FORSCHUNGSFORUM DER ÖSTERREICHISCHEN FACHHOCHSCHULEN 2024

University of Applied Sciences

Heat Shock Factor Localisation Under Different Stress Conditions

Nicole Wanko*, Thomas Czerny*, Elisabeth Riegel*

*University of Applied Science, FH Campus Wien, Section of Molecular Biotechnology, Vienna, Austria

Abstract. The heat shock response (HSR) pathway is a highly conserved cellular stress response, activated by heat and other stressors and is used by the cell to cope and adapt to proteotoxic conditions. The key player in this pathway is the heat shock factor (HSF). HSF1 is activated under stress by a conformational change and translocated into the nucleus. Inside the nucleus, HSF1 binds to heat shock elements (HSEs) and activates the transcription of heat shock proteins (HSPs), which function as molecular chaperones. Besides HSF1 a second member of this protein family exists (HSF2) ubiquitously in human cells. Activated HSFs are known to be located in so-called nuclear stress bodies (nSB), which are subnuclear organelles. We aimed to investigate the differences in the localisation of the heat shock factors HSF1 and HSF2 under different stress conditions using Western blot of nuclear and cytoplasmic extracts of U2OS and HEK293T cells, immunofluorescence, and establishment of a cell line where HSF1 was combined with the fluorescent protein msGFP2. Results showed, that HSF2 forms nSBs only after treatment with the HSP90 inhibitor geldanamycin. We could also show that there is no accumulation of HSF2 in the nucleus upon different stresses. Furthermore, the localistation into nSB happens quickly with HSF1; they could be detected after 15 minutes of heat stress. In summary, the localisation of HSF1 and HSF2 to specific compartments is cell-type and stress-dependent.

Keywords: Heat shock response pathway, heat shock factor localisation, geldanamycin, nuclear stress bodies, HSF1 and HSF2

1 INTRODUCTION

The heat shock response (HSR) pathway is a highly conserved cellular stress response that is used to cope and adapt to proteotoxic conditions. The response can be activated due to higher temperatures, as the name suggests, heavy metals and several additional stress conditions.[1,2] Additionally, the accumulation of unfolded or misfolded proteins can trigger the HSR pathway, which is mediated by its master regulator HSF1.[2] HSF1 in its inactive state exists primarily in its monomeric form. Upon stress, the transcription factor HSF1 is activated and forms a trimer which binds to heat shock elements (HSEs) of target genes in the nucleus. This binding activates the transcription of heat shock proteins (HSPs) which act as chaperones and help in the refolding and degradation of misfolded proteins. Thus, the aggregation of damaged proteins can be prevented.[3,4] Due to a nuclear localisation signal, HSF1 can be found at increased levels in the nucleus once activated. Currently, it is not known how stress is sensed, but there are indications that different stresses can activate HSF1 through different mechanisms. [3,5] In addition to HSF1 a second member of this protein family exists ubiquitously in humans (HSF2) which can modulate the HSR by forming heterooligomers with HSF1.[6] While HSF1 and HSF2 bind to similar HSE sequence motifs, they have partially shown differences in genomic occupancy.[7] Inhibitors of HSP90 like geldanamycin also induce the HSR.[8] Nuclear stress bodies (nSB) are subnuclear organelles with unknown functions that are distinct from other known nuclear bodies.[9] It has been demonstrated that HSF1 can localise to nuclear stress bodies in human cells once it is activated.[10] It was revealed that HSF2 was present in the nSB, binding to DNA dependent on HSF1.[11]

2 METHODS

2.1 Immunofluorescence

Cells were seeded on coverslips and grown to 70% confluency. The cells were heat shocked for 2 hours at 43°C or treated with 5 µM Geldanamycin for 6 hours or 24 hours for the different stress treatments. After treatment, the cells were fixated for 8 minutes with 2% paraformaldehyde (PFA) and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS). The coverslips were incubated for 2 hours in a wet chamber with the antibodies HSF1 (Cell Signaling Technology, #4356, 1:250) and HSF2 (Santa Cruz, (3E2) sc-13517, 1:50). HSF1 was stained with Alexa Fluor488 (Jackson ImmunoResearch, 711-545-152, 1:500) and HSF2 with Rhodamine (Jackson ImmunoResearch, 712-025-150, 1:200). The nuclei were stained additionally with Hoechst 33342 (1:1000). The cells on the coverslips were mounted on microscopic slides using mowiol and viewed using ZEISS LSM 900 with Airyscan 2 microscope.

2.2 Protein Extraction and Quantification

For the preparation of protein extraction cells were grown in 6 well plates. The cells were treated with heat shock for 2 hours at 43°C or 5 µM Geldanamycin for 6 or 24 hours. They

IMC Fachhochschule Krems GmbH
www.imc.ac.at
Piaristengasse 1
3500 Krems
Austria, Europe
+43 2732 802
office@imc.ac.at
Firmenbuchgericht: LG Krems an der Donau
UID: ATU 37552304
FN: 79297p

were harvested by aspirating the medium and washing the cells with 1 ml ice-cold PBS and then scraping the adherend cells, which were then collected using 2 ml ice-cold PBS. The cells were pelleted by centrifugation at 2800 rpm for 5 min at 4°C. The cells were then resuspended in 200 µl of ice-cold Buffer A [10 mM Hepes pH 7.9; 10 mM KCl; 0.1 mM EDTA; 1 mM DTT] and incubated on ice for 5 min. Then Nonident NP-40% was added to a concentration of 0.6% and after vortexing, centrifuged for 1 min at 12 000 rpm at 4°C. The supernatant collected contains the cytosolic fraction. The pellet was washed and then resuspended in 40 µl of ice-cold buffer C [20 mM HEPES pH 7.9; 400 mM NaCl; 1 mM EDTA; 1 mM DTT]. After shaking at 4°C for 15 min the cells were centrifuged at 13 000 rpm for 5 min at 4°C. The supernatant contains the nuclear fraction.

For quantification of the protein extracts, a Bicinchoninic assay (BCA) (ThermoFisher Scientific) was performed. The sample concentrations were calculated using photometric measurements at wavelength 562 nm of the samples using a standard curve derived from BSA standards.

2.3 Western Blot

12% Bis-Tris gels were blotted on a nitrocellulose membrane and blocked for 3 hours in 5% BSA in 1x TBS-T (10 mM Tris–Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, pH 8.0, 0.1% Tween 20). Incubation with the primary antibodies GAPDH (Santa Cruz, sc-47724, 1:5000), HSF1 (Cell Signaling Technology, #4356, 1:1000) and HSF2 (Santa Cruz, sc-13517, 1:5000) was performed in 5% BSA in 1x TBS-T o/n. Incubation with secondary antibody was performed for 2 hours at room temperature (RT) with Anti-rat IgG-HRP (Jackson ImmunoResearch, 712-035-150, 1:1000), Anti-rabbit IgG-HRP (Cell Signaling Technologies, #7074, 1:1000), m-IgGκ BP-HRP (Santa Cruz, sc-516102, 1:5000). Signals were detected after 5 minutes incubation with substrate solution (SuperSignal™ West Pico PLUS Chemiluminescent Substrate, Thermo Scientific) and chemiluminescence was detected in a ChemDoc station.

2.4 Creation of stable cell line

To create the stable cell line U2OS HSF1KO HSF1msGFP, lentiviral particles were produced using Takara Lenti-X™ Packaging Single Shots (VSV-G) according to user instructions with the lentiviral plasmid pLVhCMV3 HSF1msGFP2. For transduction, U2OS HSF1KO cells were seeded to 50-70% confluency and incubated for 1-2 days with 1 ml of viral particles. Thereafter, the cells were cultivated in Dulbeccos Modified Eagle Medium (DMEM), with 10% Fetal calf serum (FCS) and penicillin/streptomycin, containing 500 µg/ml G418 until all untransduced cells on a control plate had died. Single colonies were picked by trypsinizing and growing them in a 24-well plate.

For microscopy, the cells were seeded on coverslips and treated either with heat shock or geldanamycin. They then were fixated using 2% PFA, mounted on microscopic slides using mowiol and viewed using ZEISS LSM 900 with Airyscan 2 microscope.

For live-cell imaging, cells were seeded to 70% confluency and then incubated in a live-cell station for the Zeiss Axio Observer Z.1, first for 2 hours at 43°C and then at 37°C for another 6 hours with pictures taken in a 15 and 30 minute interval.

IMC Fachhochschule Krems GmbH
www.imc.ac.at
Piaristengasse 1
3500 Krems
Austria, Europe
+43 2732 802
office@imc.ac.at
Firmenbuchgericht: LG Krems an der Donau
UID: ATU 37552304
FN: 79297p

3 RESULTS

3.1 Nuclear stress body formation

Different cell lines were treated with heat shock and geldanamycin and the localisation of HSF1 and HSF2 was detected by immunofluorescence. Our results regarding the location of the heat shock factors showed that, in unstressed cells, there is no localisation into nSB with any cell lines tested while treatment with heat showed that HSF1 is capable of nSB formation in U2OS, as was previously shown for other human cells. [9] Heat shock for 2 hours at 43°C was sufficient to produce nSBs in the U2OS and HELA cell line (as seen in *Figure 1*). However, Fibroblasts (WI-38 cells) did not show formation of nSBs. Additionally, HSF2 did not localise to nSBs after heat shock in any of the cell types tested, however, treatment with 5 µM geldanamycin for 6 and 24 hours led to localisation in nSBs in U2OS. Here, HSF1 and HSF2 show overlapping signals indicating a co-localisation into nSBs. Previously it was shown that in HELA cells HSF2 form nSBs after heat shock, contradictory to our results. [9] No nSBs could be detected with any treatment in WI-38 cells, even after testing various concentrations of geldanamycin (data not shown). Therefore, we conclude that nSB formation is dependent on the cell type.

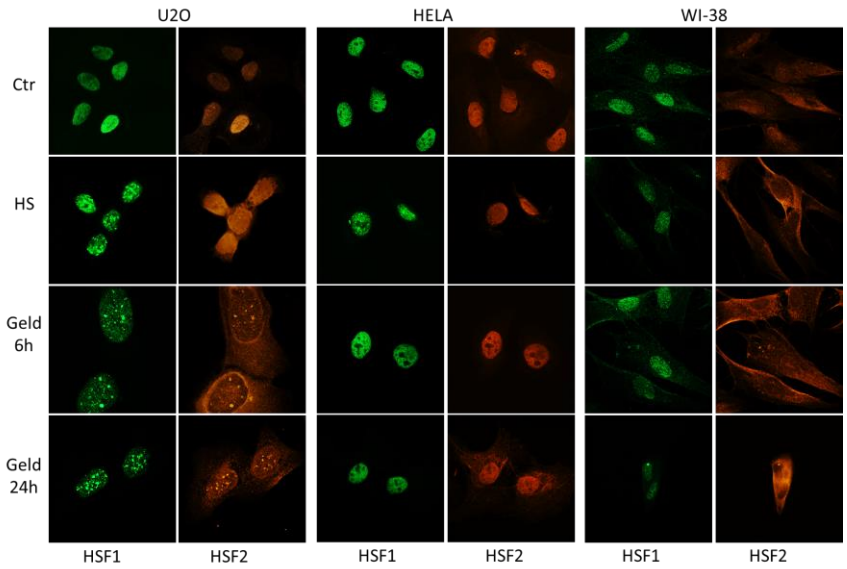

Figure 1: *Immunofluorescence detection of HSF1 and HSF2 in U2OS, HELA and WI-38 cells. Cells were treated with heat shock at 43°C for 2 hours (HS) and 5 µM geldanamycin (Geld) for 6 hours or 24 hours, fixated with 2% PFA and HSF1 stained with Alexa Fluor488 (green) and HSF2 with Rhodamine (orange). Ctr represents untreated control cells. Pictures were taken with 63x magnification.*

3.2 Location of HSF into specific departments

To additionally test the accumulation of HSF1 and HSF2 in the nucleus in stress vs non-stressed cells we performed Western blots with nuclear and cytosolic extracts of U2OS and HEK293T cells upon treatment with heat shock and geldanamycin. The Western blots

IMC Fachhochschule Krems GmbH
www.imc.ac.at
Piaristengasse 1
3500 Krems
Austria, Europe
+43 2732 802
office@imc.ac.at
Firmenbuchgericht: LG Krems an der Donau
UID: ATU 37552304
FN: 79297p

(*Figure 2*) showed that neither condition tested led to an accumulation of HSF2 in the nucleus. No difference in HSF2 levels between the treatments could be detected in both cell lines. HSF1 protein levels on the other hand increased in the nucleus with heat treatment in U2OS and with geldanamycin treatment in HEK293T cells, indicating an active contribution to gene regulation. In general, the levels of HSF1 and HSF2 in the cytosol were low compared to the nucleus, even under non-stress conditions. This was particularly pronounced for U2OS cells.

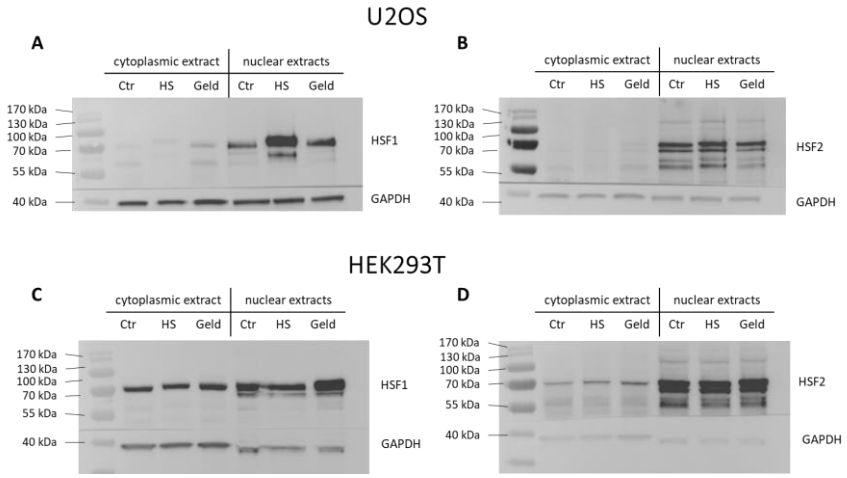

Figure 2: *Western Blot of cytoplasmic and nuclear extracts of U2OS and HEK293T cells. Cells were treated with heat at 43°C for 2 hours and geldanamycin for 6 hours before taking the extract. A) shows protein extracts taken from U2OS incubated with HSF1 antibody while B) shows the extracts incubated with HSF2 antibody. The two bands visible with the HSF2 antibody, represent the two isomers. C) shows protein extracts taken from HEK293T incubated with HSF1 antibody while D) shows the extracts incubated with HSF2 antibody. GAPDH was used as a loading control in all blots.*

3.3 Stable cell line

We established a stable cell line where the fluorescent protein msGFP2 was attached to HSF1. This cell line allows the observation of changes in the localisation of HSF1 in real time without the use of antibodies. In *Figure 3,* the formation of nSB under various conditions was demonstrated. Similar to the immunofluorescence pictures in *Figure 1*, HSF1 does not show any localisation into nSB with the control but with heat shock at 43°C and treatment with 5 µM geldanamycin.

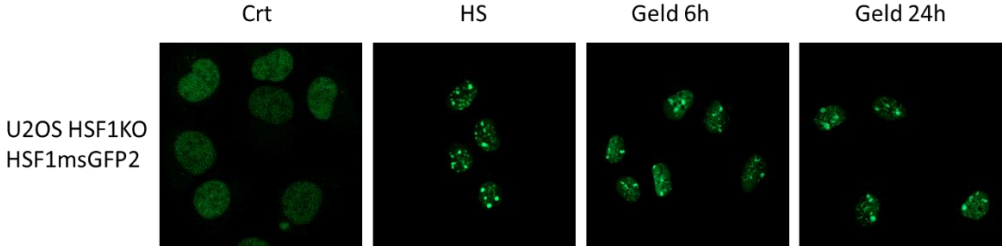

Figure 3: *U2OS HSF1KO HSF1msGFP2 cell line under microscope after treatment with various conditions. Cells were treated with heat at 43°C for 2 hours and geldanamycin for 6 hours and 24 hours and fixated on coverslips with 2% PFA. Ctr represents untreated control cells. Pictures were taken with 63x magnification.*

IMC Fachhochschule Krems GmbH
www.imc.ac.at
Piaristengasse 1
3500 Krems
Austria, Europe
+43 2732 802
office@imc.ac.at
Firmenbuchgericht: LG Krems an der Donau
UID: ATU 37552304
FN: 79297p

Additionally, we tested this cell line with live-cell imaging where the cells were observed for 8 hours and pictures were taken every 15 minutes for the first two hours during heat shock at 43°C and every 30 minutes for the remaining time (*Figure 4*). Here we could observe the localisation of HSF1 into nSB and their resolve. Already after 15 minutes, the first nSB could be detected and the resolve of the nSB could be seen 3 hours after heat shock.

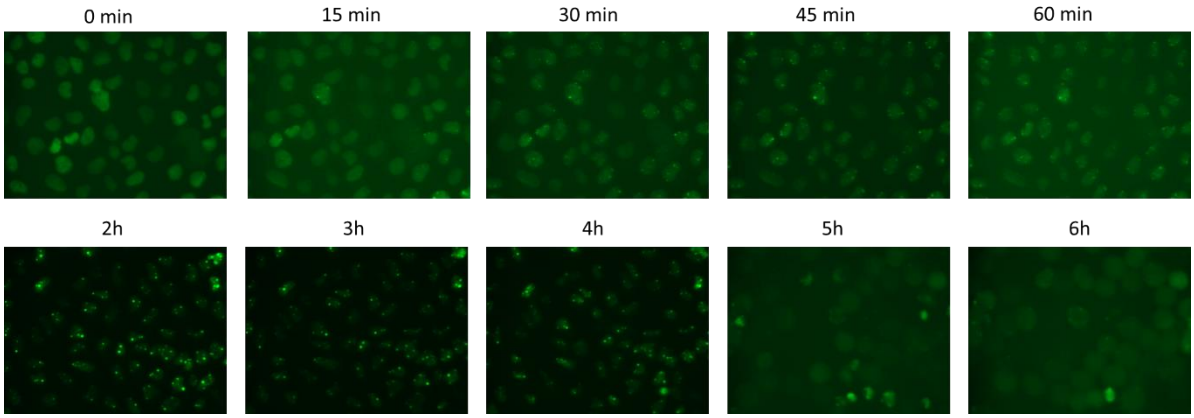

Figure 4: *Live-cell imaging of the U2O HSF1KO HSF1msGFP2 cell line. Cells were heat shocked for 2 hours at 43°C and then incubated at 37°C for another 6 hours to observe nSB formation and their resolve. Pictures were taken every 15 minutes for the first 2 hours and every 30 minutes for the rest of the time (not all pictures shown). Pictures were taken with 20x magnification.*

4 CONCLUSION

In conclusion, we can say that the formation of nSBs is dependent on cell type and stress, as we did not see any localisation into nSB with the fibroblast cell line WI-38 but they could be observed in the cancer cell line U2OS. It was previously already shown that in rodent cell lines no nSBs form.[8] Furthermore, only the HSP90 inhibitor geldanamycin could localise HSF2 into nSBs indicating that stressors can influence the formation of nSBs and HSF1 and HSF2 are differentially activated.

The dependency on cell type and stressor in the localisation of HSF1 and HSF2 to specific compartments was also demonstrated with the Western blot of nucleic and cytoplasmic extracts, thereby indicating differences in HSF regulation. While HSF1 levels increased with heat treatment in U2Os, in HEK293T they increased with geldanamycin treatment. Thereby, we see an active contribution to gene regulation of HSF1. Its increasing levels in the nucleus upon activation, can be explained by its nuclear localisation signal.[3] At the same time, HSF2 levels remain similar, independent of the stress condition.

We could also demonstrate the advantages of our established U2OS HSF1KO HSF1msGFP2 cell line. It showed the same results as the immunofluorescence pictures, allows for easier observations of the localisation of HSF1, and is more time- and cost-efficient. Furthermore, we could observe that the formation of nSB occurs rather quickly and that they resolve after the loss of the stress conditions.

IMC Fachhochschule Krems GmbH
www.imc.ac.at
Piaristengasse 1
3500 Krems
Austria, Europe
+43 2732 802
office@imc.ac.at
Firmenbuchgericht: LG Krems an der Donau
UID: ATU 37552304
FN: 79297p

5 REFERENCES

1. Lindquist, S. & Craig, E. A. THE HEAT-SHOCK PROTEINS. *Annu Rev Genet* **22**, 631–677 (1988).
2. Steurer, C. *et al.* HSF1 mediated stress response of heavy metals. *PLoS One* **13**, e0209077- (2018).
3. Anckar, J. & Sistonen, L. Regulation of HSF1 Function in the Heat Stress Response: Implications in Aging and Disease. *Annu Rev Biochem* **80**, 1089–1115 (2010).
4. Vihervaara, A. & Sistonen, L. HSF1 at a glance. *J Cell Sci* **127**, 261–266 (2014).
5. Morimoto, R. Regulation of the heat shock transcriptional response: Cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. *Genes Dev* **12**, 3788–3796 (1999).
6. Jaeger, A., Pemble, C., Sistonen, L. & Thiele, D. Structures of HSF2 Reveal Mechanisms for Differential Regulation of Human Heat Shock Factors. *Nat Struct Mol Biol* **23**, (2016).
7. Vihervaara, A. *et al.* Transcriptional response to stress in the dynamic chromatin environment of cycling and mitotic cells. *Proceedings of the National Academy of Sciences* **110**, E3388–E3397 (2013).
8. Ochel, H.-J., Eichhorn, K. & Gademann, G. Geldanamycin: The prototype of a class of antitumor drugs targeting the heat shock protein 90 family of molecular chaperones. *Cell Stress Chaperones* **6**, 105–112 (2001).
9. Biamonti, G. & Vourc'h, C. Nuclear stress bodies. *Cold Spring Harb Perspect Biol* **2 6**, a000695 (2010).
10. Sarge, K. D., Murphy, S. P. & Morimoto, R. I. Activation of Heat Shock Gene Transcription by Heat Shock Factor 1 Involves Oligomerization, Acquisition of DNA-Binding Activity, and Nuclear Localization and Can Occur in the Absence of Stress. *Mol Cell Biol* **13**, 1392–1407 (1993).
11. Alastalo, T.-P. *et al.* Formation of nuclear stress granules involves HSF2 and coincides with the nucleolar localization of Hsp70. *J Cell Sci* **116**, 3557–3570 (2003).

IMC Fachhochschule Krems GmbH
www.imc.ac.at
Piaristengasse 1
3500 Krems
Austria, Europe
+43 2732 802
office@imc.ac.at
Firmenbuchgericht: LG Krems an der Donau
UID: ATU 37552304
FN: 79297p