Examining the biological role of the HSP-90 chaperone in *C. elegans*

Doctoral dissertation

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1. INTRODUCTION

The doubling of life expectancy due to the successes of medicine also had undesirable consequences. It is necessary for the maintaining of organismal homeostasis to properly regulate the mechanisms responsible for the repair of various damages. As you age these processes might lose their responsiveness, leading to such chronic diseases like the various metabolic disorders, cancer and neurodegenerative illnesses..

One of the central regulators of protein homeostasis is the 90kDa heat-shock protein, or Hsp90, which acts through stabilizing so called client proteins with non-native structure. While many clients of Hsp90 playing roles in various signaling pathways have been identified, its potential role in the regulation of lifespan remains largely unknown.

The *Caenorhabditis elegans* roundworm is a favorite model animal of genetics and embryology, due to its genome showing high orthology with human genes. In *C. elegans* the only known member of the Hsp90 family is HSP-90, which plays a role in many processes from larval development to immunity.

It is long known about the regulators of organismal defenses that they also influence the ageing process and lifespan. One of these key regulators is the Insulin-like Growth Factor pathway, or ILS, whose decreased functionality extended lifespan in invertebrate and mammal models. In addition, the sirtuin 1 (SIRT1) protein deacetylase increased lifespan upon its activation.

2. OBJECTIVES

During my PhD thesis I examined the role of the *C .elegans* Hsp90 heat-shock protein ortholog HSP-90 as follows:

1. Examination of the biological effect of HSP-90
    - Characterizing the silencing of the *hsp-90* gene by RNAi
    - Impact of silencing on animal development and reproduction

2. Examining the role of HSP-90 in regulating lifespan
    - Effect of HSP-90 deficiency on normal and extended lifespan caused by reduced ILS
    - Identification of the potential molecular mechanism

3. Examination of the effect of HSP-90 on the stability of the SIR-2.1 protein

3. METHODS

3.1 Strains and materials

All strains used for experiments came from the Caenorhabditis Genetics Center. Animals were kept at 20°C on NGM agar (Nematode Growth Medium) poured into 60 mm Petri dishes. The required food on these culture plates was the OP50 variant of the bacterium *E. coli*.

3.2 Plates containing MG132

During experiments employing proteasome-inhibitor I added MG132 in DMSO (1% V/V) to the NGM in 10 μM final concentration using pure DMSO as control.

3.3 Crossing

3.3.1 Making the *daf-2* and *daf-16* double mutant

For my examinations I crossed the CB1370[*daf-2(e1370)*] and CF1038[*daf-16(mu86)*] mutant strains. L4 *daf-16* mutant hermaphrodites were subjected to heat-shock for 1 hour at 35°C. The progeny of *daf-2* mutant hermaphrodites impregnated by the thus generated males were heterozygous for both genes. I used allele-specific PCR for genotyping the F2 progeny produced by self-fertilization.

3.3.2 Crossing of *daf-16a::rfp* transgenic and *rle-1* mutant strains

The *DAF-16A::RFP;rle-1(*cxTi510) double mutant was created by crossing the *daf-16(mgDf50)*;*unc-119(ed3)*;lpIs12[*daf-16a::RFP + unc-119(+)*] and [*rle-1(cxTi510)*] strains. Homozygous double mutant F2 was detected using allele-specific PCR and fluoreszcence microscopy.

3.4 RNA interference

For gene silencing I used the so-called “feeding protocol”. To feed the animals we use bacteria producing the double strand RNA suitable for silencing the gene targeted. During my experiments I used RNAi bacteria to silence *hsp-90, sir-2.1* and *daf-2*.

The E. coli strains carrying RNAi were grown overnight in LB medium containing 100 μg/ml ampicillin. The culture plates contained beyond the usual composition 1 mM IPTG and 50 μg/ml ampicillin. These plates were seeded with the necessary RNAi bacteria culture, or the control bacteria containing only empty vektor. In the case of treatment starting from the egg stage I placed gravid animals on the plates for 4 hours and performed my experiments with the progeny hatching from the eggs laid. In the case of treatment starting from the L4 larval stage and animals were placed on the RNAi plates later and were kept there for 2 days before measurements. To ensure proper RNAi dosage in the case of double RNAi treatments, the single treatments were done by mixing the silencing and control bacterium strains in 1:1 ratio.

3.5 Lifespan assay

All lifespan assays were performed at 20°C. Animals were synchronized by allowing gravid adults to lay eggs for 4 hours and using the next generation for experiments after they reached young adult stage. Approximately 35 animals were transferred to each of 3 plates containing 5-fluorodeoxyuridine (FUDR) (Sigma-Aldrich) to a final concentration of 51μM. Day 0 is defined as the day the worms were placed on the FUDR plates. Every second day animals were scored by tapping with a platinum worm pick starting from day 7. Worms that crawled into the agar, onto the wall of the plate or died from

vulval bursting were censored. Every lifespan-measurement was repeated three times.

3.6 Thermotolerance assay

30 young adult animals from a synchronized population were transferred to each of 3 plates for every condition. Plates were put in an incubator preheated to 35°C for 6 hours. Then, plates were placed back into a 20°C incubator. Following a 5 hour recovery time the animals were scored every 24 hours by tapping with a platinum worm pick. All thermotolerance measurements were repeated at least three times.

3.7 Fluoreszcence microscopy

After treatments at least 50 worms per condition were placed on a 2% agarose pad, and immobilized by adding 25 mM NaN3 in M9 buffer. Pictures were taken by a Leica DMI6000B epifluorescence microscope with a DFC480 camera or by Nikon Eclipse E400 microscope with Diagnostic Instruments SPOT model 1.5.0 camera using appropriate filters.

In the case of DAF-16 localization studies, animals were sorted into three categories: ‘nuclear’ refers to animals that showed exclusively nuclear localization, ‘intermediate’ labels animals that had both nuclear and cytosolic fluorescence and ‘cytosolic’ refers to animals with solely cytosolic GFP or RFP expression.

3.8 mRNA expression analysis

mRNA from well-fed synchronized population of adult worms was isolated using GeneJET RNA Purification Kit (Thermo Scientific). The mRNA was then transcribed into cDNA by RevertAid™ Premium Reverse Transcriptase (Thermo Scientific). qPCR measurements were performed in an ABI 7300 Real-time PCR machine using Maxima™ SYBR Green/ROX qPCR Master

Mix (Thermo Scientific). Relative amounts of mRNAs were determined using the Comparative Cycle Treshold Method for quantitation and normalized to beta-actin mRNA levels.

3.9 Western Blotting

Synchronized population of animals were grown on 10 cm NGM plates with IPTG seeded with either RNAi or empty vector (EV) bacteria. Worms were washed three times using M9 buffer and frozen at -80°C. After thawing 200µl of lysis buffer (50mM This-HCl, 0.25% SDS, 1% Igepal, 150mM NaCl, 1mM EDTA, 2x Complete (Roche), pH 7.4) was added to the samples. In case of the proteasome-inhibition assays 6 M urea was also added to the buffer. After three freeze-thaw cycles samples were sonicated and centrifuged for 10 minutes at 10000g. Supernatant was used further. Samples were run in poly-acrylamide gel and transferred to nitrocellulose membrane. To control for protein content I incubated the membranes in Ponceau dye and used their pictures for densitometry. Blocking was done by incubation in TBS-T with 5% skim milk powder for 1 hour at room temperature. Primary antibodies were polyclonal SIR-2.1 and polyclonal Hsp90. Secondary antibody was HRP-labelled anti-rabbit antibody. Membranes were incubated with ECL reagent for 1 min and developed.

3.10 Dauer assay

10 gravid hermaphrodites were allowed to lay eggs on each plate for 4 hours. Then the plates were transferred to 25°C and animals were allowed to grow until the third day, when adults and dauer larvae were scored from each strain and condition.

3.11 Phenotypic characterization

10 gravid hermaphrodites were allowed to lay eggs on EV and daf-21(RNAi) plates for 1 hour in order to get a highly synchronous population of animals. The plates were kept at 20°C and progeny was scored for phenotypic differences after three days. Pictures were taken using Nikon Eclipse E400 microscope with Diagnostic Instruments SPOT model 1.5.0 camera.

3.12 Fertility assay

10 L4 hermaphrodites from each condition of each strain were placed onto treatment plates individually. Every 24 hours mothers were transferred onto new plates. Progeny was scored on the plates 48 hours after the removal of the mother. The assay was carried on till the last animal stopped laying eggs.

3.13 Statistical analysis

Statistical analysis was done by SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). Survival curves were compared using the Kaplan-Meyer log rank test. Pairwise comparisons were done using Student's t-test. Multiple comparisons were done with ANOVA. Variables were expressed as mean ± standard error of the mean (SEM). Statistical levels of significance are as follows: *:$p<0.05$, **:$p<0.01$, ***:$p<0.001$.

4. RESULTS

4.1 Characterizing the silencing of the HSP-90

4.1.1 *hsp-90* knockdown silences HSP-90 expression and induces the heat shock response

To investigate the impact of reduced HSP-90 capacity on longevity I employed a *hsp-90(RNAi)*. Assessing the impact of HSP-90 during larval development I started *hsp-90* silencing from hatching. *hsp-90(RNAi)* efficiently reduced *hsp-90* mRNA and protein levels in young adults compared to empty vector (EV) control. Consistent with its reported role in vulval development and muscle function, *hsp-90(RNAi)* fed worms exhibited a protruding vulva in ~90% of the population and a mild hypomotility, but no other developmental phenotypes. Likewise, *hsp-90* silencing from hatching neither arrested nor significantly delayed development.

To differentiate between the effects exerted by HSP-90 during larval development and adulthood, respectively, I also treated worms with *hsp-90(RNAi)* from the midst of L4 stage. As expected, no protruding vulva phenotype was detected.

hsp-90 silencing from hatching caused sterility accompanied by a lack of oocytes in the gonad, whereas that from the L4 stage reduced brood size. *hsp-90* knockdown employed from hatching or from the L4 stage similarly induced an *hsf-1* dependent *hsp-16.2* and *hsp-70* mRNA expression in young adults, consistent with a compensatory activation of the heat shock response.

Hence, *hsp-90(RNAi)* – employed either during or after larval development – is a safe approach to reduce HSP-90 capacity without compromising develoment and health.

6.1.2 Reduction in DAF-21/Hsp90 capacity limits normal lifespan

First, I measured the lifespan of N2 worms fed by either empty vector or *hsp-90(RNAi)* from hatching throughout the entire life. My results show that *hsp-90(RNAi)* shortened wild-type lifespan compared to EV. The reduction in lifespan caused by *hsp-90(RNAi)* was less pronounced, but still significant, if RNAi treatment was administered from the L4 stage throughout adulthood. The comparable lifespan reduction by *hsp-90* knockdown during and after development, respectively, suggests that *hsp-90* affects longevity independent of egg laying and fertility. The decreased lifespan, despite an increased heat shock response, which predicts and induces longevity, might be the consequence of different longevity promoting mechanism(s) impaired in the absence of an optimal HSP-90 capacity.

6.2 The effect of HSP-90 on the regulation of reduced ILS-induced lifespan

6.2.1 *hsp-90* is required from larval development for longevity conferred by reduced ILS

Increased longevity of animals with reduced ILS has been established as one of the most robust effects that prolong lifespan. Therefore I compared the lifespan of *daf-2* and *daf-2;daf-16* single and double mutant animals, respectively, grown on plates with bacteria harboring empty vector or *hsp-90(RNAi)*. *daf-2* mutation caused an increase in lifespan compared to wild-type which was entirely abrogated by *daf-16* loss of function. *hsp-90(RNAi)* treatment from hatching significantly reduced the lifespan of *daf-2* mutant worms showing a requirement for HSP-90 to fully manifest the increased longevity conferred by reduced ILS.

Next, I determined the effect of *hsp-90(RNAi)* employed from the L4 stage on the lifespan of *daf-2* mutants. In all 4 trials it extended the longevity of *daf-2* mutant animals, however, in two of them the effect was non-significant. These observations indicate a longevity supporting effect of HSP-90 primarily during, but not after, development, in both wild-type and *daf-2* nematodes.
Thus, it appears that the longevity action of HSP-90 might possess both a *daf-16* dependent as well as an independent component in both wild-type worms and those with reduced ILS.

6.2.2 *hsp-90* knockdown in neurons promotes dauer development of wild type, but in non-neuronal tissues does not affect dauer formation of *daf-2* mutants

Nematodes with lowered ILS have a high tendency to initiate an alternative developmental pathway and form dauer larvae under stressful conditions. I investigated the interference between *daf-2* and *hsp-90* in dauer development. As shown before, *daf-2* mutants grown on 25°C almost exclusively turned into dauer larvae, while having a secondary mutation in *daf-16* abrogated this effect. Silencing *hsp-90* from hatching in non-neuronal cells did not affect dauer formation in any of the strains tested. Therefore, I employed a strain that expresses the protein requiredfor RNAi in each cell including neurons, and confirmed and extended earlier findings about the neuronal requirement of sufficient HSP-90 function to bypass dauer arrest.
My findings together with the lifespan data indicate that HSP-90 in peripheral tissues affects wild-type and *daf-2* longevity that is spatiotemporally separated from its effect on development.

6.2.3 DAF-21/Hsp90 facilitates DAF-16A nuclear translocation

The fact that the impact of HSP-90 on *daf-2* lifespan is dependent on DAF-16 suggested a functional link between HSP-90 and DAF-16. An important step in DAF-16 activity in response to lowered ILS, as well as stresses including heat shock, is its nuclear translocation. First, I monitored the intracellular localization of DAF-16 in a strain containing a daf-16a/b::GFP transgene in response to *daf-2* and *hsp-90* knockdown employed from hatching. In *daf-2* silenced nematodes a large proportion of DAF-16A/B was localized in the nuclei. I found that silencing *hsp-90* partially inhibited this translocation. This result indicates that HSP-90 facilitates DAF-16 translocation in response to lowered ILS. I repeated this measurement by placing L4 larvae worms on RNAi plates and got similar results.

ILS targets two out of the three DAF-16 isoform groups, A and D/F/H, in longevity regulation. Therefore, I employed two strains that express different fluorescently tagged DAF-16 isoforms in a *daf-16* null mutant background: *daf-16a::rfp* and *daf-16d/f::gfp* and their respective *daf-2* mutant variants. RNAi treatment was employed from hatching, and DAF-16 localizaton was visualized on day 1 of adulthood.

DAF-16A::RFP showed an explicit nuclear localization while DAF-16D/F::GFP remained largely cytosolic in response to *daf-2* mutation compared to the respective strains with intact *daf-2* alleles. *hsp-90* knockdown, in accordance with its effect on DAF-16A/B::GFP, inhibited the translocation of DAF-16A::RFP, while did not affect the localization of DAF-16D/F::GFP. To gain an independent insight on the translocation of DAF-16 isoforms, I employed heat stress to induce translocation.

Heat shock induced a predominantly nuclear localization of DAF-16A/B::GFP, DAF-16A::RFP and DAF-16D/F::GFP. In response to *hsp-90(RNAi)* DAF-16D/F::GFP was still nuclear, while the nuclear localization of both DAF-16A/B::GFP and DAF-16A::RFP were abolished. I obtained similar results by RNAi treatment from L4 larval stage. These results support the requirement of HSP-90 for DAF-16A nuclear translocation.

6.2.4 HSP-90 is required for DAF-16A dependent transcriptional function

Next, I studied how *hsp-90* silencing affects the expression of various DAF-16 target genes. In the first series of experiments, in order to clearly isolate the undesired cross-talk from the other DAF-16 isoforms, I took use of the above *daf-16a::rfp* and *daf-16d/f::gfp* strains.

The induction of *sod-3* and *old-1* mRNA expression – caused by the *daf-2* mutation – were both diminished by *hsp-90(RNAi)* in the *daf-16a::rfp* strain, while they were unaffected in the *daf-16d/f::gfp* strain. I selected *scl-20* and *gst-20*, identified as specific DAF-16A targets. My experiments confirmed the upregulation of both genes by the *daf-2* mutant allele in the *daf-16a::rfp* strain and showed an efficient inhibition of *gst-20* expression by *hsp-90(RNAi)*. *lea-1* and *scl-1* were selected for our purposes as DAF-16D/F selective targets. In contrast to DAF-16A target genes, these two transcriptional targets in a *daf-16d/f::gfp* transgenic background were not inhibited by *hsp-90(RNAi)*.

To further examine a potential isoform-specific regulation I measured the expression of DAF-16A and DAF-16D/F isoform specific targets in a wild-type and mutant *daf-16* background in response to *hsp-90* silencing from the L4 stage.

Comparing the mRNA expression in a *daf-2* and *daf-16;daf-2* mutant strains, respectively, showed that the DAF-16A specific *sod-3*, *old-1*, *gst-20* and *scl-20* were induced by the *daf-2* mutation in a *daf-16* dependent manner and their expression was inhibited by silencing *hsp-90* with the exception of *scl-20*. DAF-16D/F specific target genes were supplemented by two additional genes: *col-183* and *R05D8.7*. I found that all mRNAs were efficiently induced in the *daf-2* mutant but not inhibited by *hsp-90(RNAi)*. My findings provide compelling evidence that HSP-90 specifically regulates the transcriptional activity of DAF-16A.

6.2.5 HSP-90 is not necessary for DAF-16A stability and acts upstream of its nuclear import

In *C. elegans*, the null mutation of the RLE-1 E3 ubiquitin ligase has been shown to lead to DAF-16 protein stabilization and DAF-16 dependent lifespan extension. If HSP-90 stabilized DAF-16 conformation, then reduced HSP-90 capacity would result in DAF-16 aggregation and disrupt lifespan extension in *rle-1* mutants. *hsp-90(RNAi)* did not appear to interfere with DAF-16 distribution or caused DAF-16 aggregation, while also not influencing the turnover of DAF-16 protein: I neither observed a decrease in the quantity of various fluorescently tagged DAF-16A proteins nor a compensatory upregulation of *daf-16a* mRNA upon *hsp-90* knockdown. Likewise, *rle-1* induced lifespan extension of the strain expressing solely the DAF-16A::RFP isoform still persisted in the absence of HSP-90, indicating that the stabilization of a functional DAF-16 protein does not require HSP-90. Thus, DAF-16 is unlikely to be a HSP-90 client.

When ILS signaling is ample, AKT-1 and AKT-2 kinases phosphorylate DAF-16/FOXO, which prevents its accumulation in nuclei by anchoring it to cytosolic 14-3-3 scaffold proteins. To assess if HSP-90 acts upstream or at the level of DAF-16 nuclear traffic, I employed a strain harboring the *daf-16a*^{AM}*::gfp* transgene, in which all AKT phosphorylation sites were mutated. DAF-16A^{AM}::GFP was nuclearly localized in animals possessing wild-type *daf-2*. If HSP-90 was necessary for DAF-16A to achieve its native functional conformation, then reducing HSP-90 capacity would result in an unstable DAF-16A^{AM}::GFP unable to enter the nucleus and would be degraded. Neither the quantity, nor the localization of DAF-16A^{AM}::GFP was modified by *hsp-90(RNAi)*, providing further evidence for the conformational independence of DAF-16A from HSP-90 and indicating that the nuclear import of unphosphorylated DAF-16A does not require HSP-90.

In addition, these findings made unlikely a HSP-90-dependent inhibition of nuclear export of DAF-16A. Instead, HSP-90 appears to influence DAF-16A activation upstream of its nuclear traffic.

6.2.6 HSP-90 ensures *daf-16a* dependent longevity

Based on these results I investigated how *hsp-90(RNAi)* employed from hatching affects longevity specified by individual DAF-16A and D/F isoforms in the context of reduced ILS by using strains expressing single isoforms in a daf-2;daf-16 double mutant background.

Both *daf-16a::rfp* and *daf-16d/f::gfp* transgenic strains exhibited longer lifespan compared to *daf-2;daf-16* background. *hsp-90* knockdown consistently diminished the lifespan of the DAF-16A::RFP expressing strain in all four biological replicates, while in two out of four trials it failed to

modify that of the DAF-16D/F::GFP transgenic worms. These results indicate that an optimal HSP-90 capacity from larval development plays a role in longevity through selectively ensuring DAF-16A function.

6.3 Lack of HSP-90 leads to proteasomal degradation of SIR-2.1

My colleague, Nguyen Minh Tu showed using mammalian cells that SIRT1 specifically requires Hsp90 for its stabilization, without it the protein goes through proteasomal degradation. During my PhD work, I investigated whether there is also a chaperone-client interaction between the two proteins in roundworms. I measured the changes in SIR-2.1 protein levels in response to *hsp-90(RNAi)* at 20 and 25°C. According to my results, silencing the *hsp-90* gene gén efficiently decreases the level of HSP-90 protein, while also leading to reduced SIR-2.1 protein levels. This could be observed both at 20 and at 25°C. Considering that the clients of HSP-90 often go through degradation in the proteasome in the absence of the chaperone, inhibiting this process makes it possible to investigate wether this is the case with SIR-2.1. I inhibited the functionality of proteasomal degradation by using MG132 as a proteasome-inhibitor. As a negative control, I also employed *sir-2.1(RNAi)* treatment. In these trials, *hsp-90(RNAi)* lead to around 40% reduction in the SIR-2.1 protein level, similarly to *sir-2.1(RNAi)*. Treatment with MG132 increased SIR-2.1 protein levels in animals fed by EV containing bacteria. Furthermore, MG132 restored SIR-2.1 levels to the level of EV control in combination with *hsp-90(RNAi)*, while not doing so in the case of *sir-2.1(RNAi)*. Besides, in some experiments a portion of the SIR-2.1 proteins were not able to enter the resolving gel and accumulated on top of it in response to proteasome inhibition in the EV- and *hsp-90(RNAi)*-treated samples. These observations

suggest a proteasomal degradation of SIR-2.1, which is further increased by the absence of HSP-90.

My measurements – taken together with the results of my colleague about mammalian SIRT1 – confirm the hypothesis that functionality of the SIR-2.1 protein indeed requires the presence of the HSP-90 chaperone, since there is a chaperon-client interaction between the two proteins.

5. CONCLUSIONS

During my work, I investigated the role of HSP-90, the *C. elegans* ortholog of the important human chaperone Hsp90 in the regulation of development and lifespan.

The most important new results of my doctoral thesis are as follows:

1. I demonstrated that HSP-90 is required for the proper development and fertility of *C. elegans* in a spatiotemporal manner.

2. I showed that silencing HSP-90 shortens the normal and extended lifespan caused by reduced ILS in roundworms.

3. As a possible mechanism, I revealed that HSP-90 is required selectively and upstream from intracellular localization to the translocation and transcriptional activity of the DAF-16 transcription factor isoform A.

4. I proved that the *C. elegans* SIR-2.1 requires the presence of HSP-90 as a client protein.

6. PUBLICATION LIST

Publications directly related to the thesis:

Somogyvári M, Gecse E, Sőti C. (2018) DAF-21/Hsp90 is required for *C. elegans* longevity by ensuring DAF-16/FOXO isoform A function. *Sci Rep*, 8: 12048. IF: 4,122

Nguyen MT, **Somogyvári M**, Sőti C. (2018) Hsp90 Stabilizes SIRT1 Orthologs in Mammalian Cells and C. elegans. *Int J Mol Sci*, 19: 3661. IF: 3,687

Publications not directly related to the thesis:

Burnett C, Valentini S, Cabreiro F, Goss M, **Somogyvári M**, Piper MD, Hoddinott M, Sutphin GL, Leko V, McElwee JJ, Vazquez-Manrique RP, Orfila AM, Ackerman D, Au C, Vinti G, Riesen M, Howard K, Neri C, Bedalov A, Kaeberlein M, Soti C, Partridge L, Gems D. (2011) Absence of effects of Sir2 overexpression on lifespan in *C. elegans* and Drosophila. *Nature*, 477: 482–485 IF: 36,280

Spiró Z, Arslan MA, **Somogyvári M**, Nguyen MT, Smolders A, Dancsó B, Németh N, Elek Z, Braeckman BP, Csermely P, Sőti C. (2012) RNA interference links oxidative stress to the inhibition of heat stress adaptation. *Antioxid Redox Signal*, 17: 890-901 IF: 7,189

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