FINAL PROGRESS REPORT

Heat-shock and metabolic stress responses in aging

1. SUMMARY OF ACHIEVEMENTS OF NNF-78794

The NNF-78794 project has resulted in several important observations:
-- the calorie restriction mimetic resveratrol induces chaperones, thermotolerance and lifespan extension, all of which requires the heat shock transcription factor HSF1 downstream of the sirtuin deacetylase SIR-2.1, thus providing a mechanism how protein maintenance is coupled to responsiveness in changes to the metabolic state (Tóth et al, manuscript in preparation)
-- overexpression of SIR-2.1 does not extend lifespan in *Caenorhabditis elegans* (nor in *Drosophila* melanogaster) challenging the current paradigm about the central role of sirtuins in longevity regulation (Burnett et al, manuscript under revision), however, it enhances thermotolerance via HSF1 (Tóth et al, manuscript in preparation)
-- a major mammalian sirtuin ortholog SIRT1 does not seem to activate the heat shock response and its beneficial effect observed in protein misfolding is not mediated by its deacetylase activity in mammalian cells (Arslan et al, submitted manuscript, work in progress)
-- the specific chaperone Hsp90 is required for SIRT1 folding and modulates its localization in mammalian cells (Nguyen et al, manuscript in preparation)
-- oxidative stress inhibits thermotolerance and the heat-shock response in mammalian cells and in *C. elegans* (Spiró et al, manuscript in preparation)
-- lifespan extension by complete dietary deprivation does not require classical mediators of caloric restriction (such as SIR-2.1 and AMPK), but fully depends on a cell-autonomous function of HSF1 and on the TGF-β ortholog DAF-7 (6: Dancsó et al, 2010; work in progress)
-- the antioxidant response regulator SKN-1 transcription factor is required for innate immunity and is activated *via* the PMK-1 p38 MAPK in *C. elegans* (Papp et al, manuscript in preparation)
Besides these, we have disseminated the results obtained in several interantional scientific meetings (International *C. elegans* Meeting, FEBS as well as IUBMB-FAOBMB Meetings, and 7th European Congress of Biogerontology) and in national conferences (Hungarian Biochemical Society and Membrane Transport Conferences).

2. DETAILED REPORT

Resveratrol (RSV) is a plant dietary restriction mimetic, a promising drug-candidate against cancer, ischemic injuries, cardiovascular and inflammatory diseases as well as neurodegeneration. Besides, it extends life-span from yeast to high fat diet-fed rodents (1, for the sake of simplicity, only references of high importance are cited). Its putative target is the sirtuin family of NAD^+-dependent deacetylases. There is one major sirtuin in *C. elegans* (SIR-2.1) and seven (SIRT1-7) in mammals, SIRT1 showing the greatest homology to the invertebrate protein. SIRT1 is activated under low calorie conditions and deacetylates and regulates a number of key signaling molecules including p53, FOXO and PPARgamma (2). Facilitated by the initial research in invertebrates, SIRT1 is implicated as a master regulator of metabolic stress and is a leading drug target of various pharma companies, including GSK. Recently, both RSV's effect on longevity and the role of sirtuins in its action have been challenged and evoked an intense debate (3-4). The heat shock response is a major defense mechanism guarding the proteome by modulating the levels of heat shock proteins (molecular chaperones) and is orchestrated by heat shock transcription factor-1 (HSF1). We have previously reported that resveratrol induces the heat shock response and confers thermotolerance in mammalian cells (5). Taking use of the versatile model system *C. elegans* and

mammalian cell culture, we set out to investigate how RSV and SIR-2.1 affects lifespan and stress resistance and what role the heat shock response plays in their action.

2.1. Resveratrol extends lifespan and activates the heat shock response via SIR-2.1 in *C. elegans*

Initially we observed that RSV induced all the expression of the HSP-16.2 major heat shock protein promoter, thermotolerance and confirmed that it did extend life-span in *C. elegans* at both 20ºC and 25ºC, however, at different concentrations. Though the temperature-dependent effects suggest distinct pathways or pleiotropic effects inducing damage, both temperature required functional HSF1. HSP-16.2 is a major heat shock protein in worms, its overexpression induces longevity. Intriguingly, while hsp-16.2 was necessary to induce thermotolerance, its effect was negligible on RSV-induced lifespan extension, suggesting that the entire heat shock response governed by HSF1 regulates lifespan in response to resveratrol. To elucidate the specific role of the heat shock response in RSV's effects, we studied the involvement of hsp-4, an ortholog of the major ER-resident glucose-regulated protein Grp78/BiP. Neither the expression of the HSP-4 promoter was changed, nor was it required for RSV-induced lifespan extension. Similarly, RSV did not change the autofluorescence of worms, a marker of lipofuscin, i.e. molecular damage accumulation. Moreover, using the *hsf-1(sy-441)* mutant strain possessing a basal DNA-binding property but deficient in transactivation suggests that resveratrol signaling is involved in HSF1 transactivation. Together, these data strongly imply that resveratrol neither exerts its effects via a pleiotropic induction of various stress responses (such as the UPR) nor via an accumulation of molecular damage, but instead, via a specific activation of HSF1.

Then we asked how how *sir-2.1* was involved in RSV action in *C. elegans*. Using *sir-2.1(ok434)* knockout worms and *sir-2.1(RNAi)* we observed that both the RSV-induced thermotolerance as well as the lifespan extension required SIR-2.1, while the increased thermotolerance of a hormetic pre-heat stress did not. These data suggest that SIR-2.1 is needed to induce HSF1 activation in response to resveratrol, that leads to increased survival (i.e. lifespan) in physiological conditions as well as under chronic and acute proteotoxic stress (heat shock) (manuscript in preparation).

2.2. SIR-2.1 overexpression does not extend lifespan in *C. elegans*

Our data on RSV-induced lifespan extension raised the idea that the genetic activation of SIR-2.1 would extend lifespan *via* HSF1. To investigate whether a genetic activation of SIR-2.1 would boost stress adaptation, we initially analyzed the thermotolerance and lifespan of a high-copy SIR-2.1 overexpressor strain, LG100. We, consistent with the original reports (cited in refs. 3 and 4) found that the transgene markedly induced longevity. We also observed an increased thermotolerance of the mutant. However, these effects were not seen in the double mutant harboring the *hsf-1(sy-441)* allele, suggesting that the activatable HSF1 is required for these beneficial effects to take place in *C. elegans*. In the meantime David Gems (UCL, London, UK) and other groups have found that LG100 loses its longevity after backcrossing, implying that longevity is caused by a background mutation. This finding presented at the 2010 *C. elegans* meeting have evoked an intense interest, since it may cast doubt on the crucial role of sirtuins in longevity and may lead to its therapeutic potential to be re-tested. Thus, instead of making further analysis of the questionable effect of SIR-2.1 overexpression in a SIR-2.1;HSF-1 overexpressing double transgenic strain, we focused our attention on the effect of SIR-2.1 overexpression on longevity. To systematically address the role of SIR-2.1 in lifespan regulation, an international collaboration involving seven research groups were initiated by the Gems lab. We, both independently and in blinded cross-controlled trials found that both the high copy LG100 as well as the low copy SIR-2.1

overexpressor strains (NL3909) lose their reported longevity upon backcrossing, while retain their increased SIR-2.1 levels as shown by Western blot. Longevity in the LG100 strain is coupled to a sensory neuronal mutation that is implicated in dietary restriction. These data highlight the proper design and control of genetic experiments in transgenic nematode models and suggest that SIR-2.1 overexpression does not extend lifespan in *C. elegans*. Moreover, then lack of this effect jas been confirmed in Sir2 overexpressing *Drosophila*, altogether suggesting the lack of effect of Sir2 in invertebrates (Burnett et al, manuscript under revision). Also, our data indirectly suggest that the background mutation in LG100 may be the one that extends lifespan in an HSF1-dependent manner, which may have implications in dietary restriction. Apart from a direct impact on lifespan, sirtuins may play a role in stress adaptation. In support of this, we found that both LG100 as well as NL3909 strains retain an increased thermotolerance after back-crossing, and the increased thermotolerance is abolished by RNAi-s against both *sir-2.1* and *hsf-1*. Hence, the genetic activation of SIR-2.1 induces adaptation to high temperature stress in nematodes, which suggests an important role in various pathophysiological conditions (Tóth et al, manuscript in preparation).

2.3. SIRT1 does not seem to induce the heat shock response, but protects from proteotoxicity in mammalian cells

At the time we observed a sir-2.1-dependent longevity in worms we hypothesized that SIR-2.1 would deacetylate and activate HSF1. Since dietary restriction has been linked to upregulation of self maintenance via the activation of stress responses, this interaction would provide a mechanism how the metabolic state and dietary restriction would be coupled to stress adaptation and protein homeostasis (6). To test this hypothesis and to get an insight whether the sirtuin-HSF interaction is conserved in mammals we used transient transfection of SIRT1 in COS-7 cells. Strikingly, our initial observations showed that both wild-type (wt)SIRT1 as well as the deacetylase deficient H363Y point mutant (ddSIRT1) inhibited HSF1-dependent transactivation in a reporter-based assay. Moreover, there was no significant change in the basal or heat induced level of the major heat shock protein, Hsp70. In the meantime the Morimoto group published that SIRT1 deacetylates and activates HSF1 and improves thermotolerance in HeLa cells (7). Based upon the apparent contradiction of our findings and the importance of the results we wanted to re-test this phenomenon in a more physiological setting and started collaboration with Manuel Serrano (CNIO, Spain) who engineered SIRT OE and KO mice. Using mouse embryonic fibroblasts (MEFs) we found that both SIRT1 OE and KO modestly induced Hsp70 protein expression, however, both cells displayed compromised thermotolerance compared to wild-type fibroblasts. Moreover, the transfection of human SIRT1 in SIRT1 KO MEFs inhibited, while silencing SIRT1 by an anti-sirt1 siRNA induced HSF1 transactivation in COS-7 cells. This latter, however, was not manifested at the protein and functional levels, as neither transient overexpression nor knock-down of SIRT1, respectively, affected Hsp70 protein level, and had essentially no effect on the thermotolerance in COS-7 cells. While Hsp70 protein upregulation can be a compensatory response to chronic changes in SIRT1 levels in MEFS, our results primarily show that the lack of SIRT1 does not impair, while overexpression of SIRT1 (independently of its deacetylase activity) does not seem to activate the heat shock response. Additionally, we could not detect a major change in the the acetylation status of HSF1, but this result is preliminary and requires further confirmation. The abovementioned results demonstrate that the SIRT1-HSF1 interaction may be more complex than proposed and published (7). Further studies in this direction and to elucidate other plausible modes of SIRT1 action are under way.

SIR-2.1-SIRT1 has been reported to protect from polyQ and Alzheimer's neurodegenerative misfolding diseases in worms, mammalian cells (8, 9). To utilize a physiological model of proteotoxic stress (and neurodegenerative diseases), and to further investigate the role of SIRT1 in protein homeostasis, we tested the overexpression of SIRT1 on cells expressing inert misfolded mutant proteins. We engineered two constructs: both mutant chloramphenicol acetyltransferase

(CAT) and degron-GFP destabilized variants are almost identical to their wildtype counterparts in molecular weight and sequence meaning no difference in costs of expression/degradation. Both CAT and GFP are widely used in eukaryotic cells, and no high affinity interactions of these proteins with other eukaryotic proteins have been described. As both CAT and GFP lack sequences associated with a particular disease (such as polyQ or polyA expansions), the specific mechanisms associated with disease models cannot be anticipated. Finally, both CAT and GFP is devoid of cellular functions, hence, no functional benefit is lost upon their misfolding. Thus, these factors enable us to specifically study the effects confined to protein misfolding *per se*. These constructs induced cellular toxicity and promoted cell death in response to stress. Interestingly, both wt and ddSIRT1 protected the cells to similar extent from toxicity as shown by the Annexin assay. However, this effect still persisted after an anti-hsf1 siRNA treatment. These findings suggest that SIRT1 protects from proteotoxicity, however, it is not mediated by its deacetylase. Recently, growing body of evidence shows deacetylase-independent functions of SIRT1 including neuroprotection (10, 11), however, the molecular mechanism of SIRT1 remains enigmatic. Our results extend these functions to protein misfolding, and recall tests on the involvement of SIRT1 deacetylase activity in protection from neurodegenerative disease models (8, 9), having implications on the development and use of SIRT1 activators as potential drug candidates (12) (Alper et al, submitted manuscript).

2.4. Hsp90 chaperones SIRT1

Hsp90 is an essential heat shock protein mainly involved in the folding and scaffolding of a plethora of signaling molecules, like p53, steroid receptors, protein kinases (Raf, Akt). The Hsp90 inhibitor geldanamycin induces the disruption of the Hsp90-client interactions, and results in cell proliferation arrest or cell death, hence, is a promising chemotherapeutic agent currently in clinical trials (13). Besides its role in senescence, SIRT1 is also implicated in malignant transformation. To address if SIRT1 is a client of Hsp90, first we treated cells with geldenamycin and observed that it reduced the level of SIRT1 a time- and concentration-dependent manner. Analyzing the detergent insoluble pellets of cells it turned out that geldanamycin induced the destabilization and the degradation of SIRT1 and it could be prevented by proteasome inhibition. Immunoprecipitation experiments showed that SIRT1 forms a physical complex with Hsp90 which is disrupted by geldanamycin. Interestigly, RSV enhances complex formation. Moreeover, RSV inhibits the ATP-binding of purified Hsp90 in vitro, suggesting it binds to the protein. We tested the funtions of interactors known to be in complex with both SIRT1 and Hsp90: however, we could neither detect a change in the activity of proteasome by enzyme activity measurements, nor p53 by a p21 transactivation assay. Then in light of these negative findings, instead of testing a highly complex NFκB activation, we focused our attention to the analysis of the localization of SIRT1. Immunofluorescence microscopy revealed that geldanamycin induced the relocalization of nuclear SIRT1 in the cytosol, while Hsp90 overexpression retained it in the nucleus, suggesting that Hsp90 may play a role not only in the folding, but also in the compartmentalization, of SIRT1. Thus, we conclude SIRT1 is a novel Hsp90-client protein, hence, Hsp90 may facilitate signaling of the metabolic state, and SIRT1 inactivation may contribute to the antitumor action of geldanamycin. Intriguingly, using bioinformatics on Hsp90-networks we found putative novel clients of Hsp90. Currently these assumptions are experimentally tested and being validated (Nguyen et al, manuscript in preparation).

2.5. Oxidative stress inhibits thermotolerance and the heat shock response in mammalian cells and in *C. elegans*

As RSV is a potent antioxidant, we tested its effect in the protection of the heat shock response from oxidative stress. Surprizingly, and contrary to the literature, we found that H_2O_2 treatment did not induce Hs70 expression, but inhibited heat-induced Hsp70 expression in a concentration-dependent manner. These data are consistent with recent observations (14). We found that RSV and the antioxidant N-acetyl-cystein could both eliminate this effect. As an extension of these, the inhibitory effect of H_2O_2 could also be observed in *C. elegans* and required HSF1. Currently the oxidative stress-induced signaling is being identified. These results uncover a novel element in the regulation of heat shock response/thermotolerance under oxidative conditions which may have implications in disease susceptibility and aging (Spiró et al, manuscript in preparation).

2.6. Dietary deprivation requires HSF1 and is partially mediated by DAF-7 in *C. elegans*

Dietary restriction is is the most potent environmental intervention that induces longevity. In *C. elegans* it is possible to fully withdraw the food source from adult worms, and it results in the greatest effect of lifespan. Moreover, the involvement of *hsf-1* has been reported (15). We have set up DD, and observed that it markedly extended the lifespan of worms both at ambient as well as at decreased and increased temperatures, and this effect was always dependent on *hsf-1*. Moreover, DD also induced thermotolerance in an *hsf-1*-dependent manner. Analyzing the possible signaling mechanisms, we observed that neither *sir-2.1* nor the hunger-signal sensor AMP-dependent protein kinase *aak-2* were required for dietary deprivation-induced longevity and thermotolerance. In contrast, and as reported, in our experiments both *aak-2* and *sir-2.1* was needed for resveratrol's effect. These findings confirm that dietary deprivation does not depend on classical dietary restriction mediators, like sir-2.1 or AMPK, buth similarly to resveratrol, uses HSF1 as a downstream mediator.

Signals in some dietary restriction protocols are detected by chemosensory neurons and the efficient heat shock response upon temperature upshift is initiated by thermosensory neurons. Therefore we aimed to test the possible involvement of thermosensory neurons in dietary deprivation. However, while heat-induced thermotolerance was compromised, that to dietary deprivation was retained in mutants with impaired thermosensory neuron function (6). Altogether these results show that dietary deprivation may lead to the induction of a cell-autonomous heat shock response possibly by using a novel, yet, unknown signaling mechanism(s). Indeed, we were able to show that DD-induced thermotolerance required the TGFβ ortholog DAF-7 which was not further altered by the lack of HSF1. DAF-7 is expressed in two ASI neurons, is central to the *C. elegans* dauer pathway and is involved in neuroendocrine signaling (16), hence it may be a mediator of DD-s cell non-autonomous effect of lifespan *via* HSF1. However, DAF-7 only partially mediated the DD-induced longevity, suggesting other factors are involved in DD signaling (work in progress).

2.7. SKN-1 is required for pathogen resistance in *C. elegans*

Immunity is an important determinant of longevity and resveratrol has been shown to modulate the immune response in various models (17, 1, 18). Besides, stress and immune responses are intimately linked at the cellular level. *C. elegans* is an excellent model to investigate the innate immunity, which takes place both at the initiation as well as the termination of the immune response (19). Therefore, we set up an experimental model and initiated experiments on the role of RSV and SIR-2.1 in innate immunity in the worm. Fist, we attempted to examine the effect of RSV in a killing assay by bacteria, but resveratrol *per se* inhibited bacterial growth, thus pathogen resistance was not possible to directly test. Moreover, *sir-2.1* KO mutants displayed a pathogen resistance similar to wild-type worms. Another target of resveratrol is the SKN-1 transcription factor, an ortholog of human NF-E2-related factor 2 (Nrf2), a key regulator in the antioxidant response and in

dietary restriction (20, 21). Moreover, Nrf2 is responsible for the anticancer effects of dietary restriction, where the importance of immune surveillance is implicated (22). In our preliminary experiments *skn-1(zu135)* KO mutant worms showed a diminished immune response to and an increased bacterial colonization of, *Pseudomonas aeruginosa*, a dangerous human opportunistic pathogen. We demonstared the SKN-1 nuclear localization and the SKN-1-dependent transactivation of SKN-1-target genes. Moreover, we could identify SKN-1 activation is linked to the p38-MAPK orthologous PMK-1 pathway, an important signal in *C. elegans* immunity (19). Thus, we conclude that SKN-1 is required for the pathogen resistance and this may connect oxidative stress, dietary restriction and innate immunity in *C. elegans*, that might have implications in similar human conditions (Papp et al, manuscript in preparation).

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Manuscripts related to the NNF-78794 project in progress:

1. Burnett C, Valentini S, Cabreiro F, Goss M, Somogyvári M, Piper MD, Houdinott M, Sutphin GL, Leko V, McElwee JJ, Vazquez R, Orfila A-M, Ackerman D, Riesen M, Howard K, Neri C, Bedalov A, Kaeberlein M, Sőti C, Partridge L and Gems D. Absence of effects of Sir2 over-expression on lifespan in *C. elegans* and *Drosophila*. (manuscript under revision)
2. Arslan MA, Chikina M, Csermely P and Sőti C. Misfolded proteins inhibit proliferation and promote stress-induced death in mammalian cells. (submitted manuscript)
3. Tóth ML, Somogyvári M, Dancsó B, Spiró Z, Csermely P and Sőti C. HSF-1 is an essential downstream mediator of resveratrol-induced, SIR-2.1-dependent longevity in *C. elegans.* (manuscript in preparation)
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