New molecular mechanisms of aging regulation
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Chapter 5

General discussion and future perspectives
Aging is an inevitable process across all organisms. However, its rate is not steady but instead plastic and highly regulated. Central to this regulation are transcription factors, which reside downstream of environmental influences, food intake, hormones and other signals and relay them into aging-modulating responses, i.e. regulation of stress resistance and longevity promoting genes. A good understanding of these aging-regulatory signaling pathways and their transcriptional outcomes is crucial, to be able to manipulate aging, i.e. to find therapeutics that can decelerate and ameliorate the aging process and delay age-related diseases for the sake of a healthier and longer life.

Here, I will discuss the roles of two factors that are required for the functions of the central lifespan regulatory transcription factor DAF-16/FOXO - the new lifespan regulatory transcription factor HLH-30/TFEB, and a regulator of DAF-16/FOXO, the SMK-1-containing Protein Phosphatase 4 (PP4SMK-1). Finally, I will touch upon the directions that this research may take in the future.

1- HLH-30 and DAF-16 are combinatorial transcription factors for the regulation of stress resistance and longevity under low IIS

In chapter 3, we identified a crucial role for the conserved basic helix-loop-helix transcription factor HLH-30 as a key player in pathways leading to stress resistance and longevity. HLH-30 and its closest human ortholog, Transcription Factor EB (TFEB), were previously shown to function as master regulators of lysosomal biogenesis and autophagy, which are essential and significant processes in the context of metabolism and regulation of longevity. Our study newly revealed that HLH-30/TFEB and the well-established aging-preventive transcription factor DAF-16/FOXO form a complex and work together as combinatorial transcription factors, in order to promote various anti-aging biological processes. This cross-talk enables favorable and customized transcriptional responses to nature’s various stress conditions, which allows for finer and tighter control over the organism’s stress response mechanisms, particular aspects of development, as well as prevention of aging, thus promotion of longevity.

HLH-30 and DAF-16 co-occupy the promoter regions of a significant number of target genes

In a part of this study, we looked for the promoter regions that were directly bound by DAF-16 or HLH-30, using ChIP-seq experiments. Among the target regions of the two transcription factors, a large fraction were co-occupied by both transcription factors. Looking at genes downstream of the bound promoters and taking mRNA-seq data into account, we observed that both transcription factors acted predominantly as transcriptional activators, and that the genes downstream of the co-occupied promoter regions were enriched for aging-related genes, indicating that the two transcription factors collaborate to promote expression of aging regulatory genes thus to extend the lifespan of the organism.

In order to gain more mechanistic insight into the synergy between DAF-16-HLH-30, we checked whether DAF-16 and HLH-30 showed any interdependence for their recruitment to the co-occupied promoter regions. While we observed no significant effect of DAF-16 depletion on the binding of HLH-30 to such regions, we observed a small but significant reduction in DAF-16 binding to these promoters in the absence of HLH-30. In the light of
these findings, we concluded that neither DAF-16 nor HLH-30 are essential for each other’s binding to co-bound promoters, although HLH-30 may mildly assist DAF-16’s binding to such regions. Nevertheless, as a future direction, it would still be interesting to test whether DAF-16 and HLH-30 are interdependent for Pol II recruitment, which would bring us closer to a complete mechanistic picture of this transcriptional regulatory module.

In our search for DNA sequence motifs enriched in the promoter regions occupied by DAF-16 and HLH-30, we made yet another interesting observation: DAF-16-Associated Elements (DAEs, TGATAAG) was present in promoter regions occupied by at least one of the two transcription factors. This motif is thought to be bound by PQM-1, a transcription factor that ensures baseline expression of DAF-16-regulated genes when DAF-16 is inactive (Tepper et al., 2013). Given that DAEs are also found in promoters bound by HLH-30 alone, this suggests that PQM-1 may ensure baseline-expression of HLH-30-dependent genes, too. It will be important to test this experimentally.

Other aging-regulatory transcription factors that are synergizing with DAF-16

On a different note, there are actually many transcription factors downstream of the multitude of aging-regulatory signaling pathways. We cannot exclude that others, besides HLH-30/TFEB and DAF-16/FOXO, collaborate as part of such modules. For example, DAF-16 is also known to synergize with HSF-1, essential for heat stress responses and the longevity of IIS mutants (Hsu et al., 2003; Morley, 2003), SKN-1, essential for oxidative stress responses and the longevity of TOR signaling and IIS mutants (Park et al., 2009; Tullet et al., 2008), HIF-1, important for hypoxia-induced longevity (Leiser et al., 2013; Zhang et al., 2009), or the nuclear hormone receptor DAF-12, controlling dauer formation and longevity due to the lack of a germline (Gerisch et al., 2007; Hsin and Kenyon, 1999). Distinct from the DAF-16-HLH-30 module though, in all these alternative cases, a direct physical interaction between the transcription factors has not been observed.

The human HLH-30 ortholog, TFEB, selectively binds to FOXO1 but not to FOXO3:

In our study, we showed that DAF-16 and HLH-30 physically interact and can form a complex to mediate their combinatorial downstream functions. Thus, we were curious whether this complex would also exist in humans. To test this, we conducted co-IPs between the human ortholog of HLH-30, TFEB, and the two functionally closest orthologs of DAF-16, FOXO1 and FOXO3, in HEK293T cells, in which we blocked IIS by the PI3 kinase inhibitor LY294002. Interestingly, pulldown of TFEB led to co-IP of FOXO1, whereas surprisingly, we did not observe co-IP between TFEB and FOXO3, indicating specificity of TFEB for some human FOXO paralogs over others. According to this finding, combinatorial gene regulation by DAF-16/FOXO and HLH-30/TFEB may be conserved across metazoans. Thus, it is an exciting perspective to investigate the functional significance of such module in humans, which could serve as a new and powerful mechanistic target for interventions against aging and age-related diseases.
2- PP4SMK-1 is a positive regulator of DAF-16-mediated longevity

DAF-16 is the major transcription factor downstream of insulin/IGF signaling (IIS), relaying low IIS into the expression of stress resistance and longevity promoting genes. In this role, DAF-16 does not act alone, instead, it involves several regulators and cofactors, some of which were described in previous studies. One of the most potent regulators of DAF-16 is SMK-1, which is essential for DAF-16-mediated longevity under low IIS (Wolff et al., 2006). However, little is known about the mechanism by which SMK-1 affects DAF-16 functions.

In a previous study, SMK-1 was found to be essential in IIS mutants, to promote resistance to oxidative and UV stress as well as pathogens, whereas it did not influence thermotolerance or reproduction (Wolff et al., 2006). These data suggested that SMK-1 was involved in the regulation of a subset of DAF-16/FoxO downstream functions. We also knew that SMK-1 interacted with DAF-16 genetically and that they were co-expressed in similar tissues. However, the complete list of DAF-16 target genes co-regulated by SMK-1, and how SMK-1 acted on DAF-16 had not been shown. In Chapter 4, we described a molecular mechanism where SMK-1 acted as part of a PP4 complex and regulated the transcriptional initiation of a subset of DAF-16 activated genes by dephosphorylating the transcriptional initiation/elongation factor SPT-5. This specific gene set was uniquely dependent on PP4SMK-1, as their expressions seemed to be particularly dependent on transcription initiation as a rate-limiting step.

SPT-5 is a direct target of PP4SMK-1 in the regulation of DAF-16-mediated longevity

SPT-5 is part of the 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole sensitivity-inducing factor (DSIF) complex together with SPT-4, where SPT-4 was found to be essential for the protection of SPT-5 from degradation and to promote its interaction with RNA Pol II (Ding et al., 2010). Consistently, in our large-scale purification data, we observed both SPT-4 and SPT-5 as binding partners of SMK-1. However, we only found SPT-5 as a substrate of PP4SMK-1 in our unbiased phosphoproteomics data, arguing that of the two proteins, SPT-5 is the relevant substrate. In addition, when we carried out lifespan experiments, we knocked down spt-4, spt-5, and yet another member of the SPT family, emb-5 (SUPT6H), and again only spt-5 KD lead to lifespan shortening that was specific to low IIS – the phenotype also observed upon smk-1 KD. This illustrated to us that PP4SMK-1-mediated dephosphorylation of SPT-5 but not of other members of this family specifically regulates the transcriptional initiation of the aforementioned subset of DAF-16-dependent genes to promote stress resistance and longevity.

The influence of PP4SMK-1 on SPT-5 seems to affect SPT-5’s binding to transcriptional start and end sites. We also observed that overexpression of SPT-5 led to a slight lifespan extension in wild type animals on daf-2 RNAi. However, we were unsuccessful to observe lifespan phenotypes from the expression of phospho-site mutants of SPT-5, where we disabled all phosphosites targeted by PP4SMK-1, as identified by our phosphoproteomics. This may be due to the insufficient coverage of unbiased phosphoproteomics that failed to retrieve other sites of relevance. In the future, phosphomapping on SPT-5 pulldowns should be conducted for better coverage, hopefully then leading to a more complete picture of the PP4SMK-1-targeted phosphosites of on SPT-5.

Finally, while we are the first lab to derive a functional role from the physical interaction of PP4SMK-1 and SPT-5, this interaction itself has been observed before – most importantly in
other species: For example, Spt5 was identified as an interactor of Psy2/SMK-1 in mammalian TAP tag purifications (Gingras et al., 2005) as well as in large scale purifications conducted in yeast (Gavin et al., 2002). Spt5 was even shown to be a direct interactor of Psy2/SMK-1 by yeast two-hybrid experiments (Gingras et al., 2005). Therefore, it is possible that the function of PP4SMK-1 in promoting transcriptional initiation through SPT5 is conserved across metazoans, too.

The effect of PP4SMK-1 on DAF-16 itself appears mild and indirect

When we carried out nuclear translocation assays in the absence of PP4SMK-1, we observed a slight delay of DAF-16 nuclear entry. Additionally, by ChIP-seq experiments in the absence of PP4SMK-1, we observed slightly reduced DAF-16 binding to promoter regions. Instead, DAF-16 localization slightly shifted into the gene bodies. It was proposed in a previous study that transcription factors may constitute an obstacle to Pol II during transcription (Guertin and Lis, 2010). Thus, increased localization of DAF-16 to gene bodies may fulfill such role and contribute to the overall phenotypes caused by PP4SMK-1 loss.

The above phenotypes, although likely to be physiologically relevant, appear too mild to fully explain the strong effects of PP4SMK-1 on DAF-16 target gene expression, stress resistance, and longevity. Moreover, these effects are most probably indirect and unrelated to a change in the phosphorylation status of DAF-16. We supported this hypothesis by several experiments. First, we carried out several co-IP experiments, where we tried to pull down both SMK-1 and DAF-16 at different salt concentrations, hoping to observe the more transient interactions between a phosphatase and its substrate. However, consistent with published findings (Wolff et al., 2006), we did not see any physical interaction between SMK-1 and DAF-16. We then checked whether PP4SMK-1 had a role in promoting the dephosphorylation of DAF-16 by MS-based phosphomapping, but we could not observe any significant quantitative changes in the phosphopeptide profile of DAF-16 that depended on the presence or absence of PP4SMK-1. Furthermore, we did not found DAF-16 in our unbiased phosphoproteomics data while seeking PP4SMK-1 substrates.

We eventually observed that overexpression of DAF-16 enhanced the recruitment of Pol II to promoter regions, especially in the absence of PP4SMK-1. Interestingly, this accumulation occurred upstream of the TSS, where Pol II was docked in its inactive form, awaiting transcriptional initiation (Maxwell et al., 2014). This showed that overexpression of DAF-16 was not sufficient to initiate transcription, but that instead PP4SMK-1 thereby dephosphorylated SPT-5 controlled this rate-limiting step.

Why is PP4SMK-1 dispensable for heat shock response?

There has always been one perplexing phenotype caused by loss of SMK-1, which was already reported in 2006: Although PP4SMK-1 is important for longevity and most stress resistance phenotypes of daf-2 mutant animals, it is dispensable for the increased heat stress resistance of these animals. Consistently, in this study we found that the direct substrate of PP4SMK-1, SPT-5 to be important for their oxidative stress response but dispensable for heat stress survival. Previous studies have shown that for heat shock responses there is a specific transcription factor HSF-1 helping DAF-16 to mediate these gene expression events (Hsu et al., 2003). Another explanation could be that heat shock response needs to be so rapid that transcriptional initiation is omitted as a rate-limiting step at these genes. Indeed, it has been
shown that heat shock loci are predominantly regulated at the level of elongation (Kaplan et al., 2000; Lis, 1998). This idea is supported by a recent study, in which it was shown that HSF-1 activated expression of heat shock response genes mainly by promoting Pol II pause release and transcriptional elongation, rather than Pol II recruitment and transcriptional initiation (Mahat et al., 2016) in which PP4SMK-1 and SPT-5 play important roles.

We see these observations as a conclusive explanation for why PP4SMK-1 is essential for expression of only a specific subset of DAF-16 activated genes, in the activation of which transcription initiation is the rate limiting step.

**Does PP4SMK-1 have a lifespan regulatory role in other aging-regulatory signaling pathways?**

In this study, we focused on the regulation of a subset of genes transcriptionally activated by DAF-16 under low IIS. However, there are genes transcriptionally repressed by PP4SMK-1, too, and the mechanism for this repression remains unclear and a subject of future investigations. Furthermore, PP4SMK-1 may also synergize with other transcription factors that drive expression of genes highly dependent on transcriptional initiation. To pick a random example, we tested HLH-30 under low IIS. Interestingly, we found a significant overlap between the genes regulated by PP4SMK-1 and HLH-30 in our mRNA-seq data. Additionally, we retested other conditions triggering longevity such as dietary restriction (induced by eat-2 mutants) (Panowski et al., 2007), where longevity depends on SMK-1 as well as the catalytic subunits of PP4. Another previous study showed that SMK-1 is essential for the increased lifespan of mitochondrial electron transport chain (ETC) mutants (Wolff et al., 2006). Together, these observations show that the role of PP4SMK-1 reaches far beyond IIS and DAF-16 target genes also into other aging-regulatory signaling pathways and their downstream transcription factors. Further investigation is warranted, to understand which subset of the transcriptome is affected by PP4SMK-1 in different contexts and which crucial substrate(s) PP4SMK-1 is acting on, i.e. whether it is always SPT-5 or whether there are other substrate(s) that may contribute to the lifespan regulatory roles of this phosphatase complex, too.

**SPT-5 may not be the only substrate of PP4SMK-1 for promoting DAF-mediated longevity**

So far, we have been investigating the stress resistance and longevity related phenotypes mediated by SMK-1 on the level of transcriptional regulation. We showed that PP4SMK-1 is targeting SPT-5, which in turn helps DAF-16 to initiate transcription of numerous longevity and stress response promoting genes. However, in our unbiased phosphoproteomics data we also found other possible targets, which may influence lifespan by additional means, such as splicing factors, chromatin remodelers (i.e. components of the SWI/SNF complex), transcription factors (e.g. HLH-30) or translation initiation factors (e.g. IFG-1). Several of these substrates may in fact contribute to the DAF-16-mediated phenotypes that we observe under low IIS and thus be valuable to characterize.
3. Conclusions

In summary, this thesis gives some new mechanistic insights into how the transcriptional events downstream of aging-regulatory signaling pathways are orchestrated and regulated. These complicated events not only are conferred by individual transcription factors alone but also can involve their essential cooperation, like in the case of DAF-16 and HLH-30, specific cofactors and/or coregulators, like PP$^4_{\text{SMK-1}}$, or their interaction with the chromatin landscape to trigger specific downstream processes and result in fine-tuned outcomes that promote stress resistance or longevity. For the future, it is desirable to further complete this mechanistic picture of aging regulation, so we can eventually apply educated approaches to targeting these pathways to hamper aging and age-related diseases and promote a healthier and longer life.
References:


