. 2010; Horváth et al. 2012; Tóth et al. 2013; Bartelt-Kirbach & Golenhofen 2014). Besides these, Park et al. (2005) reviewed that Rac1 is one of the key elements of heat sensing machinery under mild heat stress conditions in NIH3T3 and Rat2 fibroblasts (Park et al. 2005). In order to confirm the changes in HSR following the inhibition of Rac1 activity and membrane association, protein levels of HSP25 and HSP70 were measured in B16F10 cells. Pre-exposure of the cells to NSC or 2-Brp was performed before mild or severe heat temperatures as described in Section 3.2 Treatments.

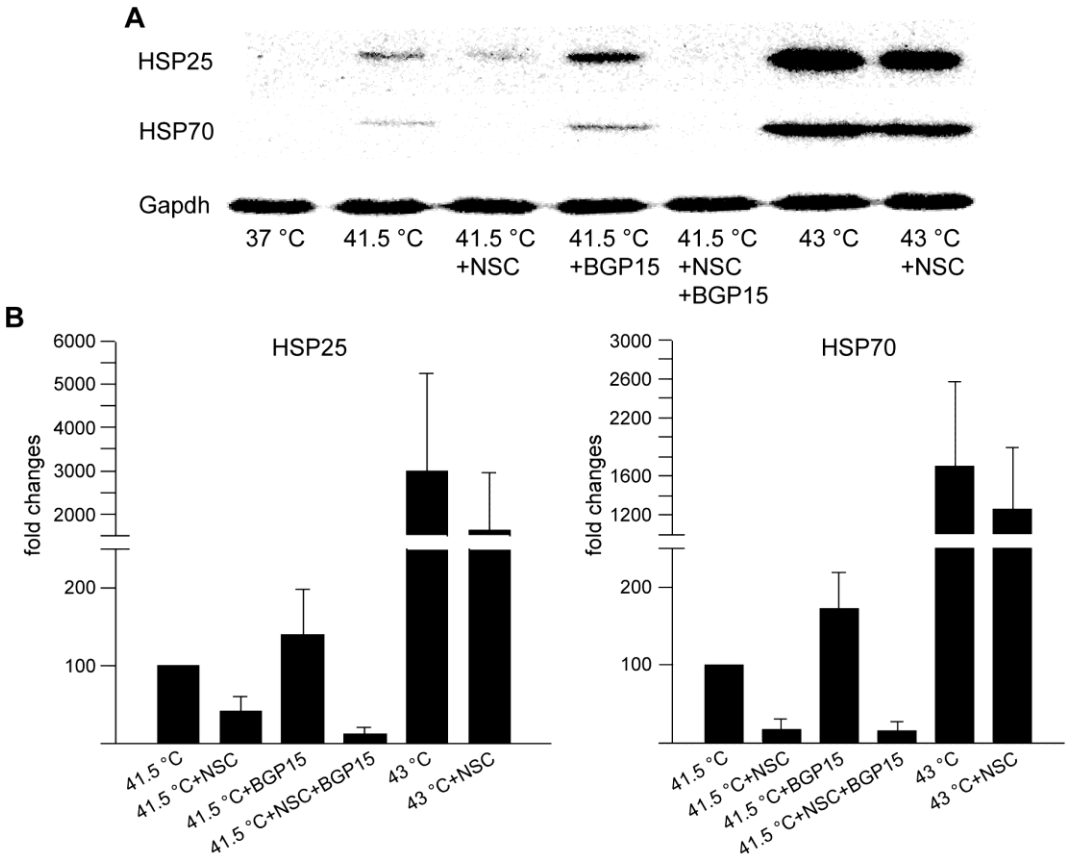


Figure 27. Effect of NSC on HSP25 and HSP70 protein levels under heat shock conditions. Cells were treated as described Figure 26 and were collected after an overnight recovery at 37 °C. (**A)** Western blotting was performed for equal amounts of total protein samples. Membranes were probed with anti-HSP25, anti-HSP70 and anti-Gapdh antibodies. (**B**) The band intensities of protein expression levels were quantitated and normalized to Gapdh. In calculations of fold changes, the protein levels of 41.5 °C samples were taken as 100. Data are means ± SD, n=3.

As is shown on Figure 27, both HSP25 and HSP70 expression levels were gradually increased with elevated temperatures. Also, combination of BGP15 and mild heat stress resulted in higher HSP25 and HSP70 expressions, than that of observed at mild heat treatment, alone. .However, the elevated expressions of both HSP25 and HSP70 were blunted by the Rac1 inhibitor NSC at either mild or severe heat treatments and the co-inducing effect of BGP15 under mild heat condition was also markedly diminished (Figure 27).

It is noteworthy that the effects of the inhibition of Rac1 function upon HSP synthesis were more pronounced under mild heat shock conditions (Figure 27B). Having correlation with Rac1 PM localization result (See in Figure 20 and Figure 21), this finding further supports the key role of Rac1 under non-proteotoxic stress conditions.

Earlier it has been shown that mild heat shock can cause disintegration on raft domains which is accompanied with an increase in the expression of the HSP27 in mGFP-GPI expressing CHO cells (Brameshuber et al. 2010). Later Gombos et al. (2011) has reported consistent findings where BGP15 administration can prevent the raft disintegration under mild heat stress in the same cell line (Gombos et al. 2011). Additionally, Langmuir monolayer experiments have demonstrated that BGP15 administration preserves the stability of Chol/SM containing domains (Gombos et al. 2011), the main components of raft platforms in mammalian cells. Connected with these observations our study suggests that BGP15 can contribute to the activation of HSP expression via Rac1 signaling pathway where Rac1 acts as a regulatory element for the reorganizations of membrane rafts under stress stimuli.

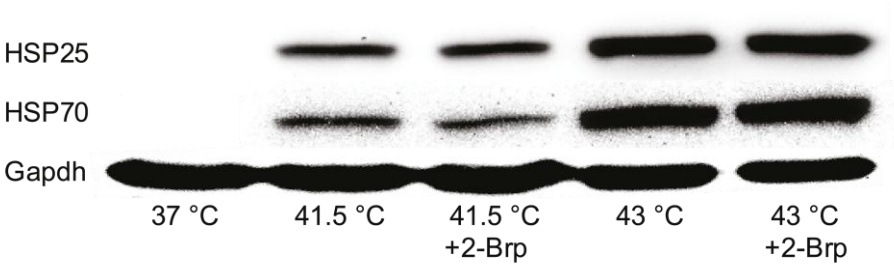


Figure 28. Representative western blotting showing the effect of 2-Brp on HSP25 and HSP70 protein levels under heat shock conditions. Treated cells were collected after an overnight recovery at 37 °C. Western blotting was performed for equal amounts of total protein samples. Membranes were probed with anti-HSP25, anti-HSP70 and anti-Gapdh antibodies.

When HSP response was studied in the presence of palmitoylation inhibitor, 2-Brp, very interestingly our results explored that this treatment had no any effect on HSP levels. This was an unexpected finding, as at mRNA levels, especially under mild heat condition, there was a strong inhibition of both gene products (Figure 28). As we did not carry out experiments to resolve this apparent discrepancy we can reason that either the life time of the mRNA was increased or there was an alteration in the translation process to compensate the decreased amount of mRNA. As noted before, we are aware of the nonspecific and pleiotropic effects of 2-Brp, so further studies are currently underway in our collaborator laboratory to create a palmitoylation-incompetent Rac1 mutant B16F10 cell.

Considering the effect of Rac1 inhibitor NSC on HSP expression levels, especially under mild heat shock conditions, next we tested the effect of NSC on other HSPs such as HSP60, HSP90 and HSP110 under mild heat conditions. All of these HSPs were abundant

proteins under normal growth conditions at 37 °C. Moreover, there was no increase in their amount upon heat treatment at 41.5 °C. Pre-exposure of cells to NSC revealed no impact under mild stress treatments on the level of any proteins, here tested (Figure 29). Based on these results, it is assumed that Rac1 specific inhibitor NSC mainly and selectively affects the inducible HSPs, such as HSP25 and HSP70, among the tested ones.

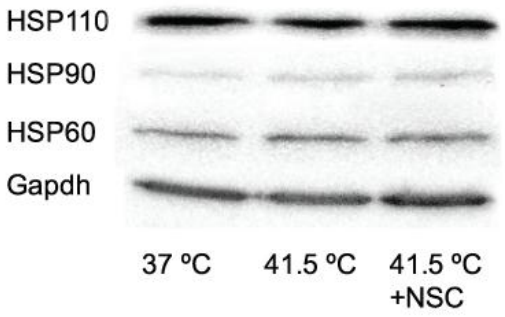


Figure 29. The effect of NSC on the expression levels of HSP60, HSP90 and HSP110. After treatments followed by overnight recovery, cells were collected and equal amounts of proteins were run for western blotting. Membranes were probed with anti-HSP60, anti-HSP90, anti-HSP110 and anti-Gapdh antibodies.

HSP60, HSP90 and HSP110 are generally found to be present at basal levels under normal growth conditions and their overexpressed levels have been shown to confer heat resistance in the cells (Oh 1999; Escribá et al. 2008). In B16F10 cells, the reason of unchanged expression levels of these HSPs at 41.5 °C may be that it is a fever-ranged mild temperature which does not create proteotoxicity. Based on all the data described in this study, we propose that the induction of the highly heat inducible HSP25 and HSP70 expressions includes Rac1 activation, but signaling pathway involving Rac1 is not the sole pathway leading to HSR. Furthermore, consistent with the assumption of Parks et al. (2005), Rac1-mediated HSR is more emphasized at fever range mild heat temperatures.

4.8. Rac1 inhibition does not affect the level of HSF1 phosphorylation

HSF1 is the primary transcriptional factor regulating the expression of the *hsp*s in vertebrates and is activated when cells experience a stress. Activation upon stress, such as elevated temperature, HSF1 is released from the chaperone complex and trimerizes. HSF1 is

then transported into the nucleus where it is hyperphosphorylated and binds to DNA containing heat shock elements (Guettouche et al. 2005). Here, we investigated the impact of heat stress at different temperatures on the extent of HSF1 hyperphosphorylation. Indeed, with western blotting using HSF1 specific antibody we detected a characteristic upward band shift which was proportionate to elevated temperatures (Figure 30). This upward shift is typical for phosphorylated proteins. As mentioned before, the level of the transcription of both *hsp25* and *hsp70* were reduced by the Rac1 inhibitors. To test the role of Rac1 on HSF1 activation, we applied both NSC and 2-Brp inhibitors to B16F10 cells prior to heat treatments. Our results showed that neither NSC nor 2-Brp modulated the heat-induced level of HSF1 phosphorylation (Figure 30).

Similar to our findings, earlier Han et al. (2001) investigated the role of Rac1 in activation of JNK and HSF1 and found that even though dominant negative forms of Rac1 (Rac1N17) exerted inhibitory effect on HSF1 activation, there was no inhibition on phosphorylation of HSF1 under heat stress conditions (Han et al. 2001). That study concluded that such a Rac1-mediated effect on heat shock gene transcription may not be directly linked to HSF1 phosphorylation. Later, Nagy et al. (2007) measured a residual inducibility for *hsp70* and *hsp25* in Hsf1-/- MEF cells and provided supportive results for the affirmation of the operation of HSF1-independent, alternative and hitherto unexplored HSP-signaling pathways (Nagy et al. 2007).

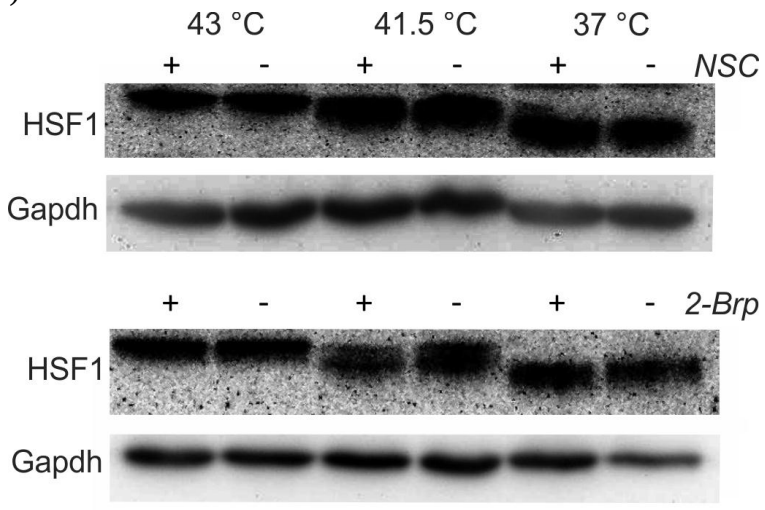


Figure 30. Effects of Rac1 inhibition on phosphorylation level of HSF1. B16F10 cells were treated or not with inhibitors (NSC or 2-Brp) prior to the indicated heat shock conditions. Immediately afterwards, samples were harvested and equal amounts were used for western blotting. Membranes were probed with anti-HSF1 and anti-Gapdh antibodies.

The DNA-binding and transactivation capacity of HSF1 depend on the regulation of multiple posttranslational modifications (phosphorylation, acetylation), protein–protein interactions and subcellular localization (Akerfelt et al. 2010). Gerber et al. (2005) reported that heat shock gene expression can be controlled at the level of promoter clearance. Hence, the expression of *hsp* genes should be particularly sensitive to the cellular level of Pol II elongation factors. Various Pol II elongation factors are recruited to sites of heat shock gene expression during heat shock (Gerber et al. 2005).

Recently it was proposed that activation of HSF1 requires a ribonucleoprotein complex containing the translation elongation factor 1α (eEF1α) and a novel non-coding RNA, called heat shock RNA-1 (HSR1), during HS (Shamovsky et al. 2006; Calderwood et al. 2010; Balogh et al. 2013). However, the signaling pathways responsible for RNA polymerase activation and the involvement of RNAs in stress sensing remain to be elucidated. Taken together, we fully agree with Akerfelt et al. (2010) that "the activation and attenuation mechanisms of HSFs require additional mechanistic insight. The roles of the multiple signal transduction pathways involved in the post-translational regulation of HSFs are only now being discovered and are clearly more complex than anticipated" (Akerfelt et al. 2010).

4.9. p38MAP Kinase is at the downstream of the Rac1-dependent stress signaling pathway

In order to interpret the different extracellular stimuli, cells need to develop sophisticated systems, so that they create and transmit the signals to respond appropriately. Among those, p38MAPK signaling is reported to have a role under stress conditions such as oxidative stress, heat stress and UV (Cuadrado & Nebreda 2010). Cellular stress involves the contribution of Rac1 activation and membrane association as upstream events of p38/SAPK2 pathway (Nègre-Aminou et al. 2002). In the present context, we intended to examine whether the p38MAPK signaling pathway is involved in Rac1 mediated HSR. For this reason, mild and severe heat shock treatments were carried out with or without the administration of Rac1 specific inhibitor, NSC. Immediately after heat shock, samples were collected and western blotting was run against p-p38MAPK antibody. Very low levels of p38MAPK phosphorylation were detected in control cells. Mild heat and mild heat combined with BGP15 did not show any increase on p38MAPK phosphorylation, however severe heat at 43 °C caused a visible elevation of p38MAPK phosphorylation levels. Interestingly and unexpectedly pre-exposure of cells to NSC significantly enhanced the p38MAPK

phosphorylation under mild heat shock conditions. By contrary, severe heat treatment did not exert any alteration on phosphorylation levels of p38MAPK in the presence of NSC (Figure 31).

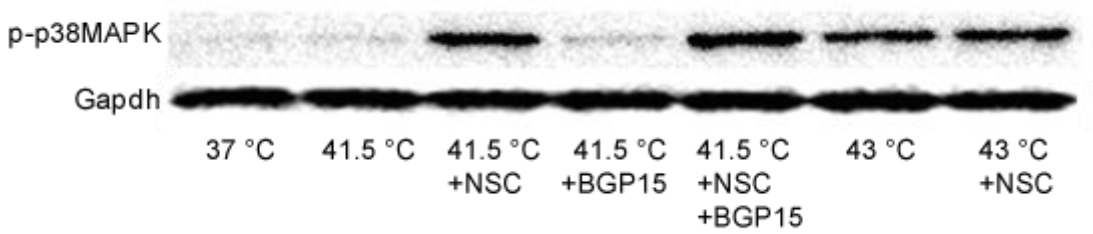


Figure 31. Effect of Rac1 inhibition by NSC on phosphorylation of p38MAPK under heat shock conditions. B16F10 cells were treated or not with BGP15 prior to the indicated heat shock conditions. Right after, samples were harvested and equal amounts were used for western blotting. Membrane was probed with anti-phospho p38MAPK antibody and normalized with anti-Gapdh antibody.

Earlier, Aminou et al. (2002) studied the role of HMG-CoA reductase inhibitor simvastatin on Rac1 activation, upon H_2O_2 mediated oxidative stress in human smooth muscle cells (SMC) (Nègre-Aminou et al. 2002). When cells were exposed to oxidative stress, Rac1 membrane localization was detected. Pre-treatment with simvastatin resulted in an inhibition of Rac1 membrane localization under oxidative stress condition. Surprisingly, oxidative stress induced phosphorylation of p38MAPK was enhanced by simvastatin pre-treatment. Simvastatin is known for inhibiting the synthesis of cholesterol and iso-prenoids, and as it inhibits farnesyl and geranyl-geranyl production, it has obviously a serious consequence on protein prenylation. As discussed above, prenylation is crucial for the proper membrane localization and thereby activity of small GTPases, including Rac1 (Henneman et al. 2010; Wei et al. 2013).

Accordingly, we investigated the impact of Rac1 activity on p38MAPK by administration of NSC, prior to heat stress. Our result underlines that inhibition of Rac1 activity by NSC eventuates an increase of p38MAPK phosphorylation (Figure 31). Importantly, Calderwood et al. (2010) reported that phosphorylation of p38MAPK induces MK2 activation, which phosphorylates HSP27 to increase its molecular chaperone function as well as HSF1 on serine 121, inhibiting its transcriptional activity (Calderwood et al. 2010). Therefore we assume that the strikingly reduced phosphorylation levels of p38MAPK due to NSC administration along with mild heat shock treatments may cause the phosphorylation of

HSF1 on this inhibitory site in the course of our studies ultimately resulting in reduced HSR. Since HSF1 has multiple phosphorylation sites (11 known up to now) therefore we cannot be sure whether the activated p38MAPK did or did not phosphorylate HSF1 on serine 121. We can also speculate that the operation of other phosphorylations/dephosphorylations at the same time can mask this particular phosphorylation in our experiments. This can well explain the fact that we were unable to detect changes while monitored the overall level of HSF1 hyperphosphorylation (Figure 30). Obviously, further detailed studies are needed to elucidate the potential function of p38MAPK pathway in the mechanism of HSF1 activation.

5. SUMMARY OF FINDINGS

The most important findings obtained during the current thesis work are summarized as follows:

- Rac1 is a membrane binding protein and stress conditions tested clearly induced Rac1 PM association. Functionally inactive Rac1 can attain membrane binding meaning that though activity requires membrane binding, but membrane binding does not go "hand in hand" with activity. Palmitoylation of Rac1 is a prerequisite for its PM localization.

- Heat stress conditions cause PM micro-domain enlargements. Palmitoylation of Rac1 can be one of the major regulatory steps for the appearance of larger, more stable membrane micro-domains, acting as stress signaling platforms, upon heat stress

- Severe heat shock conditions are resulted in F-actin filament fragmentation. Rac1 is one of the important downstream elements of cytoskeletal organization important in cell shape alterations as its inhibition saves intact F-actin and conserves the cell shape under severe heat stress conditions.

- Activation of Rac1 influences the inducible *hsp25* and *hsp70* expressions both at mRNA and protein levels under mild heat stress conditions, which also trigger PM Rac1 localization to the L_o micro-domains. Rac1 also controls the operation of BGP15-mediated HSP activation, via acting as raft-stabilizer. Rac1 specific inhibitor NSC23766 and palmitoylation inhibitor 2-Brp can diminish the elevated *hsp25* and *hsp70* expressions under both mild and severe heat shock conditions. Moreover BGP15 mediated increase at the *hsp25* and *hsp70* expression levels under mild heat conditions can be strongly attenuated by NSC23766.

- Rac1 regulates the phosphorylation levels of p38MAPK under mild heat conditions. Although data obtained doesn't confirm any change in Rac1-mediated HSF1 hyperphosphorylation but we can assume that active p38MAPK may exhibit inhibiting phosphorylation on HSF1 which in turn may result in decreased HSP response. Elucidation of the exact mechanism of Rac1-mediated HSP regulation needs therefore further investigations.

6. REFERENCES

Ahn, S. & Thiele, D.J., 2003. Redox regulation of mammalian heat shock factor 1 is essential for Hsp gene activation and protection from stress. *Genes & development*, 17, pp.516–528.

Akerfelt, M., Morimoto, R.I. & Sistonen, L., 2010. Heat shock factors: integrators of cell stress, development and lifespan. *Nature Reviews Molecular Cell Biology*, 11(8), pp.545–555.

Akunuru, S. et al., 2011. Rac1 targeting suppresses human non-small cell lung adenocarcinoma cancer stem cell activity. *PloS one*, 6(2), p.e16951.

Allen, J. a, Halverson-Tamboli, R. a & Rasenick, M.M., 2007. Lipid raft microdomains and neurotransmitter signalling. *Nature reviews Neuroscience*, 8(2), pp.128–140.

Ambily Nath, I. V et al., 2011. Diversity in transcripts and translational pattern of stress proteins in marine extremophiles. *Extremophiles*, 15(2), pp.129–53.

Anchisi, L. et al., 2012. Cholesterol homeostasis: a key to prevent or slow down neurodegeneration. *Frontiers in physiology*, 3, p.486.

Anckar, J. & Sistonen, L., 2011. Regulation of HSF1 function in the heat stress response: implications in aging and disease. *Annual review of biochemistry*, 80, pp.1089–1115.

Balasubramanian, N. et al., 2007. Arf6 and microtubules in adhesion-dependent trafficking of lipid rafts. *Nature Cell Biology*, 9(12), pp.1381–1391.

Baler, R., Welch, W.J. & Voellmy, R., 1992. Heat shock gene regulation by nascent polypeptides and denatured proteins: hsp70 as a potential autoregulatory factor. *The Journal of cell biology*, 117(6), pp.1151–1159.

Balogh, G. et al., 2013. Key role of lipids in heat stress management. *FEBS Letters*, 587, pp.1970–1980.

Balogh, G. et al., 2010. Lipidomics reveals membrane lipid remodelling and release of potential lipid mediators during early stress responses in a murine melanoma cell line. *Biochimica et biophysica acta*, 1801(9), pp.1036–1047.

Balogh, G. et al., 2005. The hyperfluidization of mammalian cell membranes acts as a signal to initiate the heat shock protein response. *The FEBS journal*, 272(23), pp.6077–6086.

Bartelt-Kirbach, B. & Golenhofen, N., 2014. Reaction of small heat-shock proteins to different kinds of cellular stress in cultured rat hippocampal neurons. *Cell stress & chaperones*, 19(1), pp.145–153.

Van Bergen en Henegouwen, P.M. et al., 1985. Studies on a possible relationship between alterations in the cytoskeleton and induction of heat shock protein synthesis in mammalian cells. *International journal of hyperthermia* , 1(1), pp.69–83.

Beuthan, J., Dressler, C. & Minet, O., 2004. Laser induced fluorescence detection of quantum dots redistributed in thermally stressed tumor cells. *Laser Physics*, 14(2), pp.213–219.

Bishop, A.L. & Hall, A., 2000. Rho GTPases and their effector proteins. *The Biochemical journal*, 348 (Pt 2), pp.241–255.

Bornfeldt, K.E., 2000. Stressing Rac, Ras, and Downstream Heat Shock Protein 70. *Circulation Research*, 86(11), pp.1101–1103.

Bouquier, N. et al., 2009. A cell active chemical GEF inhibitor selectively targets the Trio/RhoG/Rac1 signaling pathway. *Chemistry & biology*, 16(6), pp.657–666.

Brameshuber, M. et al., 2010. Imaging of mobile long-lived nanoplatforms in the live cell plasma membrane. *The Journal of biological chemistry*, 285(53), pp.41765–41771.

Brignull, H.R., Morley, J.F. & Morimoto, R.I., 2007. The stress of misfolded proteins: C. elegans models for neurodegenerative disease and aging. *Advances in experimental medicine and biology*, 594, pp.167–189.

Bukau, B. & Horwich, a L., 1998. The Hsp70 and Hsp60 chaperone machines. *Cell*, 92(3), pp.351–366.

Burridge, K. & Wittchen, E.S., 2013. The tension mounts: stress fibers as force-generating mechanotransducers. *The Journal of cell biology*, 200(1), pp.9–19.

Bustelo, X.R. et al., 2012. Rac-ing to the plasma membrane: the long and complex work commute of Rac1 during cell signaling. *Small GTPases*, 3(1), pp.60–66.

Calderwood, S.K. et al., 2010. Signal Transduction Pathways Leading to Heat shock Transcription. *Signal Transduction Insights*, 2, pp.13–24.

Castro-Castro, A. et al., 2011. Coronin 1A promotes a cytoskeletal-based feedback loop that facilitates Rac1 translocation and activation. *The EMBO journal*, 30(19), pp.3913–27.

Cates, J. et al., 2011. Sensing the heat stress by Mammalian cells. *BMC biophysics*, 4(1), p.16.

Chae, Y.C. et al., 2008. Phospholipase D activity regulates integrin-mediated cell spreading and migration by inducing GTP-Rac translocation to the plasma membrane. *Molecular biology of the cell*, 19(7), pp.3111–3123.

Choy, E. et al., 1999. Endomembrane trafficking of ras: the CAAX motif targets proteins to the ER and Golgi. *Cell*, 98(1), pp.69–80.

Chung, J. et al., 2008. HSP72 protects against obesity-induced insulin resistance. *Proceedings of the National Academy of Sciences of the United States of America*, 105(5), pp.1739–1744.

Ciocca, D.R. & Calderwood, S.K., 2005. Heat shock proteins in cancer: diagnostic, prognostic, predictive, and treatment implications. *Cell stress & chaperones*, 10(2), pp.86–103.

Civelekoglu-Scholey, G. et al., 2005. Model of coupled transient changes of Rac, Rho, adhesions and stress fibers alignment in endothelial cells responding to shear stress. *Journal of Theoretical Biology*, 232(4), pp.569–585.

Clainche, C.L.E. & Carlier, M., 2008. Regulation of Actin Assembly Associated With Protrusion and Adhesion in Cell Migration. *American Physiological Society*, 88, pp.489–513.

Clare, D.K. & Saibil, H.R., 2013. ATP-driven molecular chaperone machines. *Biopolymers*, 99(11), pp.846–859.

Clarke, J.P. & Mearow, K.M., 2013. Cell stress promotes the association of phosphorylated HspB1 with F-actin. *PloS one*, 8(7), p.e68978.

Coakley, W.T., 1987. Hyperthermia effects on the cytoskeleton and on cell morphology. *Symposia of the Society for Experimental Biology*, 41, pp.187–211.

Cordwell, S.J. & Thingholm, T.E., 2010. Technologies for plasma membrane proteomics. *Proteomics*, 10(4), pp.611–627.

Crul, T. et al., 2013. Hydroximic Acid Derivatives: Pleiotrophic Hsp Co-Inducers Restoring Homeostasis and Robustness. *Current Pharmaceutical Design*, 19(3), pp.309–346.

Cuadrado, A. & Nebreda, A.R., 2010. Mechanisms and functions of p38 MAPK signalling. *The Biochemical journal*, 429(3), pp.403–417.

Dai, C. et al., 2007. Heat shock factor 1 is a powerful multifaceted modifier of carcinogenesis. *Cell*, 130(6), pp.1005–1018.

Daugaard, M., Rohde, M. & Jäättelä, M., 2007. The heat shock protein 70 family: Highly homologous proteins with overlapping and distinct functions. *FEBS letters*, 581(19), pp.3702–3710.

Davda, D. et al., 2013. Profiling targets of the irreversible palmitoylation inhibitor 2-bromopalmitate. *ACS chemical biology*, 8(9), pp.1912–1917.

Davis, M.J. et al., 2013. RAC1P29S is a spontaneously activating cancer-associated GTPase. *Proceedings of the National Academy of Sciences of the United States of America*, 110(3), pp.912–917.

Didsbury, J. et al., 1989. Rac, a Novel Ras-Related Family of Proteins That Are Botulinum Toxin Substrates. *The Journal of biological chemistry*, 264(28), pp.16378–16382.

Dokmanovic, M. et al., 2009. Rac1 contributes to trastuzumab resistance of breast cancer cells: Rac1 as a potential therapeutic target for the treatment of trastuzumab-resistant breast cancer. *Molecular cancer therapeutics*, 8(6), pp.1557–1569.

Dominguez, R. & Holmes, K.C., 2011. Actin Structure and Function. *Annual Reviews of Biophysics*, 40, pp.169–186.

Doshi, B.M., Hightower, L.E. & Lee, J., 2009. The role of Hsp27 and actin in the regulation of movement in human cancer cells responding to heat shock. *Cell stress & chaperones*, 14(5), pp.445–457.

Dragovic, Z. et al., 2006. Molecular chaperones of the Hsp110 family act as nucleotide exchange factors of Hsp70s. *The EMBO journal*, 25(11), pp.2519–2528.

Dressler, C. et al., 2013. Microscopical heat stress investigations under application of quantum dots. *Journal of biomedical optics*, 10(4), p.41209.

Echarri, A., Muriel, O. & Del Pozo, M. a, 2007. Intracellular trafficking of raft/caveolae domains: insights from integrin signaling. *Seminars in cell & developmental biology*, 18(5), pp.627–37.

Edidin, M., 2003. Lipids on the frontier: a century of cell-membrane bilayers. *Nature reviews Molecular cell biology*, 4(5), pp.414–418.

Ellenbroek, S.I.J. & Collard, J.G., 2007. Rho GTPases: functions and association with cancer. *Clinical & experimental metastasis*, 24(8), pp.657–672.

Escribá, P. V et al., 2008. Membranes: a meeting point for lipids, proteins and therapies. *Journal of Cellular and Molecular Medicine*, 12(3), pp.829–875.

Finka, A., Mattoo, R.U.H. & Goloubinoff, P., 2011. Meta-analysis of heat- and chemically upregulated chaperone genes in plant and human cells. *Cell stress & chaperones*, 16(1), pp.15–31.

Flinder, L.I. et al., 2011. EGF-induced ERK-activation downstream of FAK requires rac1-NADPH oxidase. *Journal of cellular physiology*, 226(9), pp.2267–2278.

Frixione, E., 2000. Recurring views on the structure and function of the cytoskeleton: a 300-year epic. *Cell motility and the cytoskeleton*, 46(2), pp.73–94.

Fujimoto, M. & Nakai, A., 2010. The heat shock factor family and adaptation to proteotoxic stress. *FEBS Journal*, 277(20), pp.4112–4125.

Fulda, S. et al., 2010. Cellular stress responses: cell survival and cell death. *International journal of cell biology*, 2010, p.214074.

Gagat, M. et al., 2013. The Effect of Mild Hyperthermia on Morphology, Ultrastructure and F-Actin Organization in HL-60 Cell Line. *Medical and Biological Sciences*, 27(2), pp.19–25.

De Gannes, F.M. et al., 1998. Metabolic and cellular characterization of immortalized human microglial cells under heat stress. *Neurochemistry international*, 33(1), pp.61–73.

Gao, Y. et al., 2004. Rational design and characterization of a Rac GTPase-specific small molecule inhibitor. *Proceedings of the National Academy of Sciences of the United States of America*, 101(20), pp.7618–23.

Gao, Y. et al., 2001. Trp(56) of rac1 specifies interaction with a subset of guanine nucleotide exchange factors. *The Journal of biological chemistry*, 276(50), pp.47530–47541.

Garcia, M.P., Cavalheiro, J.R.T. & Fernandes, M.H., 2012. Acute and long-term effects of hyperthermia in B16-F10 melanoma cells. *PloS one*, 7(4), p.e35489.

Gerber, M. et al., 2005. Regulation of heat shock gene expression by RNA polymerase II elongation factor, Elongin A. *The Journal of biological chemistry*, 280(6), pp.4017–4020.

Glass, J.R., Dewitt, R.G. & Cress, A.E., 1985. Rapid Loss of Stress Fibers in Chinese Hamster Ovary Cells after Hyperthermia. *Cancer research*, 45, pp.258–262.

Golub, T. & Caroni, P., 2005. PI(4,5)P2-dependent microdomain assemblies capture microtubules to promote and control leading edge motility. *The Journal of cell biology*, 169(1), pp.151–165.

Gombos, I. et al., 2011. Membrane-lipid therapy in operation: the HSP co-inducer BGP-15 activates stress signal transduction pathways by remodeling plasma membrane rafts. *PloS one*, 6(12), p.e28818.

Goni, F.M. et al., 2009. Phase diagrams of lipid mixtures relevant to the study of membrane rafts. *Biochim Biophys Acta*, 1781(11-12), pp.665–684.

Goodwin, J.S. et al., 2005. Depalmitoylated Ras traffics to and from the Golgi complex via a nonvesicular pathway. *The Journal of cell biology*, 170(2), pp.261–272.

Greaves, J. et al., 2009. The fat controller: roles of palmitoylation in intracellular protein trafficking and targeting to membrane microdomains (Review). *Molecular membrane biology*, 26(1), pp.67–79.

Grizot, S. et al., 2001. Crystal Structure of the Rac1 - RhoGDI Complex Involved in NADPH Oxidase Activation. *Biochemistry 2001,*, 40, pp.10007–10013.

Gudheti, M. V, Mlodzianoski, M. & Hess, S.T., 2007. Imaging and shape analysis of GUVs as model plasma membranes: effect of trans DOPC on membrane properties. *Biophysical journal*, 93(6), pp.2011–2023.

Guettouche, T. et al., 2005. Analysis of phosphorylation of human heat shock factor 1 in cells experiencing a stress. *BMC biochemistry*, 6, p.4.

Gupta, S.C. et al., 2010. Heat shock proteins in toxicology: how close and how far? *Life sciences*, 86(11-12), pp.377–384.

Haigis, M.C. & Yankner, B.A., 2011. The Aging Stress Response. *Molecular Cell*, 40(2), pp.333–344.

Han, S.I. et al., 2001. Implication of a small GTPase Rac1 in the activation of c-Jun N-terminal kinase and heat shock factor in response to heat shock. *The Journal of biological chemistry*, 276(3), pp.1889–1895.

Hartl, F.U., 2011. Chaperone-assisted protein folding: the path to discovery from a personal perspective. *Nature medicine*, 17(10), pp.1206–1210.

Haynie, D.T., 2014. Molecular Physiology of the Tensin Brotherhood of Integrin Adaptor Proteins. *Proteins*.

Head, B.P., Patel, H.H. & Insel, P. A, 2014. Interaction of membrane/lipid rafts with the cytoskeleton: impact on signaling and function: membrane/lipid rafts, mediators of cytoskeletal arrangement and cell signaling. *Biochimica et biophysica acta*, 1838(2), pp.532–545.

Henneman, L. et al., 2010. Compromized geranylgeranylation of RhoA and Rac1 in mevalonate kinase deficiency. *Journal of inherited metabolic disease*, 33(5), pp.625–632.

Hernandez, E. et al., 2010. Novel Inhibitors of Rac1 in Metastatic Breast Cancer. *Puerto Rico Health Sciences Journal*, 29(4), pp.348–356.

Hetz, C., Chevet, E. & Harding, H.P., 2013. Targeting the unfolded protein response in disease. *Nature reviews Drug discovery*, 12(9), pp.703–719.

Higashi, M. et al., 2010. Visualization of the Activity of Rac1 Small GTPase in a Cell. *Acta histochemica et cytochemica*, 43(6), pp.163–168.

Hildebrandt, B. et al., 2002. The cellular and molecular basis of hyperthermia. *Critical reviews in oncology/hematology*, 43(1), pp.33–56.

Hooper, P.L. et al., 2014. The importance of the cellular stress response in the pathogenesis and treatment of type 2 diabetes. *Cell stress & chaperones*.

Horváth, I. et al., 2012. Heat shock response in photosynthetic organisms: membrane and lipid connections. *Progress in lipid research*, 51(3), pp.208–220.

Horváth, I. et al., 1998. Membrane physical state controls the signaling mechanism of the heat shock response in Synechocystis PCC 6803: identification of hsp17 as a "fluidity gene". *Proceedings of the National Academy of Sciences of the United States of America*, 95(7), pp.3513–3518.

Horváth, I. et al., 2008. Membrane-associated stress proteins: more than simply chaperones. *Biochimica et biophysica acta*, 1778(7-8), pp.1653–1664.

Horváth, I. & Vígh, L., 2010. Cell biology: Stability in times of stress. *Nature*, 463(7280), pp.436–438.

Huang, S.H. et al., 1999. Effects of hyperthermia on the cytoskeleton and focal adhesion proteins in a human thyroid carcinoma cell line. *Journal of cellular biochemistry*, 75(2), pp.327–337.

Issels, R.D., 2008. Hyperthermia adds to chemotherapy. *European journal of cancer*, 44(17), pp.2546–2554.

Itoh, R.E. et al., 2008. Phosphorylation and activation of the Rac1 and Cdc42 GEF Asef in A431 cells stimulated by EGF. *Journal of cell science*, 121(Pt 16), pp.2635–2642.

Jack, E.R. et al., 2008. Membrane interactions of peptides representing the polybasic regions of three Rho GTPases are sensitive to the distribution of arginine and lysine residues. *Molecular membrane biology*, 25(1), pp.14–22.

Jego, G. et al., 2013. Targeting heat shock proteins in cancer. *Cancer letters*, 332(2), pp.275–285.

Kampinga, H.H. et al., 2009. Guidelines for the nomenclature of the human heat shock proteins. *Cell stress & chaperones*, 14(1), pp.105–11.

Kampinga, H.H. & Craig, E.A., 2011. The Hsp70 chaperone machinery: J-proteins as drivers of functional specificity. *Nature Reviews Molecular Cell Biology*, 11(8), pp.579–592.

Keren, K. et al., 2008. Mechanism of shape determination in motile cells. *Nature*, 453(7194), pp.475–480.

Klotzsch, E. & Schütz, G.J., 2013. A critical survey of methods to detect plasma membrane rafts. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 368(1611), p.20120033.

Kovac, B. et al., 2013. Assembly of non-contractile dorsal stress fibers requires α-actinin-1 and Rac1 in migrating and spreading cells. *Journal of cell science*, 126(Pt 1), pp.263–273.

Krauthammer, M. et al., 2012. Exome sequencing identifies recurrent somatic RAC1 mutations in melanoma. *Nature genetics*, 44(9), pp.1006–1014.

Kugel, J.F. & Goodrich, J. a, 2006. Beating the heat: A translation factor and an RNA mobilize the heat shock transcription factor HSF1. *Molecular cell*, 22(2), pp.153–154.

Kultz, D., 2003. Evolution of the cellular stress proteome: from monophyletic origin to ubiquitous function. *Journal of Experimental Biology*, 206(18), pp.3119–3124..

Kültz, D., 2005. Molecular and evolutionary basis of the cellular stress response. *Annual review of physiology*, 67(1), pp.225–257.

Kumar, A. et al., 2013. Molecular dynamic simulation reveals damaging impact of RAC1 F28L mutation in the switch I region. *PloS one*, 8(10), p.e77453.

Kusumi, A. et al., 2012. Dynamic organizing principles of the plasma membrane that regulate signal transduction: commemorating the fortieth anniversary of Singer and Nicolson's fluid-mosaic model. *Annual review of cell and developmental biology*, 28, pp.215–50.

Kusumi, A. et al., 2011. Hierarchical mesoscale domain organization of the plasma membrane. *Trends in biochemical sciences*, 36(11), pp.604–615.

Kwiatkowska, K., 2010. One lipid, multiple functions: how various pools of PI(4,5)P(2) are created in the plasma membrane. *Cellular and molecular life sciences*, 67(23), pp.3927–3946.

Kwik, J. et al., 2003. Membrane cholesterol, lateral mobility, and the phosphatidylinositol 4,5-bisphosphate-dependent organization of cell actin. *Proceedings of the National Academy of Sciences of the United States of America*, 100(24), pp.13964–13969.

Lawson, C.D. & Burridge, K., 2014. The on-off relationship of Rho and Rac during integrin-mediated adhesion and cell migration. *Small GTPases*, 5(1).

Leung, A.K.L. & Sharp, P.A., 2011. MicroRNA Functions in Stress Responses. *Molecular Cell*, 40(2), pp.205–215.

Li, J. & Buchner, J., 2012. Structure, function and regulation of the hsp90 machinery. *Biomedical journal*, 36(3), pp.106–117.

Li, Y., Zhang, T. & Sun, D., 2010. New developments in Hsp90 inhibitors as anti-cancer therapeutics: mechanisms, clinical perspective and more potential. *Drug Resistance updates*, 12, pp.17–27.

Lindquist, S., 1986. The heat-shock response. *Annual review of biochemistry*, 55, pp.1151–1191.

Lingwood, D. & Simons, K., 2010. Lipid rafts as a membrane-organizing principle. *Science*, 327(5961), pp.46–50.

Literáti-Nagy, Z. et al., 2012. The HSP co-inducer BGP-15 can prevent the metabolic side effects of the atypical antipsychotics. *Cell stress & chaperones*, 17(4), pp.517–521.

Madhivanan, K., Mukherjee, D. & Aguilar, R.C., 2012. Lowe syndrome Between primary cilia assembly and Rac1-mediated membrane remodeling. *Communicative & Interactive Biology*, 5(6), pp.641–644.

Margadant, C. et al., 2011. Mechanisms of integrin activation and trafficking. *Current opinion in cell biology*, 23(5), pp.607–614.

Mátés, L. et al., 2009. Molecular evolution of a novel hyperactive Sleeping Beauty transposase enables robust stable gene transfer in vertebrates. *Nature genetics*, 41(6), pp.753–761.

Mayer, M.P. & Bukau, B., 2005. Hsp70 chaperones: cellular functions and molecular mechanism. *Cellular and molecular life sciences*, 62(6), pp.670–684.

Van Meer, G., Voelker, D.R. & Feigenson, G.W., 2008. Membrane lipids : where they are and how they behave. *Nature Reviews Molecular Cell Biology*, 9(2), pp.112–124.

Michaelson, D. et al., 2001. Differential localization of Rho GTPases in live cells: regulation by hypervariable regions and RhoGDI binding. *The Journal of cell biology*, 152(1), pp.111–126.

Michaelson, D. et al., 2008. Rac1 accumulates in the nucleus during the G2 phase of the cell cycle and promotes cell division. *The Journal of cell biology*, 181(3), pp.485–496.

Misra, A. et al., 2012. Hypoxia activated EGFR signaling induces epithelial to mesenchymal transition (EMT). *PloS one*, 7(11), p.e49766.

Mogk, A. et al., 2008. Common and specific mechanisms of AAA+ proteins involved in protein quality control. *Biochemical Society transactions*, 36(Pt 1), pp.120–125.

Moissoglu, K. et al., 2006. In vivo dynamics of Rac-membrane interactions. *Molecular biology of the cell*, 17(6), pp.2770–2779.

Morimoto, R.I., 2008. Proteotoxic stress and inducible chaperone networks in neurodegenerative disease and aging. *Genes & development*, 22(11), pp.1427–1438.

Morimoto, R.I., 1998. Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. *Genes & development*, 12(24), pp.3788–3796.

Mounier, N. & Arrigo, A.-P., 2002. Actin cytoskeleton and small heat shock proteins: how do they interact? *Cell stress & chaperones*, 7(2), pp.167–176.

Mukherjee, A. et al., 2009. Retinoic acid-induced gene-1 (RIG-I) associates with the actin cytoskeleton via caspase activation and recruitment domain-dependent interactions. *The Journal of biological chemistry*, 284(10), pp.6486–6494.

Nadeau, S.I. & Landry, J., 2007. Mechanisms of Activation and Regulation of the Heat Shock-Sensitive Signaling Pathways. In P. Csermely & L. Vigh, eds. *Molecular Aspects of the Stress Response: Chaperones, Membranes and Networks*. pp. 100–113.

Nagy, E. et al., 2007. Hyperfluidization-coupled membrane microdomain reorganization is linked to activation of the heat shock response in a murine melanoma cell line. *Proceedings of the National Academy of Sciences of the United States of America*, 104(19), pp.7945–7950.

Navarro-Lérida, I. et al., 2012. A palmitoylation switch mechanism regulates Rac1 function and membrane organization. *The EMBO journal*, 31(3), pp.534–551.

Nègre-Aminou, P. et al., 2002. Differential effect of simvastatin on activation of Rac(1) vs. activation of the heat shock protein 27-mediated pathway upon oxidative stress, in human smooth muscle cells. *Biochemical pharmacology*, 64(10), pp.1483–1491.

Niforou, K., Cheimonidou, C. & Trougakos, I.P., 2014. Molecular chaperones and proteostasis regulation during redox imbalance. *Redox Biology*, 2, pp.323–332.

Noonan, E.J. et al., 2007. Cell number-dependent regulation of Hsp70B' expression: evidence of an extracellular regulator. *Journal of cellular physiology*, 210(1), pp.201–211.

Oh, H.J., 1999. The Chaperoning Activity of hsp110. *Journal of Biological Chemistry*, 274(22), pp.15712–15718.

Ozaki, M. et al., 2000. Rac1 regulates stress-induced, redox-dependent heat shock factor activation. *The Journal of biological chemistry*, 275(45), pp.35377–35383.

Pai, S.-Y., Kim, C. & Williams, D. a, 2010. Rac GTPases in human diseases. *Disease markers*, 29(3-4), pp.177–187.

Palamidessi, A. et al., 2008. Endocytic trafficking of Rac is required for the spatial restriction of signaling in cell migration. *Cell*, 134(1), pp.135–147.

Papaharalambus, C. et al., 2005. Tumor necrosis factor alpha stimulation of Rac1 activity. Role of isoprenylcysteine carboxylmethyltransferase. *The Journal of biological chemistry*, 280(19), pp.18790–18796.

Park, H.G. et al., 2005. Cellular responses to mild heat stress. *Cellular and molecular life sciences*, 62(1), pp.10–23.

Parri, M. & Chiarugi, P., 2010. Rac and Rho GTPases in cancer cell motility control. *Cell communication and signaling*, 8, p.23.

Parton, R.G. & del Pozo, M. a, 2013. Caveolae as plasma membrane sensors, protectors and organizers. *Nature reviews. Molecular cell biology*, 14(2), pp.98–112.

Patra, S.K., 2008. Dissecting lipid raft facilitated cell signaling pathways in cancer. *Biochimica et biophysica acta*, 1785(2), pp.182–206.

Pawlik, A. et al., 2013. Hyperthermia induces cytoskeletal alterations and mitotic catastrophe in p53-deficient H1299 lung cancer cells. *Acta histochemica*, 115(1), pp.8–15.

Péter, M. et al., 2012. Nutritional lipid supply can control the heat shock response of B16 melanoma cells in culture. *Molecular membrane biology*, 29(7), pp.274–289.

Petrozzi, L. et al., 2007. Mitochondria and neurodegeneration. *Bioscience reports*, 27(1-3), pp.87–104.

Pike, L.J., 2006. Rafts defined: a report on the Keystone Symposium on Lipid Rafts and Cell Function. *Journal of lipid research*, 47(7), pp.1597–1598.

Pike, L.J., 2009. The challenge of lipid rafts. *Journal of lipid research*, 50 Suppl, pp.S323–328.

Pivovarova, A. V et al., 2005. Effects of small heat shock proteins on the thermal denaturation and aggregation of F-actin. *Biochemical and biophysical research communications*, 331(4), pp.1548–1553.

Pollard, T.D. & Cooper, J. a, 2009. Actin, a central player in cell shape and movement. *Science*, 326(5957), pp.1208–1212.

Powers, M. V & Workman, P., 2007. Inhibitors of the heat shock response: biology and pharmacology. *FEBS letters*, 581(19), pp.3758–3769.

Del Pozo, M. a et al., 2004. Integrins regulate Rac targeting by internalization of membrane domains. *Science*, 303(5659), pp.839–842.

Del Pozo, M.A. & Schwartz, M.A., 2007. Rac, membrane heterogeneity, caveolin and regulation of growth by integrins. *Trends in cell biology*, 17(5), pp.246–250.

Raucher, D. & Sheetz, M.P., 2000. Cell spreading and lamellipodial extension rate is regulated by membrane tension. *The Journal of cell biology*, 148(1), pp.127–136.

Richter, K., Haslbeck, M. & Buchner, J., 2010. The heat shock response: life on the verge of death. *Molecular cell*, 40(2), pp.253–266.

Ritossa, F., 1962. A new puffing pattern induced by temperature shock and DNP in drosophila. *Experientia*, 18(12), pp.571–573.

Roberts, P.J. et al., 2008. Rho Family GTPase modification and dependence on CAAX motif-signaled posttranslational modification. *The Journal of biological chemistry*, 283(37), pp.25150–25163.

Rocks, O. et al., 2005. An acylation cycle regulates localization and activity of palmitoylated Ras isoforms. *Science*, 307(5716), pp.1746–1752.

Rohde, M. et al., 2005. Members of the heat-shock protein 70 family promote cancer cell growth by distinct mechanisms. *Genes & development*, 70, pp.570–582.

Sahai, E. & Marshall, C.J., 2002. RHO-GTPases and cancer. *Nature reviews. Cancer*, 2(2), pp.133–142.

Saibil, H., 2013. Chaperone machines for protein folding, unfolding and disaggregation. *Nature reviews. Molecular cell biology*, 14(10), pp.630–642.

Saidi, Y. et al., 2010. Membrane lipid composition affects plant heat sensing and modulates Ca 2+ -dependent heat shock response. *Plant Signaling & Behavior*, 5(12), pp.1530–1533.

Samali, A. & Orrenius, S., 1998. Heat shock proteins:regulators of stress response and apoptosis. *Cell stress & chaperons*, pp.228–236.

Schwarz, J. et al., 2012. Serine-71 phosphorylation of Rac1 modulates downstream signaling. *PloS one*, 7(9), p.e44358.

Selye, H., 1950. Stress and the general adaptation syndrome. *British medical journal*, 1(4667), pp.1383–92.

Shamaei-Tousi, A., Halcox, J.P. & Henderson, B., 2007. Stressing the obvious? Cell stress and cell stress proteins in cardiovascular disease. *Cardiovascular research*, 74(1), pp.19–28.

Shamovsky, I. et al., 2006. RNA-mediated response to heat shock in mammalian cells. *Nature*, 440(7083), pp.556–60.

Shamovsky, I. & Nudler, E., 2008. New insights into the mechanism of heat shock response activation. *Cellular and molecular life sciences*, 65(6), pp.855–861.

Simons, K. & Ikonen, E., 1997. Functional rafts in cell membranes. *Nature*, 387(6633), pp.569–572.

Simons, K. & Sampaio, J.L., 2011. Membrane organization and lipid rafts. *Cold Spring Harbor perspectives in biology*, 3(10), p.a004697.

Simons, K. & Toomre, D., 2000. Lipid rafts and signal transduction. *Nature reviews. Molecular cell biology*, 1(1), pp.31–9.

Singer, S.J. & Nicolson, G.L., 1972. The fluid mosaic model of the structure of cell membranes. *Science*, 175(4023), pp.720–731.

Steffen, A. et al., 2013. Rac function is crucial for cell migration but is not required for spreading and focal adhesion formation. *Journal of cell science*, 126(Pt 20), pp.4572–4588.

Sun, Y. & MacRae, T.H., 2005. Small heat shock proteins: molecular structure and chaperone function. *Cellular and molecular life sciences*, 62(21), pp.2460–2476.

Symons, M., 2011. Rac1 activation comes full circle. *The EMBO journal*, 30(19), pp.3875–3877.

Szalay, M.S. et al., 2007. Stress-induced rearrangements of cellular networks: Consequences for protection and drug design. *FEBS letters*, 581(19), pp.3675–3680.

Tojkander, S., Gateva, G. & Lappalainen, P., 2012. Actin stress fibers--assembly, dynamics and biological roles. *Journal of cell science*, 125(Pt 8), pp.1855–1864.

Török, Z. et al., 2014. Plasma membranes as heat stress sensors: From lipid-controlled molecular switches to therapeutic applications. *Biochimica et biophysica acta*, 1838(6), pp.1594–1618.

Tóth, M.E., Vígh, L. & Sántha, M., 2013. Alcohol stress, membranes, and chaperones. *Cell stress & chaperones*.

Truong-Quang, B.-A. & Lenne, P.-F., 2014. Membrane microdomains: from seeing to understanding. *Frontiers in plant science*, 5, p.18.

Tsai, F.D. & Philips, M.R., 2012. Rac1 gets fattier. *The EMBO journal*, 31(3), pp.517–518.

Vallenius, T., 2013. Actin stress fibre subtypes in mesenchymal-migration cells. *Open Biology*, 3(6), pp 130001.

Vasanji, A. et al., 2004. Polarization of plasma membrane microviscosity during endothelial cell migration. *Developmental cell*, 6(1), pp.29–41.

Velaithan, R. et al., 2011. The small GTPase Rac1 is a novel binding partner of Bcl-2 and stabilizes its antiapoptotic activity. *Blood*, 117(23), pp.6214–6226.

Vereb, G. et al., 2003. Dynamic, yet structured: The cell membrane three decades after the Singer-Nicolson model. *Proceedings of the National Academy of Sciences of the United States of America*, 100(14), pp.8053–8058.

Verghese, J. et al., 2012. Biology of the heat shock response and protein chaperones: budding yeast (Saccharomyces cerevisiae) as a model system. *Microbiology and molecular biology reviews*, 76(2), pp.115–158.

Vigh, L. et al., 2007. Can the stress protein response be controlled by "membrane-lipid therapy"? *Trends in biochemical sciences*, 32(8), pp.357–363.

Vigh, L. et al., 2005. The significance of lipid composition for membrane activity: new concepts and ways of assessing function. *Progress in lipid research*, 44(5), pp.303–344.

Vígh, L. et al., 2007. Membrane-regulated stress response: a theoretical and practical approach. *Advances in experimental medicine and biology*, 594, pp.114–131.

Vigh, L., Maresca, B. & Harwood, J.L., 1998. Does the membrane's physical state control the expression of heat shock and other genes? *Trends in biochemical sciences*, 23(10), pp.369–374.

Voellmy, R., 2005. Dominant-positive and dominant-negative heat shock factors. *Methods*, 35(2), pp.199–207.

Wang, T.T. et al., 1998. Concomitant alterations in distribution of 70 kDa heat shock proteins, cytoskeleton and organelles in heat shocked 9L cells. *The international journal of biochemistry & cell biology*, 30(6), pp.745–759.

Wang, X. et al., 2006. Phosphorylation of HSF1 by MAPK-activated protein kinase 2 on serine 121, inhibits transcriptional activity and promotes HSP90 binding. *The Journal of biological chemistry*, 281(2), pp.782–791.

Webb, Y., Hermida-Matsumoto, L. & Resh, M.D., 2000. Inhibition of Protein Palmitoylation, Raft Localization, and T Cell Signaling by 2-Bromopalmitate and Polyunsaturated Fatty Acids. *Journal of Biological Chemistry*, 275(1), pp.261–270.

Wei, Y.-M. et al., 2013. Enhancement of autophagy by simvastatin through inhibition of Rac1-mTOR signaling pathway in coronary arterial myocytes. *Cellular physiology and biochemistry* , 31(6), pp.925–937.

Welch, H.C.E. et al., 2003. Phosphoinositide 3-kinase-dependent activation of Rac. *FEBS Letters*, 546(1), pp.93–97.

Welch, W.J. & Suhan, J.P., 1985. Morphological study of the mammalian stress response: characterization of changes in cytoplasmic organelles, cytoskeleton, and nucleoli, and appearance of intranuclear actin filaments in rat fibroblasts after heat-shock treatment. *The Journal of cell biology*, 101(4), pp.1198–1211.

Wennerberg, K. & Der, C.J., 2004. Rho-family GTPases: it's not only Rac and Rho (and I like it). *Journal of cell science*, 117(Pt 8), pp.1301–1312.

Wertheimer, E. et al., 2012. Rac signaling in breast cancer: A tale of GEFs and GAPs. *Cellular Signaling*, 24(2), pp.353–362.

Wettstein, G. et al., 2012. Small heat shock proteins and the cytoskeleton: an essential interplay for cell integrity? *The international journal of biochemistry & cell biology*, 44(10), pp.1680–1686.

White, M.G. et al., 2012. Mitochondrial dysfunction induced by heat stress in cultured rat CNS neurons. *Journal of neurophysiology*, 108(8), pp.2203–2214.

Wiedmann, R.M. et al., 2012. The V-ATPase-inhibitor archazolid abrogates tumor metastasis via inhibition of endocytic activation of the Rho-GTPase Rac1. *Cancer research*, 72(22), pp.5976–5987.

Wiegant, F.A.C. et al., 1987. Stress-induced Thermotolerance of the Cytoskeleton of Mouse Neuroblastoma N2A Cells and Rat Reuber H35 Hepatoma Cells Stress-induced Thermotolerance of the Cytoskeleton of Mouse Neuroblastoma N2A. *Cancer research*, 47(6), pp.1674–1680.

Wojciak-Stothard, B. & Ridley, A.J., 2003. Shear stress-induced endothelial cell polarization is mediated by Rho and Rac but not Cdc42 or PI 3-kinases. *The Journal of cell biology*, 161(2), pp.429–439.

Wolfenson, H. et al., 2010. The heel and toe of the cell's foot: A multifaceted approach for Cell Motil Cytoskeleton. *Cell Motil Cytoskeleton*, 66(11), pp.1017–1029.

Woodcock, S. a et al., 2010. Tiam1-Rac signaling counteracts Eg5 during bipolar spindle assembly to facilitate chromosome congression. *Current biology*, 20(7), pp.669–675.

Xu, B., Chelikani, P. & Bhullar, R.P., 2012. Characterization and functional analysis of the calmodulin-binding domain of Rac1 GTPase. *PloS one*, 7(8), p.e42975.

Xu, Q. et al., 2000. Mechanical Stress-Induced Heat Shock Protein 70 Expression in Vascular Smooth Muscle Cells Is Regulated by Rac and Ras Small G Proteins but Not Mitogen-Activated Protein Kinases. *Circulation Research*, 86(11), pp.1122–1128.

Xu, Q. et al., 2012. Molecular chaperones and heat shock proteins in atherosclerosis. *American journal of physiology. Heart and circulatory physiology*, 302(3), pp.H506–H514.

Xu, Y. et al., 2012. Post-Translational Modi fi cation of Human Heat Shock Factors and Their Functions: A Recent Update by Proteomic Approach. *Journal of proteome research*, 11, pp.2625–2634.

Yan, Y. et al., 2012. RAC1 GTPase plays an important role in γ-irradiation induced G2/M checkpoint activation. *Breast cancer research*, 14(2), p.R60.

Young, J.C., 2010. Mechanisms of the Hsp70 chaperone system. *Biochemistry and cell biology*, 88(2), pp.291–300.

Yuan, L. et al., 2010. ERK activation of p21 activated kinase-1 (Pak1) is critical for medulloblastoma cell migration. *Clinical & experimental metastasis*, 27(7), pp.481–491.

Zhang, Q. & Andersen, M.E., 2007. Dose response relationship in anti-stress gene regulatory networks. *PLoS computational biology*, 3(3), p.e24.

Zheng, D. et al., 2010. Abba promotes PDGF-mediated membrane ruffling through activation of the small GTPase Rac1. *Biochemical and biophysical research communications*, 401(4), pp.527–532.

7. SUMMARY

All organisms are exposed to many external challenges now and then such as elevated temperatures, oxidative stress, UV irradiation and hypoxia resulting in protein denaturation and DNA damage. Not only environmental conditions but also pathophysiological states - such as neurodegenerative diseases, diabetes and cancer - can act as stress factors. Most of these insults are also reported to target membranes and lipids. Exposure of cells to a transient, non-lethal stress results in the activation of cellular stress responses and induces a state of stress tolerance that renders them resistant to subsequent lethal insults. Thus, thermotolerant tumor cells are not only less sensitive to hyperthermia-induced cytotoxicity, but also for growth factor withdrawal, heavy metals, radiation or anticancer drugs. The state of thermotolerance is associated with the synthesis and cellular accumulation of a family of highly conserved proteins referred to as heat shock proteins (HSPs). HSPs are named according to their molecular weights: HSP100, HSP90, HSP70, HSP60 and the "small HSPs". They can reside in different compartments of the cell and can also associate with membranes via their specific protein and lipid interactions. The most prominent HSPs, HSP70 and HSP27 contribute to stress- and thermotolerance mainly through their molecular chaperone activity.

There is a growing body of evidence linking the HSP expression induced by a variety of stress conditions to changes in the lipid composition and in the architecture of membranes. The "membrane thermosensor" hypothesis postulates that besides protein denaturation or alteration in nucleic acid conformation, heat stress can be sensed through subtle changes in the fluidity and micro-domain hyperstructures of membranes influencing the operation of membrane-localized stress sensing and signaling apparatuses and hence, the expression of HSPs.

Having more information about the structure, organization and function of plasma membrane (PM) has recently opened up new doors to understand its novel role as cellular stress sensor. Lipid micro-domains are the hyperstructures of cholesterol (Chol), sphingomyelin and saturated lipid containing liquid ordered (L_o) patches of PM. Specific signaling proteins are targeted to these micro-domains as a consequence of their lipidated features. The Rho family small GTPase Rac1 is an important integrator of signals from growth factor receptors, integrins and altered signaling related to cell transformation, tumor invasion, and metastasis. Rac1 is known to promote actin assembly and have an important role in the formation of lamellipodia and membrane ruffles. Rac1 associates with Chol rich

PM micro-domains under growth factor stimuli. Rac1 is also known to drive actin polymerization which can be induced by mild hyperthermia.

In favor of membrane thermosensor model, our current working hypothesizes was that Rac1 pathway is involved in stress signaling, especially through the effect of heat stress on membrane micro-domain organization. We assumed that plasma membrane rearrangement and/or hyperfluidization caused even by mild heat stress may activate growth factor receptor tyrosine kinases by their non-specific clustering. Activation of such cell surface receptors can stimulate PI3K which in turn activates the Rac1 protein, resulting in its release from its pre-existing Rho-GDI association keeping Rac1 solubilized in cytosol. Next we suggested, that Rac1, undergoing reversible palmitoylation subsequently translocates to the Chol rich domains of PM. Finally, this surface membrane localized Rac1 pool interacts with its effector Pak1, which mediates a downstream signaling cascade to MAPKs such as p38MAPK directly affecting HSP expression.

In order to test our above described working hypothesis, first we have monitored the Rac1 PM localization, both under mild and severe heat stress conditions. By using the B16F10 mouse melanoma cells we have shown that not only its functionally active form but also the palmitoylation of Rac1 is a prerequisite for its PM localization. Next we evidenced, that exposure of cells to heat shock causes a distinct alteration of micro-domains of PM. We have shown that Rac1 palmitoylation can be one of the major regulatory steps for the observed increment of micro-domains in these cells pre-exposed to heat stress. Studying the heat shock effect on cell shape alterations, our findings have indicated that severe thermal treatments result in cell shrinkage and rounding in B16F10 melanoma. Using an additional cellular model system, we have demonstrated the F-actin fragmentation under severe heat stress in MEF cells. We also documented, that this effect can be prevented by administration of Rac1 specific and palmitoylation inhibitors. In order to test the effect of Rac1 on HSP expression, next we have analyzed the expressions of inducible *hsp25* and *hsp70* genes under both mild and severe heat shock conditions. Our results have revealed that, activation of Rac1 influences the expression of *hsp25* and *hsp70* at both mRNA and protein levels under mild heat stress conditions which also triggers PM Rac1 localization on the L_o micro-domains. The hydroximic acid derivative, BGP15 is a well-studied HSP co-inducer and was also shown to interact with membranes. We have reported that BGP15 acts as a raft stabilizer for the integrity of lipid nano-platforms. We have shown that Rac1 can also regulate the BGP15-mediated activation of HSPs. Finally, we have documented that blocking Rac1 function by its

specific inhibitor NSC under mild heat shock conditions is causing elevated phosphorylation levels of p38MAPK which is known to phosphorylate the major transactivator HSF1 on its inhibitory side to attenuate HSP expression. Although our efforts to test HSF1 hyperphosphorylation displayed no measurable changes on the overall phosphorylation pattern of HSF1, obviously further investigations are needed to elucidate whether the documented Rac1 linked activation of HSPs is HSF1-mediated or not.

8. ÖSSZEFOGLALÓ

Minden élőlény számos olyan stressztényezőnek van kitéve, mint például a magas hőmérséklet, oxidatív stressz, ultraibolya sugárzás, hipoxia, amelyek denaturálhatják fehérjéiket és károsíthatják a DNS-üket. E külső tényezők mellett kórélettani állapotok is képezhetnek stressztényezőket, mint amilyenek a neurodegenaratív betegségek, a cukorbetegség és a rák. E stressztényzők legtöbbje a lipidekre és membránokra is káros hatással van. Mindezen tényezők tekintetében egy szervezet túléléséhez alapfeltétel a káros körülményekhez való alkalmazkodóképesség. Ennek megnyílvánulása a sejtek összetett és speciális védelmi rendszere, amely összességében egy gyors és átmeneti úgynevezett stresszválasz, ami által a sejtek belső környezete stabilis marad. A hősokk indukálta stresszválaszt elsősorban a hősokkfehérjék (HSP) kifejeződése jellemzi, amelyek legfontosabb szerepe a különböző fehérjék szerkezetének megőrzése. A HSP-k elősegítik a sejtek stresszből való felépülését és hőtoleranciát nyújtanak. E fehérjék magasfokú konzerváltságot mutatnak és számos stressztényező indukálhatja termelésüket. Nevüket a molekulasúlyuk alapján kapják: HSP100, HSP90, HSP70, HSP60 és a kismólsúlyú HSP-k (sHSP). Előfordulásuk helyszíne sejten belül változó és képesek akár a membránokhoz is kötődni specifikus fehérje és lipid kölcsönhatásaik által.

Egyre több irodalmi adat utal arra, hogy a különböző stressztényezők által indukált HSP-k termelése a plazmamembrán (PM) finomszerkezeti és lipidösszetételbeli változásával asszociálható. A hőmérsékletváltozást a sejtek a fehérjedenaturáció és a nukleinsavak konformációváltozása mellett a PM fluiditásának és mikrodomén szerkezetének apró változásai által is érzékelhetik. A „membrán, mint hőérzékelő" modell szerint a fent leírt változások módosítják a PM-mel asszociált stresszérzékelést és jelátvitelt, így ezáltal a HSP-k termelését.

A PM szerkezetének, szerveződésének és funkciójának alaposabb feltérképezésével új távlatok nyíltak meg a stresszérzékelésben betöltött szerepének megértésében. A lipid mikrodomének koleszterin, szfingomielin és telített zsírsavakat tartalmazó lipidek által létrehozott rendezett szigetek a PM szerkezetében. E mikrodoménekhez (lipid "tutajokhoz") specifikus jelátviteli fehérjék kötődhetnek. A Rho fehérjecsaládba tartozó kis GTPáz Rac1 fehérje különböző jelátviteleket közvetít oly módon. Ilyenek például a növekedési faktorok receptoraitól érkező jelek, az integrinek által létrehozott, ill. a daganatok áttételeiből és betöréséből származó jelek. Ismert, hogy a Rac1 elősegíti az aktin fehérjék szerveződését, és fontos szerepet játszik a lamellipódiák és a PM fodrozottságának kialakulásában. Növekedési

faktorok jelenlétében a Rac1 koleszterinben gazdag mikrodoménekkel asszociálható, enyhe hőmérsékletnövekedés következtében pedig az aktin polimerizációját serkenti. A PM mint hőérzékelő modelljére alapozva, a jelenlegi munkahipotézisünk szerint, a Rac1 jelátviteli útvonalnak a mikrodoménekre való hatásán keresztül, a stressztényezők közvetítésében van szerepe. A PM szerkezetének és /vagy fluiditásának enyhe hőstressz következtében létrejött változása aktiválhatja a tirozin kináz növekedési faktor receptort azáltal, hogy indukálja a nem specifikus csoportosulásukat. Az ilyen sejtfelszíni receptorok aktivációja serkentheti a PI3K-t, amely aktiválja a Rac1-et. Az aktiváció pillanatában a Rac1 elszakad a Rho-GDI egységtől, amely a Rac1-et a geranil-geranil csoportjának lefedése által a citoszolban "oldottan" tartotta. Ezután a Rac1 palmitoilezésen esik át, majd átkerül a PM koleszterinben gazdag doménjéhez. Ezt követően a Rac1 kölcsönhatásba lép a Pak1 effektor molekulával, amely jelátvitelt közvetít a MAPK molekulákhoz, mint például a p38MAPK, így befolyásolva a HSP termelését.

A jelen dolgozatban tárgyalt munkánk célja az volt, hogy felderítsük a Rac1-nak a fentiek szerint vázolt szerepét emlős sejtek stressz jelátvitelében. Két fő pontja volt a kutatásainknak: a Rac1 és a PM kapcsolatából adódó funkció feltérképezése, valamint a hőstressz által kiváltott PM szerkezeti változásokkal kezdődő és HSP termelésével, valamint a citoszkeleton változásával végződő folyamatának tisztázása. Ezek felderítése érdekében megvizsgáltuk a Rac1 PM-lokalizációját enyhe és intenzív hőstressz alatt. Kimutattuk, hogy a Rac1 PM-lokalizációjához nemcsak az aktiválása szükséges, hanem a palmitoilezése is egy előfeltétel. Megállapítottuk, hogy hősokk hatására megnövekszik a B16F10 melanóma sejtvonal PM mikrodoménjeinek mérete és ennek egy fontos szabályzó tényezője a Rac1 palmitoilezése lehet. Az intenzív hőkezelés a sejtek alakját és méretét is befolyásolja, a sejtek kisebbé és gömbölyűbbé válnak hatására.

Intenzív hőkezelés alatt tanulmányozva a MEF sejteket azt találtuk, hogy fragmentálódik az F-aktin fehérje, és hogy ez a hatás megelőzhető Rac1-specifikus és palmitoilezést gátló szerek hozzáadásával.

A Rac1 HSP termelésére való hatásának feltérképezéséhez enyhe és intenzív hősokk mellett vizsgáltuk a *hsp25* és *hsp70* expresszióját. Az adataink arra utalnak, hogy enyhe hősokk mellett a a Rac1 aktiválása befolyásolja a *hsp25* és *hsp70* expresszióját hírvivő RNS és fehérje szinten egyaránt. Ez utóbbi körülmény indukálja a Rac1 PM-lokalizációját is az L_o mikrodoménekhez. A BGP15 hidroximsavszármazék egy ismert HSP-t indukáló szer, amelynek membrán és lipidkölcsönhatásait csoportunk jelenleg is vizsgálja. Nemrégiben

igazoltuk, hogy a BGP15 stabilizálja a PM lipid nano platformjait. Kísérleteinkben elsőként mutattuk ki, hogy a Rac1 képes szabályozni a BGP15 által közvetített HSP indukciót. Végezetül arra is fényt derítettünk, hogy a Rac1 funkció specifikus gátlása (NSC által) következtében, enyhe hősokk mellett, megnövekszik a p38MAPK foszforilációs szintje, ami viszont foszforilálhatja a HSF1-et, így csökkentve a HSP termelését. Annak ellenére, hogy az adataink nem mutattak változást a HSF1 teljes foszforilációs mintázatában, további vizsgálatok szükségesek ahhoz, hogy kiderítsük, vajon a Rac1 által aktivált HSP indukció a HSF1 közvetítésével zajlik-e vagy sem.

9. ACKNOWLEDGEMENT

Undertaking this PhD has been a truly life-changing experience for me and it would not have been possible to do without the support and guidance that I received from many people.

Foremost, I would like to first say a very big thank you to my supervisor Dr. Ibolya Horváth for the endless helps and encouragement she gave me. I deeply believe that her priceless advices and constructive critics are tremendously beneficial for my professional and personal improvement. I also would like to express my gratitude and thank to Prof László Vígh for providing me with a great research opportunity to accomplish my PhD project. It has been a great experience working with them.

I want to thank to the group members of Laboratory of Molecular Stress Biology for creating such a friendly atmosphere and collaborations, especially Imre Gombos and Tim Crul for their unlimited supports.

I would like to thank Dr. Ferhan Ayaydın and for Dr. László Szabo their helps with the microscopy work and useful discussions.

I would like to thank all my friends, especially Orsolya Mehi, Ildiko Stier and Istvan Vass for being by my side. Their presence in my life enriched my years in Hungary.

I would like to thank Vincent Le Gars, who has always supported me and believed in me.

Finally, my deepest thanks to my family for giving me unconditional love, understanding, helping and encouraging all the time which is necessary to ensure my success.