Chapter 1

Introduction
1. Aging

During our lives we are exposed to multifarious environmental stressors such as UV, heat, or oxidizing agents which trigger tissue deterioration caused by the accumulation of damaged macromolecules within the cell. This in turn causes functional decline in our body over time - a process that we know as aging - and it makes us more prone to age-related diseases. Although once viewed as a haphazard process, the aging process has been shown subject to regulation by a number of essential signaling pathways, thus its rate can be accelerated as well as reduced. Targeting such mechanisms to reduce the rate of aging could be a new approach to defer age-related complications and thereby improve our quality of life.

1.1 Aging-regulatory signaling pathways:

There are several signaling pathways and downstream transcription factors regulating longevity. These pathways response to nutrient changes such as dietary restriction, or even hormonal signals from reproductive system, signaling a delay or lack of fertility as well as the environmental stressors mentioned before. Many of these pathways such as insulin/IGF-1 signaling (IIS) pathway and mechanistic target of rapamycin (mTOR) signaling, are responsible for the expression of specific gene sets which are generally involved in growth and reproduction of the organism under normal dietary conditions and less stressful environment. However, when there is impairment in insulin signaling, dietary restriction condition or more stressful environment, downstream transcription factors of these signaling pathways trigger the expression of the genes that become savior of the organism by protecting cells from harsh environmental conditions and even extending their lifespan. These aging-regulatory signaling pathways are comprehensively discussed in Chapter 2.

1.2 Insulin/IGF-1 is one of the most central aging-regulatory signaling pathways:

The first pathway that was shown to have an influence on lifespan regulation is Insulin/IGF-1 signaling (IIS) pathway. It was discovered by the observation that a single gene mutation in *daf-2*, the homolog of the insulin/IGF receptor in *Caenorhabditis elegans*, led to a two-fold lifespan extension of the organism (Kenyon et al., 1993). *C. elegans* is a wonderful model organism in the study of aging, since its mean lifespan is only 2-3 weeks and even a single gene manipulation may yield a significant lifespan extension, up to 3-fold (Kenyon et al., 1993). Since *C. elegans* is mostly comprised of post-mitotic cells, there was a possibility that lifespan effects of this pathway may not be preserved in more complex organisms. However, several studies revealed that mutations in the insulin/IGF receptor increased lifespan in different organisms as well, such as *Drosophila melanogaster* and mice (Tatar et al., 2001; Blüher, Kahn and Kahn, 2003; Holzenberger et al., 2003; Taguchi, Wartschow and White, 2007), illustrating that the lifespan regulatory role of the IIS pathway is evolutionarily conserved.

In *C. elegans*, IIS is acting via phosphoregulatory signaling cascades, which negatively regulate several downstream transcription factors, such as DAF-16, a FOXO transcription factor, SKN-1, an NRF-like xenobiotic response factor (Tullet et al., 2008), and the heat-
shock transcription factor HSF-1 (Hsu, Murphy and Kenyon, 2003; Morley and Morimoto, 2004). These transcription factors genetically synergize within the nucleus to activate the expression of several stress response factors, i.e. heat shock proteins, antimicrobial peptides, glutathione S-transferases and other antioxidant proteins, proteins related to metabolism, etc – most of which are aging-preventive and thereby also extend the lifespan of the organism.

Under unstressed and well-fed conditions in C. elegans (Figure 1A), insulin is abundant and binds to the insulin/IGF-1 receptor DAF-2, which then becomes active and directly phosphorylates the phosphatidylinositol 3-kinase (PI(3)K) AGE-1 that is responsible for the conversion of phosphatidylinositol 4,5-bisphosphate (PIP₂) to phosphatidylinositol 3,4,5-trisphosphate (PIP₃). Production of PIP₃ activates 3-phosphoinositide-dependent kinase-1 (PDK-1) which in turn places activatory phosphorylations on the downstream AKT family kinases AKT-1, AKT-2 and SGK-1 (Paradis and Ruvkun, 1998; Paradis et al., 1999; Hertweck, Göbel and Baumeister, 2004). When these AKT family kinases are active, they interact and phosphorylate DAF-16. As a result, phosphorylated DAF-16 is sequestered by the 14-3-3 proteins in the cytoplasm, which prevent DAF-16 from entering the nucleus and thus render it inactive – away from its target genes (Lin, 1997; Ogg et al., 1997; Paradis and Ruvkun, 1998).

When there is an impairment in IIS, for instance, the loss-of-function mutation of DAF-2, downstream kinases are no longer active and cease to phosphorylate DAF-16. This allows DAF-16 to evade sequestration by 14-3-3 proteins and to translocate into nucleus, where it binds to its target gene promoters in order to regulate transcription of stress response genes (Figure 1B). Besides, there also exist negative regulators of IIS, including the DAF-18/PTEN lipid phosphatase and the serine/threonine phosphatase PP2A PPTR-1, counteracting AGE-1/PI3K and AKT-1 signaling, and thus assisting DAF-16 to transform to its active form (Ogg and Ruvkun, 1998; Padmanabhan et al., 2009).

In addition to DAF-16, the heat shock transcription factor HSF-1, is another downstream transcription factor required for low IIS to delay aging and extend the lifespan of C. elegans (Garigan et al., 2002; Hsu, Murphy and Kenyon, 2003) (Figure 1A, B). HSF-1 is a master regulator of heat shock protein (HSP) expression, which makes it essential for the survival under thermal stress against its resulting proteotoxic stress (Hsu, Murphy and Kenyon, 2003; Morley and Morimoto, 2004). It was also shown that in daf-2 mutants, expression of a subset of heat-shock response genes is increased under normal/unstressed conditions (Hsu, Murphy and Kenyon, 2003). These findings reveal a possibility that, to some extend, IIS pathway may affect longevity by regulating protein homeostasis.

Another transcription factor, Skinhead (SKN)-1/Nrf, is involved in the regulation of detoxification genes, particularly promoting resistance to oxidative stress in response to low IIS (Figure 1A, B). Interestingly, DAF-16 contributes to the induction of a subset of these SKN-1 target genes, pointing to an occasional combinatorial role of these two transcription factors (Tullet et al., 2008).

1.3 DAF-16/FOXO is a major mediator of the transcriptional effects of IIS

DAF-16/FOXO is involved in many physiological responses to aging-regulatory and stress signals. Consistently, under low IIS DAF-16 becomes activated and drives the expression of
downstream genes, conferring phenotypes such as increased longevity, stress resistance, or the developmental decision of dauer formation (Figure 2) (Murphy and Hu, 2013).

Figure 1. The IIS pathway uses several downstream transcription factors to regulate longevity.

A) DAF-16 and SKN-1 transcription factors are phosphorylated by AKTs and SGK-1 and remain in the cytoplasm in inactive forms under high insulin signaling conditions. HSF-1 is not regulated by the phosphorylation of these kinases but it also remains in the cytoplasm under such conditions. B) Under low insulin signaling, such as in daf-2 mutants, DAF-16 and SKN-1 cannot be phosphorylated by AKTs and SGK-1, hence they translocate into the nucleus where they bind to the promoter of stress response genes required for longevity. Similarly, HSF-1 translocates into nucleus under low IIS conditions. However, its activity is regulated by a different repression complex (Chiang et al., 2012).

*Caenorhabditis elegans* DAF-16 is a member of the Forkhead box O family of transcription factors. Its key features, such as the forkhead DNA binding domain, its preferred DNA binding motif, its regulated sequestration by cytoplasmic 14-3-3 proteins at RxRxxS/T are all conserved from *C. elegans* to mammals (Obsil and Obsilova, 2008). Therefore, it is not surprising to observe an evolutionary conservation of DAF-16 functions, e.g. the regulation of growth, metabolism, stress resistance, and longevity across metazoans (Accili and Arden, 2004). Most relevant to this thesis is the conserved role of DAF-16/FOXO for lifespan regulation.
Many studies testing for the lifespan phenotypes of mutants in the insulin/IGF–FOXO signaling axis confirm that the lifespan regulatory role of this pathway through DAF-16/FOXO is conserved in different organisms, even in humans.

In Drosophila, systemic inhibition of IIS or tissue specific increase in the activity of FOXO in adipose tissue leads to lifespan extension (Kenyon, 2010). Similarly in mice, low IGF1 levels were found to significantly associate with lifespan extension in inbred mouse strains (Yuan et al., 2009). Finally, inhibitions of the insulin and IGF1 receptors as well as their upstream regulators and downstream effectors give rise to longevity (Bartke, 2008; Kappeler et al., 2008; Selman et al., 2008; Yuan et al., 2009).

Figure 2. DAF-16/FOXO controls a myriad of downstream events of IIS.

DAF-16/FOXO is the major downstream transcription factor of IIS regulating several metabolic, stress response and longevity related processes.

In human cohorts of Ashkenazi Jew, Japanese, Italians, Germans, Chinese, Californian, and New England centenarians, mutations impairing Insulin/IGF1 receptor function and variants of AKT and FOXO3A have been found associated with longevity (Kojima et al., 2004; Lunetta et al., 2007; Suh et al., 2008; Willcox et al., 2008; Anselmi et al., 2009; Flachsbart et al., 2009; Li et al., 2009; Pawlikowska et al., 2009). In addition, FOXO1 gene variants
have been linked to longevity in American and Chinese cohorts (Lunetta et al., 2007; Li et al., 2009).

2. Regulators and co-factors of DAF-16 that modulate its transcriptional activity and specificity

As mentioned in the previous section, DAF-16 is under tight regulation by several kinases and co-factors in the cytoplasm, i.e. AKTs, SGK-1 and 14-3-3 proteins, which determine the phosphorylation status and localization of DAF-16 and in turn affect the activity of DAF-16. However, the regulation of DAF-16 activity is way beyond such mechanisms. For instance, although nuclear translocation of DAF-16 is essential for target gene expression, neither triggering nuclear localization nor overexpressing DAF-16 is sufficient to promote longevity (Henderson and Johnson, 2001; Lin et al., 2001). Moreover, the DAF-16 Binding Element (DBE), a DNA motif DAF-16 is known to bind to is present in the promoters of 78% of C. elegans’ genes (Furuyama et al., 2000; Kenyon and Murphy, 2006), whereas only a small fraction of these genes were found to be DAF-16 targets in young adults (Murphy et al., 2003; Niu et al., 2011). Hence, DBE is insufficient to predict DAF-16 target gene expression. These observations point to additional mechanisms that control or assist the transcriptional activities of DAF-16. Indeed, when DAF-16/FOXO enters the nucleus in response to reduced insulin/IGF-1 signaling, it interacts with several co-factors/regulators that are important to modulate its activity. These include regulators such as the suppressor of MEK (SMK-1/SMEK) and host cell factor-1 (HCF-1), and finally co-factors like the chromatin remodeler SWI/SNF (Wolff et al., 2006; Lapierre and Hansen, 2012; Riedel et al., 2013). These regulators are essential for the distinct downstream functions of DAF-16/FOXO.

2.1 SMK-1 is an essential regulator of DAF-16-mediated longevity under low IIS

SMK-1 is the C. elegans ortholog of the protein phosphatase 4 (PP4) regulatory subunit 3 which was first identified in Dictyostelium as a suppressor of the MEK1 null phenotype (Mendoza et al., 2005; Chen et al., 2008).

SMK-1/SMEK has several distinct roles in different organisms. It is important for DNA repair during DNA replication by dephosphorylating gamma-H2AX (Chowdhury et al., 2008), asymmetric cell division in Drosophila by modulating the localization of Miranda (Sousa-Nunes, Chia and Somers, 2009), suppression of brachyury expression in embryonic stem cells (ESCs) (Lyu, Jho and Lu, 2011), the regulation of hepatic glucose metabolism in mice via dephosphorylation of cAMP-response element binding protein regulated transcriptional coactivator 2 (CRTC2) (Yoon et al., 2010), neuronal differentiation of neuronal stem cell (NSC) by negatively regulating Par3 (Lyu et al., 2013) and prevention of the recruitment of Mbd3/NuRD complex to the target promoters of genes important for neuronal differentiation during cortical development by interacting and mediating the degradation of Mbd3 via the ubiquitin-proteasome system (Moon et al., 2017). In a recent study, PP4/SMEK1 complex was also shown to be important for promoting miRNA biogenesis, by antagonizing the MAPK cascade by dephosphorylating a core co-factor HYL1 (Hyponastic Leaves 1) in Arabidopsis (Su et al., 2017).
Additionally and interestingly, in *C. elegans* SMK-1 was shown to be an essential positive regulator of DAF-16 (Wolff *et al*., 2006). It is required for DAF-16-driven longevity and resistance to oxidative stress, to ultraviolet radiation, and to pathogens. However, it is not required for DAF-16 to promote thermotolerance, to promote dauer formation or to delay reproduction in IIS mutants (Wolff *et al*., 2006). This suggests that SMK-1 genetically interacts with DAF-16/FOXO but regulates only a fraction of DAF-16/FOXO functions – likely by influencing the expression of only a specific subset of DAF-16’s target genes. Nevertheless, the mechanism by which SMK-1 controls this subset of DAF-16/FOXO target genes has remained entirely unclear (Murphy and Hu, 2013). Further studies should be conducted to understand by which mechanisms SMK-1 influences DAF-16 functions.

### 2.2 The helix-loop-helix (HLH) transcription factor HLH-30/TFEB collaborates with DAF-16/FOXO to provide stress resistance and longevity under low IIS

HLH-30 is a member of a group of 42 helix-loop-helix (HLH) transcription factors in *C. elegans* and the closest ortholog to the mammalian transcription factor EB (TFEB) (Rehli *et al*., 1999).

*C. elegans* HLH-30 was found to induce autophagy and in this regard to also be required for lifespan extension driven by a variety of longevity promoting pathways (Lapierryre *et al*., 2013). Autophagy is a lysosome-dependent catabolic process, which is activated by starvation and negatively regulated by the mammalian target of rapamycin complex 1 (mTORC1) (Sengupta, Peterson and Sabatini, 2010). The resulting breakdown products that emerge upon autophagy are used to produce energy and to create new cellular components (Settembre *et al*., 2013). Analogous to *C. elegans*, the role of TFEB in the transcriptional regulation of autophagy and lysosomal biogenesis genes is conserved in mammals (Settembre *et al*., 2011). Moreover, it has a conserved role in the mobilization of lipid stores in response to starvation (O’Rourke and Ruvkun, 2013; Settembre *et al*., 2013).

In addition to its functions in metabolism, HLH-30/TFEB was also found to be essential for host defense against infection, both in *C. elegans* and mammals (Visvikis *et al*., 2014). By virtue of its role in stress responses, several studies have been conducted to understand the beneficial effect of this nutritionally controlled stress-response factor as a potential therapeutic target in several lysosomal and protein aggregation disorders (Decressac *et al*., 2013; Pastore *et al*., 2013; Spampanato *et al*., 2013).

Interestingly, in a recent study, where DAF-16 co-factors were identified comprehensively by large scale IP and mass spectrometry (Riedel *et al*., 2013), HLH-30 was identified as a binding partner of DAF-16. This finding brings a new perspective to a possible synergy between these two transcription factors for the regulation of stress response and longevity related genes. However, further studies were required to understand the biological significance of this physical interaction.
2.3. Other regulators and co-factors of DAF-16 in IIS longevity pathway

DAF-16/FOXO is directly regulated by several kinases in addition to AKTs, such as AMP-dependent protein kinase (AMPK) (Apfeld et al., 2004), Ste20-like kinase CST-1/MST-1 (Lehtinen et al., 2006) and e-Jun N-terminal kinase JNK-1 (Oh et al., 2005). Further important regulators are the arginine methyltransferase PRMT-1 (Takahashi et al., 2011), the E3 ubiquitin ligase RLE-1 (Li et al., 2007), the ubiquiting hydrolase MATH-33 (Heimbucher et al., 2015), β-catenin (BAR-1) (Essers, 2005), and the NAD+-dependent deacetylase SIR-2.1 (Tissenbaum and Guarente, 2001) – just to mention the most prominent examples.

3. Role of the chromatin landscape in the regulation of longevity by aging-regulatory transcription factors

Aging-regulatory signaling pathways mainly confer their phenotypes by the hands of downstream transcription factors and their gene regulatory actions. Accessibility of these target genes is strongly influenced by the epigenome at these genomic loci, thus regulating the epigenome provides powerful means to influence aging. At the same time, aging-regulatory pathways can also actively shape the epigenome, to influence genome stability and manifest their transcriptional effects.

There are several key players that influence the chromatin landscape and are known to affect aging, such as chromatin remodelers, histone modifiers and histone variants, and eventually pioneer transcription factors which are essential for the recruitment of chromatin remodelers and/or histone modifiers target loci of particular importance. In the course of cellular and organismal aging, the epigenome undergoes substantial changes characterized by the general loss of heterochromatin as well as alterations in histone marks and DNA methylation (Booth and Brunet, 2016; Pal and Tyler, 2016). This results in a profound change in the chromosomal architecture, genomic integrity, and gene expression patterns. At the same time, it has been shown that manipulation of the epigenome, e.g. by mutations in histone modifying enzymes, has the ability to change the rate of aging in the organism. In the second chapter of this thesis, the major aging-regulatory signaling pathways and their synergy between transcriptional regulation and the epigenetic landscape will be discussed extensively.

4. Overview of the thesis

4.1 Aim

The aim of this thesis is to gain a better mechanistic understanding of specific transcriptional events that regulate the lifespan and stress resistance of the organism. We used one of the most convenient model organisms, the nematode C. elegans, for this kind of research, to study one of the most important aging-regulatory signaling pathways know to date: IIS with its downstream transcription factor DAF-16/FOXO. In particular, we investigated the roles and mechanisms of an important positive regulator of DAF-16, SMK-1, as well as an important downstream effector, HLH-30. Both proteins and their underlying mechanisms may eventually serve as new and powerful targets for therapeutic approaches to counteract aging process and age-related diseases.
4.2 Outline

In the first part of the thesis in Chapter 2, we give a general overview about aging regulatory signaling pathways, their downstream transcription factors, and the interplay between their transcriptional events and the epigenome.

In Chapter 3, we characterize the distinct and combinatorial roles of the two transcription factors, DAF-16/FOXO and the helix-loop-helix transcription factor HLH-30/TFEB, under low IIS and also other aging-regulatory stimuli. We show that while fulfilling some distinct and non-overlapping functions, DAF-16/FOXO and HLH-30/TFEB can also form a complex and jointly bind and regulate a subset of their target genes. This results in sophisticated combinatorial gene regulation, yielding perfectly tuned responses to promote stress resistance, longevity, and regulate certain aspects of development.

In Chapter 4, we focus on one of the essential positive regulators of DAF-16/FOXO, SMK-1/SMEK, and illustrate one of the mechanisms through which SMK-1 regulates DAF-16-mediated downstream processes. In order to determine this mechanism, we identified the binding partners of SMK-1 by large scale IP followed by mass spectrometry and observed that SMK-1 is part of a specific Protein Phosphatase 4 (PP4) complex. By genetic and biochemical analyses we then show that PP4<sup>SMK-1</sup> promotes Pol II recruitment and subsequent transcriptional initiation at the promoter regions of a subset of DAF-16-activated genes via dephosphorylating the transcription initiation/elongation factor SPT-5/SUPT5H and thereby mediating stress resistance and longevity under conditions of low IIS.

In Chapter 5, we give an overall summary of the studies in this thesis, discuss them in the greater context of aging research, and highlight important remaining questions in the field that still need to be addressed in future studies.
References:


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