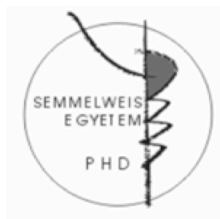


# **Exploration of the role of Hsp90 in adipogenesis**

Ph.D. Thesis

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## 1. INTRODUCTION

In recent years obesity have become a serious public health risk in the Western society. Obesity and adipose tissue dysregulation plays a major role in various human diseases, including insulin resistance, type II diabetes, hypertension, cardiovascular diseases and cancer. The peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ) is a key regulator of adipocyte differentiation and function. PPAR $\gamma$  is necessary for adipose tissue development and for systemic insulin sensitivity.

Maintenance of protein conformation and protection of protein homeostasis (proteostasis) upon stress is critical for cellular survival and function. Hsp90 is a conserved and essential molecular chaperone and protein remodelling factors involved in this process. Hsp90 binds and stabilizes thermodynamically unstable proteins. Unstable proteins may be denatured by proteotoxic stress or various proteins unstable under physiological conditions, so called Hsp90 'clients'. The majority of clients are hubs of the signal transduction network and involved in cell survival and proliferation pathways. However participation of Hsp90 in cell differentiation is largely unclear.

During my Ph.D. studies I set out to investigate the effect of Hsp90 and proteotoxic stress on adipocyte differentiation and identify the underlying molecular mechanism.

## **2. OBJECTIVES**

The specific aims were as follows:

1. To explore the connection between Hsp90 function and adipocyte differentiation.
2. To elucidate the underlying molecular mechanism.
3. To assess the impact of proteotoxic stress on adipogenesis.
4. To test whether the effect of proteotoxic stress is reversible and if it may be a novel regulatory mechanism.

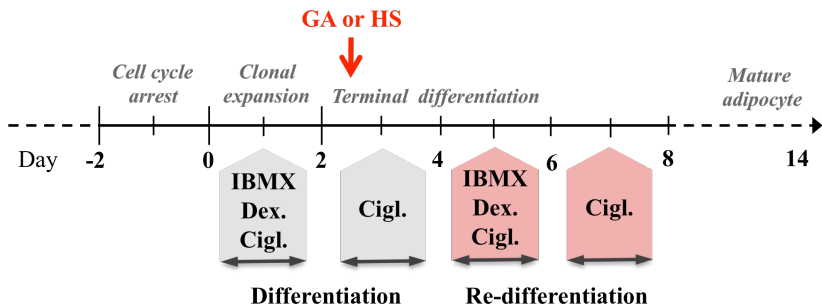
### **3. METHODS**

#### ***3.1. Cell culture (3T3-L1, HepG2)***

3T3-L1 mouse preadipocyte and HepG2 human hepatoma cells were obtained from the ATCC. Both cell types were maintained in Dulbecco's modified Eagle medium (with 4.5 mg/ml glucose), supplemented with 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5g/l glucose, 100 µg/ml streptomycin and 100 IU/ml penicillin at isobaric oxygen in 5% CO<sub>2</sub> at 37°C. 3T3-L1 and HepG2 cells were cultured in the presence of 10% bovine or 10% fetal bovine serum, respectively.

#### ***3.2. Adipocyte differentiation and treatments***

3T3-L1 cells were cultured to confluence. Differentiation was induced 2 days post-confluence (designated as day 0) by 1 µM dexamethasone, 0.5 mM isobutylmethylxanthine and 1 µM ciglitizone. After 48 hours, cells were incubated with 1 µM ciglitizone for 48 hours. Subsequently, cells were maintained in DMEM supplemented with 10% fetal bovine serum till the end of the experiments. Cells were treated with various concentrations of geldanamycin, the PI3K inhibitor LY294002, heat shock and/or the proteasome inhibitor MG132.



**Figure 1** Timescale of 3T3-L1 preadipocyte differentiation and setup of the re-differentiation experiment.

### **3.3. Oil Red O staining**

Cellular fat accumulation was monitored by Oil Red O staining on day 14, either visualized by microscopy or quantified by photometry. Cells were cultured either on coverslips or in 6-well plates. After washings and fixing cells were stained for 10 min in freshly prepared Oil Red O solution. Samples were visualized and photographed by a Nikon Eclipse E400 camera or a Alpha XD2-2T invert microscope. Lipid accumulation was determined by photometry. Optical density was measured at 500 nm by a Thermo Varioskan Flash photometer using isopropanol as blank.

### ***3.4. Cell lysis, purification of aggregates***

After treatments, cells were lysed on ice with WB lysis buffer (50 mM Tris, 300 mM NaCl, 1 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 0.1 mM EGTA, 20% glycerol, 1% NP40, 0.5 mM DTT, 2x Complete, pH 7.6). Protein concentration of the supernatants was determined by Bradford method, and equal amounts of proteins were subjected to SDS-PAGE. Detergent-insoluble pellets, containing aggregated proteins, were solubilized in urea buffer (2% SDS, 6 M urea, 30 mM Tris, pH 7.6). Equal volumes of protein extracts from each fraction were resolved by SDS-PAGE.

### ***3.5. Determination of protein concentration***

Protein concentration was determined according to Bradford by Bio-Rad reagent or by detergent-compatible BCA protein assay.

### ***3.6. Immunoprecipitation***

5x10<sup>6</sup> cells were treated by 0.25 µg/ml geldanamycin or DMSO for 2 hours on day 3. Lysis was performed in IP lysis buffer (50 mM Tris, 2 mM EDTA, 100 mM NaCl, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1% NP40, 2x Complete). PPAR $\gamma$  was immunoprecipitated by a monoclonal anti-PPAR $\gamma$  antibody. Pellets were washed five times with lysis buffer, and analyzed by SDS-PAGE and immunoblotting with anti-Hsp90 and anti-PPAR $\gamma$  antibodies.

### ***3.7. SDS-PAGE and Western blotting***

Equal amounts of proteins were subjected to SDS-PAGE. Western blotting was performed by transfer to nitrocellulose membrane and by blocking in 5% (w/v) skim milk powder. Blots were probed with the appropriate primary antibodies overnight at 4 °C and incubated with peroxidase-conjugated secondary antibodies for an hour at room temperature, and developed using ECL detection. The protein levels were determined by densitometric analysis of the Western blots using Image J, and normalized to the corresponding  $\beta$ -actin level.

### ***3.8. Cell viability***

Cell viability was assayed by trypan blue exclusion in a hemocytometer.

### ***3.9. mRNA expression analysis***

mRNA was prepared and was reverse transcribed using the GeneJET RNA Purification Kit, and the RevertAid™ cDNA Synthesis Kit, respectively. Sequences of primer sets for mouse PPAR $\gamma$ 2, adiponectin, lipoprotein lipase (LPL), GLUT4, aP2 and 28S rRNA were described in work of Ezure and Amano. Quantitative PCR was performed in an ABI 7300 System by using Maxima SYBR Green/ROX qPCR Master Mix according to the

manufacturer's instructions. Relative amounts of mRNA were determined using the Comparative CT Method for quantification and were normalized to 28S rRNA mRNA levels.

### ***3.10. Statistical analysis***

Data were compared by Student's *t* test. Variables were expressed as mean  $\pm$  standard deviation (SD). Statistical significance was indicated as follows:

\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .



## 4. RESULTS

### *4.1. Hsp90 inhibition impedes differentiation and survival of 3T3-L1 cells*

To investigate the effect of Hsp90 on adipogenesis, mouse 3T3-L1 preadipocytes, a widely used model was employed. To exclude the effect of Hsp90 on cell proliferation, treatments were performed on day 3 at the terminal differentiation phase of adipogenesis. In response to hormonal stimulation, 3T3-L1 cells terminally differentiate into mature adipocytes in two weeks and accumulate triglycerides in lipid droplets, readily stained by Oil Red O. Two structurally unrelated inhibitors of Hsp90 ATP binding, geldanamycin (GA) binding to the N-terminal domain and novobiocin targeting the C-terminal domain totally suppressed adipogenesis in a concentration-dependent manner ( $p < 0.001$ ).

Inhibition of differentiation may arise as a consequence of cell death. To test this possibility I measured cell viability and differentiation in parallel experiments on day 14. Low GA concentrations selectively inhibited adipogenesis (56 nM: 80% differentiation inhibition,  $p < 0.001$ ; without significant cell death). Higher GA concentrations and longer treatments caused cell death and impeded viability of both preadipocytes and mature adipocytes. This result is consistent with the essential role of Hsp90 in cell survival. The tenfold difference between the 50% inhibitory concentration of differentiation and viability, respectively ( $IC_{50_{diff}} = 16.38$  nM vs.  $IC_{50_{viab}} = 163.6$  nM,  $p < 0.001$ ) suggests that differentiation promoting effect of Hsp90 is a consequence of other than its pro-survival mechanism(s).

#### ***4.2. Hsp90 inhibition selectively depletes PPAR $\gamma$ protein levels***

Next, I investigated potential molecular mechanisms behind the GA-induced inhibition of adipogenesis. In search for a potential Hsp90 client, protein levels of main regulators of terminal differentiation were determined by Western blot in response to a 20-hr GA treatment on day 3. Under these conditions GA partially decreased the protein level of Akt, a known Hsp90 client ( $p < 0.01$ ), whereas did not impinge on the PPAR $\gamma$  co-operator C/EBP $\alpha$  p42 level. Importantly GA and novobiocin treatment entirely depleted protein levels of both isoforms of PPAR $\gamma$  in a concentration-dependent manner (IC50 values for PPAR $\gamma$ 1 and PPAR $\gamma$ 2: 51.0 and 40.8 nM, respectively,  $p < 0.001$ ). Hence, PPAR $\gamma$  (and Akt) may mediate the adipogenesis promoting effect of Hsp90. GA treatment also decreased PPAR $\gamma$  protein levels in HepG2 human hepatocytes. Thus, Hsp90 stabilizes PPAR $\gamma$  protein in mammalian cells.

#### ***4.3. Inhibition of the Hsp90-PPAR $\gamma$ interaction triggers destabilization and proteasomal degradation of PPAR $\gamma$***

Hsp90 binds and stabilizes clients in an ATP-dependent manner. To address whether PPAR $\gamma$  and Hsp90 interact I immunoprecipitated endogenous PPAR $\gamma$  protein from control and GA-treated 3T3-L1 cells. Hsp90 was detected in a physical complex with PPAR $\gamma$ , which was disrupted by GA treatment, suggesting an ATP-dependent interaction.

Hsp90 clients are destabilized and undergo proteasomal degradation in response to Hsp90 inhibition. To examine this, 3T3-L1 cells were treated with

GA and/or the proteasome inhibitor MG132, then detergent soluble (stable) and insoluble (aggregate) fractions were isolated. GA caused the complete disappearance of PPAR $\gamma$  and Akt from the detergent soluble supernatant. A parallel treatment by the proteasome inhibitor MG132 induced accumulation of both PPAR $\gamma$  and Akt in the detergent insoluble pellet, demonstrating a destabilization-induced aggregation of PPAR $\gamma$  (and Akt). Thus, inhibition of ATP-dependent function of Hsp90 causes destabilization and proteasomal degradation of PPAR $\gamma$ .

#### ***4.4. Hsp90 function is required for PPAR $\gamma$ transcriptional output and for survival of mature adipocytes***

Next, we investigated how Hsp90 inhibition affects PPAR $\gamma$ -driven transcription. To this end, expression of PPAR $\gamma$  target mRNAs was determined from control and GA treated cells, by reverse transcription and quantitative PCR on day 4 and at the end of adipocyte differentiation on day 14. GA treatment prevented the induction of PPAR $\gamma$ -dependent target mRNAs important for autonomous (GLUT4, aP2) and for systemic (adiponectin) functions of adipocytes ( $p < 0.001$ ). Moreover, GA treatment inhibited the expression of PPAR $\gamma 2$  mRNA itself ( $p < 0.001$ ), suppressing a positive auto-regulatory loop. PPAR $\gamma$  target genes exhibited virtually identical GA concentration dependence and IC<sub>50</sub> values, and these values are similar to the IC<sub>50</sub> value of PPAR $\gamma$  protein depletion (IC<sub>50</sub> values: *PPAR $\gamma 2$*  39 nM; *GLUT4* 41.9 nM; *aP2* 85.5 nM; *adiponectin* 43.8 nM). Assessing the protein levels of PPAR $\gamma$  and the master adipokine, adiponectin revealed a parallel decline in

GA-treated mature adipocytes. Hence, Hsp90 function is necessary for PPAR $\gamma$  stability and ensures the transcriptional response of PPAR $\gamma$ , which creates and maintains the functional characteristics of mature adipocytes. Thus, PPAR $\gamma$  is a novel Hsp90 client.

#### ***4.5. Proteotoxic stress halts adipogenesis via abrogating the Hsp90-PPAR $\gamma$ complex***

Recent evidence confirms that Hsp90 binds thermodynamically unstable proteins. As increasing the amount of misfolding proteins may overload Hsp90 capacity, I investigated how proteotoxic stress interferes with PPAR $\gamma$  stability and adipogenesis. Strikingly, a transient moderate heat shock at 43°C on day 3 in 3T3-L1 cells induced a quantitative depletion of PPAR $\gamma$ , contrasting the unchanged level of C/EBP $\alpha$  p42 isoform. Lysates from heat treated cells were separated to detergent soluble and insoluble fractions, respectively. I observed that the entire amount of PPAR $\gamma$  (and Akt) was rapidly misfolded and sedimented in the detergent-insoluble pellet upon heat shock, even when proteasome function was not pharmacologically inhibited. Hsp90 largely preserved its solubility, indicating a loss of interaction with PPAR $\gamma$  following by destabilization and aggregation of PPAR $\gamma$ . The transient folding defect of PPAR $\gamma$  in response to a moderate heat shock significantly inhibited PPAR $\gamma$ -dependent transcription and led to the cessation of adipocyte differentiation, while up to 120 minutes it did not largely interfere with their survival.

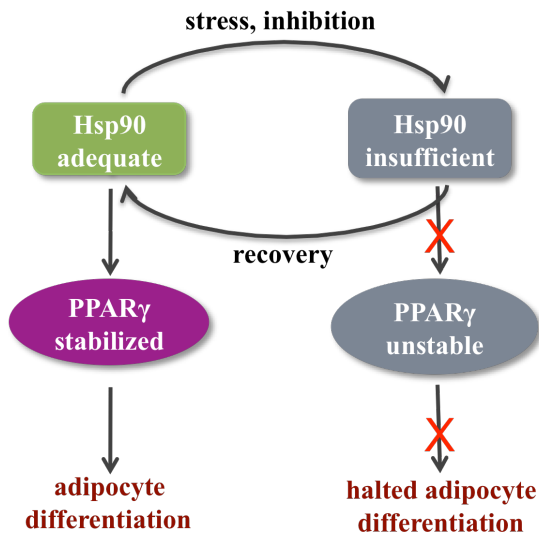
In physiological conditions, ~30% of newly synthesized proteins are mistranslated, misfolded and degraded by the proteasome. Therefore, I increased misfolded protein load by partial proteasome inhibition as an independent proteotoxic challenge. Indeed, partial inhibition of proteasome resulted in a selective destabilization of PPAR $\gamma$ , inhibition of PPAR $\gamma$  target gene expression, as well as an impaired adipogenesis. The above observations demonstrated that global proteotoxic stresses exert similar effects to selective Hsp90-inhibition and abrogate all PPAR $\gamma$  folding, function and adipogenesis.

#### ***4.6. Recovery from stress allows the continuation of adipogenic program by the restoration of PPAR $\gamma$ function***

To investigate whether the ability to differentiate is reversibly restored upon attenuation of stress, after a heat shock or GA treatment on day 3, 3T3-L1 preadipocytes were re-exposed to the stimuli inducing differentiation on day 5. Cells were able to restore differentiation at a progressively decreasing level with increasing doses of GA and heat- shock treatments.

To assess the relationship between PPAR $\gamma$  stability and adipocyte differentiation I selected two conditions (112 nM GA and 2h heat shock) characterized by maximal differentiation inhibition along with minimal cell death, to circumvent the influence of residual PPAR $\gamma$  activity and extensive cell death, respectively, on differentiation in response to the repeated stimulus. After 2-hr heat shock at 43°C and 112 nM overnight GA treatment quantitative inhibition of adipogenesis were observed. Intriguingly, re-exposure of cells to the differentiation medium led to a successful

differentiation of the majority of the heat shocked, but not GA-treated cell population. This phenomenon was in line with the restoration of stabilized PPAR $\gamma$  protein levels in response to the hormonal treatments after recovery from heat shock, but not after GA treatment, respectively. This result is consistent with intracellular accumulation of, and sustained inhibition of Hsp90, by GA. Neither GA nor heat shock affected total Hsp90 protein level. These data suggest that recovery from stress allows the restoration of Hsp90 capacity, PPAR $\gamma$  stability and the continuation of the adipogenic program.



**Figure 2 Model of the stress-responsive regulation of PPAR $\gamma$  and adipogenesis.**

## 5. CONCLUSIONS

1. My study revealed a critical requirement of (ATP-dependent function of) Hsp90 for the differentiation and survival of 3T3-L1 adipocytes. Hence, Hsp90 might be a novel therapeutic target in obesity and the metabolic syndrome. However, further *in vivo* studies could help assess its feasibility and the requirement of adipose-specific targeting.
2. I identified the adipose master regulator PPAR $\gamma$  as an Hsp90 client, which provides a molecular mechanism underlying the regulation of adipocyte differentiation by Hsp90. Whether the Hsp90-PPAR $\gamma$  interaction has significance in extra-adipose tissues and various (patho)physiological processes (such as inflammation, neurodegeneration, cancer or ageing) modulated by PPAR $\gamma$  requires future research.
3. I demonstrated that general proteotoxic stresses induce the destabilization and aggregation of PPAR $\gamma$  (and Akt) and halt adipogenesis.
4. I observed that upon relief from stress the differentiation program was continued by the restoration of PPAR $\gamma$  function. My findings reveal a novel, immediate and dynamic regulatory mechanism acting directly at the level of protein conformation. The Hsp90-PPAR $\gamma$  interaction modulates cellular function and phenotype in a stress-responsive manner and may facilitate the selection of cells with greater fitness to benefit the organism. Whether this mechanism might be generalized to other tissues and Hsp90 clients is an exciting opportunity to explore.

## 6. PUBLICATION LIST

### 6.1. Publications related to the thesis

**Nguyen M.T.**, Csermely P., Sőti C. (2013) Hsp90 chaperones PPAR $\gamma$  and regulates differentiation and survival of 3T3-L1 adipocytes. *Cell Death Differ.* 20: 1654-1663

**IF: 8.371**

Citation (total/independent): 1/1

Dancsó B., Spiró Z., Arslan M.A., **Nguyen M.T.**, Papp D., Csermely P., Sőti C. (2010) The heat shock connection of metabolic stress and dietary restriction. *Curr. Pharm. Biotechnol.*, 11, 139-145.

**IF: 3.455**

Citation (total/independent): 8/7

### 6.2. Publications not directly related to the thesis

Spiró Z., Arslan M.A., Somogyvári M., **Nguyen M.T.**, Smolders A., Dancsó B., Nemeth N., Elek Zs., Braeckman B.P., Csermely P. and Sőti C. (2012) RNA Interference Links Oxidative Stress to the Inhibition of Heat Stress Adaptation. *Antioxid. Redox. Signal.*, 17(6):890-901

**IF: 7.189**

Fábián T.K., Sőti Cs., **Nguyen, M.T.**, Csermely P., Fejérdy P.: (2008) Expected functions of salivary HSP70 in the oral cavity. *International Journal of Medical and Biological Frontiers.* 14: 289-308.



*Fábián T.K., Fejérdy P., Nguyen M.T., Sőtí Cs., Csermely P.* Potential immunological functions of salivary Hsp70 in the mucosal and periodontal defence mechanisms. (Review). *Arch. Immunol. Ther. Exp.* 2007; 55: 91-98.

**IF: 1.689**

Citation (total/independent): 20/13

*Fábián G., Müller O., Kovács Sz., Nguyen M.T., Fábián T.K., Csermely P., Fejérdy P.* (2007) Attitude toward death. Does it influence dental fear? *Ann. N.Y. Acad. Sci.* 1113: 39-349.

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Citation (total/independent): 11/6

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