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DECIPHERING MECHANISMS THAT REGULATE AGING IN *CAENORHABDITIS ELEGANS*

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Cover illustration: Made by the author. Nile red staining of fat reserves in *daf-2(e1370)* mutant *C. elegans*. Fat reserves are displayed in green.

Deciphering mechanisms that regulate aging in *Caenorhabditis elegans*

Thesis for Doctoral Degree (Ph.D.)

By

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"The pursuit of science is a never-ending journey into the unknown, fueled by curiosity and guided by reason."

- *Neil Armstrong*

Popular science summary of the thesis

Aging: the new frontier. For a long time, it was believed that aging represented an inevitable path we all had to follow. Even today, openly suggesting that aging is a process we can control tends to provoke skepticism and concern in many. At the same time, certain influencers are swaying the opinions of the public by investing substantial funds and engaging in diverse therapies to preserve their youth. Even within the scientific community, viewpoints differ. Some claim we're close to finding the fountain of youth and consider aging a complex disease, while others urge caution, stating that we are far from fully understanding aging and developing rejuvenating therapies. Regardless, the stage is set for confronting aging, as we are facing the challenges posed by the aging human population, the rising socio-economic burden, and the prevalence of age-associated diseases. Still, who is right, and how close are we really to unlocking the secrets of aging? Despite the heated debate surrounding the promise of rejuvenating therapies in humans, compelling evidence suggests that we can control and modify aging in various model organisms. In this thesis, I provide a brief overview of aging research followed by studies describing novel mechanisms of aging, focusing specifically on aging in *Caenorhabditis elegans* – a popular model system for this type of work. The findings within this thesis reveal previously unknown regulators of aging, aiding in the ongoing quest to unravel the mystery of aging.

Abstract

Aging is a multifaceted and poorly understood process characterized by physiological changes that culminate in the decline of an organism's functions. The research field of Biology of Aging aims to provide insight into this process, thereby contributing to the development of therapies that alleviate age-related symptoms in humans. This thesis provides a concise overview of research in this field, primarily focusing on the model organism *Caenorhabditis elegans*. The thesis also encompasses the studies conducted during my PhD, uncovering novel regulators and mechanisms of aging in *C. elegans*. Additionally, it examines the role of primary cilia in neuron differentiation. We anticipate that these studies will deepen our understanding of aging and certain age-associated diseases, thus facilitating the development of anti-aging therapies.

List of scientific papers

I. Exploring the interplay between DAF-16/FOXO and BAF-1/BANF1 in the regulation of aging
II. Mapping of transcriptionally relevant chromatin accessibility changes reveals LIN-39 as a driver of longevity in *Caenorhabditis elegans* with reduced insulin/IGF-like signaling
III. Transcriptomics-Based Screening Identifies Pharmacological Inhibition of Hsp90 as a Means to Defer Aging
IV. Primary cilia promote the differentiation of human neurons through the WNT signaling pathway

Contents

List of abbreviations

ACh	Acetylcholine
AMPK	AMP-activated protein kinase
ATAC	Assay for transposase-accessible chromatin
BAF-1	Barrier-to-autointegration factor 1
ChIP	Chromatin immunoprecipitation
CR	Caloric restriction
DBE	DAF-16 Binding Elements
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
FOXO	Forkhead box O
FPLD	Familial partial lipodystrophy
FUDR	5-fluoro-2′-deoxyuridine
GABA	Gamma-aminobutyric acid
HGPS	Hutchinson-Gilford progeria syndrome
HOX	Homeobox
H_2O_2	Hydrogen peroxide
IE	Inside end
IIS	Insulin/IGF-1 signaling
LIN-39	abnormal cell LINeage 39
MAD	Mandibuloacral dysplasia
ME	Mosaic end
MNs	Motor neurons
mRNA	Messenger RNA
mTOR	Mechanistic target of rapamycin
NGM	Nematode growth medium
NGPS	Nestor-Guillermo progeria syndrome

NR	Nile Red
OA	Oleic acid
OE	Outside end
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol 3,4,5-trisphosphate
PI3K	Phosphatidylinositol 3-kinase
RD	Restrictive dermopathy
RdRPs	RNA-dependent RNA polymerases
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen species
RT	Room temperature
Seq	Sequencing
siRNA	Small interfering RNA
t-BOOH	Tert-butyl hydroperoxide
TF	Transcription factor
Tn	Transposase
UV	Ultraviolet
VRKs	Vaccinia-related kinases

1 Introduction

Aging is a complex and poorly understood process characterized by physiological changes that ultimately lead to the functional decline of an organism. While aging was historically viewed as an inevitable natural process, modern research has revealed that specific cellular pathways influence the rate of aging (Zainabadi, 2018). The initial insights into the malleability of aging rates were associated with the effects of caloric restriction (CR). CR, achieved by reducing caloric intake to a level that avoids starvation, has been demonstrated to extend lifespan in numerous model organisms (Chapman, T, 1996; Jiang et al., 2000; Klass, 1977; S.-J. Lin et al., 2002; Weindruch et al., 1986; Weindruch & Walford, 1982). Subsequent studies have confirmed these findings also in dogs and non-human primates, and have suggested potential health benefits of CR in humans (Fontana et al., 2004; Kealy et al., 2002; Lane et al., 2000). Another factor that sparked interest in aging research was the identification of premature aging disorders in humans like Cockayne Syndrome or Hutchinson-Gilford Progeria Syndrome, characterized by phenotypic features reminiscent of human aging (Cockayne, 1936; Thomson & Forfar, 1950; Tollefsbol & Cohen, 1984). These early observations were pivotal in realizing that aging might be a regulated process and susceptible to interventions.

2 Literature review

1.1 Common biological theories of aging – a brief overview

Theories of aging have emerged with the aim of elucidating the reasons and mechanisms behind the aging process. While evolutionary theories of aging delve into the purpose of aging from an evolutionary standpoint, modern theories focus on unraveling the mechanisms underlying the aging process. Below are brief descriptions of some of the most prominent theories of aging (Jin, 2010; Lipsky & King, 2015; Vijg & Kennedy, 2016).

Evolutionary theories of aging

One of the first evolutionary theories was postulated by August Weisman, and it states that aging has evolved to remove older individuals from a population (Weismann et al., 1891). By sacrificing older individuals, resources can be redirected to support the growth and reproduction of younger individuals, thereby ensuring the survival of the species. However, this raises questions about whether natural selection would support the emergence of aging within a population. Natural selection tends to favor traits that enhance an organism's survival and reproductive success, which does not align well with aging having been selected for as a "cleansing" mechanism.

Peter Medawar proposed that aging is a consequence of the accumulation of genomic mutations over an organism's lifespan, resulting in a functional decline (Medawar, 1952). Before reproduction, organisms are subject to natural selection, which helps filter out harmful mutations. However, after reproducing, there is less evolutionary pressure to eliminate these harmful mutations, allowing them to accumulate and hinder the organism's survival. While this theory proposes a mechanism that could drive the aging process, it lacks conclusive evidence to support mutations as the primary cause of aging. In addition, it does not consider

other well-established factors contributing to aging, overlooks the short or long lifespans of certain species that do not show correlating alterations in mutation load, and hence does not provide an adequate answer to the fundamental question of why organisms age.

George Williams introduced the "theory of antagonistic pleiotropy", which suggests that genes exhibit pleiotropic effects, conferring benefits early in life to ensure an organism's survival while imposing detrimental effects later in life (Williams, 1957). These genes are selected for by evolution and are not eliminated by natural selection despite their negative consequences in old age, as they offer advantages for survival early in life, thus creating a trade-off. However, the theory of antagonistic pleiotropy falls short in addressing all aspects of aging, as it oversimplifies the process by primarily attributing it to gene roles and genetic trade-offs. Additionally, it assumes a universal pattern of aging across species and lacks sufficient empirical evidence.

"The disposable soma theory", proposed by Thomas Kirkwood, suggests that organisms face a constant trade-off between investing resources into repair and maintenance versus reproduction (Kirkwood, 1977). While allocating resources to survival and reproduction is essential for species continuity, it comes with a cost: without sufficient investment in somatic repair mechanisms, damage will accumulate over time, leading to functional decline and the eventual inability to support reproduction. Interestingly, studies in model organisms like *C. elegans* and *D. melanogaster* have demonstrated that removal of the germline extends the lifespan of these animals, providing support for the disposable soma theory (Flatt et al., 2008; Hsin & Kenyon, 1999). However, the theory does not describe the cellular mechanisms that enable organisms to prioritize resources for somatic repair over reproduction. It also overlooks observations such as the beneficial effects of CR on lifespan and instances when reproduction is positively correlated with lifespan in certain species (Van Den Heuvel et al., 2017). Furthermore, the theory fails to address the longer life expectancy of females over males in some species, despite females investing more energy into reproduction.

Programmed theories of aging

Programmed theories of aging propose that genes regulate the progression of life by undergoing selective expression depending on distinct life phases (Jin, 2010; Lipsky & King, 2015). Expressed genes ensure optimal conditions tailored to the requirements of specific life phases, such as development, growth, maturation, and aging. As organisms transition between life phases, gene expression patterns change to induce cellular adaptations specific to each phase. This process will continue and eventually lead to aging of an organism, culminating in its death.

The role of telomeres in aging can be explained within the framework of programmed theories. Telomeres may serve as a biological clock that regulates the number of cell divisions before reaching replicative senescence, defined by the Hayflick limit. Upon entering this state, cells undergo a full portfolio of age-induced detrimental changes that impede their function.

However, it's also possible that only a subset of cells undergoes programmed changes, thereby impacting the functioning of other systems within an organism. According to the "neuroendocrine theory", the neuroendocrine system coordinates the functioning and aging of distant parts of an organism through the secretion of neurohormones. Examples of events programmed by neurohormones include puberty and menopause.

Another aspect to consider is the possible programmed decline of the immune system over time, which is evident from the atrophy of the thymus. This atrophy might represent a pivotal stage in immune function decline, contributing to increased disease susceptibility and age-related decline. Dysregulated immune responses, leading to chronic inflammation, provide evidence that the immune system contributes to the aging process (Rozemuller et al., 2005).

Still, all of these theories face certain limitations as they inadequately account for variations in lifespan among members of the same species, overlook the influence of the environment on the aging process, or fail to provide a clear evolutionary explanation for why aging would be beneficial.

Stochastic theories of aging

These theories posit that aging results from the accumulation of stochastic changes over an organism's lifespan (Jin, 2010; Lipsky & King, 2015). If left unrepaired, these changes can impair the functioning of cells and organs. Consequently, the initial blueprint for the proper functioning of diverse body systems is lost, leading to a loss of homeostasis and the inability of an organism to function effectively.

An example of such a theory is the "wear and tear theory," proposed by August Weismann, which compares living organisms to machines (Weismann et al., 1891). This theory proposes that, similar to machines, organisms accumulate damage over time due to environmental insults and the repetitive use of their systems, which may not be designed to sustain certain processes indefinitely. A notable critique of this theory is its oversight of cellular repair mechanisms that can potentially reverse accumulated damage. Moreover, maintaining organisms in secure and protected environments does not consistently alter their aging pattern nor increase their maximal lifespan.

"The rate of living theory" explains that animals possess a finite amount of calories, and that their lifespan is determined by the rate at which they expend these calories. Accordingly, organisms with a fast basal metabolism and high oxygen consumption rates are expected to have shorter lifespans, while those with a slow basal metabolism and low oxygen consumption rates are inclined to live longer. Supporting this theory, are the observations that smaller mammals with fast metabolism typically exhibit shorter lifespans compared to larger mammals with slower metabolism. Furthermore, poikilothermic animals demonstrate an extended lifespan when environmental temperatures rise, as they expend fewer calories to regulate their body temperature. However, this theory fails to explain cases of longevity observed in species with high metabolic rates, and it overlooks the diverse energy allocation strategies evolved by organisms.

"The free radical theory", proposed by Denham Harman, suggests that free radicals damage cells and impair their function (Harman, 1956). Reactive oxygen species (ROS), such as the superoxide anion, hydroxyl radical, and hydroperoxyl radical, are highly reactive molecules generated as byproducts of cellular metabolism. ROS induce oxidative damage in cellular macromolecules like DNA, proteins, lipids, and carbohydrates, ultimately causing a decline in cellular function. However, antioxidants and detoxifying enzymes can mitigate the detrimental effects of ROS. Critics of this theory point out that ROS also play valuable roles in cellular processes, such as cellular signaling, immune reactions, and senescence. Furthermore, while supplementing diets with antioxidants has extended the lifespan of some animals, this phenomenon cannot be reproduced in all contexts.

1.2 Emergence of Aging Research

The first evidence for the genetic basis underlying lifespan regulation emerged from studies in the nematode *Caenorhabditis elegans*. Early studies identified inbred and mutant strains exhibiting varying lifespans, laying the foundation for comprehending the role of genetic factors in determining longevity (Johnson & Wood, 1982; Klass, 1983). Shortly thereafter, it was revealed that some of the previously identified long-lived mutants harbored a hypomorphic mutation in the *age-1* gene, which encodes a component of the phosphatidylinositol 3-kinase (PI3K) complex (Friedman & Johnson, 1988). A multitude of subsequent studies has confirmed that single-gene mutations can indeed influence lifespan in a variety of model organisms, ranging from yeast to mice (Holzenberger et al., 2003; Kaeberlein et al., 1999; Kenyon, Chang, & Gensch, 1993). Discovery of such genes prompted the identification of key aging-related factors and signaling pathways, including the insulin/insulin-like growth factor 1 (IGF-1) signaling (IIS) pathway, signaling through the mechanistic target of rapamycin (mTOR), and the sirtuin proteins. These pathways' conserved roles in regulating aging have been extensively investigated across model organisms. They have proven pivotal in

understanding the interplay between metabolism and the aging process, and have unveiled promising therapeutic targets.

Another important aspect of aging research concerns the molecular mechanisms underlying accelerated-aging syndromes, like Hutchinson-Gilford progeria syndrome (HGPS), Werner syndrome, Cockayne Syndrome, or more recently described Nestor-Guillermo progeria syndrome (NGPS). These diseases accelerate the onset of aging symptoms in humans, and hence provide good models to study various aspects of the aging process and to identify novel aging regulators. A thorough understanding of the mentioned genetic pathways and diseases may shed light on the mechanisms driving aging and facilitate the development of aging-preventive or even rejuvenating therapies. Additionally, such therapies may alleviate or defer the onset of age-related pathologies, such as Alzheimer's and Parkinson's disease, sarcopenia, type-2 diabetes, and cancer (Franceschi et al., 2018).

1.3 The model organism *Caenorhabditis elegans*

Caenorhabditis elegans, a widely used model organism, is a free-living nematode. Selected as a laboratory animal for its numerous advantages (which I will discuss in detail below), it has been employed in research across various fields. *C. elegans* has been particularly favored for genetic studies, as it was one of the first multicellular organisms to have its genome sequenced, complemented by its ease of cultivation in laboratory settings on agar plates (The C. elegans Sequencing Consortium, 1998). Moreover, analysis using different similarity thresholds suggested that approximately 40–80% of *C. elegans* proteins have orthologs in humans, thus implying significant translational potential (Kim et al., 2018; C.-H. Lai et al., 2000).

In the lab, *C. elegans* consume a non-pathogenic strain of *Escherichia coli* as their food source and reproduce rapidly, yielding approximately 300 offspring per worm within a reproductive cycle of just 3.5 days at room temperature (RT)

(Meneely et al., 2019). Additionally, they have the capability for both self-fertilization and cross-fertilization. Adult worms, measuring just 1 mm in length, are easily observable using a stereo microscope. The transparency of both the eggshell and body of these animals facilitates the observation of embryogenesis and the entire developmental process of the worm. Furthermore, it enables cell lineage tracing and tracking of fluorescently labeled molecules or reporters using fluorescence microscopy techniques. Animals exhibit two distinct sexes: hermaphrodites, possessing two X chromosomes, and males, characterized by a single X chromosome. Males emerge spontaneously due to the loss of one X chromosome, estimated to occur in approximately one out of every 600 worms. They are readily discernible from hermaphrodites by the morphology of their tails. Males are relevant in genetic research, serving to introduce novel alleles into populations, thereby generating cross-progeny with unique traits. In hermaphrodites, both spermatogenesis and oogenesis occur, resulting in the production of sperm and oocytes. Following fertilization within the spermatheca, oocytes develop into zygotes and embryos, until these are eventually laid as eggs. After the completion of embryogenesis, worms hatch from eggs and undergo a developmental progression through the distinct larval stages, denoted as L1 to L4 (Figure 1). Throughout these stages, they experience significant growth and maturation. In response to adverse conditions, L2 worms have the capability to enter a dauer larval stage, characterized by a thin and inert body and reduced metabolic activity. When conditions improve, the animals can transition out of this "emergency" state, undergo molting into the L4 stage, and subsequently progress into adulthood. Once they reach adulthood, wild-type *C. elegans* have a lifespan of only approximately 2-3 weeks at RT, making them a convenient model for aging studies.

As worms age, they exhibit age-associated changes including tissue degeneration, decreased mobility, and cessation of reproductive activity – just like humans (Garigan et al., 2002; C. Huang et al., 2004; Hughes et al., 2007). Another rationale for utilizing this model organism in longevity studies is the conservation of

numerous genes and biological pathways associated with aging across different species, ranging from *C. elegans* to humans (Fischer et al., 2022; Kenyon, 2001). Hence, *C. elegans* can be utilized for the investigation of genome maintenance genes and mechanisms implicated in accelerated-aging syndromes, such as Cockayne, Bloom, and Werner syndrome (M. H. Lee et al., 2002; S.-J. Lee et al., 2004; Wicky et al., 2004). Finally, *C. elegans* has played a crucial role in elucidating conserved molecular and cellular changes associated with aging, referred to as the "hallmarks of aging" (López-Otín et al., 2013).

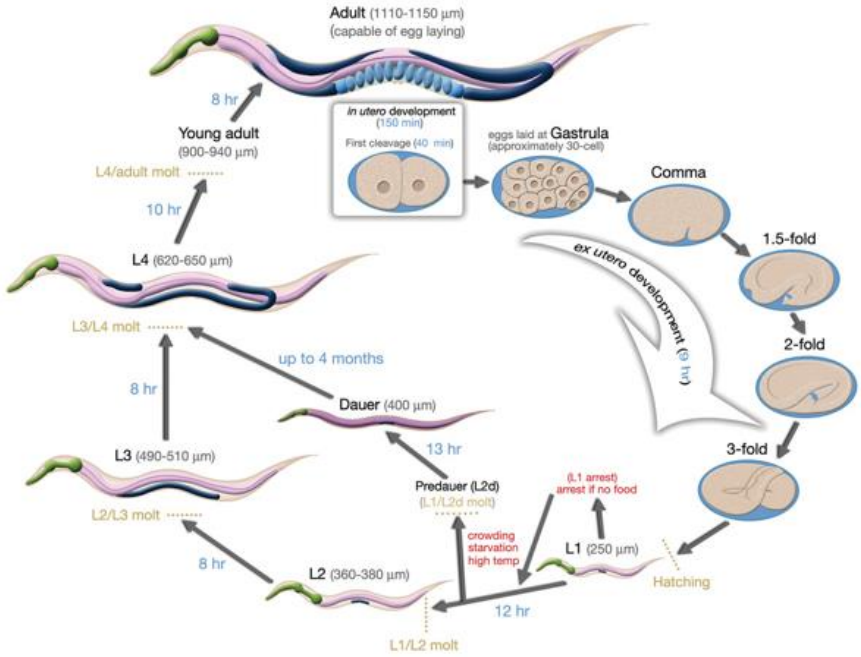

Figure 1. (taken from (Meneely et al., 2019)). The life cycle of *C. elegans* commences with embryogenesis occurring within laid eggs. Upon completion, worms hatch and progress through developmental stages L1 to L4, eventually maturing into adult nematodes. In adverse conditions, they can enter an alternative state to the L3 stage, known as the dauer state.

1.4 Hallmarks of aging

Hallmarks of aging encompass interconnected cellular and molecular features that commonly manifest during the aging process (López-Otín et al., 2023). While a direct causal role between these hallmarks and the aging process remains unproven, extensive studies support their involvement in aging. Each hallmark must meet three criteria: (1) It should be characteristic of physiological aging (2) Its experimental exacerbation should accelerate aging (3) Therapeutic interventions targeting the hallmark should extend healthy lifespan and delay aging. The current hallmarks of aging include genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, disabled macroautophagy, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, altered intercellular communication, chronic inflammation, and dysbiosis (Figure 2). These hallmarks can be classified hierarchically into three groups: primary, antagonistic, and integrative hallmarks. Primary hallmarks result from the progressive accumulation of damage in macromolecules or organelles, such as DNA damage or lipid peroxidation. Primary hallmarks can trigger detrimental misadaptations of antagonistic hallmarks, shifting their nature from beneficial to deleterious. For example, at low levels, reactive oxygen species (ROS) play a beneficial role by mediating cell signaling and promoting cell survival. However, when ROS levels become excessive, they can lead to cellular damage. The final category, termed Integrative hallmarks, arises when damage and alterations inflicted by primary and antagonistic hallmarks can no longer be compensated for and begin to impair the functioning of tissues and organs. Such events can lead to chronic inflammation or exhaustion of stem cells.

Many factors and mechanisms associated with the hallmarks of aging have been linked to the regulation of longevity through studies in *C. elegans*. Notable examples include chromatin remodelers and histone modifiers (Guillermo et al., 2021; Hamilton et al., 2005; M. Huang et al., 2022; Samuelson et al., 2007), regulators of autophagy and mitophagy (Hansen et al., 2008; Meléndez et al., 2003; Palikaras et al., 2015), the ubiquitin-proteasome pathway (Ghazi et al., 2007;

W. Li et al., 2007), various unfolded protein response pathways (Henis-Korenblit et al., 2010; Houtkooper et al., 2013; A. L. Hsu et al., 2003; Morley & Morimoto, 2004; Walker & Lithgow, 2003), and components of nutrient-sensing pathways (Apfeld et al., 2004; Friedman, 1988; Kenyon, Chang, Gensch, et al., 1993; Vellai et al., 2003).

Deregulated nutrient sensing is recognized as one of the hallmarks of aging, emerging from aging-induced changes within the intricate nutrient sensing network. This network comprises an extensive array of molecular pathways involving extracellular ligands, their corresponding receptors, and intracellular signaling cascades. For instance, insulin and insulin-like growth factors act as ligands and bind to specific cellular receptors, thus triggering the PI3K-AKT signaling cascade. In conditions of nutrient abundance, these sensors and pathways stimulate cellular growth, proliferation, and anabolic processes, enabling cells to harness nutrients for energy production and biosynthesis. Conversely, under nutrient scarcity, these sensors and pathways induce catabolic mechanisms like autophagy and fatty acid oxidation to produce energy and maintain homeostasis. Age-associated alterations in this network can result in metabolic dysregulation, potentially compromising cellular function and accelerating the aging process. The main signaling pathways comprising the nutrient sensing network include AMP-activated protein kinase (AMPK), mTOR, sirtuins, and the IIS pathway.

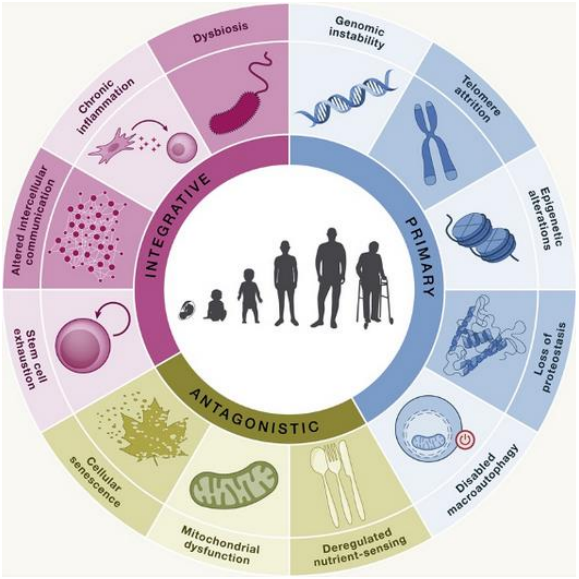

Figure 2. (taken from (López-Otín et al., 2023)). The framework comprises the current 12 hallmarks of aging, namely: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, disabled macroautophagy, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, altered intercellular communication, chronic inflammation, and dysbiosis. These hallmarks are categorized into three groups: primary, antagonistic, and integrative.

1.5 Insulin/IGF-1 signaling (IIS) pathway

The role of IIS pathway in aging regulation was discovered by studying a long-lived *C. elegans* strain with a hypomorphic mutation in the gene *age-1*, which turned out to be a component of the PI3K complex (Friedman & Johnson, 1987). Subsequently, Kenyon and colleagues found that the longevity-regulating function of IIS depends on the activity of the transcription factor (TF) DAF-16, which is orthologous to the FOXO family of TFs found across metazoans (Obsil & Obsilova, 2008).

The IIS pathway gained increased attention following the revelation of its conserved role in regulating aging across species, including *D. melanogaster* (Tatar et al., 2001) and mice. Regarding the latter, it has been reported that reduced IIS, caused by partial inactivation of IGF-1R in the embryonic brain, impairs growth but leads to delayed mortality and longer mean lifespan in mice (Kappeler et al., 2008). Another study showed that mice with a knock-out of the insulin receptor substrate 1 (IRS-1) are long-lived and experience a later onset of multiple age-related symptoms, including skin, immune, bone and motor dysfunction (Selman et al., 2008). Studies with human cohorts have also demonstrated that genetic variation in the FOXO3A exhibited a significant correlation with human longevity (Flachsbart et al., 2009; Willcox et al., 2008). Collectively, these studies suggested that elucidating the mechanisms through which DAF-16 regulates longevity in *C. elegans* holds potential for rapid charting of the IIS- and DAF-16/FOXO-related aging-regulatory mechanisms and the development of anti-aging therapies for humans.

Signaling through the IIS pathway occurs upon binding of insulin/IGF-1-like peptides to the insulin/IGF receptor (called DAF-2 in *C. elegans*, Figure 3A), which in turn triggers the receptor's intrinsic tyrosine kinase activity (Ullrich et al., 1985). This leads to the recruitment and activation of the kinase AGE-1/PI3K, which converts phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP3 recruits 3-phosphoinositide-dependent kinase-1 (PDK-1) to the plasma membrane, enabling PDK-1 to phosphorylate and thereby activate the downstream AKT family of kinases, including AKT-1, AKT2 and SGK-1 (Paradis & Ruvkun, 1998). This AKT family of kinases is responsible for the phosphorylation of several TFs, including DAF-16/FOXO. When phosphorylated, DAF-16 is bound by the cytoplasmic 14-3-3 proteins PAR-5 and FTT-2, which sequester DAF-16 to the cytoplasm. This prevents DAF-16 from entering the nucleus and driving the expression of its target genes (Paradis & Ruvkun, 1998). Besides DAF-16, the IIS pathway also negatively regulates the TFs SKN-1 and HSF-1 (A. L. Hsu et al., 2003; Tullet et al., 2009). Furthermore, it is worth noting that IIS

is negatively regulated by the lipid phosphatase DAF-18/PTEN and the serine/threonine phosphatase PP2A^{PPTR-1}, which counteract AGE-1/PI3K and AKT-1 signaling, respectively.

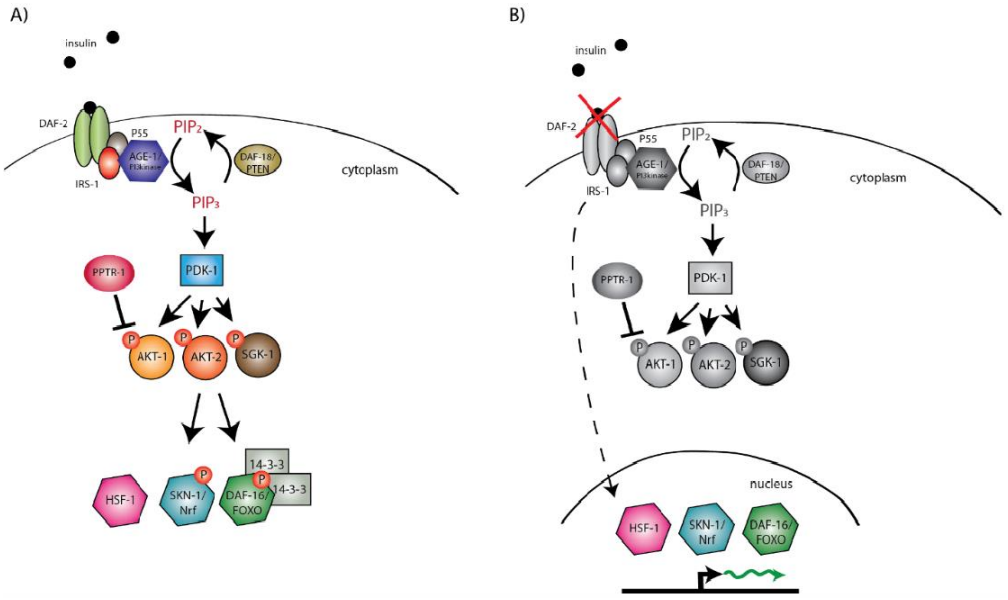

Figure 3. (A) The IIS pathway. IIS negatively regulates DAF-16/FOXO by phosphorylation. (B) Reduced IIS results in the activation of DAF-16/FOXO and its nuclear entry (adapted from a figure by Christian Riedel).

To reduce IIS in the lab, it is very common to use worm strains containing a conditional loss-of-function mutation in *daf-2*, which reduces signaling through the pathway and results in the eventual phosphorylation of DAF-16 (Figure 3B). Consequently, DAF-16 is no longer sequestered by 14-3-3 proteins and is able to translocate into the nucleus, where it will regulate the expression of genes promoting longevity, stress resistance (e.g. heat shock proteins, antimicrobial peptides, or antioxidant proteins), dauer formation, fat metabolism, and other processes (Figure 4) (Zečić & Braeckman, 2020).

Figure 4. Groups of genes regulated by DAF-16/FOXO (adapted from a figure by Christian Riedel)

To this day, it remains unclear how DAF-16 selectively regulates the expression of its target genes in response to specific stimuli. It has been reported that 78% of all *C. elegans* genes contain one or more DAF-16 Binding Elements (a specific DNA sequence that serves as the binding site for DAF-16; DBE) in their 5-kb upstream region (Kenyon & Murphy, 2006). Therefore, it should be assumed that additional mechanisms of regulation are involved in the transcriptional control of DAF-16 target genes. Such mechanisms may involve the synergy between DAF-16 and additional TFs, enabling DAF-16 to selectively activate or repress a specific group of genes. This hypothesis gains support from the observation that artificially induced nuclear translocation of DAF-16 is insufficient to promote longevity (K. Lin et al., 2001). Furthermore, DAF-16 is known to cooperate with a variety of interaction partners to regulate the expression of its target genes, leading to specific physiological outcomes like longevity or stress resistance (A. L. Hsu et al., 2003; Lapierre & Hansen, 2012; J. Li et al., 2008; S. Li et al., 2019; X.-X. Lin et al.,

2018; Pekec et al., 2022; Riedel, 2013; Robida-Stubbs et al., 2012; Sen et al., 2020; Wolff et al., 2006). All this evidence argues that DAF-16 requires assistance from additional proteins, the identity of which may often still be unknown, to influence the rate of aging and thus promote longevity.

1.6 Barrier-to-autointegration factor 1

Barrier-to-autointegration factor 1 (BAF-1) is an essential and highly conserved protein that is found across metazoans, spanning from *C. elegans* to humans (Cai et al., 1998; Zheng et al., 2000a). It is a highly mobile protein and localizes to two compartments, the nucleus and cytoplasm. These two BAF-1 pools are considered distinct and do not interchange (Haraguchi et al., 2007; Shimi et al., 2004). BAF-1 is known to bind components of the nuclear lamina, including lamin, LEM-domain proteins, and histones (De Oca et al., 2005; Furukawa, 1999; Laguri et al., 2001; K. K. Lee et al., 2001; Mansharamani & Wilson, 2005). Despite broader localization, it is enriched at the nuclear envelope and this enrichment is regulated by the kinase VRK-1, a member of the vaccinia-related kinases (VRKs) family. The phosphorylation of BAF-1 at residues Ser-4, Thr-2, and Thr-3 impedes its binding to DNA and reduces its ability to homodimerize or interact with LEM-domain proteins (Bara et al., 2014; Bengtsson & Wilson, 2006; Gorjánácz et al., 2007a; Nichols et al., 2006). BAF-1 is dephosphorylated by phosphatase PP2A (Asencio et al., 2012). In solution, BAF-1 forms homodimers (Cai et al., 1998; Umland et al., 2000) which oligomerize into hexamers or dodecamer and bind DNA nonspecifically (Zheng et al., 2000b). As a result, BAF-1 oligomers can bind and bridge different DNA regions, resulting in condensation, looping and higher-order chromatin structure formation (Bradley et al., 2005; Margalit et al., 2005; Umland et al., 2000; Zheng et al., 2000a). Despite its small size (~10 kDa), BAF-1 is indispensable for numerous cellular processes, presumably relying on interactions with diverse binding partners (Montes De Oca et al., 2009). These processes include epigenetic regulation (de Oca et al., 2011), gene expression (Cox et al., 2011; Holaska et al., 2003; Margalit et al., 2007; X. Wang et al., 2002), chromosome

segregation, post-mitotic nuclear envelope assembly (Gorjánácz et al., 2007b; Haraguchi et al., 2001; Margalit et al., 2005), stress (Bara et al., 2014; Cenni et al., 2020) and DNA damage response (Bolderson et al., 2019; Dittrich et al., 2012; Montes De Oca et al., 2009), immunity (Ibrahim et al., 2011, 2013; M. S. Lee & Craigie, 1994, 1998), repair of nuclear ruptures (Denais et al., 2016; Halfmann et al., 2019; Young et al., 2020). Lastly, several studies implied BAF-1's involvement in carcinogenesis (T.-C. Lai et al., 2010; J. Li et al., 2018; Ren et al., 2020; G. Zhang, 2020).

1.7 Néstor-Guillermo Progeria Syndrome

Néstor-Guillermo Progeria Syndrome (NGPS) is a premature aging syndrome that is caused by the coding mutation (c.34G>A [p.A12T]) in BANF1, the human orthologue of BAF-1 (Fisher et al., 2020; Paquet et al., 2014). This residue is expected to have an important biological role, as it is conserved across most metazoans (Puente et al., 2011). However, there exists no clear understanding on how the A12T mutation affects BANF1 function and causes NGPS. One study reports that this mutation decreases BANF1 stability (Puente et al., 2011), while a second study proposes that the A12T mutation impairs BANF1 binding to DNA, consequently causing nuclear structural aberrations (Paquet et al., 2014). A recent study reported that the A12T mutation decreased BAF-1's binding affinity for lamin A/C, resulting in reduced efficiency of lamin A/C recruitment to nuclear rupture sites (Janssen et al., 2022). Surprisingly, this did not impact the repair of nuclear ruptures but heightened the likelihood of re-ruptures. Still, the frequency of re-ruptures was significantly increased in only one of the two currently available NGPS patient cell lines, suggesting the involvement of additional mechanisms in NGPS pathology.

Another potential mechanism could be associated with oxidative stress, which is known to trigger the re-localization of BANF1 from the nuclear lamina to intranuclear speckles (Cenni et al., 2020). BANF1 acts as a negative regulator of

poly [ADP-ribose] polymerase 1 (PARP-1), a protein essential for repairing oxidative DNA damage and DNA strand breaks (Bolderson et al., 2019). Overexpression of BANF1 inhibits PARP-1, resulting in inefficient repair and the accumulation of oxidative DNA damage. However, this effect was observed only under conditions of BANF1 overexpression, while no discernible phenotype was noted when BANF1 was depleted. The mutated A12T variant of BANF1 displays increased binding affinity for PARP-1, consequently impeding the repair of oxidative DNA lesions in NGPS patient cell lines. However, these findings were based on experiments involving the induction of oxidative stress with hydrogen peroxide (H_2O_2), without demonstrating their relevance under physiological conditions. Furthermore, no significant difference in the amount of oxidative DNA damage was observed between control and NGPS cell lines under physiological conditions, leading to speculation about the extent of PARP-1's involvement in NGPS.

Patients with NGPS experience aging symptoms from early childhood, which is a defining hallmark of progerias. The most commonly known progerias are caused by defects in DNA repair mechanisms or by aberrant nuclear envelope dynamics and structure, prominent examples of which are Cockayne syndrome and HGPS, respectively (Ramírez et al., 2007). The clinical features of NGPS patients have strong similarities to these progerias (Cabanillas et al., 2011). Patients show normal development until 2 years of age (Figure 5). Afterwards, they develop an aged appearance, growth retardation, decreased subcutaneous fat, thin limbs, and stiff joints (Puente et al., 2011; Ramírez et al., 2007). Interestingly, NGPS patients do not show signs of cardiovascular impairment, diabetes mellitus, or hypertriglyceridemia like HGPS patients. However, they develop profound skeletal abnormalities. Another major difference is that NGPS patients reach early adulthood, while Cockayne and HGPS patients die around their teenage years (Puente et al., 2011; Ramírez et al., 2007). The molecular mechanisms underlying the differing symptoms between NGPS and other progerias remain unclear, as does the potential role of BANF1 in these other progerias. Human fibroblasts from patients diagnosed with progeroid syndromes such as HGPS, Mandibuloacral

dysplasia (MAD), Dunnigan-type familial partial lipodystrophy (FPLD), and restrictive dermopathy (RD) exhibit an imbalance in BANF1 distribution towards a nuclear localization (Capanni et al., 2010, 2012). Additionally, certain mutations causing HGPS, MAD, and RD impair the binding of BANF1 to lamin A/C (Samson et al., 2018). These observations suggest a potentially shared underlying mechanism among laminopathies that involves dysfunction of BANF1.

Figure 5. Appearance of patient with NGPS at 1 and 24 years of age. Progeroid features, such as facial abnormalities, started developing after 2 years of age.

1.8 Homeodomain protein LIN-39

Homeobox (Hox) genes encode TFs distinguished by a conserved homeodomain, which enables direct DNA binding and thereby regulation of target genes essential for determining cellular fate (McGinnis & Krumlauf, 1992; Quiring et al., 1994). Hox genes are expressed in specific regions of the body and play an essential role in patterning the anteroposterior axis in metazoans (Krumlauf, 1994; Lawrence & Morata, 1994; Loker et al., 2021; McGinnis & Krumlauf, 1992; Salser & Kenyon, 1994). Impairing the function of these genes leads to "homeotic transformations", resulting in developmental abnormalities such as the loss of particular structures or alterations in the identity of body parts or segments (Lewis et al., 1999). The *C.*

elegans genome contains a cluster of 6 Hox genes situated on chromosome III (Aboobaker & Blaxter, 2003). Included within this cluster are the anterior Hox gene *ceh-13*, along with two midbody genes *lin-39* and *mab-5*, and three posterior Hox genes *egl-5*, *nob-1*, and *php-3*. *lin-39* is required for patterning of the central body region of *C. elegans* (Bürglin & Affolter, 2016; B. B. Wang et al., 1993), migration of QR descendants (Clark et al., 1993), formation of VC neurons (Potts et al., 2009; Salser et al., 1993), fate specification of serotonergic and male-specific CA and CP neurons (Clark et al., 1993; Kalis et al., 2014; Salser et al., 1993), and vulval development (Maloof et al., 1999). In addition, LIN-39 serves as a terminal selector, playing a crucial role in defining the identity of specific motor neurons (MNs) (Hobert, 2021). Mechanistically, LIN-39 coordinates the expression of "neuron-type-specific gene batteries," thereby ensuring the proper establishment of anatomical and structural neuronal properties. The nervous system of the adult *C. elegans* hermaphrodite encompasses 302 neurons (Hobert, 2010). More than half of these neurons are cholinergic, with a majority belonging to the class of MNs that utilize acetylcholine (ACh) for synaptic transmission (Pereira et al., 2015). *lin-39* is primarily expressed in MNs, where it acts as a terminal selector. Consequently, LIN-39 defines and preserves the identity of cholinergic neurons through regulation of cholinergic gene expression (Feng et al., 2020, 2022; Hobert, 2021). This is particularly the case in the MNs of the ventral nerve cord. *lin-39* is continuously expressed in 70% of cholinergic MNs from development to adulthood, suggesting its functional requirement in these neurons throughout the lifespan of *C. elegans*. In the context of aging research, a single study suggested that ACh signaling influences DAF-16 nuclear translocation, implying a potential role of ACh signaling in promoting longevity under reduced IIS (McIntyre et al., 2021). At the same time, elevating synaptic ACh levels was shown to accelerate the aging process of neuromuscular junctions in mice (Sugita et al., 2016). Still, the exact mechanisms by which ACh signaling affects IIS and aging overall remain incompletely understood.

3 Research aims

Studies (I-III) were initiated to identify novel mechanisms of aging regulation in *C. elegans*, with the potential conservation of these mechanisms holding promise to deepen our understanding of aging regulation in humans. Such findings could reveal specific targets, further advancing the development of potential anti-aging therapies.

The aims of the studies I and II were to explore mechanisms by which reduced IIS, and its downstream component DAF-16, regulate longevity in *C. elegans*. This led to the discovery of novel regulators of aging, namely BAF-1 and LIN-39. Intriguingly, IIS, DAF-16, BAF-1 and LIN-39 are all conserved, arguing that the mechanisms described in these studies should affect aging in humans as well. This is particularly evident in the case of BAF-1, given its established role in NGPS.

The aim of study III was to screen for aging-preventive compounds by building age-predictive models from publicly available human transcriptomics data, and then to validate their anti-aging properties in *C. elegans*. This approach holds promise for uncovering new compounds with anti-aging properties, also amongst already FDA-approved drugs, potentially expediting their repurposing or at least their transition to clinical use.

The aim of study IV was to assess the importance of ciliary signaling for neuron differentiation. The obtained findings may also contribute to elucidating the mechanisms underlying ciliopathies, as well as certain neurodevelopmental conditions and disorders.

4 Materials and methods

RNA interference (RNAi) by feeding

In *C. elegans*, RNAi serves as a valuable tool for efficiently knocking down genes of interest. RNA interference (RNAi)-induced phenotypes typically exhibit milder effects than those observed with gene knockouts, making RNAi particularly valuable for studying essential genes. Delivery of double stranded RNA (dsRNA) can be achieved via microinjection or feeding (Conte et al., 2015; Fire et al., 1998; Timmons et al., 2001). Microinjection allows for precise administration of dsRNA to specific tissues using a needle, while feeding involves ingestion of dsRNA contained within bacteria. In the studies encompassed in this thesis, we opted for RNAi induction by feeding due to its simplicity and efficiency compared to microinjection. Furthermore, feeding *C. elegans* with dsRNA-producing bacteria allows for simultaneous RNAi induction in a large population of worms, facilitating downstream biochemical studies. The RNAi bacterial clones utilized in this study were sourced from commercially available Ahringer (Kamath et al., 2003) and Vidal (Rual et al., 2004) libraries, collectively targeting approximately 90% of predicted protein-coding genes based on the WS244 genome release.

After ingesting RNAi bacteria, *C. elegans* absorbs the dsRNA in its intestine and releases it into the fluid-filled body cavity surrounding internal tissues (Raman et al., 2017). The dsRNA-selective importer SID-1 enables the entry of dsRNAs into the cells of all tissues (Winston et al., 2002). Afterwards, the dsRNA is processed as described in the classical RNAi pathway. Long dsRNA is first bound by RDE-4, the dsRNA-binding component of the Dicer complex. This leads to the recruitment of the endonuclease DCR-1, the cleavage of long dsRNAs into short dsRNAs, and finally the generation of siRNAs (Ketting et al., 2001; Raman et al., 2017; Tabara et al., 2002). These siRNAs are bound by the Argonaute RDE-1, a constituent of the RNA-induced silencing complex (RISC), and are matched with their corresponding target mRNAs. This process triggers the synthesis of secondary siRNAs by RNA-dependent RNA polymerases (RdRPs), amplifying the initial RNAi signal (Sijen et al., 2001; Steiner et al., 2009; Tabara et al., 1999). Ultimately, Argonautes with RNase H activity utilize the newly generated siRNAs to cleave homologous mRNA targets (Corrêa et al., 2010; Yigit et al., 2006).

In addition to inducing systemic RNAi, we employed several tissue-specific RNAi strains in our studies. These strains were created through tissue-specific *sid-1* or *rde-1* transgene rescue in the respective RNAi-deficient mutant backgrounds. By using these strains, we were able to selectively induce RNAi in specific tissues of *C. elegans*, including the nervous system, the intestine, muscles, and the hypodermis.

Lifespan assays

Lifespan assays are survival assays during which a synchronized population of *C. elegans* is monitored and the death time of each individual worm is recorded. Conducting such an assay can facilitate the discovery of aging-regulatory genes, pathways, and drugs that modulate lifespan. Throughout the assay, censoring and mortality events are typically documented every 2–3 days using a platinum wire (Hamilton et al., 2005). Dead or censored worms are removed from the assay to prevent confusion during subsequent scoring. To determine the status of a worm, gentle touching of the head and tail is conducted. If the worm shows no response and fails to move, it is scored as "dead". It's important to note that worms may crawl off plates or burrow into the agar, and in such cases, they should be excluded from the analysis. Once all death incidents are documented, the data is utilized to generate Kaplan-Meier survival curves. These curves depict the probability of survival over various time intervals. To compare survival curves of different *C. elegans* populations, a log-rank test is employed. This statistical test assesses whether the difference in survival times between two groups is significant (Kishore et al., 2010).

Before beginning a lifespan assay, it's crucial to synchronize the *C. elegans* population to ensure uniform age among all animals at the start of the assay. This synchronization process involves bleaching gravid worms, collecting the released eggs, and incubating them in M9 media until hatching. At the L1 stage, most worms' growth halts due to nutrient deprivation until they encounter a food source. Once feeding resumes, the isogenic worm population is expected to grow at similar rates, and all animals are expected to be of a comparable age. To prevent progeny from hatching, 5-fluoro-2′-deoxyuridine (FUDR) is administered at the late L4 stage, after somatic structures have formed. FUDR acts as an inhibitor of thymidylate synthase, which is necessary for DNA synthesis (Rooney et al., 2014). In our studies, all the lifespan assays were conducted on a solid surface, utilizing

nematode growth medium (NGM) agar plates seeded with various strains of *E. coli* as a food source. Worms were typically cultured on plates containing live bacteria, except during the drug-screening lifespan assay. For this assay, worms were initially grown on plates with live bacteria until the late L4 stage. Subsequently, they were transferred to plates containing heat-killed bacteria, which are unable to metabolize administered drugs (Beydoun et al., 2021). This setup was selected because exposing worms to killed bacteria during development can induce physiological changes, such as delayed development and reduced brood size (Szewczyk et al., 2006). After reaching the late L4 stage, *C. elegans* strains were treated with FUDR and then incubated at 20°C for the duration of the assay.

Stress resistance assays

These assays are a type of survival assays, conducted in the presence of internal or external stressors. The stressors can be categorized as abiotic (such as environmental conditions) or biotic (such as pathogens). In our studies, we conducted abiotic stress resistance assays, to evaluate the resistance of *C. elegans to* heat, oxidative, and ultraviolet (UV)-induced stress. Stress resistance has been linked to longevity in several model organisms (Dues et al., 2016; Murphy, 2006; Murphy et al., 2003). Enhanced stress resistance may thus accompany the activation of longevity-promoting mechanisms, which decrease the rate of aging. These assays were chosen because we conducted research on *daf-2* mutants, which exhibit prolonged longevity alongside increased resistance to heat, oxidative, and UV-induced stress (Honda & Honda, 1999; Lithgow et al., 1995; Murakami & Johnson, 1996).

Heat shock can be induced in *C. elegans* by exposing the nematodes to temperatures higher than those typically used for cultivation. Usually, heat stress resistance assays are performed at temperatures ranging from 32°C to 35°C, at which *C. elegans* start dying within several hours. Exposure to such high temperatures alters animal physiology and damages macromolecules. Neuronal degeneration and necrotic cell death are examples of heat-induced cellular defects in *C. elegans* (Kourtis et al., 2012; Labbadia & Morimoto, 2015). In order to maintain homeostasis and cope with proteotoxic stress, *C. elegans* can employ heat shock transcription factor-1 (HSF-1) and DAF-16 to upregulate chaperone expression and thus reduce the accumulation of abnormal proteins (A.-L. Hsu et al., 2003; Morley & Morimoto, 2004). In our studies, heat resistance assays were

performed at 32°C with an automated Lifespan machine due to the short lifespan of animals at this temperature (Stroustrup et al., 2013). Automated lifespan assays are less laborious than manually performed ones, and worms are not exposed to stress induced by platinum wire usage. The machine captures photos at specified time intervals to monitor the movement of worms over time. The photos are later processed to compute survival data for each individual worm, which are used to generate Kaplan-Meier survival plots.

Oxidative stress resistance assays are used to determine the lifespan of *C. elegans* exposed to oxidizing agents. These assays facilitate the identification of factors or interventions that either enhance or inhibit the response to oxidative stress. In our studies, we conducted this assay in the presence of a highly reactive and toxic organic peroxide called tert-butyl hydroperoxide (t-BOOH). The metabolism of t-BOOH by cytochrome P450 generates peroxyl and alkoxyl radicals, initiating free radical chain reactions that can be highly damaging to cells (Davies, 1989; Riley, 1994). These radicals pose a significant threat to cellular components. Additionally, detoxification of t-BOOH leads to the depletion of glutathione, which is oxidized to its disulfide form. This process contributes to the inhibition of mitochondrial enzymes, further impairing cellular functions (Crane et al., 1983). Given that exposure to t-BOOH drastically shortens the lifespan of *C. elegans*, we employed the automated Lifespan machine for scoring and data collection.

In UV stress resistance assay nematodes are subjected to UV radiation within the wavelength range of 100–400 nm. Exposure to UV radiation profoundly impacts the biology of macromolecules, inducing various mutagenic and cytotoxic DNA lesions, such as cyclobutane-pyrimidine dimers and 6-4 photoproducts (Rastogi et al., 2010). The assay can thus reveal which factors are required for *C. elegans* to cope with UV stress and indicate if they are involved in the regulation of DNA repair. We have induced UV stress by exposing worms to 1800 J/m2 of radiation. Censoring and mortality events were scored with a platinum wire (Hamilton et al., 2005).

Staining fat reserves in *C. elegans*

C. elegans serves as a valuable model for lipid research, as it harbors many conserved fat metabolism genes that may have implications for human adiposity (Klapper et al., 2011; Y. Zhang et al., 2013). Currently, over 400 genes have been annotated as involved in lipid metabolism, and various techniques have emerged for evaluating the lipid content of *C. elegans* (Ashrafi et al., 2003). Lipid stores can be stained using various dyes and monitored with microscopy-based techniques. Popular dyes include Nile Red, Oil Red O, BODIPY, and Sudan Black. Among these options, we opted for Nile Red (NR) due to its widespread usage, staining consistency, straightforward staining procedure, and compatibility with standard fluorescence microscopy (Elle et al., 2010; Klapper et al., 2011).

NR, also known as 9-diethylamino-5H-benzo[α]phenoxazine-5-one, is a lipophilic lysochrome dye. It selectively stains neutral lipids like triglycerides or cholesterol esters while exhibiting minimal interaction with surrounding tissues (Escorcia et al., 2018). NR enables the selective staining of intracellular lipid droplets due to its property of emitting light with a blue shift proportional to the hydrophobicity of the environment (Greenspan & Fowler, 1985). Consequently, the abundance of neutral lipids can be quantified by detecting yellow-gold fluorescence (excitation, 450–500 nm; emission, around 520 nm), while filtering out the red fluorescence (excitation, 515–560 nm; emission, greater than 590 nm) (Greenspan et al., 1985). In our studies, we conducted NR staining on fixed samples, since previous reports indicated that live *C. elegans* fed with NR displayed staining of lysosome-like granules rather than lipid droplets (O'Rourke et al., 2009). Samples were stained after fixation with isopropanol, and imaging of NR-stained worms was conducted using the FITC/GFP channel.

Assay for Transposase-Accessible Chromatin with high-throughput sequencing (ATAC-seq)

Chromatin accessibility is regulated by the epigenetic code, which is determined by epigenetic modifications such as DNA methylation, histone modifications, and nucleosome positioning. Some regions of chromatin are silenced and consequently inactivated, while others are readily accessible and transcriptionally active (Daugherty et al., 2017; Klemm et al., 2019). Therefore, chromatin accessibility plays a crucial role in determining gene expression, thus influencing

diverse biological and pathological conditions, including development, aging, and oncogenesis (Bozukova et al., 2022; Simon et al., 2014; Stergachis et al., 2013).

Various genome-wide analysis methods, such as micrococcal nuclease digestion with sequencing (MNase-seq) and chromatin immunoprecipitation with sequencing (ChIP-seq), can unveil site-specific epigenetic modifications or be utilized to map TF-binding sites (Landt et al., 2012; Zaret, 2005). Nonetheless, acquiring reliable results typically requires a substantial input material, often ranging from tens to hundreds of millions of cells. Additionally, the library preparation protocols are intricate and time-consuming (Grandi et al., 2022). Moreover, the identification of heterogeneous cellular populations may be compromised, potentially rendering rare cellular subtypes indistinguishable from the background (Buenrostro et al., 2015). To overcome these limitations while facilitating comprehensive chromatin profiling, ATAC-seq was developed.

ATAC-seq employs artificially engineered, hyperactive Tn5 transposase preloaded with sequencing adapters to map accessible chromatin regions (Grandi et al., 2022). Due to the reduced accessibility of compacted DNA, successful transposition is less likely to occur at such chromatin regions. Therefore, amplifiable DNA adapters necessary for high-throughput sequencing are preferentially inserted into regions of accessible chromatin. It has been demonstrated that ATAC-seq offers a reproducible and sensitive genome-wide assessment of chromatin accessibility, even when starting with as few as 500–5000 nuclei (Buenrostro et al., 2013). ATAC-seq is compatible with various methods for cell separation and isolation and can be performed with cells irrespective of their origin.

The ATAC-seq protocol can be segmented into three main parts: cell lysis, transposition, and amplification. Transposition is carried out by the Tn5 transposase (Figure 6), which has been engineered to exhibit enhanced activity through the introduction of three mutations. These mutations cause increased transposase binding to outside end sequences, blocked synthesis of the inhibitor, and altered dimerization potential of the transposase (Goryshin & Reznikoff, 1998). The sequencing adapters, which are bound by the transposase, are synthetic oligonucleotides containing a single 19-bp hyperactive derivative (mosaic end, ME) of Tn5 end elements. In the transposition process, the hyperactive transposase binds to a ME adaptor, fragments the target DNA, and catalyzes the end-joining of the adaptor to the 5' end of the target DNA (Buenrostro et al., 2013;

Reznikoff, 2008). Using a transposase for adaptor insertion, compared to conventional library construction protocols, requires fewer steps and reduces input requirements. This is because of the highly efficient conversion of DNA into sequencing-compatible material (Adey et al., 2010).

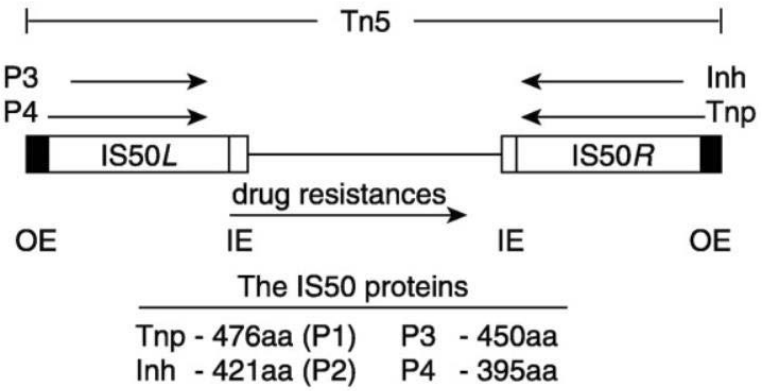

Figure 6. (taken from (Goryshin & Reznikoff, 1998)). Tn5 is a composite transposon with two IS50 elements flanking the three antibiotic resistance genes. The IS50R encodes the active transposase (Tnp) and the trans-dominant negative Inhibitor (Inh). The IS50L encodes truncated forms of these two proteins, which are not required for the transposition process. Another crucial element for transposition is the inverted 19-bp sequences that define the ends of Tn5, termed outside end (OE) sequences. Additionally, there exists a distinct 19-bp sequence known as the inside end (IE) sequence, which is also recognized by Tnp. While IE plays a role in IS50 transposition (an OE-IE event), it does not partake in Tn5 transposition.

5 Results

Studies I and II focus on two proteins downstream of reduced IIS, namely BAF-1 and LIN-39, and show that both are required for the longevity under reduced IIS. They rely on DAF-16 for their function, operating within the same genetic pathway. Additionally, they exert their effects within the nervous system, and in the case of BAF-1, also in the intestine—tissues in which DAF-16 promotes longevity as well. Despite these similarities, BAF-1 and LIN-39 exhibit significant differences. For instance, only BAF-1 physically interacts with DAF-16 and it is not a TF in itself, lacking high sequence specificity for DNA binding and a transactivating domain. In contrast, LIN-39 functions as a conventional sequence-specific TF. Also, BAF-1 influences longevity throughout the entire lifespan of *C. elegans*, while LIN-39 specifically regulates longevity during development, providing a compelling example of a developmental determinant that impacts aging.

Study I: Exploring the interplay between DAF-16/FOXO and BAF-1/BANF1 in the regulation of aging

After establishing BAF-1's role in regulating lifespan from specific tissues, our attention shifted to understanding the underlying mechanisms. Utilizing ATAC-seq, we observed that both BAF-1 and DAF-16 play essential roles in a substantial portfolio of chromatin opening events occurring under IIS. Notably, the regions co-opened by these two proteins were enriched in enhancer elements. To assess how these chromatin accessibility changes impact the transcriptome, we conducted mRNA-seq experiments. Intriguingly, both BAF-1 and DAF-16 were necessary for the upregulation of a large set of genes induced in *daf-2* mutants. Upon closer examination of these genes, we found them to be involved in diverse cellular processes, including the dauer-promoting TGF-β signaling pathway and fatty acid metabolism.

Next, we investigated if dysregulation of these pathways leads to detectable phenotypic changes in *C. elegans*. Lack of not only DAF-16 but also BAF-1 significantly reduced the dauer formation capacity of *daf-2* mutants and resulted in the depletion of fat reserves. Given that lipodystrophy is a feature of NGPS, we delved deeper into the role of fat metabolism and its contribution to the accelerated aging observed in *daf-2* mutants that lack BAF-1. Notably, a reduction

in BAF-1 levels significantly altered the abundance of major lipid classes in *daf-2* mutants. Supplementing the *C. elegans* diet with OA replenished the fat reserves and partially rescued the shortened lifespan observed in animals with reduced IIS lacking BAF-1.

Study II: Mapping of transcriptionally relevant chromatin accessibility changes reveals LIN-39 as a driver of longevity in *Caenorhabditis elegans* with reduced insulin/IGF-like signaling

Like BAF-1, LIN-39 is also essential for opening a substantial number of regions that become accessible under reduced IIS. Annotation of these regions revealed their enrichment in enhancers. After associating these regions with their proximal genes, we observed that the expression of many predicted LIN-39-regulated genes is changing during aging in neurons. In other words, our results suggest that LIN-39 regulates longevity under reduced IIS by promoting the expression of neuron-specific genes whose expression declines with age.

To assess whether LIN-39 specifically influences longevity within a particular class of neurons, we compared genes proximal to enhancers opened by LIN-39 with transcriptomic signatures of specific neuron classes. Interestingly, LIN-39-induced genes most significantly overlapped with gene expression profiles of cholinergic and GABAergic neurons. These findings inspired downstream experiments, revealing that reducing ACh signaling, but not GABA signaling, partially suppressed the lifespan-shortening phenotype induced by the lack of LIN-39 in *daf-2* mutants. Based on these observations, we concluded that the lack of LIN-39 leads to dysregulated ACh signaling, causing detrimental effects that accelerate aging under reduced IIS.

Finally, we provide evidence that both LIN-39 and DAF-16 regulate longevity under reduced IIS through the same pathway, and that they synergize to facilitate the opening of a set of enhancers under reduced IIS.

Study III: Transcriptomics-Based Screening Identifies Pharmacological Inhibition of Hsp90 as a Means to Defer Aging

In this study, we utilized an *in silico* approach to identify potential compounds with aging-preventive properties, which we refer to as geroprotectors. Subsequently, we evaluated the effects of these compounds on the healthspan and lifespan of *C. elegans*.

To screen for potential geroprotectors, we first constructed a model capable of assessing biological age based on the transcriptomic profile of tissue samples. To achieve this, we turned to the GTEx Consortium, which contains a diverse set of human tissue-specific transcriptomic data from donors of various chronological ages. After obtaining age-predictive models, we applied them to The Connectivity Map database, which comprises over 6,000 human transcriptomes of cell lines treated with various compounds. Our rationale was that if a specific compound demonstrates anti-aging properties, it will induce gene expression changes leading to a shift in the predicted age of these cells towards a younger state. This approach led to the identification of potential aging-preventive compounds, which we ranked based on their geroprotective potential.

Among the highest-ranking candidate compounds, several of them extended the lifespan of *C. elegans*. Notably, monorden, rapamycin, LY-294002, and valproic acid exhibited the most prominent effects. Monorden, believed to act as an Hsp90 inhibitor, had not been previously described as an aging-preventive compound. To evaluate whether inhibition of the Hsp90 chaperone indeed regulates aging, we knocked down *daf-21*, the gene encoding Hsp90. This approach significantly extended the lifespan of *C. elegans*, confirming that this chaperone is indeed involved in the regulation of aging. Additionally, knockdown of *daf-21* suppressed the lifespan-extending properties of monorden, further validating that monorden affects aging through inhibition of Hsp90.

Finally, we assessed whether treatment with monorden also influences the healthspan of *C. elegans*. We determined this by conducting a thrashing assay, which measures physical motility based on swimming movements. Monorden significantly increased the thrashing rate of both young and old animals, indicative of an improved healthspan.

Study IV: Primary cilia promote the differentiation of human neurons through the WNT signaling pathway

This study was conducted in collaboration with Dr. Peter Swoboda's lab at the Karolinska Institute, with the goal of assessing the involvement of primary cilia in neuron differentiation. While the study was led by the Swoboda lab, the Riedel lab contributed with specific experiments.

By utilizing LUHMES cells, we observed an increase in the percentage of ciliated cells after cell cycle exit. The maximum number of ciliated cells occurred on day 3 post exit, followed by a steady decrease as the maturation process concluded (on day 6). To assess the role of cilia in neuron differentiation, we mutated *RFX2*, a gene required for ciliogenesis during vertebrate development. This mutation resulted in structural anomalies and impaired neuronal function. Specifically, *RFX2-/-* cilia could not support axon growth as efficiently as wild-type cilia.

To provide further evidence for the importance of cilia in neuron differentiation, we utilized siRNAs to silence two essential genes involved in ciliary assembly and maintenance: *IFT88* and *IFT172*. Knocking down these genes impaired axon outgrowth and reduced the number of neurons successfully reaching differentiation stage 3. Furthermore, neurons lacking IFT88 or IFT172 exhibited fewer branched axons than their corresponding wild-type counterparts.

6 Discussions and future perspectives

The studies within this thesis unveil novel insights into the regulation of aging in *Caenorhabditis elegans*. Given the complexity of aging, our findings in this model organism shall serve as foundational understanding, which can be translated to more complex models. Hopefully, this research will inspire further studies, ultimately leading to a comprehensive understanding necessary for the development of therapies aimed at delaying or alleviating aging symptoms in humans.

The IIS pathway, as one of the most prominent and well-conserved aging-regulatory pathways, was at the focus of two of our studies. The major downstream component of this pathway, the TF DAF-16/FOXO, has been extensively studied due to its conserved aging-regulatory role and potential implication in human longevity. This thesis provides novel insights into how DAF-16 confers its longevity-regulating role under reduced IIS in *C. elegans*, and shall facilitate the discovery of analogous mechanisms in humans. Our studies support the hypothesis that DAF-16 synergizes with additional factors to regulate the expression of its target genes. We show that in *C. elegans*, DAF-16 requires BAF-1/BANF and LIN-39 to regulate aging under reduced IIS. BAF-1 and LIN-39 seem to be involved in the regulation of longevity primarily in the nervous system and the intestine, or solely in the nervous system, respectively. Previous research has indicated that IIS and DAF-16/FOXO influence aging predominantly in both the nervous system and the intestine, despite DAF-16/FOXO being present and impacting gene expression across various tissues of the organism. IIS orchestrates the metabolism of *C. elegans* in response to environmental cues, promoting survival and longevity under non-favorable conditions. Consequently, it is plausible that both IIS and DAF-16 influence aging via the nervous system, due to its neuroendocrine capabilities, as well as through the intestine, a metabolic tissue used for energy utilization and storage. Gaining a deeper understanding of the tissue-specific mechanisms through which IIS and DAF-16 regulate aging may offer novel insights into the aging processes within each tissue, shedding light on their unique characteristics and potential differences. Additionally, it may elucidate how these tissues communicate to collectively govern the overall aging process. Ultimately, these discoveries could aid in uncovering new and potentially

tissue-specific roles of IIS and FOXO factors in humans, revealing their likely role in the regulation of aging.

In terms of mechanism, we have explored how BAF-1 and LIN-39 might be involved in the regulation of aging in the nervous system and the intestine, or the nervous system, respectively. We showed that BAF-1 and LIN-39 are essential for a significant portfolio of chromatin remodeling events occurring under reduced IIS. This aligns with previous studies indicating that alterations in the chromatin landscape impact the aging rate of diverse organisms. We presented evidence that DAF-16 cooperates with both BAF-1 and LIN-39 to facilitate the opening or maintenance of accessible regions under reduced IIS, thereby establishing a chromatin environment primed for transcription. The synergy between DAF-16 and BAF-1, or LIN-39, exemplifies a mechanism that enables DAF-16 to selectively regulate its target genes in response to specific stimuli. In the context of aging regulation, DAF-16 is known to influence diverse cellular processes that contribute to longevity, some of which, as we have demonstrated, depend on BAF-1 and LIN-39. Specifically, DAF-16 promotes longevity under reduced IIS by depending on BAF-1 to initiate dauer-related transcriptional programs and regulate lipid metabolism, and on LIN-39 to modulate ACh signaling. Since IIS, DAF-16, BAF-1, and LIN-39 are all conserved across metazoans, our findings may well be relevant for aging in humans. It has been established that alterations in lipid metabolism and ACh signaling are implicated in the aging process. For instance, lipodystrophy is a characteristic of premature aging syndromes such as NGPS and HGPS, whereas cholinergic neurons undergo age-related detrimental changes. Finally, it remains uncertain whether FOXO transcription factors interact with human orthologs of BAF-1 and LIN-39 to affect chromatin organization, and whether this function is tissue-specific. These mechanisms could potentially influence the aging process in humans or explain certain aspects of NGPS.

In Study III, we developed an aging clock using transcriptomic data to assess biological age. This clock was then employed to identify compounds with potential anti-aging properties. Notably, the Hsp90 inhibitors monorden and tanespymicine emerged as top candidates. We confirmed that monorden extends lifespan in wild-type *C. elegans* by targeting the Hsp90 chaperone. Hsp90 inhibitors are known to effectively trigger the cytosolic unfolded protein response in an HSF-1-dependent manner. Since HSF-1 regulates aging in *C. elegans* and its partial loss of function suppresses the lifespan extension induced by Hsp90 inhibition, the lifespan-extending effects of monorden seem to be mediated by HSF-1.

Upregulation of heat stress responses might thus reduce the rate of aging by improving proteostasis. HSF-1 and DAF-16 are known to co-regulate the expression of heat-shock genes, implying their synergy in the regulation of the response to heat stress. It would be valuable to confirm whether compounds like monorden, or Hsp90 inhibitors in general, promote HSF-1-dependent DAF-16 functions. Additionally, DAF-16 and HSF-1 may cooperate to induce chromatin remodeling, potentially required for the transcriptional activation of their target genes. Ultimately, this synergy may be crucial for achieving metabolomic outcomes necessary to support heat stress resistance and longevity. A deeper understanding of how these aging regulators promote longevity, and the potential pivotal role of certain metabolites in this process, could lead to the identification of novel therapeutic targets or metabolites that could alleviate the symptoms of aging. In general, Hsp90 inhibitors show potential for decelerating the aging process in humans, considering their documented role in alleviating chronic inflammation through the suppression of excessive immune cell activation, as well as their ability to act as senolytic agents. Therefore, Hsp90 inhibition may offer geroprotection through various mechanisms in humans, resulting in improved proteostasis, reduced chronic inflammation and reduced number of senescent cells.

In study IV, we assessed the influence of cilia and ciliary signaling on nervous system development using the LUHMES cell model, which enables the differentiation of proliferating neuronal precursor cells into neurons within one week. We provided novel evidence for cilia and ciliary signaling in promoting neuron maturation during the early differentiation phase, wherein cilia facilitate axon outgrowth and regulate subsequent axon branching. To explore the impact of ciliary alterations on neuron differentiation, we mutated the gene *RFX2* and suppressed the expression of two essential ciliary genes, *IFT88* and *IFT172*, resulting in deregulated differentiation and the presence of abnormal cilia.

Eight *RFX* genes exist in humans, with LUHMES cells and neurons prominently expressing several RFX family members. These include RFX1-3 and RFX5, previously not associated with ciliogenesis. While RFX2 plays a pivotal role in ciliogenesis, the interplay between RFX1-3 in this process has not been extensively studied. We observed significant differential expression of ciliary genes, including relevant ciliary signal transduction genes, between wild-type and *RFX2* knockout neurons at various stages of differentiation. Surprisingly, *RFX2* knockout cilia were not truncated but significantly longer than wild-type cilia. This contrasts with

previous studies in different organisms, where reduced *RFX2* expression resulted in shorter cilia (Bisgrove et al., 2012). This could be due to those studies utilizing a transient downregulation of *RFX2* gene expression, unlike the gene knockout approach employed in our study. The observed elongation of cilia following RFX2 loss, along with the early upregulation of *RFX2* expression during neuronal differentiation, suggests RFX2's involvement in negatively regulating certain aspects of ciliogenesis at the initiation of neuronal differentiation. RFX1 and RFX3 may participate in regulating ciliogenesis after day 1, when ciliary signaling starts to promote the differentiation process. We hope that future studies will delve into this topic further, shedding light on the involvement and significance of cilia for the proper functioning of the nervous system.

7 Conclusions

C. elegans serves as a valuable model for aging research, offering insights that may shed light on the mechanisms of aging in humans. In studies (I-III), we have described novel mechanisms and proposed interventions that impact the aging rate of *C. elegans*, with potential therapeutic implications for humans.

One of the key regulators of aging is the TF DAF-16/FOXO, which is a downstream component of the conserved and well-studied IIS pathway. DAF-16 relies on its interaction partners to modulate the expression of its target genes, thereby impacting various cellular processes in *C. elegans*. In the context of aging, DAF-16 depends on BAF-1, which it physically binds, and LIN-39, to promote longevity under reduced IIS (Studies I and II). BAF-1 and LIN-39 emerge as previously unrecognized regulators of aging in *C. elegans*, functioning within the same tissues where DAF-16 exerts its longevity-regulating effects, namely the nervous system and the intestine. BAF-1 promotes longevity in both tissues, while LIN-39 does so exclusively in the nervous system. Mechanistically, BAF-1 and LIN-39 cooperate with DAF-16 to orchestrate the remodeling of the chromatin landscape, eventually impacting the transcriptome under reduced IIS. Ultimately, this influences specific DAF-16-driven physiological outcomes. Notably, DAF-16 and BAF-1 regulate fat metabolism and dauer formation, while DAF-16 and LIN-39 modulate ACh signaling under reduced IIS. Lack of BAF-1 or LIN-39 perturbs lipid metabolism and ACh signaling, respectively, which in turn impairs the longevity of *daf-2* mutants. Overall, DAF-16 achieves proper target gene regulation through BAF-1 and LIN-39, ultimately promoting longevity under reduced IIS. These findings hold promise for elucidating certain aspects of human aging and NGPS pathology.

In Study III, we present a novel approach to uncover geroprotective compounds, and propose Hsp90 inhibition as a promising therapeutic direction for delaying aging and addressing age-related complications. Specifically, we report that two Hsp90 inhibitors, monorden and tanespimycin, slow down the aging process and extend healthspan in *C. elegans*. Inhibition of Hsp90 with monorden induces the unfolded protein response, thereby improving protein homeostasis, known to be compromised in aging. This is supported by observations indicating that monorden enhances the survival of *C. elegans* exposed to proteotoxic stress, likely via mechanisms mediated by HSF-1. We hope that future studies will explore

the full potential of Hsp90 inhibitors for postponing aging and mitigating age-related complications in humans.

In study IV, we show that cilia are required for the proper axon outgrowth, branching and arborization during the early differentiation of neurons. Furthermore, we have pinpointed time-dependent signals and elucidated the mechanisms through which ciliary signaling orchestrates, enhances, and contributes to anatomical changes. Ciliary malfunctions may result in an inability to precisely translate signaling events into anatomical changes, potentially contributing to neurodevelopmental conditions and disorders.

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