Investigating the role of prostaglandin signals from adult germ stem cells on somatic aging delay of *Caenorhabditis elegans* at cold temperatures

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Alireza Noormohammadi

aus Mashhad, Iran

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Berichterstatter: Dr. David Vilchez

Prof. Dr. Aleksandra Trifunovic

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باغها را گرچه دیوار و در است
از هواشان راه با یکدیگر است
شاخ از دیوار سر بر می کشد
میل او بر باغ دیگر می کشد
باد می آرد پیام آن بدین
وه ازین پیک و پیام نازنین
شاخه ها را از جدایی گر غم است
ریشه ها را دست در دست هم است

هوشنگ ابتهاج

Two gardens by a wall and gate are severed, cleft.

And yet the air connects them. They are less bereft.

Over the wall the branches grow, a greeting send,

A gesture of affection tendered to a friend.

The wind conveys the thankful tidings in reply,

How lovely are the breeze-born tidings wafted high.

Though boughs bewail the parting, burning at the heart,

Yet hand in hand the roots remain, not apart.

Houshang Ebtehaj

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Abbreviations:

germ stem cell (GSC)
Heat Shock Factors (HSFs)
3-dimentional (3D)
Heat Shock Proteins (HSPs or chaperones)
small HSP (sHSP)
ubiquitin-proteasome system (UPS)
adult stem cells (ASCs)
hematopoietic stem cells (HSCs)
Caenorhabditis elegans (*C. elegans*)
primordial germ cell (PGC)
distal tip cells (DTCs)
heat shock response (HSR)
dehydroepiandrosterone sulphate (DHEAS)
H_2S releasing nonsteroidal anti-inflammatory drugs (HS-NSAID)
hydrogen sulfide (H_2S)
prostaglandin E2 (PGE2)

Abstract

Organisms are continuously being challenged by the alterations in environmental conditions. The ability of organisms to detect these alterations and maintain internal integrity is called homeostasis. By the ever-changing environmental conditions, organisms evolutionary also need to distribute their limited energy resources to secure the production of healthy progeny. By the re-allocation of their resources towards generating healthy progeny, resources are less available for the maintenance of soma, leading to a progressive demise of the soma. In these lines, germline removal in many animals is an effective mechanism to promote longevity.

Here we challenge this trade-off concept of soma and germline by addressing an intriguing question: how does germline impinge upon aging under favorable conditions? We hypothesize that under such conditions the need for a compromised allocation of resources reduces, thus reproductive capacity can be extended, without a considerable charge on somatic deterioration. Considering lifespan extension as a positive outcome, a moderate reduction in body temperature can induce a significant lifespan extension in distinct animals including *C. elegans*. Although the pro-longevity effects of reducing body temperature were originally reported over a century ago, little is known about the mechanisms underlying this process. Conventionally, it is thought that longevity results from a passive thermodynamic process. However, discovery of cold-sensitive TRPA-1 channel together with other studies challenge this passive thermodynamic effect of cold temperature on lifespan.

In this study, we found that germline-lacking worms have a diminished lifespan at low temperatures when compared with wild-type worms, indicating a role of the germline in cold-induced longevity. Low temperature delays the exhaustion of the germ stem cell (GSC) pool during adulthood, resulting in an extended reproductive capacity. Additionally, we found a role of thermosensory AFD neurons and TRPA-1 channel in maintenance of the GSC pool with age. Notably, cold-induced longevity is blocked by chemical and genetic interventions that impair adult GSC proliferation. Conversely, these interventions do not affect lifespan at higher temperatures. Robust proliferation of adult GSCs induces the

expression of CBS-1 in somatic tissues such as the intestine, which leads to the enhanced production of hydrogen sulfide (H_2S), a gaseous pro-longevity signaling molecule. Importantly, ectopic expression of *cbs-1* in the intestine extends lifespan at standard and warm temperatures. Additionally, we found that GSCs modulate somatic tissues at cold temperatures to induce longevity by production and release of prostaglandin E2 (PGE2). Whereas loss of adult GSCs reduces somatic *cbs-1* expression and cold-induced longevity, application of exogenous PGE2 rescues these phenotypes. Taken together, our results indicate that adult GSCs communicate with somatic tissues via prostaglandin signals to extend longevity under favorable environmental and physiological conditions such as cold temperature. This process coordinates extended reproductive capacity and long lifespan under such conditions, without compromising on either the germline or the soma.

Zusammenfassung

Organismen müssen sich diversen Umwelteinflüssen anpassen, was zur Veränderung ihres normalen Gleichgewichtszustandes führen kann. Der Prozess, durch den Organismen diese Veränderungen wahrnehmen und aufrechthalten, nennt man Homöostase. Organismen steht nur eine limitierte Menge an Energie zur Verfügung, die sie nicht nur zur Anpassung an die Umwelt verwenden müssen, sondern auch für die Fortpflanzung. Ressourcen, die normalerweise für die Aufrechterhaltung eines gesunden Somas gebraucht werden, werden für eine erfolgreiche Fortpflanzung umgeleitet, was zum stetigen Verfall des Somas führt. Das Entfernen der Keimbahn in diesen Organismen führt zu der Verlängerung ihrer Lebensdauer.

In unserer Arbeit wollen wir das Gleichgewicht zwischen Soma und Keimbahn weiter untersuchen und stellen die spannende Frage: Wie verhindert die Keimbahn das Altern unter optimalen Umweltbedingungen? Unsere Hypothese besagt, dass bei idealen Umweltbedingungen keine Verteilung von reduzierten Ressourcen zwischen Soma und Fortpflanzung nötig ist, was zu einer Verlängerung der Fortpflanzungsfähigkeit führt, ohne dass das Soma negativ beeinflusst wird. In bestimmten Organismen, wie in dem Fadenwurm *C. elegans,* kann die Verlängerung des Lebens bereits durch die Reduzierung der Körpertemperatur erreicht werden. Die Beobachtung, dass kalte Temperaturen die Lebensdauer verlängern, ist seit über einem Jahrhundert bekannt. Aber wie genau dieses Ergebnis erreicht wird, ist bisher unbekannt. Generell wurde bisher angenommen, dass das Phänomen mit passiven thermodynamischen Prozessen zu tun hat. Allerdings hat die Entdeckung eines kalt-sensitiven Kanals, TRPA-1, diesen passiven thermodynamischen Effekt in Frage gestellt.

Unsere Studie zeigt, dass Würmer, welche keine Keimbahnen besitzen, bei kalten Temperaturen eine geringere Lebenserwartung haben verglichen mit Wildtyp Würmern. Diese Beobachtung führt zu der Vermutung, dass Keimbahnen die Langlebigkeit bei kalten Temperaturen beeinflussen. Kalte Temperaturen verlangsamen die stetige Abnahme der Keimbahn-Stammzellen im Erwachsenenalter, was zur Verlängerung der Vermehrungsfähigkeit führt. Die Erhaltung eines gewissen Pools an Keimbahn-Stammzellen wird von den AFD

Nervenzellen und dem TRPA-1 Kanal, deren Aufgabe die Temperaturwahrnehmung ist, gesteuert. Die chemische oder genetische Blockade erwachsener Keimbahn-Stammzellen hebt die in kalten Temperaturen beobachtete Langlebigkeit auf. Im umgekehrten Fall wird bei gleicher Blockade in warmen Temperaturen die Langlebigkeit nicht beeinflusst. Keimbahn-Stammzellen, die sich stark vermehren, induzieren die Expression von CBL1, einem Protein, welches im somatischen Gewebe (hier Darm) produziert wird. Eine hohe Synthese dieses Proteins verstärkt ebenfalls die Produktion von Schwefelwasserstoff (H_2S), ein gasförmiges und lebensverlängerndes Signalmolekül. Interessanterweise führt die verstärkte Expression von *cbs-1* im Darm zur Verlängerung der Lebensdauer bei normalen und warmen Temperaturen. Zusätzlich zeigten unsere Ergebnisse, dass Keimbahn-Stammzellen bei kalten Temperaturen durch die Produktion und Freisetzung von Prostaglandin E2 in somatisches Gewebe die Lebensdauer verlängern. Ein Verlust von Keimbahn-Stammzellen im Erwachsenenstadium führt zu der Reduktion von *cbs1* und die Langlebigkeit bei kalten Temperaturen kann nicht mehr beobachtet werden. Die Einführung von PGE2 von außen hebt diesen Phänotyp wieder auf. Zusammenfassend zeigen unsere Ergebnisse, dass Keimbahn-Stammzellen über die Prostaglandin-Produktion der somatischen Gewebe Langlebigkeit bei optimalen und kalten Temperaturen auslösen. Dieser Prozess erlaubt eine verlängerte Fruchtbarkeitszeitspanne und verlängerte Lebensdauer, ohne das Soma oder die Keimbahn negativ zu beeinflussen.

1: Introduction

1.1: Aging and its Hallmarks

1.1.1: Homeostasis and the disposable soma theory

Organisms are continuously being challenged by the alterations in environmental conditions. Myriad of processes acting at molecular or organismal level keep the biological systems functional. These include molecular processes like DNA repair, or also at organismal level the metabolic changes which contribute to the systems' stability (Pellettieri & Sanchez Alvarado, 2007; Rando, 2006). The ability of organisms to detect the environmental changes and maintain robust steady state internal environment is called homeostasis. Since different tissues greatly differ in their metabolic activities, exposure to nutrients and environmental challenges, homeostasis in individual organs can vary noticeably (Amit, Winter, & Jung, 2016). Chronic diseases arise when biological systems deviate from the balanced state and are thus considered as homeostatic failure (Kotas & Medzhitov, 2015).

Because of the ever-changing environmental conditions, organisms evolutionary need to allocate their limited energy resources in a way that could ensure the production of healthy progeny. In other words, given limited resources, the more the organisms invest on body maintenance and repair, the less they have resources for reproduction and maintaining healthy progeny, and vice versa (Ljubuncic & Reznick, 2009). Aging occurs when the damage to the organism accumulates and the repair mechanisms are less capable of correcting them. The distinction between somatic and reproductive cells is very important because the somatic cells are necessary for the maintenance of one single organism during the life, but the reproductive cells are vital over generations to transfer the biological data from the preceding generations to the next and thus the key element in disposable soma theory is the trade-off between the maintenance of soma and reproduction, (Figure 1) (Kirkwood, 2005; Ljubuncic & Reznick, 2009), and according to these, aging is a by-product of natural selection (Le Bourg, 2001). Organisms have a reproduction probability of zero at birth and they reach the peak at youth. Afterwards, the reproductive capacity decreases because of various external or internal causes. This means that damages or mutations that

arise at young age can severely affect the organism and its progeny but the mutations at older ages are much less probable to transfer to progeny and less critical for natural selection (Le Bourg, 2001).

Figure I scheme showing how organisms should allocate resources between fitness of soma and reproduction (from (Kirkwood, 2005).

1.1.2: Hallmarks of aging

World population has significantly increased during the last century and is ever increasing, mainly due to the improvement in life quality and medical care. Human beings have always been eager to inquire the secrets of aging and find ways to slow down its pace, but only recently we have access to the technologies and methods required for deciphering this mystery in a systematic manner.

Despite lots of well-developed theories on the process of aging, having a single comprehensive explanation of biological aging is still beyond the reach. Generally spoken, aging is a complex biological process and is defined as a time-dependent continuous loss of physiological integrity, which eventually leads to the individual's demise (Lopez-Otin, Blasco, Partridge, Serrano, & Kroemer, 2013; Rebelo-Marques et al., 2018).

Having the recent advancements in hand, there are two main streams of explanation on the process of aging: the programmed aging and the damage or error-based theories. The first theory claims an intrinsic biologic programmed deterioration of the cells, both structural and functional. The other proposes that accumulation of damages in living organisms leads to aging and death

(Piedrafita, Keller, & Ralser, 2015; Rebelo-Marques et al., 2018). To better understand the aging and its causes, Lopez-Otin and colleagues proposed nine cellular and molecular candidate hallmarks that are generally contributing to the aging process. These hallmarks are: 1) genomic instability, 2) telomere attrition, 3) epigenetic alterations, 4) loss of proteostasis, 5) deregulated nutrient sensing, 6) mitochondrial dysfunction, 7) cellular senescence, 8) stem cell exhaustion, and 9) altered intercellular communication, (Figure 2) (Lopez-Otin et al., 2013; Rebelo-Marques et al., 2018). In scope of this work, hallmarks 4, 8 and 9 are the most relevant.

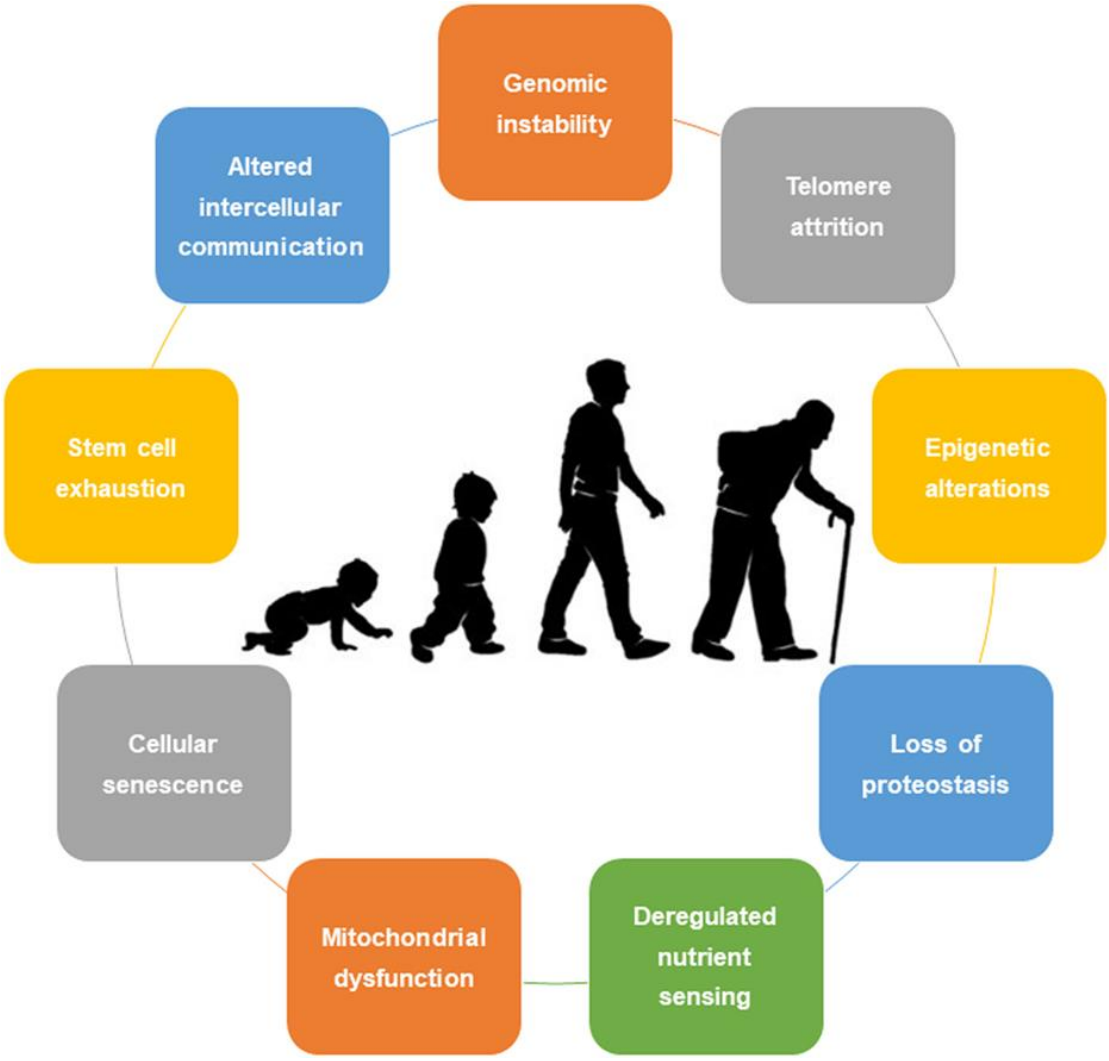


Figure II: The scheme shows the nine hallmarks of aging: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing,

mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication (from (Rebelo-Marques et al., 2018).

1.1.2.1: Loss of proteostasis

Eukaryotic cells possess a highly complex and interconnected proteome, ranging from about 6,000 proteins in fungi to more than 10,000 proteins in human cells, and yet different proteins number and profile in varying cell types and tissues of the same individual (Hipp, Kasturi, & Hartl, 2019; Kulak, Geyer, & Mann, 2017). With such a huge network of proteins, cells have to develop very accurate mechanisms that ensure the quality of protein synthesis and folding, their 3D structure and their degradation. The orchestrated action of proteins, proteolytic systems and their regulators lead to a balanced working state of proteome, referred to here as protein homeostasis (Balch, Morimoto, Dillin, & Kelly, 2008).
Most proteins throughout their lifetime need to be folded to a 3-dimentional (3D) form to serve their function in the cell, with some exceptional proteins that have disordered regions and they acquire their 3D structure only after interaction with other partner molecules (Dunker, Silman, Uversky, & Sussman, 2008; Hipp et al., 2019). However, Proteins must go through complex energy scenery during folding, and because of their complexity and various stable states, this puts them at the risk of adopting a stable but non-native structure (Hipp et al., 2019).
To reduce the number of non-native misfolded proteins, the proteome network possesses molecular chaperones. Under stress conditions that might increase protein misfolding, group of transcription factors known as heat shock factors (HSFs) bind the promoter regions of specific genes that encode heat shock proteins (HSPs or chaperones) and this results in induction of more HSPs and thus better quality control under such conditions (Anckar & Sistonen, 2011; Zheng et al., 2016). These molecular chaperones are required for the function of proteome network and can be broadly divided into the DNAJ/HSP40, chaperonin/HSP60, HSP70, HSP90 and small HSP families (Haslbeck, Franzmann, Weinfurtner, & Buchner, 2005; Y. E. Kim, Hipp, Bracher, Hayer-Hartl, & Hartl, 2013). Chaperones have the ability to work alone or in combination with

various co-chaperones to regulate the interactions in the cells (Powers, Morimoto, Dillin, Kelly, & Balch, 2009). Cells establish these systems in a balanced manner to restore the structure of misfolded proteins and to prevent the cells from accumulation of damaged proteins.

Despite various quality control mechanisms for protein folding and maintenance, there are always a number of proteins with misfolding fate and cells need to avenge them. Protein degradation is the key way to stop accumulation of the misfolded proteins. The major protein degradation mechanisms are the ubiquitin-proteasome system and the autophagy machinery (Dikic, 2017). While UPS degradation system autophagy pathway are both ATP-dependent, autophagy pathway enables the degradation of proteins or whole organelles. In the case of both pathways molecular chaperones have a pivotal role in detecting the misfolded proteins and changing their conformation to a degradable state (Hipp et al., 2019; Tekirdag & Cuervo, 2018).

When organisms age, the capacity to keep a fully functional proteostasis decreases and this is characterized by appearance of protein aggregations in different tissues (Labbadia & Morimoto, 2015). Thus, aging is directly linked to an impaired proteostasis and loss of this capacity can lead to the onset of age-related neurological diseases such as Alzheimer's or Parkinson's and Huntington's diseases (Koga, Kaushik, & Cuervo, 2011; Powers et al., 2009).

1.1.2.2: Altered intercellular communication

Organisms are constantly exposed to different environmental and physiological stimuli or stresses that lead to the accumulation of damage in the cells and age-associated dysfunction of tissues (van Oosten-Hawle & Morimoto, 2014). Besides the cell-cell communications that enable the pass of information between the cells, organisms have developed pathways to transfer the signals between far reaching tissues called cell non-autonomous interactions. This type of communications includes endocrine, neuroendocrine or neural signaling and plays an important role in the biology of aging (Rosello-Diez, Madisen, Bastide, Zeng, & Joyner, 2018), (Lopez-Otin et al., 2013). In endocrine signaling, specific signaling molecules known as hormones are released from cells that transmit

long distances e.g. via bloodstream or neurons to reach the target cells (Russell & Kahn, 2007). In neuroendocrine signaling hormones are released from neural cells which then affect other cell types (Berendzen et al., 2016). Neuronal signaling is the process that enables the transfer of neurotransmitters from the axons of neurons and these neurotransmitters bind the dendrites of the next neurons to relay the signal (Oetjen et al., 2017).

Evidences from different studies show that age-related alterations in one tissue can affect other tissues. For example senescent cells that cease dividing can damage their surrounding and induce senescence in neighbor cells through gap-junction contacts (Nelson et al., 2012). Conversely, manipulations that extend lifespan in one tissue, can delay the age-related symptoms in other tissues. Durieux and colleagues (Durieux, Wolff, & Dillin, 2011) showed that stress response pathways in one tissue could detect mitochondrial perturbations in distal tissues, which proves the importance of communications between the tissues. These findings enable us to enhance medical methods for restoration of age-associated decay via pharmacological or nutritional interventions.

1.1.2.2.1: Altered intercellular communications in *Caenorhabditis elegans*

Caenorhabditis elegans (*C. elegans*) as a model organism holds great potential for studies on biology of aging. This is on one side due the many molecular pathways and processes that are conserved in higher organisms as well as the short lifespan and feasibility of working with this model organism. One of the research areas that *C. elegans* can be helpful as a model organism is in deciphering the molecular mechanisms that regulate signaling pathways. The trans-cellular signaling in *C. elegans* that affect the aging can be categorized in two groups. One group is the direct signaling between non-neuronal tissues where secreted signaling molecules can directly activate or inhibit the transcription factors in receiver cell. The second group is the indirect signaling of a secreted molecule. Here, the somatic tissues secret factors that enter the nervous system and can affect the other cells or tissues through neuroendocrine signaling (Figure 3) (van Oosten-Hawle & Morimoto, 2014). The main signaling pathways that have been

studied in *C. elegans* are the Insulin/IGF-1, TOR signaling and germline specific signaling pathways (Lapierre & Hansen, 2012).

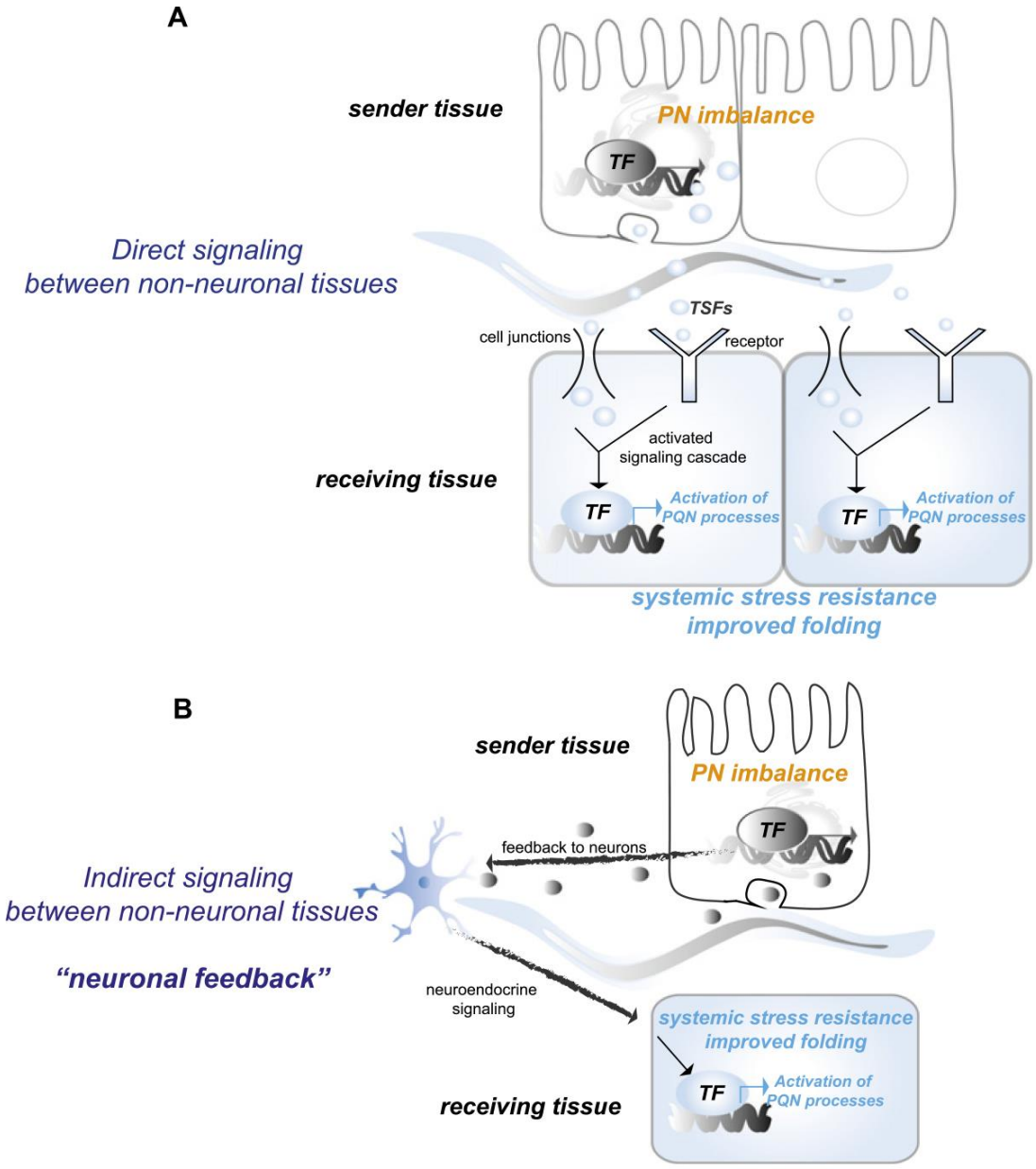


Figure III: Inter-tissue signaling mechanisms in *C. elegans*. (A) Direct signaling between non-neuronal tissues. Signals from the sender tissue triggers transcription factors activation or stress factors. These factors are taken up with receiver tissues are initiates a response. (B) Indirect signaling between non-neuronal tissues via the neurons. Signals from the sender tissue reach the receiver tissue via neuronal cells, which activates signaling pathways to modify the transcriptional programs in receiver tissue (from (van Oosten-Hawle & Morimoto, 2014).

Insulin/IGF-1 signaling

Aging research in *C. elegans* initiated with pioneering discoveries that the lifespan of *C. elegans* can be significantly extended up to double times through mutations in *daf-2* or *age-1* genes (Friedman & Johnson, 1988; Kenyon, Chang, Gensch, Rudner, & Tabtiang, 1993). Both of these genes are key players of insulin/IGF-1 signaling and these studies also reveal the importance of peptide hormones as modulators of aging (Kenyon, 2010).

Activation of DAF-2 initiates a cascade of phosphorylation events that activates multitude kinases. Theses kinases eventually inactivate transcription factor DAF-16 by blocking its entrance into the nucleus (Lapierre & Hansen, 2012). Mutations in *daf-2* or general reduction in insulin/IGF-1 signaling remove this blocking and DAF-16 can enter the nucleus where it can induce the expression of lifespan-extending genes or the genes that increase the resistance to various stresses (Murphy, 2006).

The importance of DAF-16 expression in neuronal or intestinal cells to activate the longevity phenotype is well studied. It is shown that reduction of DAF-2 activity in neurons activates the DAF-16 in a cell-autonomous manner and leads to a reduction in INS-7 production. Consequently, the reduced INS-7 amounts increase DAF-16 activity in intestine (Murphy, Lee, & Kenyon, 2007). This signaling pathway, which is also called FOXO-to-FOXO, shows that hormonal signaling is responsible for regulation of several cell and molecular processes in *daf-2* long-lived mutant (Murphy et al., 2007).

TOR signaling

The TOR (target of rapamycin) pathway coordinates growth and reproduction in response to environmental inputs including the availability of nutrients and growth factors (Saxton & Sabatini, 2017). The TOR protein, which is important for nutrient sensing, mediates the metabolic response to dietary restriction and thus essential for pro-longevity effects by dietary restriction (Fontana, Partridge, & Longo, 2010). TOR exists in two forms, TORC1 and TORC2 and its inhibition

can extend lifespan in different organisms in a conserved manner (Laplante & Sabatini, 2012).

Inhibition of TORC1 in *C. elegans* induces autophagy due to increased expression of *unc-51*, which is an upstream activator of autophagy via PHA-4 (Lapierre, Gelino, Melendez, & Hansen, 2011). TOR can also regulate ins-7 expression suggesting that it can modulate aging through hormones. Besides it can also regulate the target genes of *daf-16* and *skn*-1 (Lapierre & Hansen, 2012). Despite these discoveries on the effect of TOR on the aging in *C. elegans*, the molecular mechanisms through which it works remain to be unraveled.

Germline Signaling

Regulation of lifespan by germline has been reported in different animals including flies, mice and *C. elegans* (Kenyon, 2010). In *C. elegans*, germline is the tissue responsible for production of the progeny. Besides, the germline can communicate with other tissues through signaling pathways to modulate aging. Studies on various organisms show that removing the germline will extend the lifespan of individuals and their resistance to a variety of stresses, because the resources are liberated from reproduction and merely dedicated to the fitness of the soma. In *C. elegans* and the fruit fly *Drosophila* if the germline precursor cells are removed, there will be a lifespan extension up to 40-60% (Kenyon, 2010; Khodakarami, Saez, Mels, & Vilchez, 2015). This extended longevity is not simply because of sterility. In fact the onset of signals at germline proliferating cells are responsible for the modulation of longevity (Khodakarami et al., 2015; M. C. Wang, O'Rourke, & Ruvkun, 2008). It is shown that removal of germline cells can extend the lifespan up to 60% but this extension can be neutralized by concomitant removal of somatic gonad, showing that there are different signaling pathways initiating from germline or somatic gonads with opposite effects on aging (Hsin & Kenyon, 1999).

Opposing to the insulin/IGF-1 pathway that influences DAF-16 expression in intestine and neurons, germline removal induces DAF-16 expression in intestine, which is a target tissue for longevity signals that initiate from germline (Kenyon, 2010). The activation of DAF-16 by germline removal requires the adaptor

protein KRI-1 as well as the transcription factor TCER-1 in order to extend lifespan. KRI-1 is needed for induction of TCER-1 in the intestine of germline lacking worms and nuclear localization of DAF-16 (Berman & Kenyon, 2006). Loss of the germline can also influence the other tissues by steroid signaling and yet other undiscovered signals that might activate *tcer*-1 gene expression (Kenyon, 2010). Besides, the microRNA *mir-71*, which is expressed in neuronal cells, can cell-non-autonomously regulate DAF-16 localization in the intestine (Boulias & Horvitz, 2012), supporting a role of different DAF-16 activators for longevity phenotype in germline lacking worms (Figure 4) (Lapierre & Hansen, 2012).

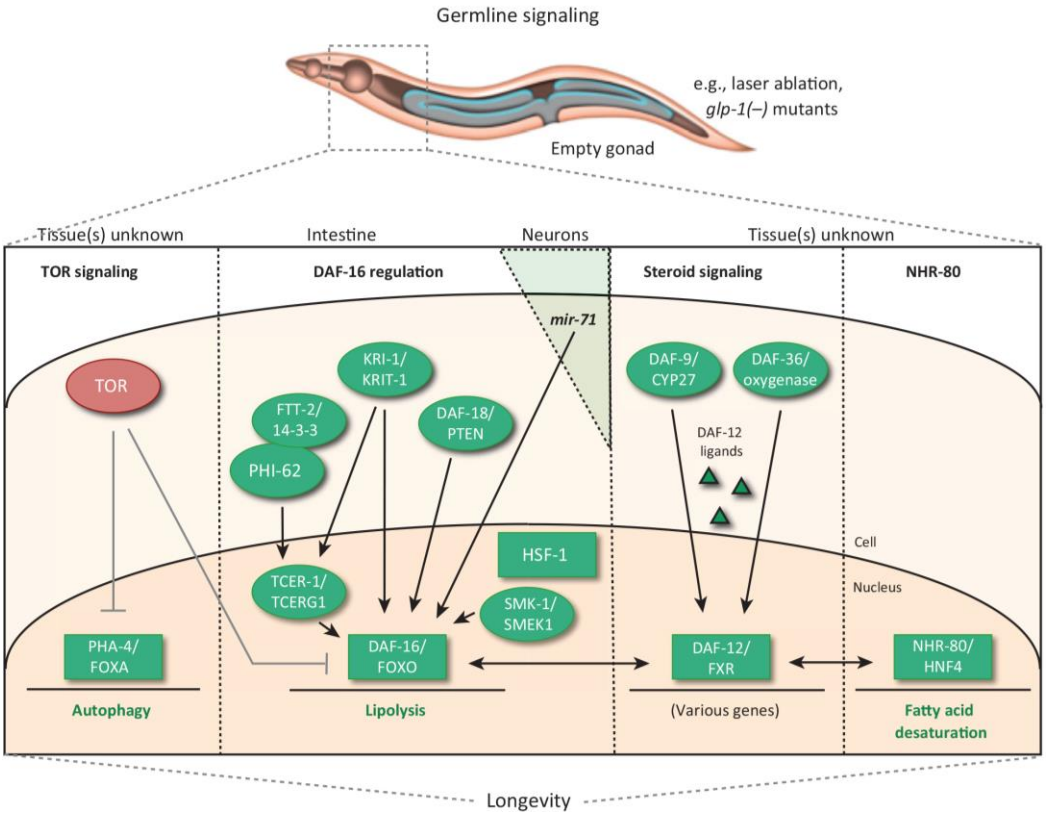


Figure IV: Germline signaling regulates C. elegans longevity. Lifespan extension in germline lacking worms depends on different signaling mechanisms: reduction in TOR signaling, DAF-16/FOXO regulation, increase in steroid signaling by DAF-36/DAF-9/DAF-12 pathway and increase in NHR-80/HNF-4 signaling, which elevates fatty-acid desaturation (from (Lapierre & Hansen, 2012).

Besides these regulators of DAF-16 in intestine, which lead to longevity phenotype, there is also a steroid signaling pathway that affects DAF-16 localization and is necessary for lifespan extension of germline ablated worms. The key components of this steroidal pathway are DAF-9, DAF-36 and DAF-12. DAF-9 (ortholog of the mammalian cytochrome P450) and DAF-36 (Rieske like oxygenase) are important for the synthesis of various ligands related to nuclear hormone receptor DAF-12. DAF-12 interacts with DAF-16-DAF-9 complex and in response to germline absence, triggers steroid signals that induce DAF-16 activity in the intestine (Dowell, Otto, Adi, & Lane, 2003; Gerisch et al., 2007).

1.2: *C. elegans* anatomy and life cycle

C. elegans is a transparent, free-living soil worm that lives in different parts of the world and feeds mainly from the bacteria. *C. elegans* can be either self-fertilizing hermaphrodite (XX) or male (XO) with males less frequent (Kuwabara, 1999). *C. elegans* like other nematodes has a cylindrical body shape that attenuates towards the end. The typical nematode body profile has two tubes, the outer tube and the inner tube, which are separated by the pseudocoelomic space. The outer tube includes cuticle, hypodermis, excretory systems, neurons and muscles. The inner tube consists of pharynx, intestine and gonads (Altun, 2009).
C. elegans is an important model organism in many biological studies such as cell biology, neuroscience, genetics, proteomics and aging for many reasons. It can be grown fast in large numbers on plates containing bacteria and can be frozen and defrosted when needed. They produce a large number of progeny and with their short life cycle, it is easy to track changes in short time in a large number of individuals. *C. elegans* is amenable to crossing and its reproduction cycle is 3 days under optimal conditions. Although *C. elegans* is a simple organism, it has many molecular signaling and mechanisms resembling higher animals, even humans (Brenner, 1973; Byerly, Cassada, & Russell, 1976).

1.2.1: *C. elegans* germline and its proliferation

A main quality of the life continuum in sexually reproducing animals is the establishment of germline and its passing from one generation to the next. Germline development in *C. elegans* theoretically has three steps: specification, growth and maintenance. During early embryogenesis, potential germline is created with the birth of P4 cell, the first primordial germ cell (PGC), which has a distinct fate from somatic cells (Figure 5) (Hubbard & Greenstein, 2005; Sulston, Schierenberg, White, & Thomson, 1983). During early gastrulation, P4 starts dividing and produces two PGCs, called Z2 and Z3 and these cells start dividing after hatching (Sulston et al., 1983). Thus L1 larvae have two primordial germ cells (Z2 and Z3) flanked by two somatic gonad precursors (Z1 and Z4) (J. Kimble & Hirsh, 1979; Pazdernik & Schedl, 2013). At mid-L1 phase, Z2 and Z3 cells start producing germ cells. Z1 and Z4 also proliferate to 12 cells by the end of L1: two distal tip cells (DTCs) that are essential for germline proliferation, and 10 proximal cells that generate the hermaphrodite somatic gonad primordium (J. Kimble & Hirsh, 1979).

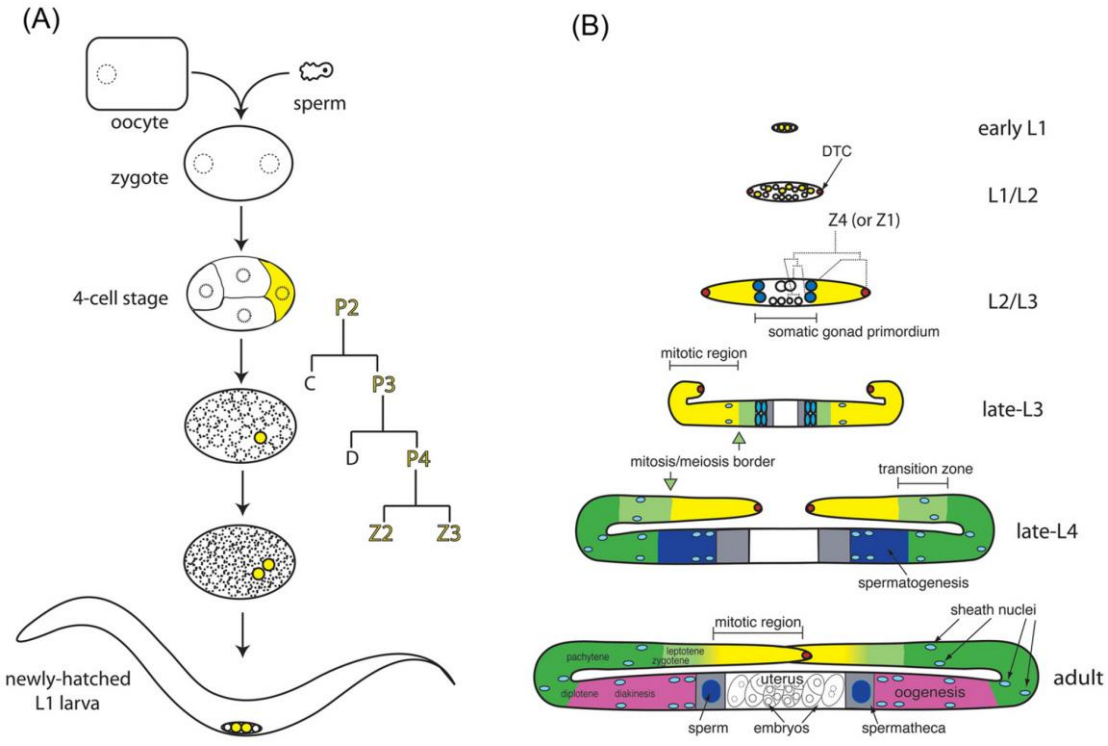


Figure V: representation of gonadogenesis. (A) Oocyte and sperm produce the zygote and thus embryonic development initiates. (B) Post-embryonic hermaphrodite gonad development (from (Hubbard & Greenstein, 2005).

During the L4 stage, germ cells boost the amplification up to four-fold and at this time the hermaphrodite germline begins to look like its adult form. Germ cells close to the DTC proliferate mitotically, hence this zone is called proliferative or mitotic zone, while the cells away from DTC enter the meiotic division (Pazdernik & Schedl, 2013). DTCs not only control proliferation of germline at larval stages, but they also maintain and control the germline mitosis in adulthood. Ablation of DTCs forces the germ cells to enter meiosis (Feng et al., 1999; J. E. Kimble & White, 1981).

In adult *C. elegans*, the germline has a well-developed mitotic region and a transition zone (Figure 5). In young adult worms, the mitotic region has almost 250 germ cells and cells with this quality reach up to 19-20 cells away from the DTC (Hansen, Hubbard, & Schedl, 2004; Lamont, Crittenden, Bernstein, Wickens, & Kimble, 2004). The germ cells in adult mitotic region continuously replenish themselves and also produce the differentiated gametes and thus perfectly fit the stem cells criteria, namely self-renewing and production of differentiated cells (Watt & Hogan, 2000).

The germline proliferation at larval stage and its maintenance at adulthood are controlled by Notch signaling (Seydoux & Schedl, 2001) , which seems to be a conserved signaling among organisms (Calvi et al., 2003). Key players of Notch signaling are the signaling ligand (LAG-2), the receptor (GLP-1) and transcription factors (LAG-1 and LAG-3). GLP-1 signaling in the germline negatively regulates initiation of meiosis in response to signals from DTC. Removal of the components of Notch signaling leads to meiosis initiation of germ cells (Doyle, Wen, & Greenwald, 2000; Petcherski & Kimble, 2000).

The initiation of meiotic cell cycle at the germline is controlled by a set of RNA regulatory proteins. The main regulators of meiosis initiation are GLD-1, GLD-2, GLD-3 and NOS-3, all of which are active in post-transcriptional gene regulation (Eckmann, Crittenden, Suh, & Kimble, 2004; Hansen et al., 2004; Hubbard & Greenstein, 2005). GLD-1 belongs to the RNA-binding proteins that repress the

activity of mRNAs that encode critical proteins for germline mitosis (M. Lee, Cram, Shen, & Schwarzbauer, 2001). Among them is the *glp-1* mRNA, which is important for Notch signaling (Marin & Evans, 2003a). GLD-2 and GLD-3 proteins bind to each other in vitro and they assist the entry to meiosis (Eckmann et al., 2004). NOS-3, which belongs to Nanos family of zinc finger proteins, represses the translation of proteins in mitotic cycle (Kraemer et al., 1999).

Several P-granule components are essential for germline development and proliferation. One group is the PGL proteins family like PGL-1 and PGL-3. These proteins bind to the RNA and mutations in one or both of them affects the germline proliferation at high temperatures (L. Kawasaki et al., 2004). The other group is the GLH proteins family which are the *C. elegans* equivalent of DEAH-box helicase. Among them, GLH-1 and GLH-4 proteins are critical for germilne proliferation (Kuznicki et al., 2000b). IFF-1 protein from IFF proteins family, which is the *C. elegans* eukaryotic initiation factor 5A homologue, is also essential for germline proliferation and its depletion leads to under-proliferation of germline (Hanazawa et al., 2004).

MOG proteins are other critical proteins for germline proliferation and oogenesis (Graham & Kimble, 1993). MOG proteins are all nuclear. MOG-1, MOG-4 and MOG-5 proteins are part of DEAH-box RNA helicases, the *C. elegans* homologue of PRP16, PRP2 and PRP22, respectively (Puoti & Kimble, 2000). MOG proteins may operate in gld-1/nos-3 branch or through a separate regulatory pathway. Depletion of these proteins affects the normal proliferation of germ cells and double mutants of a *mog* gene and *gld-3* leads to tumorous germline (Belfiore, Pugnale, Saudan, & Puoti, 2004).

1.2.2: *C. elegans* nervous system

The nervous system in hermaphrodite *C. elegans* is its most complex tissue, including 302 neurons and 56 glial cells that is more than 30% of all somatic cells of an individual. These neurons belong to two independent nervous systems: a large somatic nervous system (282 neurons) and the smaller pharyngeal nervous system (20 neurons) (Sulston & Horvitz, 1977; Sulston et al.,

1983). Most of these neurons are located in the head and they belong to at least 118 different neuron types according to their topology and synaptic branches pattern (White, Southgate, Thomson, & Brenner, 1986) and thus the well expanded neuronal system of *C. elegans* makes it a proper model organism for neurobiology and neuronal studies.

The neurons in *C. elegans* can be divided to four categories by their functionality: (1) motor neurons that control crawling and swimming together with muscles; (2) sensory neurons that are specialized for sensing the environmental inputs; (3) interneurons that serve the connection of other neurons; and (4) polymodal neurons that have multiple functions (B. L. Chen, Hall, & Chklovskii, 2006; Hall, Lints, & Altun, 2005).

C. elegans like other animals need to be aware of its environment and detect the food sources as well as potential threats. The sensation of environmental clues including physical stimuli, temperature, chemical and toxic substances, oxygen levels, light and pH are in the realm of sensory neurons (Bargmann, 2006).

1.2.2.1: Thermosensation:

Temperature as an environmental factor, affects the behavior and longevity in both poikilotherms and homeotherms. Animals have evolved different mechanisms to sense the temperature and to adapt to its changes. Animals can sense the temperature via molecular thermosensors like TRP channels, keratinocytes and tissues like intestine (Xiao, Liu, & Xu, 2015). *C. elegans* can live and reproduce in a narrow range of permissive temperatures (~12-15°C) to restrictive temperatures (~26°C) (Hedgecock & Russell, 1975). *C. elegans* is able to detect temperature shift and this is reflected in its thermotactic behavior and lifespan. *C. elegans* lives shorter when shifted from the standard culturing temperature (20°C) to higher temperatures, whereas lower temperature (e.g., 15°C) dramatically extends lifespan (S. J. Lee & Kenyon, 2009; Xiao et al., 2013). When grown at a temperature range from 16°C to 25°C and placed afterwards at a temperature gradient, worms tend to migrate to their growth temperature and they scatter away from the temperatures at which they were previously starved (Hedgecock & Russell, 1975). The thermotactic behavior in *C. elegans* can be

evolutionary important for it to escape the adverse environments and to position itself in the upper soil levels, away from exposure to sun (de Bono & Maricq, 2005).

C. elegans is equipped to different thermosensors; AFD neurons are the main thermosensors, while the amphid AWC and ASI, FLP and PHC neurons have supportive roles (Beverly, Anbil, & Sengupta, 2011; Mohammadi, Rodgers, Kotera, & Ryu, 2013; Ohnishi, Kuhara, Nakamura, Okochi, & Mori, 2011). Animals in which the AFD neurons are ablated are athermotactic. According to the thermosensory neural circuit, AFD and AWC neurons sense the temperature and then thermal information is transferred to AIY neurons. Subsequently this information is transferred to AIZ and RIA interneurons for further processing (Fig. 6) (Ohnishi et al., 2011). By ablation of AIY neuron worms become cryophilic and move towards colder temperatures, while removing the AIZ neurons makes the worms thermophilic and they tend to move to warmer temperatures (Mori, 1999).

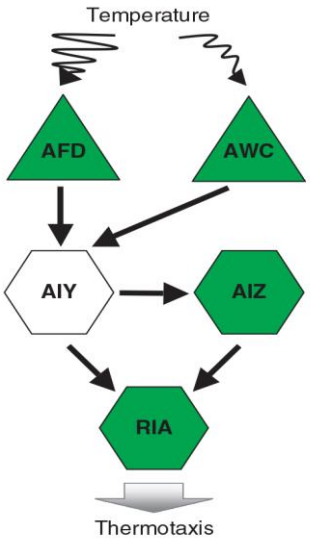


Figure VI: The thermotaxis neural circuit: AFD and AWC sensory neurons sense the temperature, and then they pass the thermal information to AIY interneuron. The information is further conveyed to AIZ and RIA interneurons leading to thermotaxis (from (Ohnishi et al., 2011).

C. elegans is sensitive to severe cold or hot temperatures. When worms are exposed to prolonged high temperatures (30°C), the heat shock response (HSR) is activated and worms stop laying egg and continue reproduction when they get back to normal temperatures (Aprison & Ruvinsky, 2014; Lithgow, White, Melov, & Johnson, 1995). It is shown that at cold or high temperatures, different neural circuits can be activated. In *C. elegans* two different channel protein families in distinct neurons can detect temperatures. First are the cGMP-dependent TAX-2/TAX-4 channels, which become activated in AFD neurons upon acute heat. Thermosensation in AFD neurons also needs receptor-type guanylyl cyclases (GCY-8, GCY-18 and GCY-23) (Inada et al., 2006; Kuhara, Inada, Katsura, & Mori, 2002). The second group is thermal-gated TRP channels. TRP channels were first cloned in Drosophila and they form a family of evolutionary conserved cation channels that have important roles in chemosansation, mechanosensation, osmosensation, photosensation and thermosensation in plenty of organisms (Venkatachalam & Montell, 2007). ThermoTRPs, a subset of TRP proteins has temperature sensitivity and are important for thermosensation in lots of animals ranging from *C. elegans* to humans (Xiao et al., 2015). These channels are detected in various animals like *C. elegans* (17 members), Drosophila (13 members) and mammalians (28 members) (Fowler & Montell, 2013; Venkatachalam & Montell, 2007; Xiao et al., 2015). The thermosensitivity of TRP channels is species-specific. For instance, TRPA-1 works as a heat-sensitive channel in flies and snakes, but as a cold-sensitive channel in *C. elegans* and mice (Xiao et al., 2015).

1.2.2.2: Mechanisms of thermosensation and its longevity regulation effects

Temperature modulates various physiological and behavioral processes like aging, reproduction, development and circadian rhythm (Clarke & Portner, 2010; Xiao et al., 2015). Modest changes in temperature can have significant effects on the properties of any living organism such as metabolism, behavior and lifespan. Early studies on the effect of temperature on aging focus on ectothermic animals that rely on external heat source and poikilothermic animal, whose body

temperature can vary depending on environmental temperature (Keil, Cummings, & de Magalhaes, 2015).

Studies in *Drosophila melanogaster* show lifespan is negatively correlated to temperature and that modest temperature changes over a long time can influence lifespan (Keil et al., 2015; Walford & Liu, 1965). In *D. melanogaster*, reducing the temperature from 27°C to 21°C, doubles the lifespan. In *C. elegans* a 5°C temperature drop can extends the lifespan up to 75%, consistent between 20°C to 15°C and 25°C to 20°C shifts (Miquel, Lundgren, Bensch, & Atlan, 1976; Van Voorhies & Ward, 1999). Studies on insects also confirm the effect of temperature change on lifespan. For example in wasps *Trichogramma platneri*, 5°C temperature decrease leads to 72% lifespan extension (McDougall & Mills, 1997).

Although the studies about the effect of temperature on lifespan of vertebrates is technically harder, but there are some controlled studies of its effect. These studies show that ectotherms tend to live longer at cold temperature. In fish species like *Sander vitreus* or *Cottus bairdii* longevity depends on temperature (Clarke & Portner, 2010). In short-lived fish *Cynolebias*, 5°C or 6°C temperature reduction extends the lifespan 43% or 75% respectively (Liu & Walford, 1966). Most of the studies about temperature in homeotherms (animals that can maintain a constant body temperature) are done in mice. Only 0.5°C lowering the core body temperature of mice induces 20% longevity (Conti et al., 2006). In Ames dwarf mouse, which lives one year longer than the wild-type mice, general lower body temperature seems to play a critical role in lifespan extension (Bartke & Brown-Borg, 2004). Lower body temperature is also related with extended lifespan in human beings and other mammals. In humans, men seem to have a slightly lower body temperature (36.2°C vs. 36.4°C) but women still live longer (Waalen & Buxbaum, 2011) which shows other key players of aging regulation in humans. Studies on healthy men in ages between 16 to 95 shows three key biomarkers for longer lifespan: lower temperatures, less insulin levels and higher dehydroepiandrosterone sulphate (DHEAS) levels (Roth et al., 2002).

Historically the regulation of aging by temperature is explained with its effect on the level of thermodynamics, a theory that is called rate-of-living-theory (Demetrius, 2013). Temperature is thought to change the metabolic rates and

that lower temperatures reduce the rate of biochemical reactions of living organisms resulting in extended lifespan. In these lines, higher temperatures increase the metabolism rate and thus accelerating the aging. Besides, temperature increase can extend the rate of molecular chaos and damage, which in turn shortens lifespan (Demetrius, 2013).

In mice with lower core body temperature, an increase in energy expenditure efficiency is observed (Conti et al., 2006), which in turn can affect different mechanisms in cells including oxidative stress and DNA damage (Farmer & Sohal, 1987). In young male *D. melanogaster*, two hours of heat shock activates HSP70 heat shock proteins until 10-51 days after the heat shock, suggesting the potential role of heat shock response (van Oosten-Hawle & Morimoto) in longevity effect of temperature (Sarup, Sorensen, & Loeschcke, 2014).

Recent studies challenge the view that temperature modulation of longevity is a passive thermodynamic process as suggested by rate-of-living theory. These studies support a process, which is regulated by active molecular signaling (Xiao et al., 2015). In this line, themosensory neurons of *C. elegans* are important for the sensing of the temperature and mutations in these neurons reduce the expression of *daf-9*, which in turn shortens the lifespan even further at higher temperatures. DAF-9 is an important gene required for the synthesis of ligands that inhibit the nuclear hormone receptor DAF-12, and mutations of *daf-12* suppress the short lifespan of thermosensory mutants at warm temperatures (S. J. Lee & Kenyon, 2009).

In *C. elegans* there are two distinct neuroendocrine signaling circuits by which the worms sense cold and warm temperatures separately (B. Zhang et al., 2018). The cold sensing circuit depends on the small neurotransmitters glutamate and serotonin for lifespan extension, whereas the anti-longevity warm sensing circuit relies on insulin-like neuropeptides (B. Zhang et al., 2018). Both circuits regulate the DAF-16/FOXO expression in gut but with opposite outcome, one extending and the other shortening the lifespan (Figure) (B. Zhang et al., 2018).

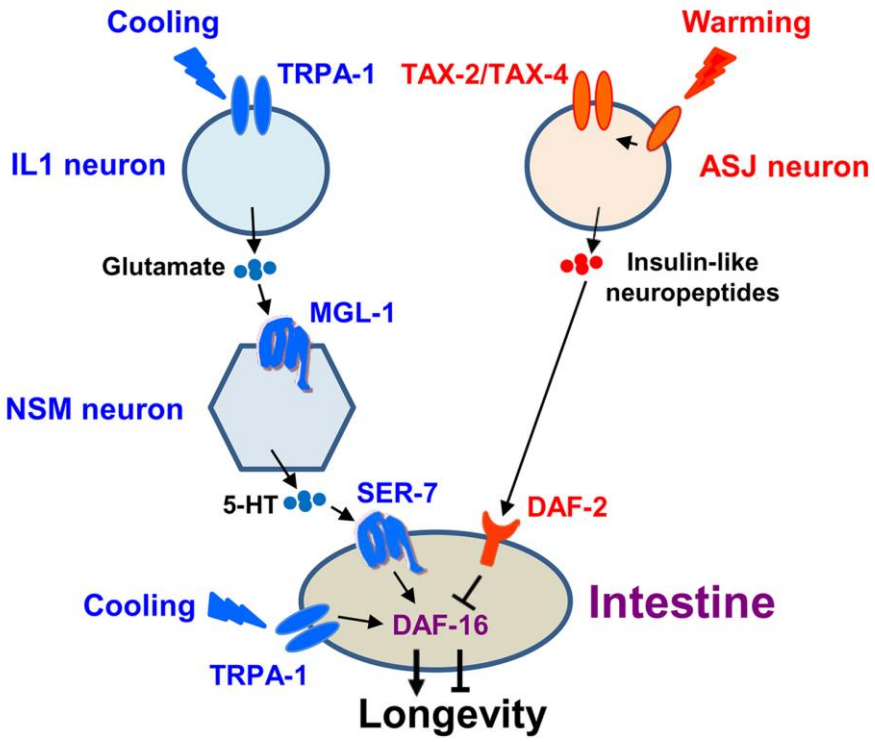


Figure VII: A schematic model illustrating the two separate neuron-intestine signaling circuits that transform environmental temperature inputs into opposing longevity outputs by differentially regulating DAF-16 in the intestine. Cold temperatures can also activate TRPA-1 channel in intestine independent of the neuronal circuit to promote DAF-16 (from (B. Zhang et al., 2018).

Recent studies in *C. elegans* show that the cold-sensitive TRPA-1 channel detects low temperature during adulthood, leading to a calcium ion influx that activates DAF-16/FOXO transcription factor and this promotes lifespan extension (Xiao et al., 2013; B. Zhang et al., 2015). TRPA-1 can promote lifespan at 15°C and 20°C but it has no effect at higher temperatures (25°C), showing that it is specifically working at lower temperatures. TRPA-1 recruits a signaling cascade including calcium influx, PKC-2 (calcium sensitive protein kinase C/, SGK-1 (DAF-16/FOXO kinase), DAF-16 transcription factor (Figure 8) (Xiao et al., 2013). Interestingly, SGK-1 and the upstream mTORC2 can also modulate *C. elegans* lifespan (Mizunuma, Neumann-Haefelin, Moroz, Li, & Blackwell, 2014). Although TRPA-1 is expressed in multiple tissues, overexpression of this TRP channel in either neurons or intestine is sufficient to extend lifespan at standard (20°C) and cold

temperatures (15°C) (B. Zhang et al., 2015). Expression of TRPA-1 in a single sensory neuron, IL1, is sufficient to extend lifespan at low but not high temperatures (B. Zhang et al., 2018).

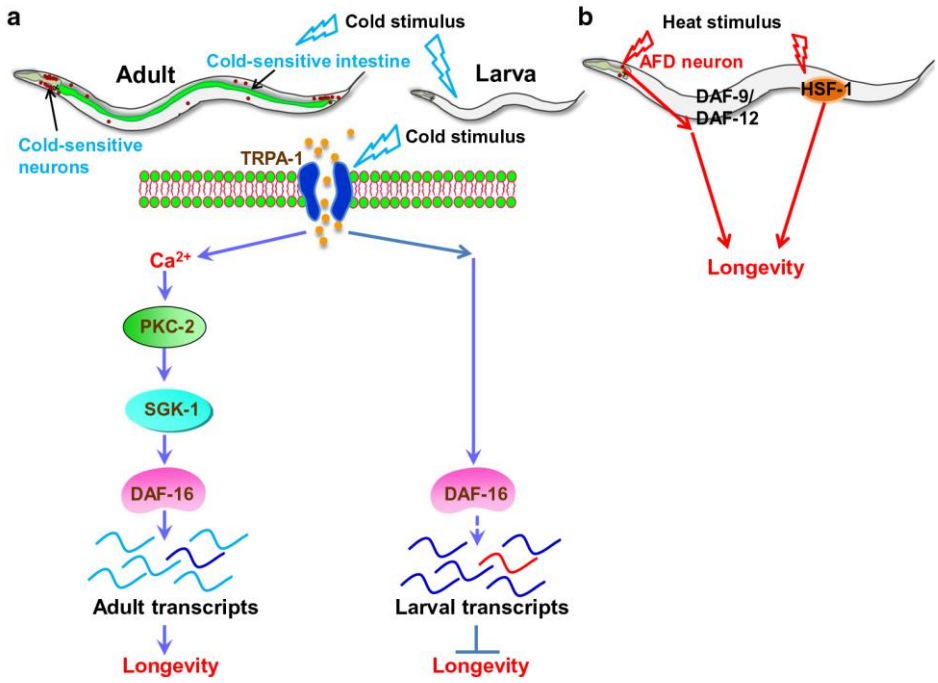


Figure VIII: Molecular mechanisms showing temperature modulation of longevity in C. elegans. a) TRPA-1 in adult worms can promote lifespan at lower temperatures via DAF-16 activation. At larval stage TRPA-1 can shorten the lifespan by differentially regulation of DAF-16 target genes. b) AFD neuron detects the higher temperatures and transmits the signal to DAF-9/DAF-12. This cell-non-autonomous signaling counteracts the lifespan shortening effect of higher temperatures. HSF-1 in both adults and larva can detect the heat and extend lifespan via heat shock response (from (Xiao et al., 2015).

Unlike the cold sensitive neurons, warm sensory neurons are more studied. Among the warm sensing neurons, AFD neurons are better characterized. But the role of AFD neurons seems to be more maintaining the lifespan rather than shortening it (S. J. Lee & Kenyon, 2009). AFD neurons also mediate a cell-non-autonomous heat shock response (Sugi, Nishida, & Mori, 2011) and ablation of AFD neurons further reduces the lifespan at high temperatures, proving their role in inhibiting detrimental effects of higher temperatures on lifespan (Xiao et al., 2015). To promote or maintain the normal lifespan at higher temperatures, AFD neurons need DAF-9 and nuclear hormone receptor DAF-12, but not the DAF-16 transcription factor. Besides, HSF-1 can also act independent of AFD

neuron to extend the lifespan (Figure 8) (Jeong, Artan, Seo, & Lee, 2012; S. J. Lee & Kenyon, 2009).

In *C. elegans,* TRPA-1 can be directly activated by temperature reduction (<20 °C) in intestine and PVD neuron, but on the other side TAX-2/TAX-4 channel in AFD neurons require intercellular signaling for activation and they are not temperature-gated per se (Ramot, MacInnis, & Goodman, 2008). Other warm sensing neurons like ASJ take advantage of TAX-2/TAX-4 channel for signal transduction (B. Zhang et al., 2018). Ablation of ASJ neurons blocks the output of these neurons, resulting in a temperature-dependent lifespan extension. These worms show a longer lifespan at 25°C but a normal lifespan at 20°C, opposite of the effect of cold sensitive neurons IL1 (B. Zhang et al., 2018).

In *D. melanogaster*, back and forth temperature shift from cold to warm has similar effects as when the worms are kept at cold temperature, suggesting that not the exposure alone, but the physiological adaptations to temperature change is important for lifespan extension (Rikke & Johnson, 2004). Temperature reduction also utilizes different metabolic pathways that suppress autoimmunity in older ages (Rikke & Johnson, 2004). In *D. melanogaster*, the lower developmental temperature enhances resistance to starvation, oxidative and heat stresses (K. Kim, Lin, & Park, 2010). Overall, these results show that the temperature effect on longevity is not a passive but rather an active process at cell and molecular levels.

Yet, another important element for longevity effect of temperature is due to correct protein folding. Proper protein folding is essential for cellular physiology (Labbadia & Morimoto, 2015) and temperature can affect the protein folding. DAF-41/ZC395.10, the *C. elegans* homolog of p23 co-chaperone/prostaglandin E synthase 3 regulates the longevity of *C. elegans* in a temperature dependent way (Horikawa, Sural, Hsu, & Antebi, 2015). Mutations in *daf-41* extend lifespan at high temperatures (25°C) but these worms are short-lived at lower temperatures. Lifespan extension at 25°C works through daf-16/FOXO and hsf-1, while short-lived phenotype relies on the daf-12/FXR steroid receptor (Horikawa et al., 2015).

Other studies also show that autophagy is required for lifespan extension at lower temperatures (15C) in *C. elegans*. The adiponectin receptor AdipoR2

homolog PAQR-2 detects temperature reduction and increases the biosynthesis of two ω-6 polyunsaturated fatty acids, γ-linolenic acid and arachidonic acid. These fatty acids induce autophagy in the epidermis, which delays collagen age-dependent decline and increases lifespan (Y. L. Chen et al., 2019). Low temperature induces the autophagy and epidermal specific autophagy is responsible for lifespan extension, which is associated with collagen maintenance at low temperature. Knocking down the autophagy genes *bec-1* (*C. elegans* ortholog of ATG6/VPS30/beclin1), *let-512* (*C. elegans* ortholog of VPS34), and *epg-1* (*C. elegans* ortholog of Atg13) shortens lifespan at lower temperature but not at higher temperatures (Y. L. Chen et al., 2019). Collectively, these data show that genetic and molecular pathways are essential for lifespan regulation at either high or low temperatures.

1.3: H_2S and its role in longevity:

Aging research is a multidisciplinary research field and among them is the investigation of gasotransmitters. These gases diffuse across the membrane freely and have roles in signal transduction. Apart from nitric oxide (NO) and carbon monoxide (CO), hydrogen sulfide (H_2S) was the third gasotransmitter discovered (Perridon, Leuvenink, Hillebrands, van Goor, & Bos, 2016). Until the 1990s research on H_2S was focused on its toxicology. In the recent years, however, evidences hint towards other physiological roles and many suggest positive effects on the manifestation of several diseases, including age-related ones (R. Wang, 2012).

Hydrogen sulfide is produced either endogenously or by exogenous intake. Endogenously, it is produced in cells with the aid of three enzymes: cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS) and 3-mercapto-pyruvate sulfurtransferase (3MST) (Kabil & Banerjee, 2014). The CSE and vitamin B6-dependent CBS enzymes are localized in the cytoplasm and translocate to mitochondria to enhance mitochondrial H_2S production under stress conditions (Fu et al., 2012). The sulfur-containing amino acids methionine and cysteine are the main source of endogenous H_2S production (R. Wang, 2002).

Exogenous H_2S is produced by bacteria in mouth or gastrointestinal tract and can penetrate the membrane without facilitation of channels (Perridon et al., 2016).

Nowadays, hydrogen sulfide is known as an important signaling molecule involved in different molecular pathways and pathologies. H_2S can affect the maintenance of proteostasis. Treatment with NaHS inhibits increased protein synthesis and aggregation in brains of Zucker diabetic rats (Talaei et al., 2014). Application of NaHS increases glucose intake in cardyomyocytes and also increases the phosphorylation of different insulin/IGF-1 signaling components like PI3K and Akt (Xue et al., 2013). The insulin/IGF-1 signaling pathway can be impaired by H_2S because it inhibits insulin secretion from pancreatic beta cells by stimulation of ATP-sensitive potassium channels (Patel & Shah, 2010).

H_2S improves mitochondrial function by increasing the production of antioxidants, reducing the levels of superoxides and ROS products (Perridon et al., 2016). H_2S can increase mitochondrial ATP production in muscle cells with impaired ATP production, and under stress conditions cells use H_2S as an inorganic energy source to keep ATP production in mitochondrial respiratory chain (Hildebrandt & Grieshaber, 2008). H_2S also protects mitochondrial genome from damage and preserves its integrity against dysfunction (Guo et al., 2012).

The effects of H_2S on aging are also partially studied. Different studies support this idea that H_2S has direct effect on aging and age-related diseases. H_2S levels in amygdala of aged rats are significantly less than healthy adult rats and application of H_2S or NaHS reversed the age-associated amygdalar synaptic plasticity and fear memory deficits, suggesting its benefit for therapeutic approaches (Zhan et al., 2018). When exposed to H_2S, *C. elegans* worms lived longer and are more heat resistant (Miller & Roth, 2007). These phenotypes are independent of insulin signaling pathway, mitochondrial dysfunction or dietary restriction, but require SIR-2.1 activity to sustain longevity (Miller & Roth, 2007). Loss of germline in *C. elegans* triggers H_2S production and mitochondrial biogenesis as both H_2S and ROS are required for lifespan extension induced by low levels of superoxide-generator paraquat and by the mutation that blocks respiration (Wei & Kenyon, 2016).

Both CBS and CSE enzymes are expressed in prostate and H_2S drugs may be beneficial against prostate cancer (Y. Zhang et al., 2013). H_2S releasing

nonsteroidal anti-inflammatory drugs (HS-NSAID) can inhibit the growth of different cancer cells like breast, pancreatic, prostate, lung and leukemia cancer cells by stopping cells proliferation and apoptosis induction (Y. Zhang et al., 2013).

2. Materials and Methods

2.1 Materials

List of primers used for qPCR assays with *C. elegans* extracts.

Gene	Forward (5' → 3')	Reverse (5' → 3')
cdc-42	CTGCTGGACAGGAAGATTACG	CTCGGACATTCTCGAATGAAG
pmp-3	GTTCCCGTGTTCATCACTCAT	ACACCGTCGAGAAGCTGTAGA
mog-5	ATTGCTACGAATATTGCCGAAAC	CTGCGGCTTGTGAGATTGG
mog-4	TGCTGATGAAGTTGGATGCAA	TGTGCAATCCTCGAAACGAA
mog-1	CTCAAGCTTCCATTGATCTCAAAC	GAAATACGCAGAGCAAATGCAT
pgl-1	ACTTTTTTGGTTTCATCCATTTCAC	CCCACCGAAATCCACAATTT
pgl-3	GCCGGATTCTTTGGTGGAT	AAAAAGAGAACCTAAAACTTGTGAATACG
glh-1	GATGGTTGGAGTGATAGCGAAAG	CCGAAACCGCCTCCACTAC
glh-4	ACTGTGCTGTTTAGTGCATCCTTCT	TAACCCTCTTTGACGAACTTTGG
iff-1	TGGCTTCGGAGGGTGGAT	TGATCTTCGGACATACTCTCTATGGT
iff-2	ACGCCCATGCAAGATCGT	AGCGTGACCGTGCTTTCC
eif-2A	AGGAAAGCATTCCAACTCAAAAAG	CGCCACTCGTTGCTTGAGA
gld-1	TGTTCAATCCAAAGAGCAGAAGTATC	CAGGCTCTTGTCCATCGATGA
mrp-6	GAGTGGATTCTTCTCGTGGATCA	CGGAAACCATCCCGTTCTC
daf-41	AAACAAGCTTCACTTCCAAGGAA	ACGGATGCAGGATCGATTTC
cal-5	GGATGATTTGAAGGGAATTTTTAGAG	CGCAGTTCTTCCCTTTGAATG
cct-2	CCAAAGGGAATGGACAAAATTC	TTGATTCCTCCTGCGCTTTC
cct-5	ATGAAGGAGCAGAAGGTTATCGA	AACCACCTGAGTGGCCAATG
cct-6	CTTCACGAAATGGCGATTCA	CGTCGAAGCCTTGGCAAT
cct-8	CGGACCAAATGGAATGAACAA	GTCGTTGGTGACGAAGAGCTT
hint-1	GATTCGGATGCTGCGCTTA	AGCTGCTTTGCAACCTTTGAA
fkb-6	CCGAGATGCCATGAAGTTGTT	GAGCGGCGGCCTTATTCT
hsp-1	CGGAGATGCTGCCAAGAATC	TTGGCATCGAAAACAGTGTTATG
ama-1	CGGAGGAGATTAAACGCATGTC	GCTTTCCGTTCTCGTAGACTTCTG
cbs-1	AGCTTTCCAGTTTGCTGAAGAGA	CCGGCGGTCACAAAAACTT
nlp-24	AAATGCTCATCAGTTGCCAGAA	GGTTGGTGAGTGGAATTTGTACTTATT

List of Kits

Name	Source
Addgene FireLab Kit	Addgene
Fugene	Promega
HiSpeed Plasmid Maxi Kit	Quiagen
Normocin	InvivoGen
Pierce BCA Proetin Assay Kit	Fisher Sientific
Puerlink Mini Kit	Life Technologies
Qiagen LongRange 2Step RT-PCR Kit	Quiagen
Qiagen Plasmid Plus Midi Kit	Quiagen
Qiagen-tip 20	Qiagen
QIAquick PCR purification Kit	Quiagen
QIAzol Lysis Reagent	Quiagen
Quanta Q Flex Reverse Transcription Kit	VWR
RNA Bee	Gentaur
SsoAdvanced SYBR Green Supermix	Bio-Rad
Zymoclean Gel DNA Recovery Kit	Zymo

List of chemicals

Name	Reference
2-Mercaptoethanol	Sigma
2-Propanol	Sigma
5-Bromo-2`Deoxyuridine	Sigma
Aceton	Sigma
Adenosine 5′-triphosphate disodium salt hydrate	Sigma
Ammonium Bicarbonate	Sigma
Ammoniumsulfate	Carl Roth
Calcium Chloride Dihydrate	Sigma
Chloroform	Sigma
Citric Acid	Sigma
Coomassie Brillantblau G-250	VWR
Cycloheximide	Sigma
D-(+)-Galactose	Sigma
D-(+)-Glucose	Sigma
Deoxycholic Acid Sodium	Sigma
Dibutyryl Camp Sodium Salt	Sigma
Diethyl Pyrocarbonat	Sigma
Dimethl Pimelimidate Dihydrochloride	Sigma
Dl-Dithiothreitol	Sigma
DMSO	Sigma
EDTA	Sigma
Edta Solution Ph 8,0	VWR
Ethanol 99,5% (Denat. 1% Mek)	VWR
Ethanol Pure	Sigma
Ethanolamine Free Base	Sigma
Formaldehyde, 10%, Methanol Free	Polyscience
Glacial Acetic Acid	Sigma
Glycerol, 500 Ml	LifeTechnologies
Glycin	Sigma
Hydrochloric Acid	Sigma
Hydrogen Peroxide 30%	Sigma
Isopropanol	Sigma
Kaliumhydrogenphospat	Roth
Kanamycin Sulfate	LifeTechnologies
L-Ascorbic Acid,	Sigma
L-Ascorbic Acid, Reagent Grade	Sigma
LB Broth	Merck Millipore
Magnesium Chloride Hexahydrate	Sigma

Methanol	VWR
Methylviologen Hydrat 98% (Paraquat)	Sigma
Mops	Carl Roth
Mowiol	Sigma
N-Acetyl-L-Cysteine	Sigma
N-Ethylmaleimide	Sigma
N,N-Dimethylformamid	Sigma
Nonfat-Dried Milk From Bovine	Sigma
Peptone	BD
Phenol Chloroform	Sigma
Phenylmethylsulfonyl fluoride (PMSF)	Sigma
Poly-L-Ornithine Hydrobromide	Sigma
Ponceau S Solution	Sigma
Potassium Acetate	Sigma
Potassium Chloride	Fisher
Potassium Dihydrogen Phosphate	ALFA
Potassium Hydrogen Phosphate Dibasic	ALFA
Potassium Hydroxide	Sigma
Proteinase K, Recombinant	Sigma
Rubidium Chloride	Sigma
SDS- sodium dodecyl sulfate	Sigma
Sodium 4-Phenylbutyrate	VWR
Sodium Acetate, Anhydrous	Sigma
Sodium Azide	Sigma
Sodium Azide	VWR
Sodium Chloride	Sigma
Sodium Fluoride	Sigma ALFA
Sodium Hydrogen Phosphate Dibasic	Sigma
Sodium Hypochlorite Solution	Sigma
Sodium Orthovanadate	Sigma
Sodium Selenite	Sigma
Sucrose	Sigma
Tetracycline Hydrochloride	Sigma
Thapsigargin	Sigma
Transferin, Human, Recombinant	Sigma
Trichloroacetic Acid	VWR
Tris 1M Ph 7,5	Sigma
Triton X-100	Sigma
Trizma Base	Sigma
Trypan Blue Solution (0,4%)	Sigma
Tunicamycin	Biochemica
Tween® 20	

List of electrical devices

Instrument	Manufacturer
BINDER CB-150	BINDER GmbH
Mini-Protean TGX, 10% ;10W; 10	Bio-Rad
Mini-Sub Cell GT System W/ 7x7	Bio-Rad
Mini-Trans-Blot Module	Bio-Rad
C1000 Touch Thermal Cycler	Bio-Rad
S1000 Thermal Cycler	Bio-Rad
Mini-PROTEAN Tetra Cell	Bio-Rad
PowerPac Basic HC	Bio-Rad
Eppendorf 5430R, 5424, 5810R	Eppendorf
Thermomixer Comfort	Eppendorf
Hanna Instruments HI3220	HANNA Instruments GmbH
Heidolph unimax 1010	Heidolph Instruments GmbH
ScanLaf Class 2 Mars	Labogene
VTX-3000L Mixer Uzusio	LMS Co., Ltd
Shaking platform RM S-30V	M. Zipper GmbH
MIR-554-PE Cooled Incubator	Panasonic
EnSpire Multimode Plate Reader 2300	Perkin Elmer
Precisa XB4200C	Precisa Gravimetrics AG
Dri-Block DB-2D	Techne
NanoDrop 8000	ThermoFisher Scientific
Fusion Solo	Vilber Lourmat
SteREO Discovery.V8	ZEISS
ZEN imaging software	ZEISS
Imager. Z1	ZEISS
ZEISS Apotome .2	ZEISS
Ec-Plan-Neofluar 20X-40X	ZEISS
Achro-plan 10X	ZEISS
Axiocam 506 mono	ZEISS
Axio Zoom.V16	ZEISS

2.2: METHODS

2.2.1: *C. elegans* strains and maintenance

C. elegans strains were grown and maintained on standard nematode growth media (NGM) seeded with *E. coli* (OP50) (Brenner, 1974). Wild-type (N2), CB4037 (*glp-1(e2141)*III), SS104 (*glp-4(bn2)*I), CF512 (*fer-15(b26)*II;*fem-1(hc17)*IV) (*fer-15* is also named *rrf-3*), TQ233 (*trpa-1(ok999)*IV), GN112 (pgIs2[*gcy-8p*::TU#813 + *gcy-8p*::TU#814 + *unc-122p*::GFP + *gcy-8p*::mCherry + *gcy-8p*::GFP + *ttx-3p*::GFP]), JN215 (*iff-1(tm483)*III/hT2 [*bli-4(e937)* let-?(*q782*) qIs48](I;III)), PR767 (*ttx-1(p767)*V), PY1283 (*ttx-1(oy29)* oyIs17 [*gcy-8p::*GFP + *lin-15*(+)]V), BA837 (*spe-26(it112)IV),* CB4108 (*fog-2(q71)*V), JK560 (*fog-1(q250)*I) and JK1466 (*gld-1(q485)/dpy-5(e61) unc-13(e51)*I) were provided by the *Caenorhabditis* Genetics Center (CGC) (University of Minnesota), which is supported by the NIH Office of Research Infrastructure Programs (P40 OD010440). CF2424 (PR767 (*ttx-1(p767)*V) outcrossed 4 times to wild-type N2) was a gift from S.J. Lee. AA1724 (*pges-2(ok3316)*IV) was a gift from A. Antebi and was generated by outcrossing the RB2421 strain (*pges-2(ok3316)*IV) 4 times to wild-type N2. RB2421 was made by the *C. elegans* Gene Knockout Project at the Oklahoma Medical Research Foundation. TQ6891 (*xuEx1670*[*aqp-6p::trpa-1::sl2::yfp*]; *trpa-1(ok999)*IV) was a kind gift from X.Z. Shawn Xu.

For tissue-specific RNAi experiments, we used either *rde-1* or *sid-1* mutant animals in which wild-type *rde-1* or *sid-1* genes have been rescued using tissue-specific promoters, respectively. Tissue-specific knockdown was confirmed in the original publications. In WM118 strain (*rde-1(ne300)*V; neIs9[*myo-3p*::HA::RDE-1 + *rol-6(su1006)*], RNAi treatment is only effective in muscle (Yigit et al., 2006). In VP303 strain (*rde-1(ne219)*V; kbIs7[*nhx-2p*::*rde-1* + *rol-6(su1006)*], RNAi treatment is only effective in intestine (Espelt, Estevez, Yin, & Strange, 2005). In AMJ345 strain (jamSi2[*mex-5p*::*rde-1*(+)]II; *rde-1(ne219)*V), transgene rescues effective RNAi treatment in both intestine and germline (Marre, Traver, & Jose, 2016). TU3401 strain (*sid-1(pk3321)*V; uIs69[pCFJ90(*myo-2p*::mCherry) + *unc-119p*::*sid-1*]) was used for neuron-

specific knockdown (Calixto, Chelur, Topalidou, Chen, & Chalfie, 2010). All the aforementioned tissue-specific RNAi-sensitive strains were provided by the CGC. For tissue-specific RNAi experiments in the germline alone, we used DCL569 strain (mkcSi13[*sun-1p::rde-1::sun-1 3'UTR* + *unc-119*(+)]II; *rde-1(mkc36)*V) (Zou et al., 2019). DCL569 was a kind gift from Di Chen.

For the generation of worm strains DVG146–DVG147 (N2, *ocbEx130*[*sur-5p::ZC373.1*, *myo-3p::GFP*] and N2, *ocbEx131*[*sur-5p::ZC373.1*, *myo-3p::GFP*]), a DNA plasmid mixture containing 70 ng µl^{-1} of the plasmid *sur5-p::cbs-1* and 20 ng µl^{-1} pPD93_97 (*myo3-p::GFP*) was injected into the gonads of adult N2 hermaphrodite animals, using standard methods[85]. GFP-positive F_1 progeny were selected. Individual F_2 worms were isolated to establish independent lines. Following this method we also generated the worm strains DVG162 (N2, *ocbEx145*[*rgef-1p::ZC373.1*, *myo3-p::GFP*]), DVG163 (N2, *ocbEx146*[*rgef-1p::ZC373.1*, *myo3-p::GFP*], DVG166 (N2, *ocbEx149*[*gly-19p::ZC373.1*, *myo-3p::GFP*]), DVG167 (N2, *ocbEx150*[*gly-19p::ZC373.1*, *myo-3p::GFP*]), DVG173 (N2, *ocbEx156*[*myo-3p::ZC373.1*, *myo-3p::GFP*]) and DVG174 (N2, *ocbEx157*[*myo-3p::ZC373.1*, *myo-3p::GFP*]). Control worms DVG9 (N2, *ocbEx9[myo3p::GFP]*) were generated by microinjecting N2 worms with 20 ng µl^{-1} pPD93_97 (Noormohammadi et al., 2016). DVG186 (*glp-1(e2141)*III; *ocbEx150*[*gly-19p::ZC373.1*, *myo-3p::GFP*]) was generated by crossing DVG167 to CB4037.

For the generation of *cbs-1* transcriptional reporter line DVG156 (N2, *ocbEx140*[*ZC373.1p::GFP*, pRF4(*rol-6*)]), a DNA plasmid mixture containing 50 ng µl^{-1} of the plasmid pDV184 and 30 ng µl^{-1} pAD126 (pRF4(*rol-6*)) was injected into the gonads of adult N2 hermaphrodite animals (Mello, Kramer, Stinchcomb, & Ambros, 1991). Roller-phenotype-positive F1 progeny were selected. Individual F2 worms were isolated to establish independent lines. Similarly, we generated the *cbs-1* transcriptional reporter line DVG185 by injecting a mixture of the plasmid pDV184 and pAD126 into the gonads of DCL569 germline-specific RNAi strain.

To generate DVG177 strain (*ocbEx160*[*cbs-1p*::GFP, pRF4(*rol-6*)]; *pges-2(ok3316)*IV), DVG156 was crossed to AA1724. Roller-phenotype-positive F1 progeny were selected and transferred to individual plates. Screening

of *pges-2(ok3316)* homozygote worms was done in F2 progeny by PCR using the following primers:

TGAACGCTGAGCATCCATAG and CGCCCGTTTTCTTTTAATG.

2.2.2: RNAi constructs

RNAi-treated strains were fed *E. coli* (HT115) containing an empty control vector (L4440) or expressing double-stranded RNAi. *pgl-1*, *pgl-3*, *glh-1*, *mog*-4, *mog-5*, *iff-1*, *iff-2*, *ifg-1*, *ife-2*, *rps-15*, *rsks-1*, *cct-2*, *cct-6*, *cct-8*, *hint-1*, *hsp-1*, *fkb*-6, *ttr-6*, *cal-5* and *gld-1* RNAi constructs were obtained from the Vidal RNAi library. *glh-4*, *mog-1*, *eif-2A*, *cct-5*, *ama-1*, *cbs-1*, *nlp-24*, *daf-41* and *mrp-6* RNAi constructs were obtained from the Ahringer RNAi library. All constructs were sequence verified. For each RNAi, we assessed the knockdown efficiency at the distinct temperatures the RNAi was used.

2.2.3: Lifespan studies

Synchronized animals were raised and fed OP50 *E. coli* at 20 °C or 25 °C until day 1 of adulthood. Then, worms were transferred onto plates with HT115 *E. coli* carrying empty vector or RNAi clones. 96 animals were used per condition and scored every day or every other day (Noormohammadi et al., 2016). From the initial worm population, the worms that are lost or burrow into the medium as well as those that exhibit 'protruding vulva' or undergo bagging were censored. *n*= total number of uncensored animals/total number (uncensored+censored) of animals observed in each experiment. Lifespans were conducted at the temperatures indicated in the corresponding figure. For non-integrated lines DVG9, DVG146, DVG147, DV162, DV163, DVG166, DVG167, DVG173 and DVG174, GFP-positive worms were selected for lifespan studies. PRISM 6 software was used to determine median lifespan and generate lifespan graphs. OASIS software was used for statistical analysis to determine mean lifespan (J. S. Yang et al., 2011). *P* values were calculated using the log-rank (Mantel–Cox) method. The *P* values refer to experimental

and control animals in a single experiment. In the main text, each graph shows a representative experiment.

2.2.4: Construction of *cbs-1 C. elegans* expression plasmids

To construct the *cbs-1 C. elegans* ectopic expression plasmid (pDV163), pPD95.77 from the Fire Lab kit was digested with SphI and XmaI to insert 3.6KB of the *sur-5* promoter. The resultant vector was then digested with KpnI and EcoRI to excise GFP and insert a multi-cloning site. ZC373.1a (*cbs-1*) was PCR amplified from cDNA to include 5′ NheI and 3′ NotI restriction sites then cloned into the aforementioned vector. To generate neuronal-specific ectopic expression *cbs-1* plasmid (pDV188), pMS1 plasmid (*rgef-1p::rpn-6.1::unc-54* 3'UTR) with an inserted multi-cloning site was digested with 5′ NheI and 3′ NotI restriction sites. Then, *cbs-1* was PCR amplified from cDNA to include 5′ NheI and 3′ NotI restriction sites and cloned into pMS1. Following the same strategy, *cbs-1* was cloned into pMS2 (*gly-19p::rpn-6.1::unc-54* 3'UTR) to construct intestinal-specific expression plasmid (pDV189). To generate muscle-specific expression plasmid (pDV190), pMS3 plasmid (*myo-3p::rpn-6.1::unc-54* 3'UTR) was digested with 5′ XbaI and 3′ AscI restriction sites. All constructs were sequence verified. pMS1-3 were a gift from A. Dillin.

2.2.5: Construction of cbs-1 transcriptional reporter construct

To construct pDV184, pPD95.77 from the Fire Lab kit was digested with SalI and BamHI. The promoter region and part of exon 1 of ZC373.1a (*cbs-1*) was PCR amplified from N2 gDNA to include −1428 to 187 and then cloned into the aforementioned vector using the same enzymes.

2.2.6: *cbs-1* transcriptional reporter experiments and imaging

DVG156 worms were grown at 20 °C until day 1 of adulthood and then transferred onto plates with *E. coli* (HT115) expressing empty vector or double-stranded RNAi. Worms were then cultured until day 7 at the indicated temperatures in the respective figure. For FUdR treatment experiments, day 1

adults were transferred onto plates with *E. coli* (HT115) expressing empty vector covered with 100 µg ml^{-1} FUdR. Adult worms were then grown until day 5 at the indicated temperatures under FUdR treatment. In rescue experiments, worms were transferred at day 5 of adulthood into plates with PGE2 (Tocris, #363-24-6) spotted on top of the bacterial lawn. For imaging, adult worms were immobilized using 0.1% Azide in M9 buffer and covered with cover slip. Images of whole worms were acquired with Zeiss Axio Zoom.V16 fluorescence microscope. To quantify GFP fluorescence, worms were outlined and quantified using ImageJ software.

2.2.7: Body size imaging and quantification

Images of worms were acquired with Zeiss Axio Zoom.V16 microscope. To quantify body size, the whole worm was outlined and quantified using ImageJ software.

2.2.8: Bromodeoxyuridine (BrdU) proliferation assays

Worms were incubated with 33mM solution of BrdU (Sigma-Aldrich) in S medium for 2h at 20 °C. Worms were washed with EBT buffer (1X egg buffer, 0.2 % Tween 20, 20 mM Sodium Azide). Worms were then decapitated to extract germline on a coverslip and covered with a poly-lysine-coated microscope slide. The coverslip was removed and the slide was fast frozen on dry ice. Then, the slide was fixed in methanol for 2 min at -20 °C and washed with PBST (1X PBS and 0.1% Tween 20). DNA was denatured in 2M HCl for 45 min at room temperature followed by washing with PBST. After blocking for 30 min in PBST 10% donkey serum, anti-BrdU antibody (Abcam, ab6326, RRID:AB_305426) was added (1:250) followed by overnight incubation in a humid chamber. Anti-Rat IgG secondary antibody (Life Technologies 1744742) was added (1:500) for 2 h at room temperature. Finally, slides were mounted with Precision coverslip (Roth) using DAPI fluoromount-G (Southern Biotech 0100-20).

2.2.9: Egg counting

Synchronized animals were raised and fed OP50 *E. coli* at 20 °C or 25 °C until late L4 stage. Number of eggs during the self-reproductive period was measured by singly plating late L4 worms and cultured them at the distinct temperatures indicated in the corresponding figures. Each adult worm was then transferred to a new plate every 24 h and the previous plate was kept at the respective temperature for another 24 h when the number of alive progeny, visible as L1 larvae was scored to assess percentage of viable eggs. This procedure was repeated until no living progenies were counted.

For egg counting after the self-reproductive period, worms were grown at the distinct temperatures indicated in the corresponding figures for 8 days during adulthood. Then, worms were singly plated and mated for 48 h at the indicated temperatures with young males which were raised at 20°C. After mating, females were transferred to a new plate every 24 h and the previous plate were kept at the respective temperature for another 24 h when the number of living progeny was scored. This procedure was repeated until no living progenies were counted.

2.2.10: H_2S detection

H_2S production capacity was measured following the lead sulfide method (Hine et al., 2015). First, worms were homogenated in lysis buffer (PBS supplemented with 10 mM L-cysteine and 1 mM pyridoxal 5'-phosphate hydrate (PLP)) using a Precellys 24 homogenizer (Bertin technologies). 300 μg of the lysate was transferred onto a well of a 96-well plate. Then, lead acetate paper was placed directly over the well for detection of H_2S at 37°C.

2.2.11: Western blot

Worms were lysed in protein lysis buffer (50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 0.25% sodium deoxycholate, 1 mM EDTA and protease inhibitor (Roche)) using a Precellys 24 homogenizer. Worm lysates were centrifuged at 10,000 rpm for 10 min at 4 °C and the supernatant was collected. Protein concentrations were determined with standard BCA protein assay (Thermoscientific). 30 μg of total protein was separated by SDS-PAGE,

transferred to nitrocellulose membranes (Millipore) and subjected to immunoblotting. Western blot analysis was performed with anti-PGES2 antibody (Bioss, bs-2639R, 1:500; RRID:AB_10860215) and α-tubulin (Sigma, T6199, 1:5,000; RRID:AB_477583).

2.2.12: *C. elegans* germline and gut immunostaining

Worms were washed with EBT buffer (1X egg buffer, 0.2 % Tween 20, 20 mM Sodium Azid), decapitated to extract germline and intestine on a coverslip and covered with a poly-lysine-treated microscope slide. The slide was fast frozen on dry ice, fixed in methanol for 2 min at -20 °C and washed with PBST (1X PBS and 0.1 % Tween 20). Immunostaining was processed by blocking with 10 % donkey serum in PBST for 30 min followed by overnight incubation with anti-PGES2 antibody (Bioss, bs-2639R, 1:100; RRID:AB_10860215) at room temperature. Alexa Fluor 488 goat anti-rabbit IgG (Life Technologies 1408830, 1:500) was added for 2 h. Slides were mounted with DAPI fluoromount-G (Southern Biotech 0100-20).

2.2.13: PGE2 measurements

We quantified PGE2 levels using a PGE2 ELISA Kit (amsbio, AMS.E-EL-0034), following manufacturer's instructions. *fer-15;fem-1* control sterile worms were lysed in protein lysis buffer (50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 0.25% sodium deoxycholate, 1 mM EDTA and protease inhibitor (Roche)) using a Precellys 24 homogenizer. Worm lysates were centrifuged at 10,000 rpm for 10 min at 4°C and supernatant was collected. Protein concentrations were determined with standard BCA protein asaay (Thermoscientific). 3 mg of total protein were used for measurement.

2.2.14: Oxygen consumption

Oxygen consumption rates were measured using an Oroboros Oxygraph 2k (Oroboros Instruments GmbH). 300 adult worms were used for each measurement. Data was analyzed using DatLab7 software (Version 7 3.0.3).

2.2.15: Motility assay

At day 6 of adulthood, worms were transferred to a drop of M9 buffer and after 30 s of adaptation the number of body bends was counted for 30 s. A body bend was defined as change in direction of the bend at the mid-body (Brignull, Moore, Tang, & Morimoto, 2006).

2.2.16: Sample preparation for label-free quantitative proteomics and analysis

fer-15;fem-1 control sterile worms were lysed in urea buffer (8 M urea, 2 M Thiourea, 10 mM HEPES, pH 7.6) by sonication and cleared using centrifugation (13,000 rpm, 10 min). Supernatants were reduced (1 mM DTT, 30 min), alkylated (5 mM iodoacetamide (IAA), 45 min) and digested with trypsin at a 1:100 w/w ratio after diluting urea concentration to 2 M. One day after, samples were cleared (16,000*g*, 20 min) and supernatant was acidified. Peptides were cleaned up using stage tip extraction (Rappsilber, Ishihama, & Mann, 2003). The liquid chromatography tandem mass spectrometry (LC-MS/MS) equipment consisted out of an EASY nLC 1000 coupled to the quadrupole based QExactive instrument (Thermo Scientific) via a nano-spray electroionization source. Peptides were separated on an in-house packed 50 cm column (1.9 µm C18 beads, Dr. Maisch) using a binary buffer system: A) 0.1% formic acid and B) 0.1% formic acid in acetonitrile. The content of buffer B was raised from 7% to 23% within 120 min and followed by an increase to 45% within 10 min. Then, within 5 min buffer B fraction was raised to 80% and held for further 5 min after which it was decreased to 5% within 2 min and held there for further 3 min before the next sample was loaded on the column. An applied voltage of 2.2 kV ionized eluting peptides. The capillary temperature was 275°C and the S-lens RF level was set to 60. MS1 spectra were acquired using a resolution of 70,000 (at 200 m/z), an Automatic Gain Control (AGC) target of 3e6 and a maximum injection time of 20 ms in a scan range of 300-1750 Th. In a data dependent mode, the 10 most intense peaks were selected for isolation and fragmentation in the HCD cell using normalized collision energy of 25 at an isolation window of 2.1 Th. Dynamic exclusion was enabled and set to 20 s. The MS/MS scan properties were: 17.500 resolution at 200 m/z, an AGC target of 5e5 and a maximum injection time of 60 ms. All label-free proteomics data sets were analyzed with

the MaxQuant software (Cox & Mann, 2008) (release 1.5.3.8). We employed the LFQ mode (Cox et al., 2014) and used MaxQuant default settings for protein identification and LFQ quantification. All downstream analyses were carried out on LFQ values with Perseus (v. 1.5.2.4) (Cox & Mann, 2012).

2.2.17: RNA isolation and sequencing

RNA from extruded germlines of day 6-adult N2 worms was extracted using RNAbee (Tel-Test Inc.). Libraries were prepared using the TruSeq Stranded mRNA Library Prep Kit. Library preparation started with 1 µg total RNA. After selection (using poly-T oligo-attached magnetic beads), mRNA was purified and fragmented using divalent cations under elevated temperature. The RNA fragments underwent reverse transcription using random primers followed by second strand cDNA synthesis with DNA Polymerase I and RNase H. After end repair and A-tailing, indexing adapters were ligated. The products were then purified and amplified (20 µl template, 14 PCR cycles) to create the final cDNA libraries. After library validation and quantification (Agilent 2100 Bioanalyzer), equimolar amounts of library were pooled. The pool was quantified by using the Peqlab KAPA Library Quantification Kit and the Applied Biosystems 7900HT Sequence Detection System. The pool was sequenced on an Illumina HiSeq 4000 sequencer with a paired- end (2x75bp) protocol.

RNA-seq data were analysed using a QuickNGS pipeline (Wagle, Nikolic, & Frommolt, 2015). Basic read quality check was performed using FastQC and read statistics were obtained with SAMtools. The basic data processing consists of a splicing-aware alignment using Tophat2 (D. Kim et al., 2013) followed by reference-guided transcriptome reassembly with Cufflinks2 (Trapnell et al., 2012). Read count means, fold change and *P*-values were calculated with DEseq2 (Anders & Huber, 2010) and gene expression for the individual samples was calculated with Cufflinks2 (Trapnell et al., 2012) as FPKMs, using in both cases genomic annotation from the Ensembl database version 87.

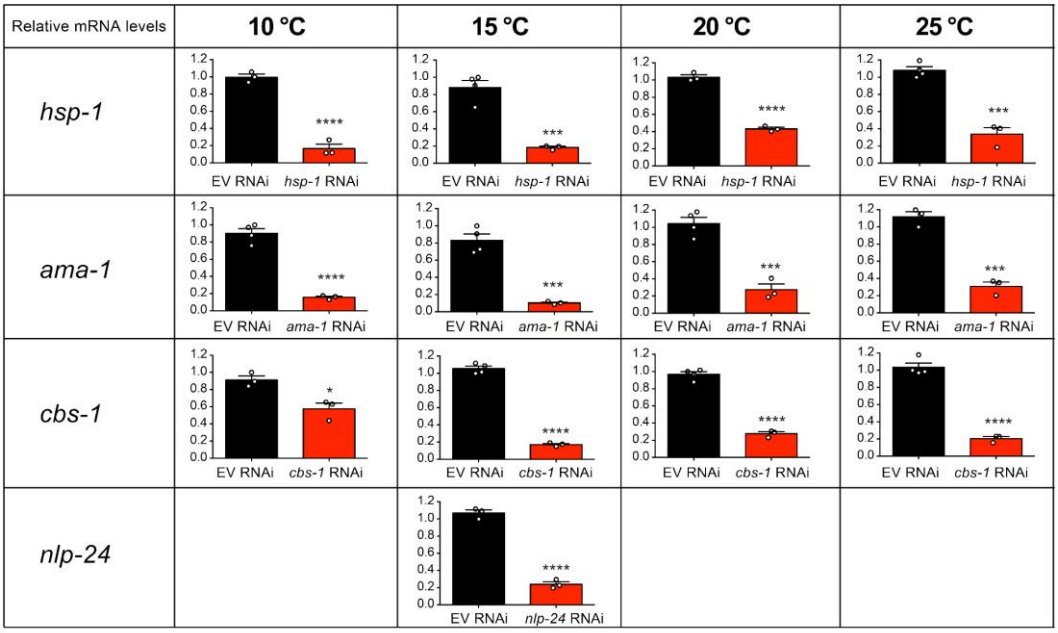
2.2.18: Quantitative RT–PCR

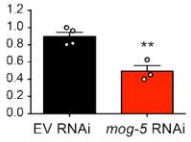
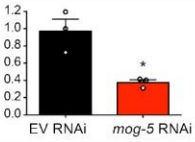
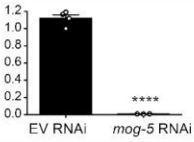
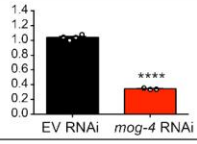
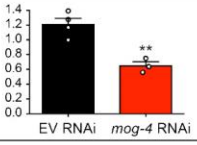
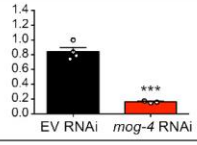
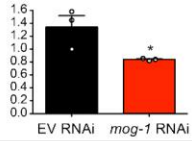
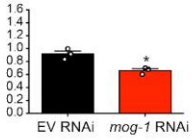
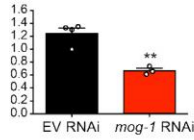
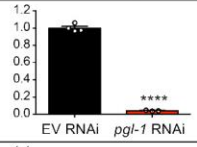
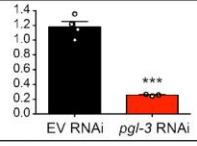
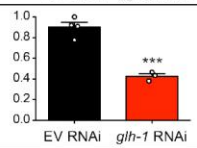
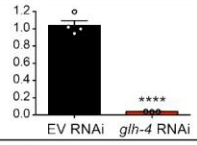
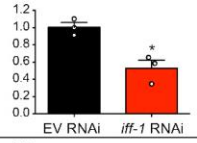
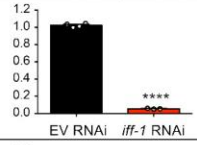
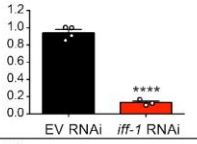
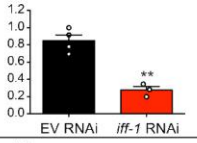
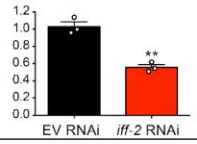
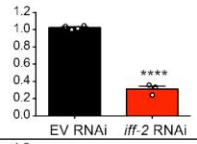
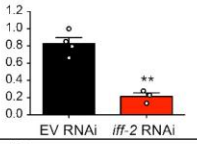
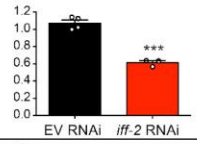
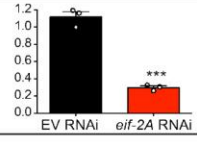
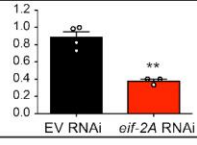
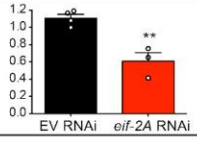
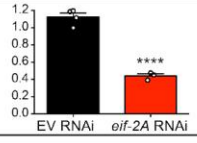
Total RNA was isolated from ~2,000 synchronized day 5-adult worms using RNAbee (Tel-Test Inc.). cDNA was generated using qScript Flex cDNA

synthesis kit (Quantabio). SybrGreen real-time qPCR experiments were performed with a 1:20 dilution of cDNA using a CFC384 Real-Time System (Bio-Rad) following the manufacturer's instructions. Data were analysed with the comparative $2\Delta\Delta C_t$ method using the geometric mean of *cdc-42* and *pmp-3* as housekeeping genes (Hoogewijs, Houthoofd, Matthijssens, Vandesompele, & Vanfleteren, 2008).

2.2.19: Knockdown levels at the indicated temperatures.

We performed qPCR analysis of day 5-adult wild-type worms to assess knockdown efficiency of the distinct RNAi used in this study. For each RNAi, we tested the temperatures at which the RNAi was used in the manuscript. Graphs represent the relative expression to Empty Vector (EV) RNAi control (mean ± s.e.m. (n= 3-4)). All the statistical comparisons were made by Student's t-test for unpaired samples. P-value: *(P<0.05), **(P<0.01), ***(P<0.001), ***(P<0.0001).



Relative mRNA levels	10 °C	15 °C	20 °C	25 °C
mog-5				
mog-4				
mog-1				
pgl-1				
pgl-3				
glh-1				
glh-4				
iff-1				
iff-2				
eif-2A				

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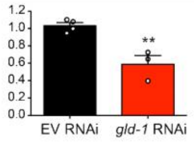
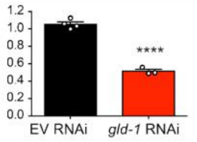
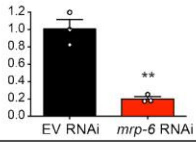
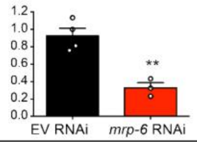
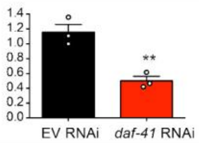
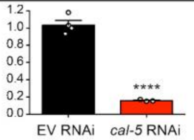
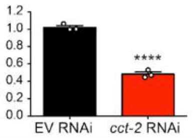
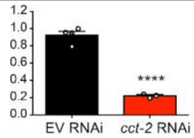
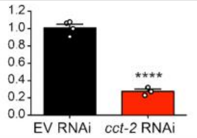
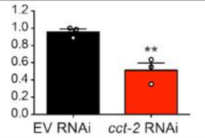
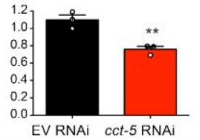
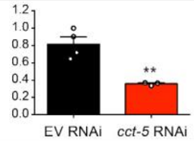
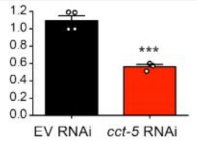
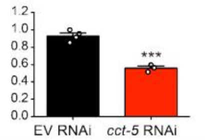
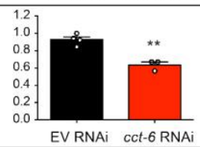
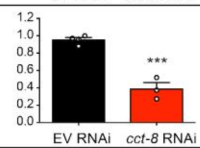
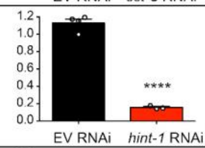
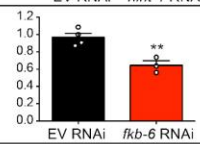
gld-1				
mrp-6				
daf-41				
cal-5				
cct-2				
cct-5				
cct-6				
cct-8				
hint-1				
fkb-6				

3: RESULTS

3.1: The germline is required for longevity at cold temperatures

In *C. elegans,* lowering the body temperature leads to a dramatic lifespan extension. With this observation we asked if germ cells have any role in this phenotype. For this, we first examined *glp-1(e2141)* worm strain. These mutant worms develop into adulthood without having a germline because at restrictive temperature (25°C) the proliferation of germ cells is ceased during developmental stage (Priess, Schnabel, & Schnabel, 1987). Thus, we raised *glp-1(e2141)* mutant larvae at 25°C to obtain adult worms lacking a germline and then shifted them to the distinct temperatures. As broadly reported (Arantes-Oliveira, Apfeld, Dillin, & Kenyon, 2002; Berman & Kenyon, 2006), *glp-1* germline-lacking worms are long-lived at 20°C compared with wild-type germline-possessing animals (**Fig. 1a)**. Strikingly, we discovered that at lower temperatures (*i.e.*, 15°C, 10°C) *glp-1* mutants lived significantly shorter when compared with wild-type worms (**Fig. 1a)**. Our results show that the pro-longevity effects of lower temperatures decline in *glp-1* germline-lacking mutants (**Fig. 1b)**, but the fact that these mutants are long-lived at 20°C makes it hard to interpret these observations.

To further check that cold-induced longevity demands the existence of germline, we used *glp-4(bn2)* mutant worms, another germline-lacking strain that has no lifespan extension relative to wild-type worms at 20°C (Curran, Wu, Riedel, & Ruvkun, 2009; Syntichaki, Troulinaki, & Tavernarakis, 2007; TeKippe & Aballay, 2010) (**Fig. 1c**). At low temperature (15°C) *glp-4* germline-lacking worms did not show a cold-induced longevity phenotype, resembling *glp-1* mutants (**Fig. 1b, c**). To assess that this phenotype is not a consequence of sterility per se, we examined a third mutant strain, namely *fer-15(b26/;fem-1(hc17)*, which is also sterile but still has an active proliferating germline when raised at the restrictive temperature (25°C) during development (**Fig. 2a, b**). Unlike the *glp-1* and *glp-4* mutants strains, we found that low temperature at adulthood increased lifespan of the *fer-15(b26/;fem-1(hc17)* strain and these sterile worms lived longer when compared with germline-lacking *glp-1* worms at low temperature (**Fig. 2c**). We also examined further sterile strains, which have spermatogenesis failure but still possess proliferating germline and produce oocytes, such as *fog-1*, *fog-2* and

spe-26 mutant strains and we observed that low temperature also extends lifespan of these mutant strains (Andux & Ellis, 2008; Barton & Kimble, 1990; Hubbard & Greenstein, 2005; Schedl & Kimble, 1988) (**Fig. 1d, e**). Collectively, our results show that the depletion of the germ cells and not sterility impairs the cold-induced longevity effect.

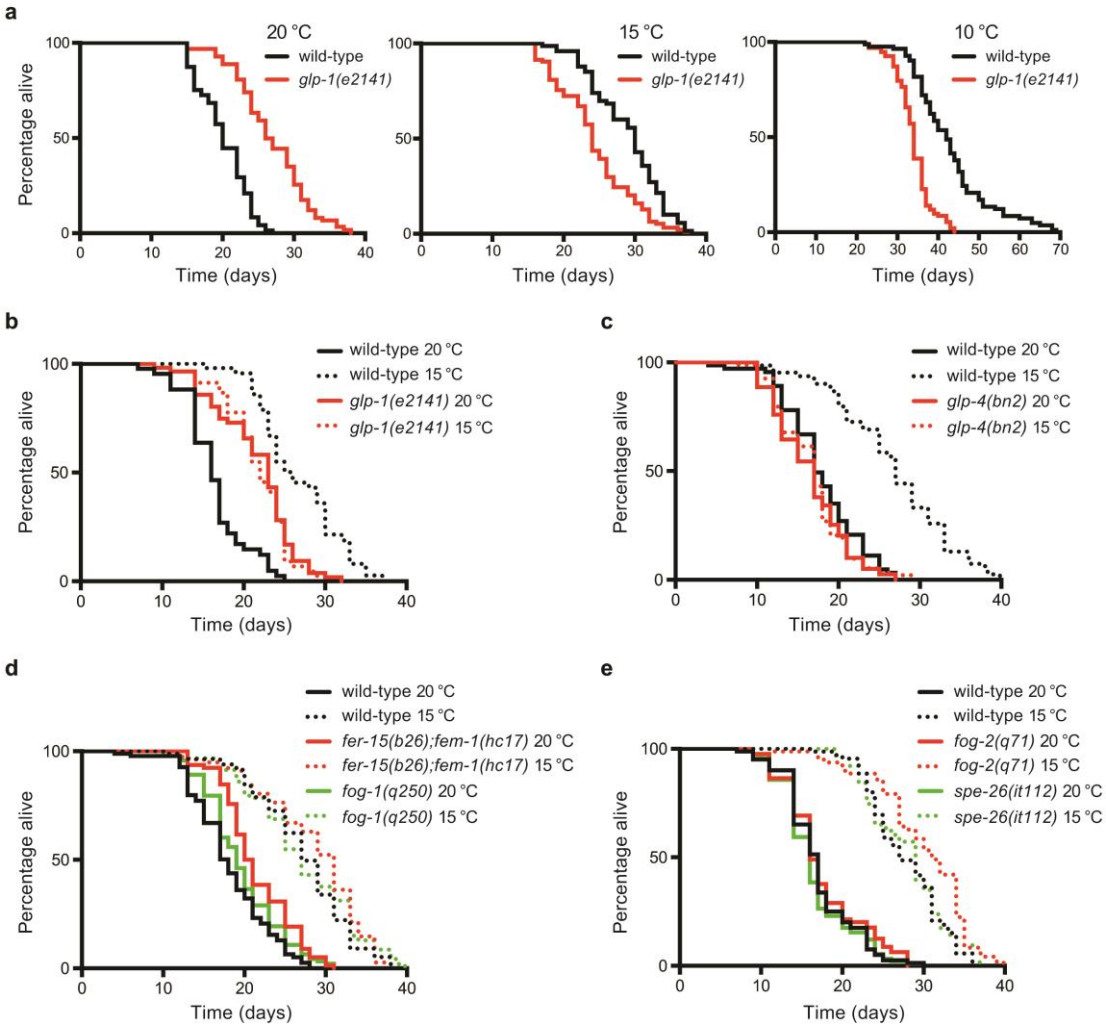


Figure 1. The germline is required for longevity at cold temperatures.

a, Germline-lacking worms (*glp-1(e2141)*) are long lived when compared with wild-type (N2) strain at 20°C (*N2* 20°C mean ± s.e.m: 20.15 ± 0.40, *glp-1* 20°C: 26.90 ± 0.58, P<0.0001). In contrast, germline-lacking worms are short lived at lower temperatures (15°C and 10°C) when compared with wild-type worms (*N2* 15°C: 29.01 ± 0.58, *glp-1* 15°C: 24.39 ± 0.56, P<0.0001; *N2* 10°C: 42.94 ± 1.05, *glp-1* 10°C: 33.95 ± 0.46, P<0.0001). **b,** Lower temperature (15°C) extends lifespan of wild-type worms (*N2* 20°C mean ± s.e.m: 16.40 ± 0.60, *N2* 15°C: 26.73 ± 0.77, P<0.0001) but not *glp-1* germline-lacking worms (*glp-1* 20°C: 21.59 ± 0.66, *glp-1* 15°C: 21.78 ± 0.60, P= 0.6396). Wild-type worms are long lived compared with *glp-1* mutant worms at cold temperature (*N2* 15°C versus *glp-1* 15°C, P<0.0001). **c,** Lower temperature (15°C) extends lifespan of wild-type

worms (*N2* 20°C mean ± s.e.m: 17.83 ± 0.58, *N2* 15°C: 26.81 ± 0.90, P<0.0001) but not *glp-4(bn2)* germline-lacking worms (*glp-4* 20°C: 16.44 ± 0.49, *glp-4* 15°C: 16.71 ± 0.45, P= 0.7379). Wild-type worms are long lived compared with *glp-4* mutant worms at cold temperature (*N2* 15°C versus *glp-4* 15°C, P<0.0001). **d,** Temperature reduction (15°C) extends lifespan of sterile *fer-15(b26);fem-1(hc17)* and *fog-1(q250)* mutant worms with a proliferating germline. *N2* 20°C mean ± s.e.m: 18.17 ± 0.53 versus *N2* 15°C: 27.20 ± 0.67, P<0.0001; *fer-15;fem-1* 20°C: 21.42 ± 0.50 versus *fer-15;fem-1* 15°C: 28.46 ± 0.74, P<0.0001; *fog-1* 20°C: 19.62 ± 0.49 versus *fog-1* 15°C: 27.22 ± 0.70, P<0.0001. **e,** Temperature reduction (15°C) extends lifespan of sterile *fog-2(q71)* and *spe-26(it112)* mutant worms with a proliferating germline. *N2* 20°C mean ± s.e.m: 17.01 ± 0.48 versus *N2* 15°C: 27.79 ± 0.60, P<0.0001; *fog-2* 20°C: 16.55 ± 0.46 versus *fog-2* 15°C: 27.94 ± 0.62, P<0.0001; *spe-26* 20°C: 17.51 ± 0.54 versus *spe-26* 15°C: 29.96 ± 0.67, P<0.0001. P-values: log-rank test, n= 96 worms/condition.

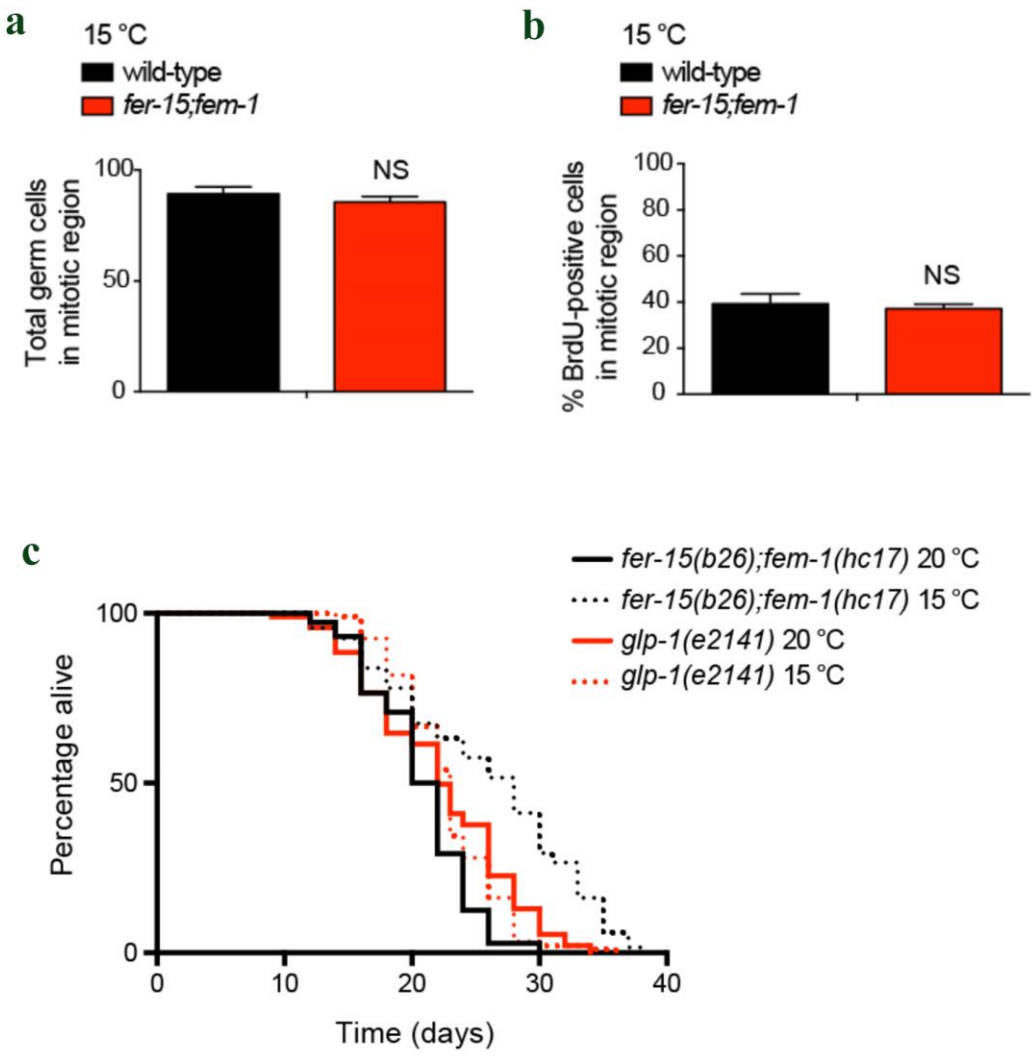


Figure 2. The total number of germ cells and percentage of proliferating cells within the mitotic region are not affected in fer-15;fem-1 sterile mutants. Low temperature during adulthood increases lifespan of control sterile fer-15;fem-1 mutants whereas it does not extend lifespan of germline-lacking worms.

a, Graph represents the total number of germ cells in the mitotic region quantified by DAPI staining (mean ± s.e.m., 16 germlines scored per condition from 2 independent experiments). **b,** Graph represents the percentage of BrdU-positive cells/total nuclei within the mitotic region (mean ± s.e.m., 16 germlines scored per condition from 2 independent experiments). **c,** Temperature reduction (15°C) extends lifespan of control sterile *fer-15;fem-1* mutants with proliferating germline (*fer-15;fem-1* 20°C mean ± s.e.m: 20.44 ± 0.44, *fer-15;fem-1* 15°C: 26.90 ± 0.89, P<0.0001) but not *glp-1* germline-lacking worms (*glp-1* 20°C: 22.23 ± 0.60, *glp-1* 15°C: 22.73 ± 0.42, P= 0.6075). Sterile control worms are long lived compared with *glp-1* mutant worms at cold temperature (*fer-15;fem-1* 15°C versus *glp-1* 15°C, P<0.0001). Statistical comparisons were made by Student's t-test for unpaired samples. NS= not significant.

3.2: Cold temperature extends proliferation of GSCs and delays reproductive aging

In the previous experiments, we raised all worm strains at the same temperature (25°C) until adulthood and then transferred them to the distinct temperatures. Thus our results show that the pro-longevity effect of germline at cold temperatures is particularly important during adulthood. In fact, transferring the wild-type *C. elegans* to lower temperature (15°C) only at adulthood was enough to extend the lifespan after development at higher temperatures (25°C or 20°C) (**Fig. 3a, b)** (B. Zhang et al., 2015). Accordingly, we hypothesized that cold-induced longevity is determined by temperature-associated changes in the adult germline. The germline of adult *C. elegans* includes a mitotic zone at the distal end followed by zones containing early mitotic stage cells to maturing gametes, extending proximally (Hubbard & Greenstein, 2005; J. Kimble & Crittenden, 2005) (**Fig. 4a**). During gametogenesis and cell death events, the adult germ cells are constantly lost, but still the proliferating germ stem cells (GSCs) at the mitotic region are able to self-renew and provide new cells that enter meiosis. These cells replenish the germline and produce gametes (Crittenden, Leonhard, Byrd, & Kimble, 2006). To check the effect of age and temperature in proliferation of adult germline, we grew wild-type larvae at 20°C until adulthood and then transferred them to different

temperatures. We did not notice a change at the total number of germ cells at the mitotic zone with age or temperature change (**Fig. 4b, c**). Instead, low temperature slowed down the age-associated decrease in the percentage of mitotic cells, which were labeled with bromodeoxyuridine (BrdU) (**Fig. 4b, d**). Additionally, we found that at cold temperature the proliferation of germ cells is active for an extended fraction of the animal's mean lifespan (**Fig. 4e**). At 25°C, the percentage of proliferating germ cells (BrdU-positive cells) was significantly reduced at day 5 to less than 10% whereas the organismal mean lifespan was 13.6 days (**Fig. 4d, e**). At 20°C, worms had a mean lifespan of 19.6 days with a strong decline in proliferating germ cells at day 10. However, at 15°C adult worms delayed the acute decline in proliferating germ cells until day 23, with their mean lifespan of 28 days. Similarly, at 10°C worms had still a high percentage of proliferating germ cells until day 37, close to their mean lifespan of 43 days (**Fig. 4d, e**). Therefore, these results indicate that cold temperatures extend the self-renewing ability of GSCs during aging, securing the pool of proliferating cells within the mitotic zone.

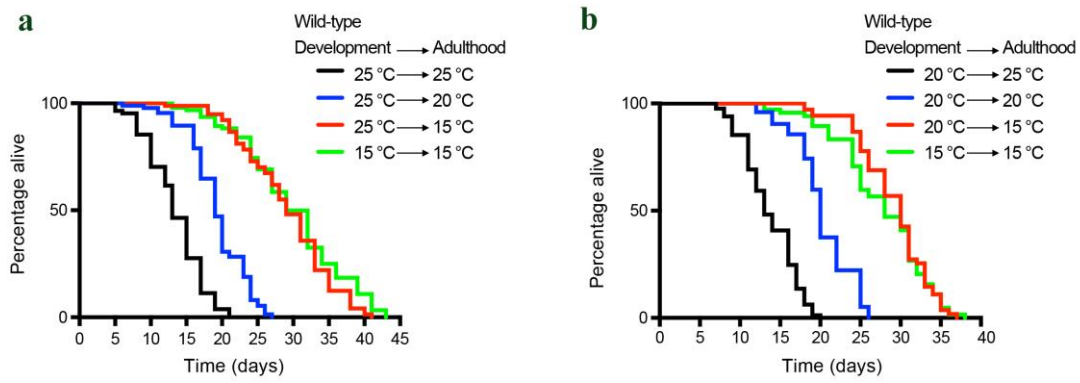


Figure 3. Low temperature during adulthood is sufficient to extend lifespan after development at 25°C or 20°C.

a, Wild-type larvae were raised at 25°C and then adult worms were shifted to the indicated temperatures. Exposure to low temperature (15°C) during adulthood extends longevity (log rank, P<0.0001, n= 96 worms/condition). 25°C (development and adulthood) mean ± s.e.m: 13.38 ± 0.43, shifted to 20°C after development: 19.28 ± 0.44, shifted to 15°C after development: 29.07 ± 0.72, 15°C (development and adulthood): 29.89 ± 0.76. **b.** After development at 20°C, adult wild-type worms were shifted to the indicated temperatures. Exposure to low temperature (15°C) during adulthood is sufficient to extend longevity (log rank, P<0.0001, n= 96 worms/condition). 20°C

(development and adulthood) mean ± s.e.m: 20.25 ± 0.44, shifted to 25°C after development: 13.66 ± 0.37, shifted to 15°C after development: 29.11 ± 0.53, 15°C (development and adulthood): 26.65 ± 0.70.

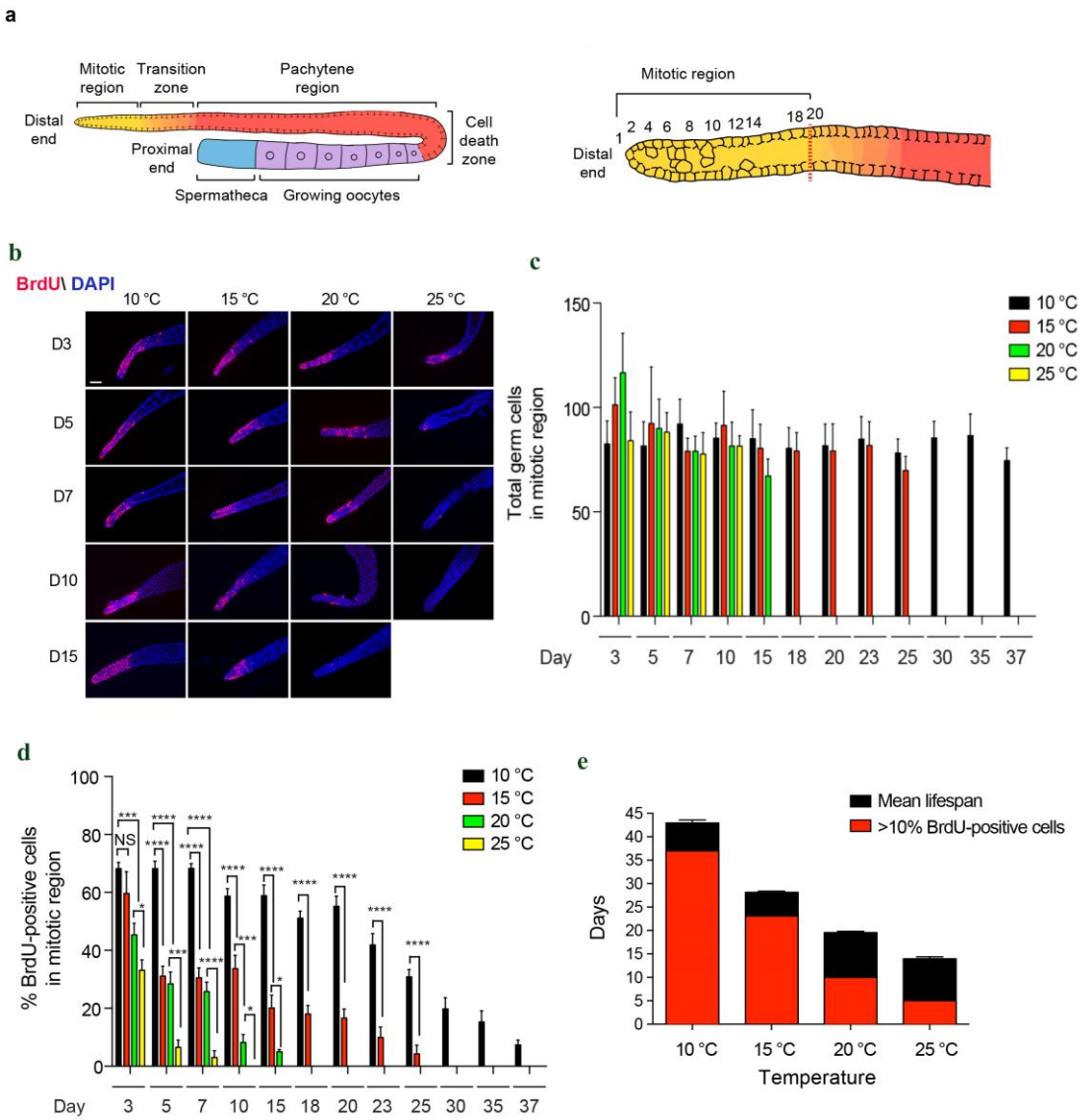


Figure 4. Cold temperature delays exhaustion of GSCs and reproductive aging. The total number of germ cells within the mitotic region did not change with age or temperature.

a, The left panel shows a schematic representation of a young adult hermaphrodite germline (one of two gonad arms is shown). Germ cells are derived from a proliferating GSC population located at the mitotic region. Proliferating germ cells enter meiosis and move proximally from the mitotic region to the meiotic zones (transition zone, pachytene). GSCs generate sperm during larval stages, and then switch to oocyte production during adulthood. Germ cell apoptosis occurs essentially in the germline

loop region (death cell zone), as developing oocytes exit the pachytene region. Right, the mitotic region is represented. Red dashed line corresponds to the point at which multiple germ cells exhibit an early meiotic phenotype, considered the boundary between the mitotic region and the transition zone (row 20 of cells from the most distal cell). **b,** Bromodeoxyuridine (BrdU) staining of proliferating germ cells. Wild-type *C. elegans* were shifted to the distinct temperatures during adulthood and the germlines were extruded after 2 h BrdU treatment at the indicated day of age (e.g., Day 3= D3). Cell nuclei were stained with DAPI. Scale bar represents 20 μm. The images are representative of 4 independent experiments. **c,** Graph represents the total number of germ cells in the mitotic region quantified by DAPI staining (mean ± s.e.m., 15-20 germlines scored per condition from 3 independent experiments). We did not examine the germline after day 15 at 25°C, day 18 at 20°C, day 25 at 15°C and day 37 at 10°C as the percentage of BrdU-positive cells was below 10% at the indicated ages/temperatures. Wild-type larvae were raised at 20°C until adulthood and then shifted to the indicated temperatures. Statistical comparisons were made by Student's t-test for unpaired samples. No significant differences were found. **d,** Graph represents the percentage of BrdU-positive cells/total nuclei within the mitotic region (mean ± s.e.m., 15-20 germlines scored per condition from 3 independent experiments). We did not examine the germline after day 15 at 25°C, day 18 at 20°C, day 25 at 15°C and day 37 at 10°C as the percentage of BrdU-positive cells was below 10% at the indicated ages/temperatures. **e,** Graph represents the average ± s.e.m. of the mean lifespan at 10°C (n= 10 independent experiments), 15°C (n= 30), 20°C (n= 19) and 25°C (n= 14). The graph also indicates until what day of the respective mean lifespan is germ cell proliferation maintained at each temperature (>10% BrdU-positive cells in the mitotic region).

Besides sustaining the pool of proliferating cells, GSCs also generate differentiating gametes. More than half of the developing oocytes undergo apoptosis and the rest of the cells progress to terminal differentiation and fertilization; thus GSCs are also essential to replenish the pool of oocytes (Jaramillo-Lambert, Ellefson, Villeneuve, & Engebrecht, 2007; Morita & Tilly, 1999). The first germ cells during development differentiate into spermatocytes, producing in a limited number of sperms (240-320 sperms), and the following germ cells generate oocytes, which can be then self-fertilized and ovulated in hermaphrodite worms (Andux & Ellis, 2008; L'Hernault, 2006) (**Fig. 4a**). At standard temperature, the first 4-5 days of adulthood is the peak of reproduction in hermaphrodite *C. elegans* worms and then the supply of self-sperm exhaust during the first 4-5 days of adulthood (Hodgkin & Barnes, 1991). Likewise, self-reproduction of worms at 15°C takes place until day 5 (**Fig. 5a**). At 10°C although worms extended their self-reproductive period until day 8 of adulthood (**Fig.**

5a), but the total number of eggs laid by hermaphrodites was not increased at cold temperatures (**5b**). Most of these eggs hatched in all the temperatures tested (**Fig. 5c**). However, self-reproduction period is not enough to assess total reproductive capacity of worms because self-progeny brood sizes are limited by the number of self-sperm (Hodgkin & Barnes, 1991). To avoid this limitation, we mated day 8 adult hermaphrodites at the distinct temperatures with young males for 48 h to replenish the pool of sperm. At cold temperatures (10°C and 15°C), worms extended the reproduction (egg laying) for the following 9 and 5 days after mating, respectively (**Fig. 6a**). In contrast, worms at 20°C laid eggs only within the first 2 days after meeting external sperms and at 25°C we did not observe any further eggs. Notably, the total number of eggs laid at low temperatures (10°C and 15°C) was significantly more when compared with 20°C (**Fig. 6b**). Most of these eggs were hatched into viable larvae (**Fig. 6c**). All together, these results indicate that cold temperature sustains the pool of GSC during adulthood, leading to an improved maintenance of the proliferating germline region and reproductive capacity with age.

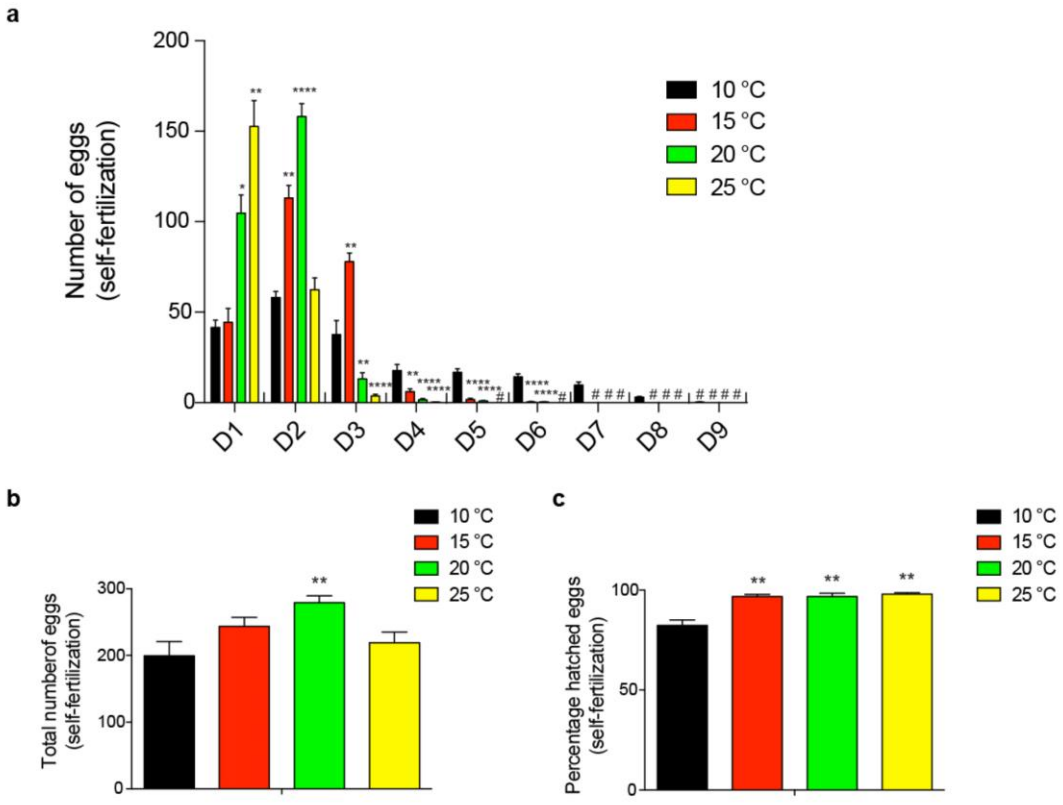


Figure 5. Exposure to different temperatures during adulthood does not have strong effects on self-fertility.

a, Number of eggs laid per wild-type hermaphrodite every 24 h during the self-reproductive period at different temperatures (mean ± s.e.m., n= 63 worms scored per condition from 3 independent experiments). Worms were exposed to the indicated temperatures during adulthood. (#) no eggs were laid. **b,** Total number of eggs laid at different temperatures during the self-fertilization period (mean ± s.e.m., n= 63 worms scored per condition from 3 independent experiments). **c,** Percentage of hatched eggs at different temperatures (mean ± s.e.m., n= 4 independent experiments). Almost all the eggs produced by hermaphrodites at 15°C, 20°C and 25°C were viable (96-98%). Despite a slight but significant decrease at 10°C, most of the eggs were also viable (82%) at this temperature. Statistical comparisons were made by Student's t-test for unpaired samples. P-value: *(P<0.05), **(P<0.01), ****(P<0.0001).

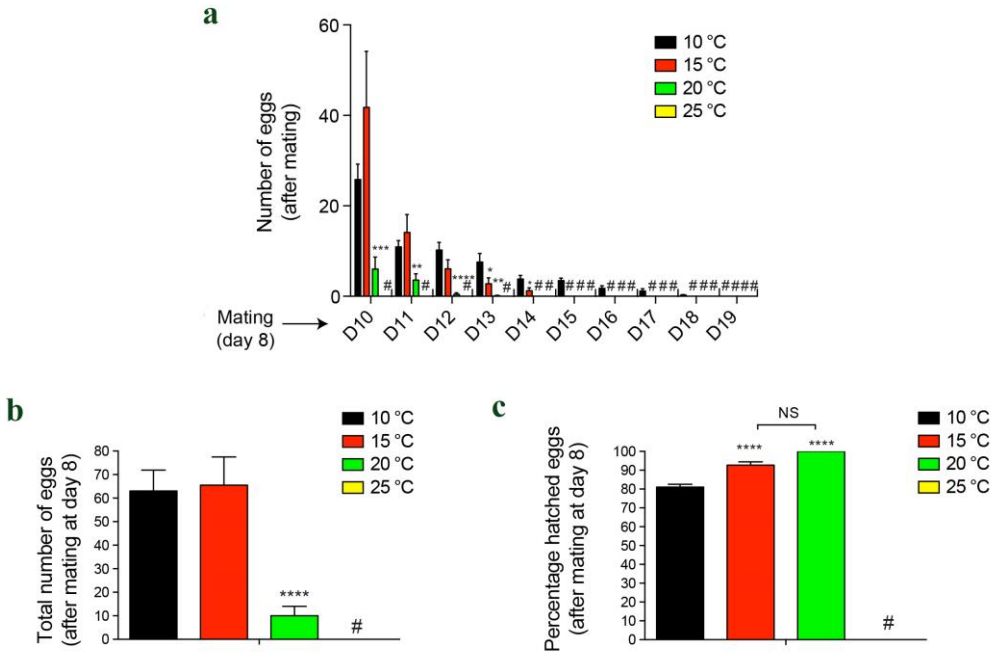


Figure 6. Cold temperature sustains the pool of GSC during adulthood, leading to an improved maintenance of the proliferating germline region and reproductive capacity with age.

a, Adult worms were cultured at the indicated temperature during adulthood. After the self-reproductive period, worms were mated at day 8 of adulthood with young males raised at 20°C and kept at the respective temperatures. Graph represents the number of eggs laid per worm every 24 hours after mating (mean ± s.e.m., n= 24 worms scored per condition from 3 independent experiments). (#) no eggs were laid. **b,** Total number of eggs laid per worm at different temperatures after mating at day 8 of adulthood (mean ± s.e.m., n= 24 worms scored per condition from 3 independent experiments). (#) no eggs were laid by mated worms at 25°C. **c,** Percentage of hatched eggs at different

temperatures (mean ± s.e.m., n= 24 worms scored per condition from 3 independent experiments). The percentage of viable eggs after mating is similar at 15°C and 20°C. Although egg hatching decreases at 10°C, most of the eggs (81%) were still viable. (#) no eggs were laid. Statistical comparisons were made by Student's t-test for unpaired samples. P-value: *(P<0.05), **(P<0.01), ***(P<0.001), ****(P<0.0001). NS= not significant.

To determine if maintenance of GSCs correlates with the pro-longevity effect of cold temperatures, we transferred worms from higher temperature to lower temperature at different adult stages. Remarkably, when the worms at 20°C were transferred to lower temperature at day 10 of adulthood, cold-induced longevity was dramatically decreased (**Fig. 7a**), correlating with the acute decline in germline proliferation at 20°C (i.e., <10% BrdU-positive cells). Likewise, worms showed a diminished ability to live longer under cold temperature once they experienced 25°C until day 5 of adulthood (**Fig. 7b**), correlating with the demise of germline proliferation at this temperature (**Fig. 4d**). Despite a reduction in the length of cold-induced longevity, worms possessing low percentage of proliferating germ cells could still exhibit a partial lifespan extension when shifted to cold temperature (**Fig. 7a, b**). In these lines, we found that shifting the worms to cold temperature after an acute decrease in germline proliferation partially recovers this phenotype (**Fig. 7c**).

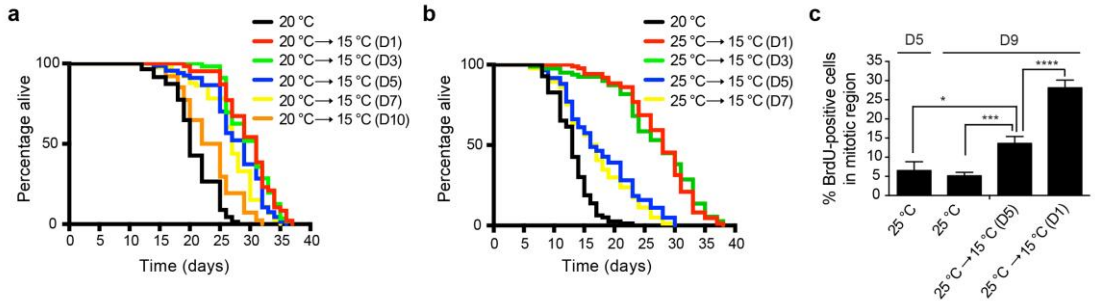


Figure 7. Maintenance of GSCs correlates with the pro-longevity effect of cold temperatures.

a, Lifespan analysis of worms at 20°C shifted to lower temperature (15°C) at the indicated days (D). 20°C mean ± s.e.m: 20.72 ± 0.43, shifted to 15°C at D1: 29.85 ± 0.52 (P<0.0001), shifted to 15°C at D3: 29.86 ± 0.49 (P<0.0001), shifted to 15°C at D5: 27.75 ± 0.58 (P<0.0001), shifted to 15°C at D7: 26.92 ± 0.53 (P<0.0001), shifted to 15°C at D10: 23.26 ± 0.48 (P<0.0001). Cold-induced longevity is decreased when worms are shifted to lower temperature at day 10 (shifted to 15°C at D10 versus shifted to 15°C at

D1-7, P<0.0001). **b,** Lifespan analysis of worms at 25°C shifted to 15°C at the indicated days (D). 25°C mean ± s.e.m: 13.09 ± 0.35, shifted to 15°C at D1: 27.43 ± 0.60 (P<0.0001), shifted to 15°C at D3: 26.90 ± 0.80 (P<0.0001), shifted to 15°C at D5: 17.74 ± 0.68 (P<0.0001), shifted to 15°C at D7: 16.98 ± 0.62 (P<0.0001). Cold-induced longevity is decreased when worms are shifted to lower temperature at day 5 (shifted to 15°C at D10 versus shifted to 15°C at D1-3, P<0.0001). Worms shifted to 15°C at either day 5 or day 7 exhibit a similar lifespan (P<0.2661). **c,** Graph represents the percentage of BrdU-positive cells/total nuclei within the mitotic region (mean ± s.e.m., 16-20 germlines scored per condition from 2 independent experiments). Worms were maintained at 25°C until BrdU analysis (day 5 and 9) or shifted to 15°C at the indicated days before BrdU analysis at day 9 of adulthood.

Somatic tissues such as thermosensory neurons perceive the changes in temperature (Garrity, Goodman, Samuel, & Sengupta, 2010; Glauser et al., 2011; Xiao et al., 2013; B. Zhang et al., 2018), thus we asked whether these cells modulate germline maintenance at cold temperature. To extend the lifespan, cold-sensitive channel TRPA-1 detects temperature decline in intestine and neurons. Loss of function of *trpa-1* reduces the length of cold-induced longevity (Xiao et al., 2013; B. Zhang et al., 2015) (**Fig. 8a**). Lack of *trpa-1* at early adulthood stages, did not affect proliferation of germline at cold temperature (**Fig. 8b, c**). However, at older age germline proliferation dramatically diminished in *trpa-1*-lacking mutants when compared with wild-type worms, whereas the total number of germ cells within the mitotic region remained similar (**Fig. 8b, c**). Previous studies show that cold-sensitive IL1 neurons express *trpa-1* (Kindt et al., 2007) and these neurons are required for the longevity phenotype induced by low temperature (B. Zhang et al., 2018). Since transgenic expression of *trpa-1* in IL1 neurons alone is sufficient to rescue the short lifespan of *trpa-1*-lacking mutants at cold temperatures (B. Zhang et al., 2018) (**Fig. 8a**), we tested whether it also ameliorates the decline in germline proliferation. Indeed, ectopic expression of *trpa-1* in IL1 neurons rescued the diminished germline proliferation of *trpa-1* mutants at cold temperature (**Fig. 8b, c**). Since loss of *trpa-1* does not completely blocked the pro-longevity effect of cold temperatures (Xiao et al., 2013), we hypothesized that other thermosensory neurons might also contribute to this phenotype. Among these other thermosensory neurons are a pair of thermoreceptors neurons, called AFD, that are required for temperature-related behaviors (Garrity et al., 2010) but

they do not express TRPA-1 channel (Kindt et al., 2007). To check the potential role of AFD neurons at modulation of cold-induced longevity, we used worms expressing reconstituted caspase-3 under AFD-specific promoter to ablate these pair of neurons (Glauser et al., 2011). As with *trpa-1* mutants, we grew AFD-ablated worms at 20°C during development and then transferred them to distinct temperatures at adulthood to assess lifespan. Notably, we found that AFD-ablated transgenic worms lived significantly shorter at cold temperature when compared with wild-type animals (**Fig. 9a, b**). Conversely, ablation of AFD neurons at 20°C and 25°C did not affect lifespan (**9c, d**). To further examine the role of AFD neurons in cold-induced longevity, we checked two distinct strains with mutations in the *ttx-1* gene that specifically disrupt AFD neurons structure and function (Perkins, Hedgecock, Thomson, & Culotti, 1986; Satterlee et al., 2001). Both *ttx-1* mutant strains showed short lifespan when lived at cold temperatures (10°C or 15°C) during adulthood (**Fig. 9e, f, g, h**). On the contrary, the *ttx-1* mutants showed no significant lifespan change relative to wild type worms at either 20°C or 25°C (**Fig. 10a-d**). In both AFD-ablated and *ttx-1* mutant worms, we observed a decline in germline proliferation at cold temperature, especially at older age (**Fig. 10e, f**). Altogether, these results indicate that low temperature detection by thermosensory neurons such as IL1 and AFD neurons delays GSC exhaustion.

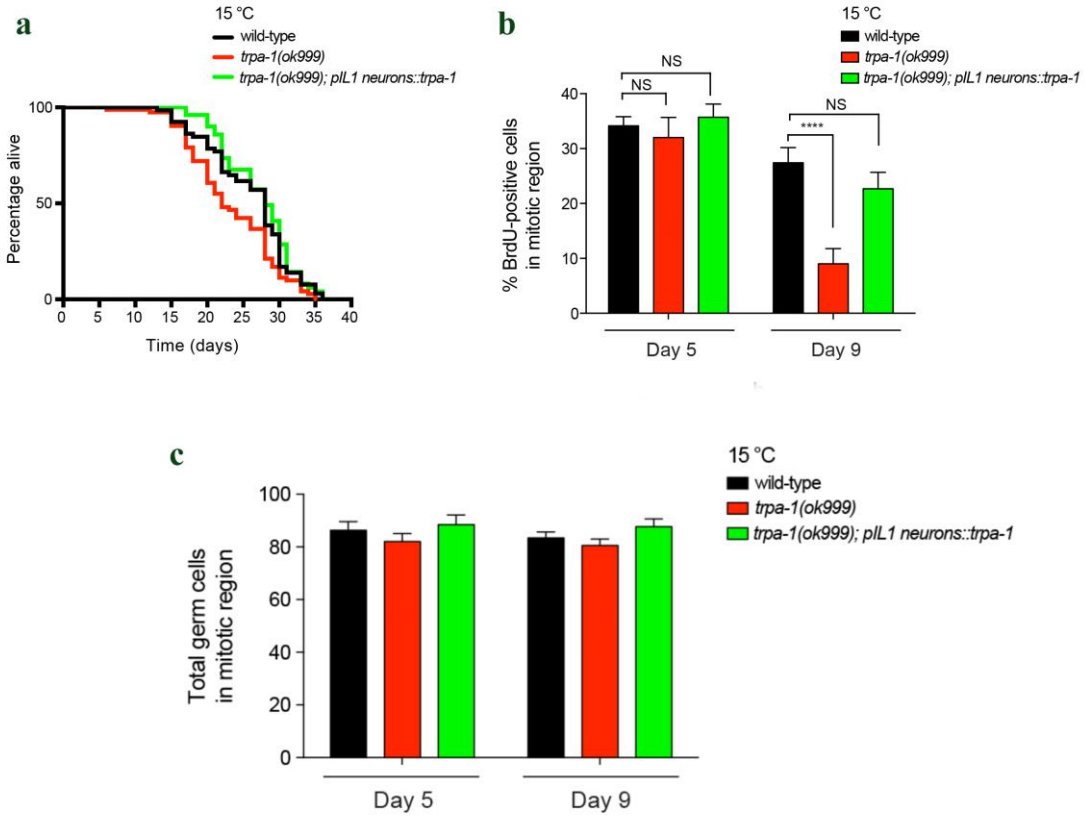


Figure 8. Thermosensory neurons regulate cold-induced longevity and GSC proliferation. Loss of trpa-1 channel does not affect the total number of germ cells within the mitotic region.

a, Ectopic expression of *trpa-1* in IL1 neurons driven by *aqp-6* promoter is sufficient to rescue the short-lived phenotype of *trpa-1* mutants at 15°C. *N2* wild-type mean ± s.e.m: 25.97 ± 0.73, *trpa-1(ok999)*: 23.34 ± 0.72 (P=0.0127), *trpa-1(ok999);pIL1::trpa-1*: 27.70 ± 0.70 (P=0.4163). **b,** Ectopic expression of *trpa-1* in IL1 neurons is sufficient rescues the low germline proliferation phenotype of *trpa-1* mutant worms at 15°C. Graph represents the percentage of BrdU-positive cells/total nuclei in the indicated strains at day 5 and 9 of adulthood (mean ± s.e.m., 15 germlines scored per condition from 2 independent experiments). **c.** Graph represents the total number of germ cells in the mitotic region quantified by DAPI staining in the indicated strains at day 5 and 9 of adulthood (mean ± s.e.m., 15 germlines scored per condition from 2 independent experiments). Statistical comparisons were made by Student's t-test for unpaired samples. No significant differences were found.

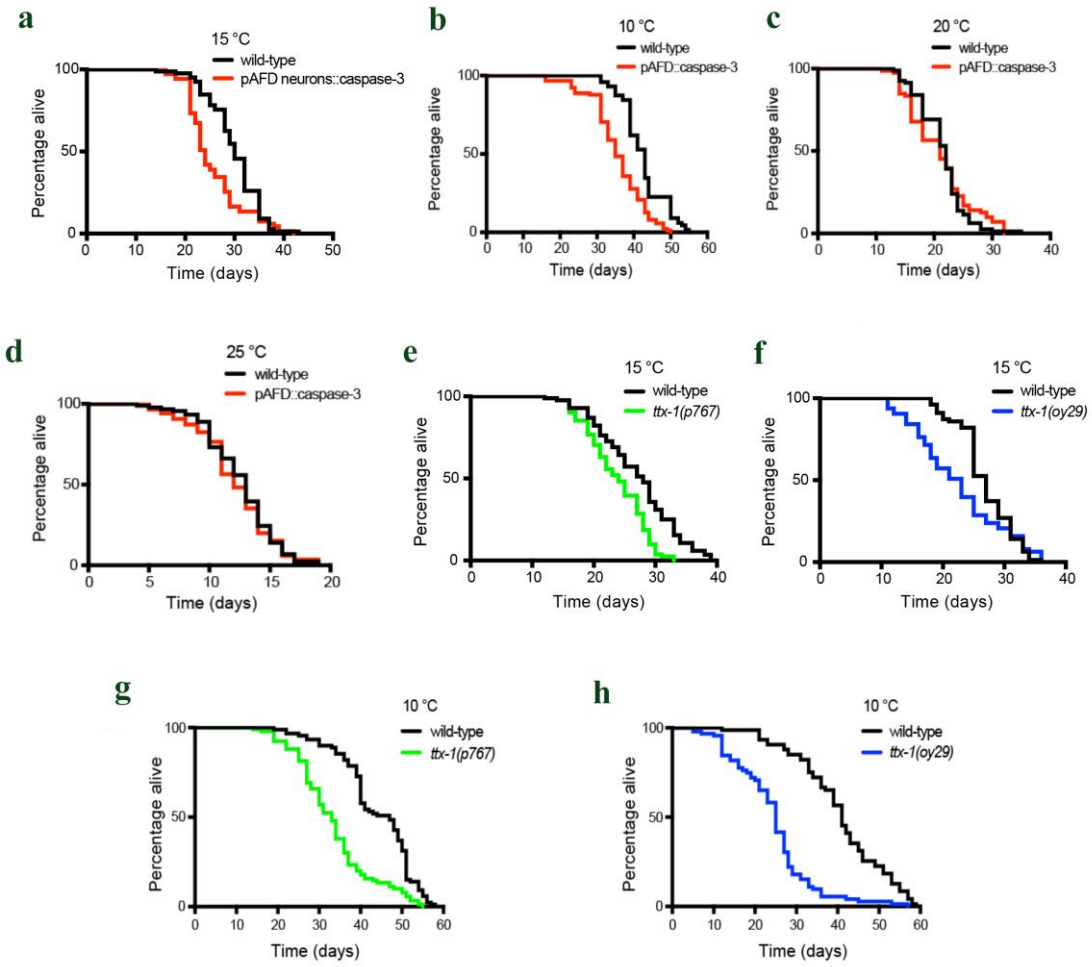


Figure 9. Ablation of AFD neurons diminishes cold-induced longevity. ttx-1 mutant worms are short-lived at cold-temperature.

a, AFD ablation by driving reconstituted caspase-3 under the control of the AFD-specific *gcy-8* promoter reduces cold-induced longevity at 15°C (*N2* wild-type mean ± s.e.m: 29.88 ± 0.58, pAFD::caspase-3: 25.61 ± 0.69, P= 0.0002). **b,** AFD-ablated transgenic worms live shorter when compared with wild-type strain at 10°C (N2 wild-type mean ± s.e.m: 42.51 ± 0.70, pAFD::caspase-3: 35.55 ± 0.77, P< 0.0001). **c,** AFD-ablated transgenic worms do not live shorter when compared with wild-type strain at 20°C (N2: 21.24 ± 0.45, pAFD::caspase-3: 20.85 ± 0.64, P= 0.6869). **d,** Lifespan of AFD-ablated transgenic worms compared with wild-type strain at 25°C (N2: 12.57 ± 0.30, pAFD::caspase-3: 12.16 ± 0.34, P= 0.6240). P-values: log-rank test, n= 96 worms/condition. **e,** *ttx-1(p767)* mutant worms are short-lived at 15°C (*N2* mean ± s.e.m: 27.05 ± 0.69, *ttx-1(p767)*: 23.64 ± 0.54, P< 0.0001). **f,** *ttx-1(oy29)* mutant worms are short-lived at 15°C (*N2* mean ± s.e.m: 26.81 ± 0.50, *ttx-1(oy29)*: 22.49 ± 0.91, P= 0.0267). **g,** *ttx-1(p767)* mutant worms live shorter when compared with wild-type strain at 10°C (N2 wild-type mean ± s.e.m: 42.51 ± 0.70, *ttx-1(p767)*: 35.55 ± 0.77, P< 0.0001). **h,** *ttx-1(oy29)* mutant worms live shorter when compared with wild-type strain at 10°C (N2 wild-type mean ± s.e.m: 40.71 ± 1.23, *ttx-1(oy29)*: 24.35 ± 1.10, P< 0.0001).

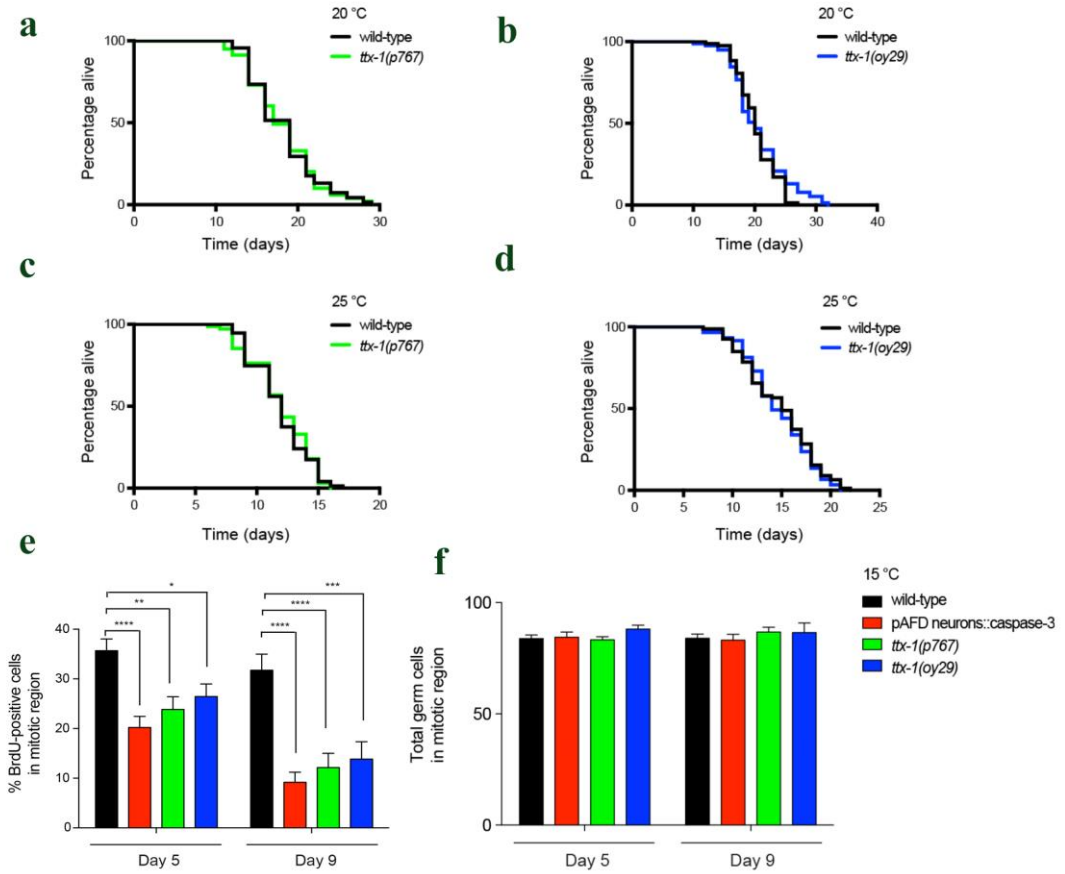


Figure 10. ttx-1 mutant worms are not short-lived at standard and warm temperatures. In both AFD-ablated and ttx-1 mutant worms germline proliferation declines at cold temperature but total number of germ cells within the mitotic region remains unchanged.

a, Lifespan of *ttx-1(p767)* worms compared with wild-type strain at 20°C (N2 mean ± s.e.m: 18.21 ± 0.49, *ttx-1(p767)*: 18.11 ± 0.56, P= 0.9486). **b,** Lifespan of *ttx-1(oy29)* worms compared with wild-type strain at 20°C (N2 mean ± s.e.m: 20.24 ± 0.36, *ttx-1(oy29)*: 20.60 ± 0.51, P= 0.2874). **c,** Lifespan of *ttx-1(p767)* worms compared with wild-type strain at 25°C (N2 mean ± s.e.m: 11.84 ± 0.26, *ttx-1(p767)*: 12.00 ± 0.32, P= 0.8799). **d,** Lifespan of *ttx-1(oy29)* worms compared with wild-type strain at 25°C (N2 mean ± s.e.m: 14.80 ± 0.41, *ttx-1(oy29)*: 14.65 ± 0.44, P= 0.3118). P-values: log-rank test, n= 96 worms/condition. **e,** Graph represents the percentage of BrdU-positive cells/total nuclei in the indicated strains at day 5 and 9 of adulthood (mean ± s.e.m., 15-20 germlines scored per condition from 3 independent experiments). In lifespan experiments, P-values: log-rank test, n= 96 worms/condition. **f.** Graph represents the total number of germ cells in the mitotic region quantified by DAPI staining in the indicated strains at day 5 and 9 of adulthood (mean ± s.e.m., 15-20 germlines scored per condition from 3 independent experiments). Statistical comparisons were made by Student's t-test for unpaired samples. No significant differences were found. In Figure **e**, statistical comparisons were made by Student's t-test for unpaired samples. P-value: *(P<0.05), **(P<0.01), ***(P<0.001), ****(P<0.0001). NS= not significant

3.3: Proliferation of adult Germ Stem Cells regulates cold-induced longevity

Our findings with germline-lacking mutant worms showed that the germline is essential for pro-longevity effect of cold temperature. Additionally, we found that during adulthood low temperature maintains robust proliferation of GSCs. Having these results, we asked whether adult GSCs have a direct role in cold-induced longevity. To address this, we grew the worms at 20°C until adulthood with a normal proliferative germline and then inhibited adult GSCs function by different means. Since the only pool of proliferating cells in adult worms is the germ cells within the mitotic region (Joshi, Riddle, Djabrayan, & Rothman, 2010; J. Kimble & Crittenden, 2005), treatment with 5-fluoro-2′deoxyuridine (FUdR), an inhibitor of DNA synthesis, particularly affects these cells in adult worms. Although FUdR treatment initiated during adulthood dramatically reduced proliferation of GSCs at either 20°C or 15°C (**Fig. 11a**), it blocked only the lifespan extension induced by cold temperature (15°C), and did not affect the lifespan at 20°C (**Fig. 11b**).

To further check the need for adult proliferating germ cells in cold-induced lifespan extension, we silenced distinct regulators of GSC proliferation. First, we knocked down *mog* family genes (*mog-1*, *mog-4* and *mog-5*). These genes encode DEAH-box RNA helicases required for robust proliferation of germ cells during germline development (Graham & Kimble, 1993; Puoti & Kimble, 1999, 2000). When we silenced these genes in young adults after normal development of the germline, we observed diminished proliferation of germ cells within the mitotic zone (**Fig. 11c-g**). Notably, knocking down the *mog* genes during adulthood decreased the lifespan extension induced by low temperature (15°C) (**Fig. 12a**) but it did not reduce lifespan at 20°C and 25°C (**Fig. 12b, c**). Apart from *mog* genes, other RNA-binding proteins such as PGL-1, PGL-3 and GLH-1 are also required for maintenance of germline proliferation. These proteins are components of P-granules, a germline-specific RNA/protein complex (I. Kawasaki et al., 2004; Kawasaki et al., 1998; Kuznicki et al., 2000a). However, the effect of these specific P-granule components on germline proliferation is sensitive to temperature and their individual loss can reduce proliferation of germ cells at high temperature (25°C), but not at lower temperatures (I.

Kawasaki et al., 2004; Kawasaki et al., 1998; Kuznicki et al., 2000a). Interestingly, knockdown of *pgl* genes slightly extends lifespan of wild-type worms at high temperature (Curran et al., 2009). In contrast to *mog* genes, we found that knockdown of *pgl-1* alone in adult worms did not reduce germline proliferation (**Fig. 11c-d**) and cold-induced longevity at 15°C (**Fig. 12d**). Similarly, knockdown of *glh* genes did not alter the lifespan extension induced by low temperature (**Fig. 12d**).

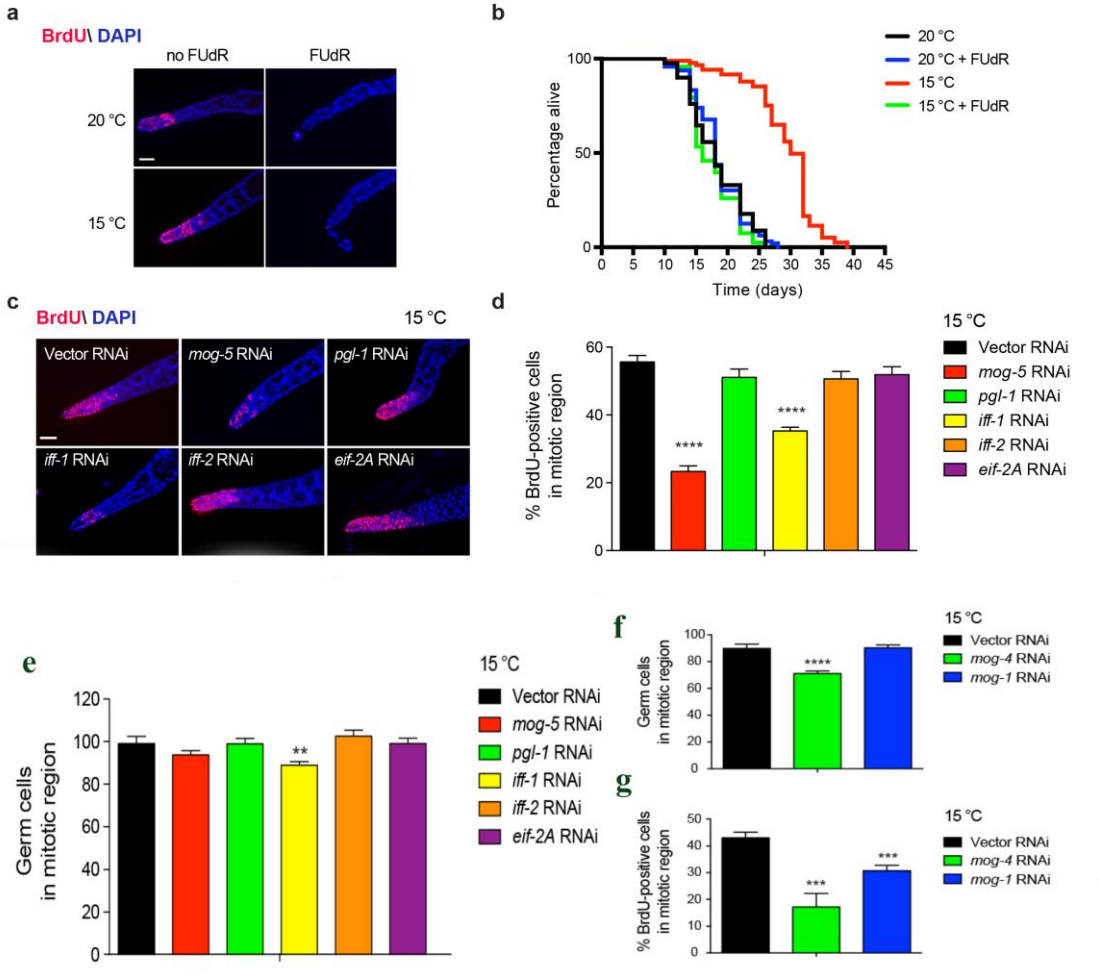


Figure 11. Robust proliferation of adult GSCs determines cold-induced longevity. Total number of germ cells within the mitotic region upon knockdown during adulthood of distinct regulators of GSC proliferation remains not affected but the proliferative germ cells within the mitotic region diminish upon downregulation of specific regulators of GSC.

a, BrdU staining of germline from wild-type *C. elegans* treated with 100 µg ml^{-1} FUdR only during adulthood at the indicated temperatures after development at 20°C. Worms were examined at day 5 of adulthood. Cell nuclei were stained with DAPI. Scale bar

represents 20 µm. The images are representative of 3 independent experiments. **b,** FUdR treatment during the entire adulthood period does not affect lifespan of wild-type worms at 20°C temperature (20°C mean ± s.e.m: 18.23 ± 0.49, 20°C + FUdR: 18.28 ± 0.40, P= 0.8617). However, FUdR treatment reduces lifespan extension induced by cold temperature (15°C: 29.08 ± 0.60, 15°C + FUdR: 17.49 ± 0.40, P<0.0001). **c,** BrdU staining of the germline from wild-type *C. elegans* fed with the indicated RNAi from day 1 of adulthood. Worms were examined at day 5 of adulthood. Cell nuclei were stained with DAPI. Scale bar represents 20 µm. The images are representative of 3 independent experiments. **d,** Graph represents the percentage of BrdU-positive cells/total nuclei (mean ± s.e.m., 33-36 germlines scored per condition from 3 independent experiments). **e,** Graph represents the total number of germ cells in the mitotic region quantified by DAPI staining (mean ± s.e.m., 33-36 germlines scored per condition from 3 independent experiments). Wild-type worms were examined at day 5 of adulthood. **f,** Graph represents the total number of germ cells in the mitotic region quantified by DAPI staining (mean ± s.e.m., 11 germlines scored per condition from 2 independent experiments). **g,** Graph represents the percentage of BrdU-positive cells/total nuclei (mean ± s.e.m., 11 germlines scored per condition from 2 independent experiments). Statistical comparisons were made by Student's t-test for unpaired samples. P-value: **(P<0.01), P-value: ***(P<0.001), **** (P<0.0001).

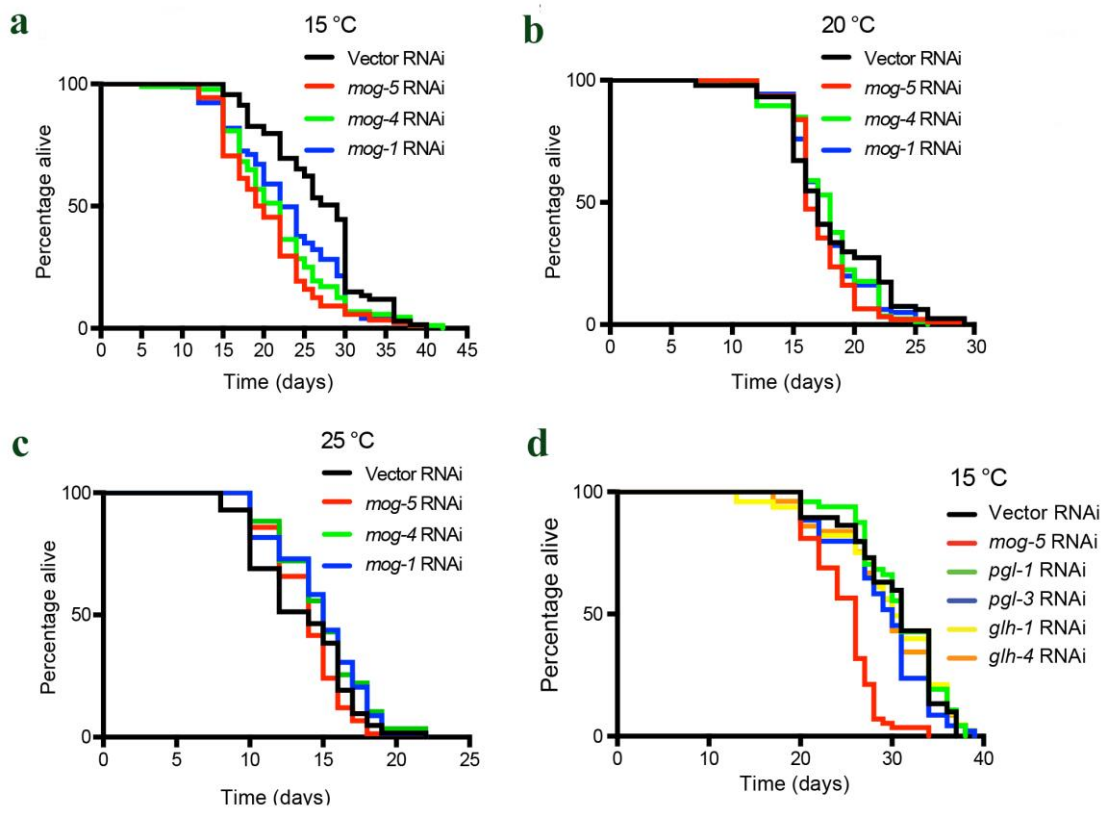


Figure 12. Knockdown of mog genes during adulthood decreased the lifespan extension induced by low temperature (15°C) but not at warm temperatures.

a, Knockdown of *mog* genes reduces the long lifespan induced by cold temperature (15°C) in N2 wild-type worms. All RNAi treatment was initiated at day 1 of adulthood.

Empty vector RNAi mean ± s.e.m: 26.60 ± 0.74, *mog-5* RNAi: 20.52 ± 0.63 (P<0.0001); *mog-4* RNAi: 21.83 ± 0.69 (P<0.0001); *mog-1* RNAi: 22.87 ± 0.76 (P= 0.0020). **b,** Knockdown of *mog* genes does not reduce lifespan of N2 wild-type worms at 20°C (RNAi was initiated at day 1 of adulthood). Empty vector RNAi mean ± s.e.m: 17.96 ± 0.46, *mog-5* RNAi: 17.13 ± 0.28 (P= 0.0653), *mog-4* RNAi: 17.68 ± 0.34 (P= 0.3608), *mog-1* RNAi: 17.58 ± 0.34 (P= 0.2567). **c,** Knockdown of *mog* genes does not reduce lifespan of N2 worms at 25°C (RNAi was initiated at day 1 of adulthood). Empty vector RNAi mean ± s.e.m: 13.50 ± 0.42, *mog-5* RNAi: 13.93 ± 0.27 (P= 0.7344), *mog-4* RNAi: 14.88 ± 0.27 (P= 0.0420), *mog-1* RNAi: 14.76 ± 0.36 (P= 0.0685). **d,** Knockdown of *pgl* and *glh* genes does not suppress the lifespan extension induced by cold temperature (15°C) in N2 worms. RNAi was initiated at day 1 of adulthood. Empty vector RNAi mean ± s.e.m: 30.35 ± 0.85, *mog-5* RNAi: 24.92 ± 0.44 (P<0.0001), *pgl-1* RNAi: 30.96 ± 0.65 (P= 0.6424), *pgl-3* RNAi: 28.95 ± 0.72 (P= 0.2590), *glh-1* RNAi: 29.64 ± 0.94 (P= 0.9853), *glh-4* RNAi: 29.57 ± 0.81 (P= 0.7474).

These results show a link between proliferation of germline and cold-induced longevity during adulthood, but it is important to note that expression of *mog* genes is not limited to germline and these genes are also expressed in somatic tissues. To avoid the potential direct effects of silencing the *mog* genes on somatic tissues, we checked other genes that are important for the maintenance of germline proliferation. In these lines, we knocked-down the orthologues of the translational initiation factor EIF5A (*i.e.*, *iff-1*, *iff-2*), which is duplicated in *C. elegans*. Whereas *iff-2* is only expressed in somatic tissues (Hanazawa et al., 2004), *iff-1* is specifically expressed in the germline and it is particularly abundant in the distal gonads where mitotic divisions occur (Hanazawa et al., 2004). In fact, *iff-1* is essential for germline proliferation at either larvae or adult stages (Green et al., 2011; Hanazawa et al., 2004). We showed that *iff-1* RNAi initiated during adulthood was sufficient to reduce the proliferation of germ stem cells (**Fig. 11c-e**). Notably, knockdown of *iff-1* during adulthood shortened lifespan extension induced by low temperatures (*i.e.*, 10°C and 15°C) (**Fig. 13a, b**), but did not reduce lifespan at higher temperatures (*i.e.*, 20°C and 25°C) (**Fig. 13c, d**). Conversely, loss of *iff-2* during adulthood did not impair proliferation of germline (**Fig. 11c, d**) and cold-induced longevity (**Fig. 13a, b**). Upon *eif-2A* RNAi, a different translational initiation factor, worms showed a normal proliferating germline (**Fig. 11c, d**) and they had a lifespan extension at cold

temperature similar to control worms (**Fig. 13a-d**). Altogether, our data suggest that maintenance of adult GSC proliferation determines cold-induced longevity.

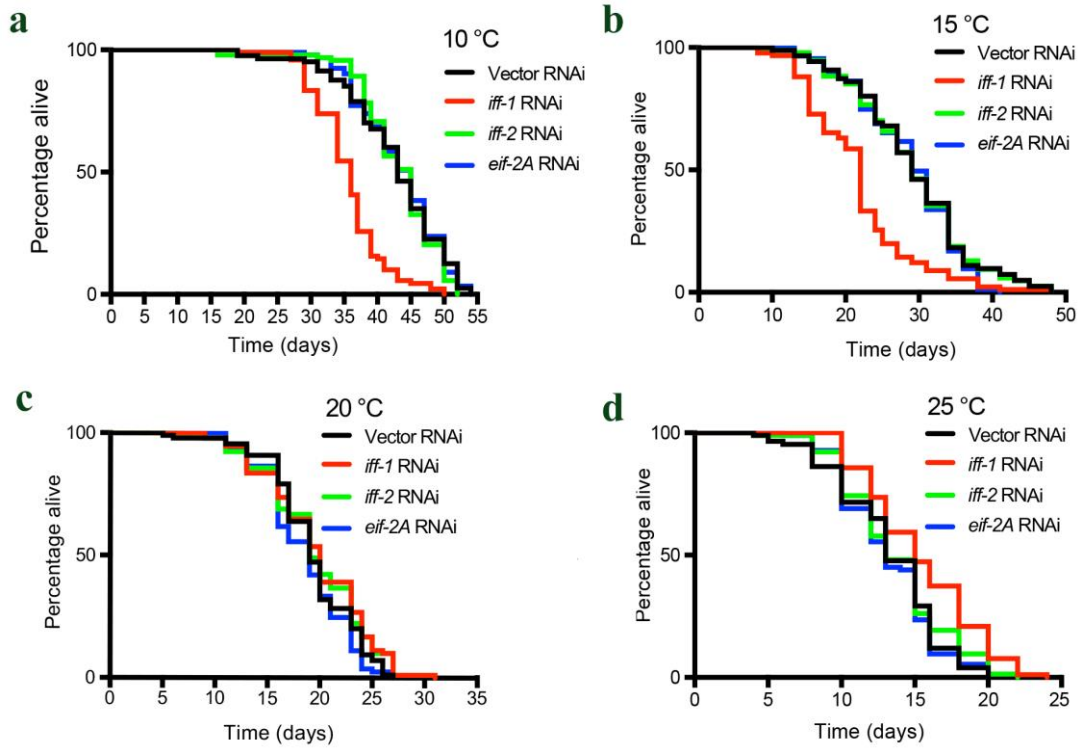


Figure 13. Knockdown of iff-1 during adulthood shortened lifespan extension induced by low temperatures (10°C and 15°C), but did not reduce lifespan at higher temperatures (20°C and 25°C). Loss of iff-2 during adulthood did not impair cold-induced longevity.

a, Loss of *iff-1* reduces the long lifespan induced by cold temperature (10°C) in N2 wild-type worms. RNAi was initiated at day 1 of adulthood. Empty vector RNAi mean ± s.e.m: 42.45 ± 0.84, *iff-1* RNAi: 35.52 ± 0.54 (P<0.0001), *iff-2* RNAi: 43.00 ± 0.66 (P= 0.6186), *eif-2A* RNAi: 43.11 ± 0.68 (P= 0.8526). **b,** Loss of *iff-1* cold-induced longevity at 15°C in N2 worms. RNAi was initiated at day 1 of adulthood. Empty vector RNAi mean ± s.e.m: 29.19 ± 0.86, *iff-1* RNAi: 21.66 ± 0.76 (P<0.0001), *iff-2* RNAi: 29.24 ± 0.85 (P= 0.8227), *eif-2A* RNAi: 28.53 ± 0.74 (P= 0.3853). **c,** *iff-1* RNAi does not reduce lifespan of N2 worms at 20°C (RNAi starting at day 1 of adulthood). Empty vector RNAi: mean ± s.e.m: 19.33 ± 0.45, *iff-1* RNAi: 19.79 ± 0.51 (P= 0.1317), *iff-2* RNAi: 19.61 ± 0.50 (P= 0.2137), *eif-2A* RNAi: 18.65 ± 0.44 (P= 0.2061). **d,** *iff-1* RNAi (starting at day 1 of adulthood) moderate lifespan extension of N2 worms at 25°C. Empty vector RNAi mean ± s.e.m: 13.23 ± 0.40, *iff-1* RNAi: 15.36 ± 0.39 (P<0.0001), *iff-2* RNAi: 13.69 ± 0.42 (P= 0.4603), *eif-2A* RNAi: 13.19 ± 0.33 (P= 0.6935). In lifespan experiments, P-values: log-rank test, n= 96 worms/condition.

Prompted by these findings, we checked how temperature affects *iff-1* mutants. These mutants develop into young adult worms with few germ cells

(~40 germ nuclei) when compared with wild-type worms (~2000 germ nuclei) (Hanazawa et al., 2004), thus resembling *glp-1* mutants. Similar to *glp-1* worms (**Fig. 1b**), *iff-1* germline-less mutants had also lifespan extension at 20°C while they lived shorter than the wild-type strain at 15°C (**Fig. 14a, b**). Although the impairment of GSC proliferation induced by *iff-1* knockdown at the adult stage is not equivalent to literal ablation of the germline in either *iff-1* or *glp-1* mutants, these results further support a role of the germline in cold-induced longevity. In these lines, *iff-1* RNAi during adulthood did not further reduce the lifespan of distinct germline-lacking strains (*glp-1* and *glp-4*) at cold temperature (**Fig. 14c, d**). As a formal test of the specific role of adult GSCs, we also knock-downed *iff-1* in adult sterile worms with a proliferating germline (*fer-15;fem-1*) and found a decrease in cold-induced longevity (**Fig. 14e**). These results not only further support the link between adult GSCs and cold-induced longevity, but also show that sterility per se does not affect the longevity phenotype. In fact, *iff-1* RNAi during adulthood diminished proliferation of GSCs and cold-induced longevity (**Fig. 11d and 13a, b**), but did not strongly affect the total number of eggs laid by self-fertilizing hermaphrodite worms (**Fig. 14f, g**). Since maintenance of germline proliferation is required for cold-induced longevity, we asked whether over-proliferation of germline could further extend lifespan at low temperature. Mutations in *gld-1*, a tumor-suppressor factor, promote the germ cells to reenter the mitotic cycle and to over-proliferate (Francis, Barton, Kimble, & Schedl, 1995; Francis, Maine, & Schedl, 1995). Similarly, knockdown of *gld-1* also promotes over-proliferation of the germline (Marin & Evans, 2003b). Eventually, these over-proliferating germ cells resemble a tumorous state that kills the worms at early adulthood stages. Thus, these worms exhibit a short lifespan at 20°C (Pinkston, Garigan, Hansen, & Kenyon, 2006) (**Fig. 15a**). Similarly, both *gld-1* mutant worms and *gld-1* RNAi from either development or adulthood kill *C. elegans* early in life at lower temperature (**15b**). Altogether, these results indicate that cold-induced longevity is determined by an extended maintenance of the healthy pool of proliferating GSCs, while abnormal over-proliferation of these cells is detrimental to the animal.

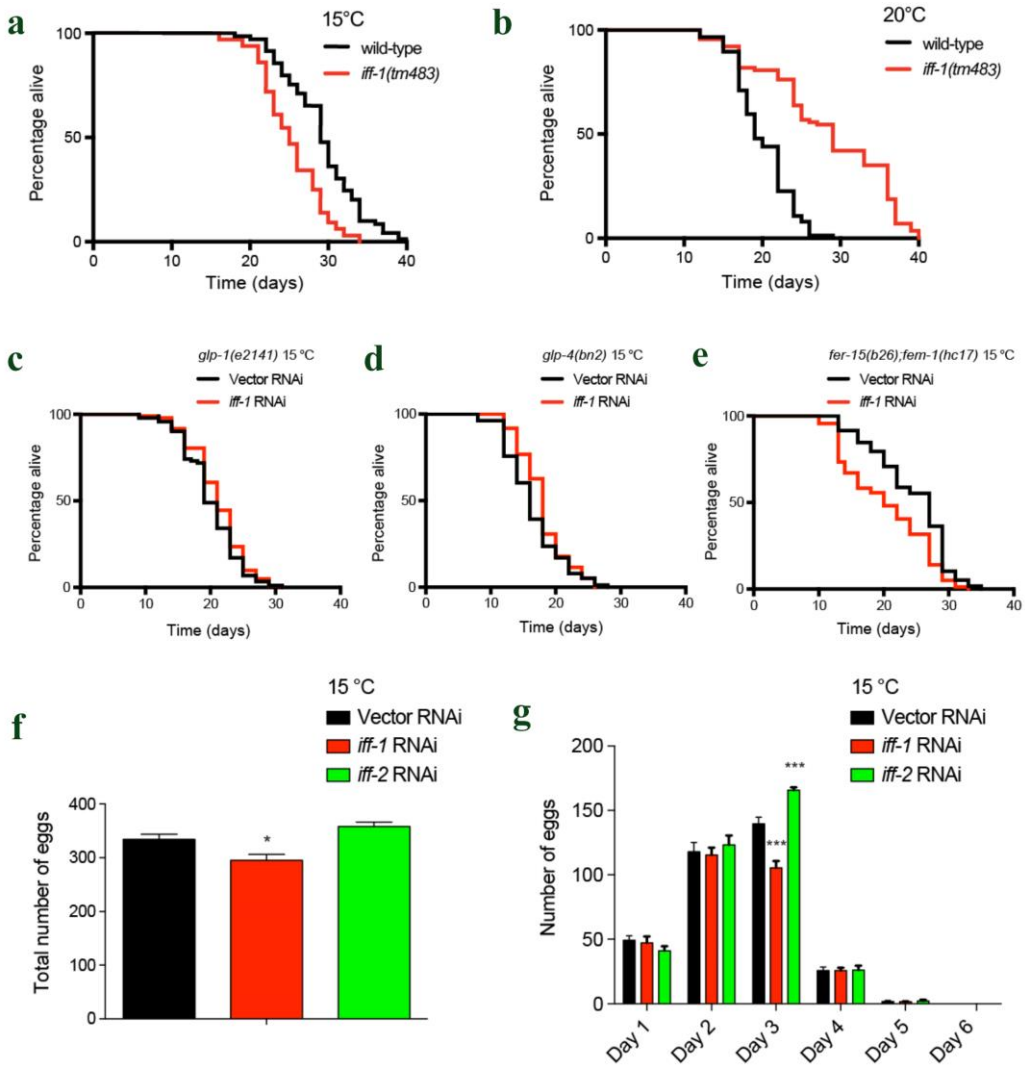


Figure 14. iff-1 germline-less worms are short-lived at 15°C, whereas they are long-lived at 20°C. Knockdown of iff-1 does not further shorten the lifespan of germline-lacking worms at 15°C, but reduces cold-induced longevity in sterile worms with a proliferating germline. Knockdown of iff-1 does not strongly affect the number of eggs laid by self-fertilizing hermaphrodites.

a, *iff-1(tm483)* germline-lacking mutant *C. elegans* are short lived compared with N2 wild-type worms at cold temperature (log rank, P<0.0001). *N2* 15°C mean ± s.e.m: 29.24 ± 0.57, *iff-1* 15°C: 25.29 ± 0.49 (n= 96 worms/condition). **b,** *iff-1* germline-lacking mutant worms are long lived at 20°C (log rank, P<0.0001). *N2* 20°C mean ± s.e.m: 19.88 ± 0.42, *iff-1* 20°C: 28.14 ± 0.88 (n= 96 worms/condition). **c,** Knockdown of *iff-1* during adulthood does not reduce lifespan of *glp-1(e2141)* germline-lacking worms at 15°C (log rank, P= 0.1538). Empty vector RNAi: mean ± s.e.m: 20.09 ± 0.45, *iff-1* RNAi: 21.08 ± 0.46 (n= 96 worms/condition). **d,** Loss of *iff-1* does not reduce lifespan of *glp-4(bn2)* germline-lacking worms at 15°C (log rank, P= 0.0827). Empty vector RNAi: mean ± s.e.m: 16.45 ± 0.49, *iff-1* RNAi: 17.92 ± 0.40 (n= 96 worms/condition). **e,** Loss of *iff-1* during adulthood decreases longevity induced by cold temperature (15°C) in sterile worms with a proliferating germline (*fer-15(b26);fem-1(hc17)*). Empty vector RNAi: mean ± s.e.m: 24.35 ± 0.74, *iff-1* RNAi: 20.35 ± 0.72 (n= 96 worms/condition; log rank,

P= 0.0005). **f,** Total number of eggs laid at 15°C during the self-reproductive period (mean ± s.e.m., n= 30 worms scored per condition from 3 independent experiments). RNAi was initiated during adulthood of wild-type worms. **g,** Number of eggs laid every 24 h during the self-reproductive period at 15°C (mean ± s.e.m., n= 30 worms scored per condition from 3 independent experiments). Statistical comparisons were made by Student's t-test for unpaired samples. P-value: P-value: *(P<0.05), ***(P<0.001).

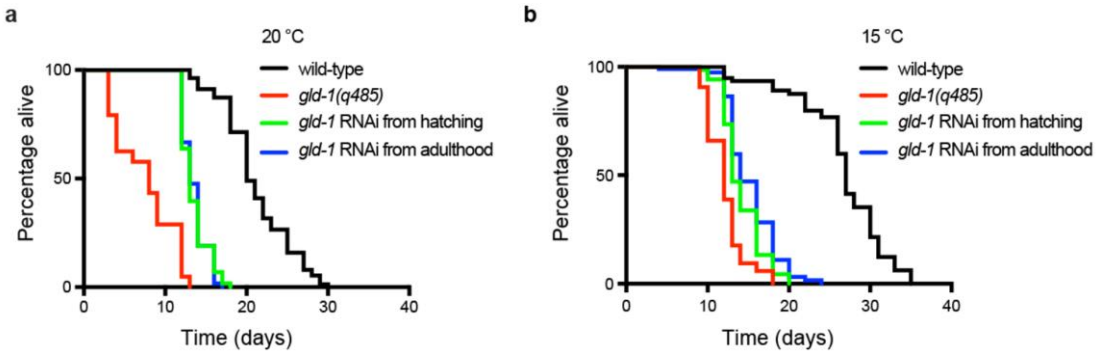


Figure 15. Over-proliferation of the germline caused by either tumorous gld-1 mutations or gld-1 knockdown kills C. elegans early in life.

a, *gld-1(q485)* allele and *gld-1* RNAi shortens lifespan at 20°C (n= 96 worms/condition). Wild type + empty vector RNAi: 21.17 ± 0.47; *gld-1(q485)* + empty vector RNAi: 7.54 ± 0.43, P<0.0001; wild-type + *gld-1* RNAi from hatching: 13.50 ± 0.21, P<0.0001; wild-type + *gld-1* RNAi from adulthood: 13.54 ± 0.18, P<0.0001. **b,** *gld-1(q485)* allele and *gld-1* RNAi shortens lifespan at 15°C (n= 96 worms/condition). Wild type + empty vector RNAi mean ± s.e.m: 26.58 ± 0.65; *gld-1(q485)* + empty vector RNAi: 12.09 ± 0.24, P<0.0001; wild-type + *gld-1* RNAi from hatching: 14.10 ± 0.30, P<0.0001; wild-type + *gld-1* RNAi from adulthood: 15.17 ± 0.38, P<0.0001.

3.4: Adult proliferating germ cells promote *cbs-1* expression in the intestine to extend lifespan

Our results suggest that adult proliferating germ cells directly regulate cold-induced longevity. Thus we asked whether these proliferating germ cells promote lifespan extension through regulation of somatic tissues. Although worms showed a slight increase in motility at low temperature, we did not observe impairments in germline-lacking mutant worms (*glp-1*, *glp-4*) when compared with either wild-type or control sterile *fer-15;fem-1* worms (**16a**). Similarly, inhibition of adult germline proliferation upon *iff-1* or *mog-5*

knockdown did not affect motility at cold temperature (**16b**). We also measured worms' body size and found that neither germline ablation nor inhibition of adult germline proliferation by RNAi treatment had a notable effect on the animal's size (**Fig. 16c-f**). In *C. elegans*, metabolic rates increase with temperature and are often negatively correlated with longevity (Van Voorhies & Ward, 1999). We checked the rate of oxygen consumption as an indicator of metabolic rates and found a reduced oxygen consumption of wild-type worms at cold temperature (15°C) (**Fig. 16g**). In fer-15;fem-1 sterile worms, despite the cold-induced lifespan extension (**Fig. 1d),** oxygen consumption increased at cold temperature (**Fig. 16g***),*. In glp-1 germ-lacking mutants, metabolic rates did not change significantly at lower temperature when compared with worms at 20°C (**Fig. 16g**). More importantly, in iff-1 RNAi-treated worms the short lifespan did not correlate with an increase in metabolic rates at 15°C (**Fig. 16h**). Indeed, *iff-1* RNAi-treated worms had a decrease in oxygen consumption when compared with control worms, resembling *iff-2* RNAi treatment, which does not affect cold-induced longevity (**Fig. 16h**). Thus, these results suggest that inhibition of adult GSC proliferation does not have a remarkable effect on features such as body size, motility or metabolic rates, indicating that these cells regulate cold-induced longevity by distinct mechanisms.

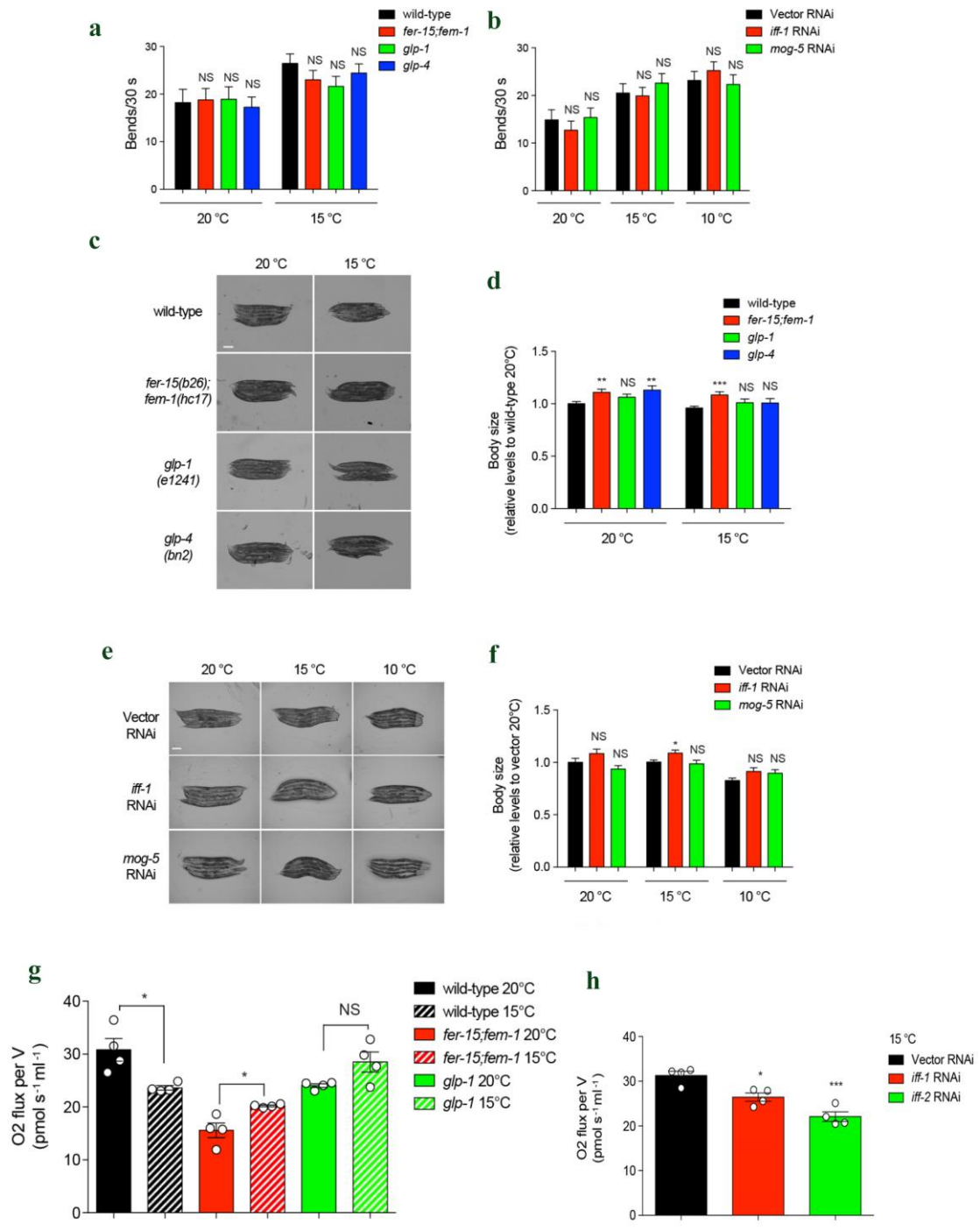


Figure 16. Ablation of germline and inhibition of adult germline proliferation do not affect motility or body size at cold temperature. Inhibition of adult germline proliferation does not increase metabolic rates.

a, Bar graphs represent the mean ± s.e.m. (n=25) of thrashing movements over a 30 s period. We examined wild-type, sterile *fer-15(b26);fem-1(hc17)* and *glp-1(e2141)/glp-4(bn2)* germline-lacking worms on day 6 of adulthood. No significant differences were found when sterile and germline-lacking worms were compared with wild-type worms at the same temperature. **b,** Graph represents the mean ± s.e.m. (n=25 per condition from 2 independent experiments) of thrashing movements/30 s at day 6 of

adulthood. RNAi treatment was initiated at day 1 of adulthood. No significant differences were found when *iff-1* or *mog-5* RNAi-treated worms were compared with empty vector control worms at the same temperature. **c,** Differential interference contrast images of wild-type, sterile *fer-15(b26);fem-1(hc17)* and *glp-1(e2141)/glp-4(bn2)* germline-lacking worms on day 6 of adulthood. Scale bar, 200 µm. Images are representative of two independent experiments. **d,** Quantification of body size at the indicated temperatures. Graph represents the mean ± s.e.m. relative to wild-type 20°C (15-22 worms per condition from 2 independent experiments). No significant differences were found in body size when germline-lacking worms were compared with wild-type worms at 15°C. **e,** Differential interference contrast images of wild-type worms on day 6 of adulthood. Worms were shifted to the indicated temperatures and RNAi treatment at day 1 of adulthood. Scale bar, 200 µm. Images are representative of two independent experiments. **f,** Quantification of body size on the indicated RNAi treatment and temperatures. Graph represents the mean ± s.e.m. relative to Vector RNAi 20°C (11 worms per condition from 2 independent experiments). *iff-1* or *mog-5* RNAi-treatment did not have a notable effect on body size when compared with empty vector control at the same temperature. **g,** Graph represents oxygen consumption (pmol s^{-1} ml^{-1}) of wild-type, sterile *fer-15(b26);fem-1(hc17)* and *glp-1(e2141)* germline-lacking worms cultured at the indicated temperatures during adulthood (mean ± s.e.m., n= 4). Oxygen consumption was measured at day 5 of adulthood. **H,** Graph represents oxygen consumption (pmol s^{-1} ml^{-1}) of wild-type worms treated with *iff-1* and *mog-5* RNAi from adulthood at 15°C (mean ± s.e.m., n= 4). Oxygen consumption was measured at day 6 of adulthood. All the statistical comparisons were made by Student's t-test for unpaired samples. P-value: *(P<0.05), ** (P<0.01), ***(P<0.001). NS= not significant.

To gain further insights about changes in somatic tissues induced by GSCs at low temperature, we applied a quantitative proteomics approach. We identified 233 down-regulated and 225 up-regulated proteins at 20°C when compared with *C. elegans* cultured at cold temperature (15°C). We hypothesized that inhibition of germ cells proliferation during adulthood at 15°C triggers similar modifications in lifespan regulators to those induced by temperature increase. Thus, we examined the proteome of *iff-1* RNAi-treated worms at cold temperature (15°C). Other than IFF-1 levels itself, quantitative proteomics analysis revealed that 152 other proteins are significantly changed upon *iff-1* knockdown during adulthood at 15°C. Among them, 27 proteins were also up-regulated at 20°C (**Fig. 17a**). Similarly, 27 proteins were down-regulated in both *iff-1* RNAi treated worms and 20°C condition (**Fig. 17a**). To examine whether these proteins modulate lifespan extension at cold temperatures, we conducted an RNAi screen. Notably, knockdown of several genes decreased cold-induced longevity as we validated in independent experiments (**Fig. 17b-e**). Particularly, genes that encode proteins

that are increased at cold-temperature and down-regulated by loss of *iff-1* (**Fig. 17a**). The most significant effect was induced by knockdown of distinct subunits of the chaperonin TRiC/CCT complex (i.e. *cct-2*, *cct-5*, *cct-6*) (**Fig. 17b**). Since loss of a single subunit of TRiC/CCT complex is sufficient to impair the assembly of the complex (Spiess, Meyer, Reissmann, & Frydman, 2004), our results suggest that increased TRiC/CCT assembly is required for lifespan extension at cold temperature. Besides the *cct* subunits, knockdown of either the hsp70 chaperone *hsp-1* or the RNA polymerase II *ama-1* reduced also lifespan extension at cold temperature (**Fig. 17c, d**). Similarly, up-regulated level of CBS-1, the orthologue of human cystathionine β-synthase (CBS) (Hine et al., 2015; Vozdek, Hnizda, Krijt, Kostrouchova, & Kozich, 2012), was required for the full extent longevity phenotype at 15°C (**Fig. 17e**). Similarly knockdown of *cct* subunits, *hsp-1*, *ama-1* and *cbs-1* at cold temperature (10°C) reduced the lifespan extension phenotype (**Fig. 17f**). In contrast, knockdown of these genes at higher temperatures (20°C and 25°C) did not shorten lifespan (**Fig. 17g, h**). Whereas these factors regulated cold-induced longevity in wild-type worms possessing a proliferating germline, their knockdown did not further reduce the short lifespan of germline-lacking worms (*glp-1*) at 15°C (**Fig. 17i**). Altogether, our results indicate that high expression of *cct* subunits, *hsp-1*, *ama-1*, and *cbs-1* are required in the cold-induced longevity mediated by proliferating germ cells.

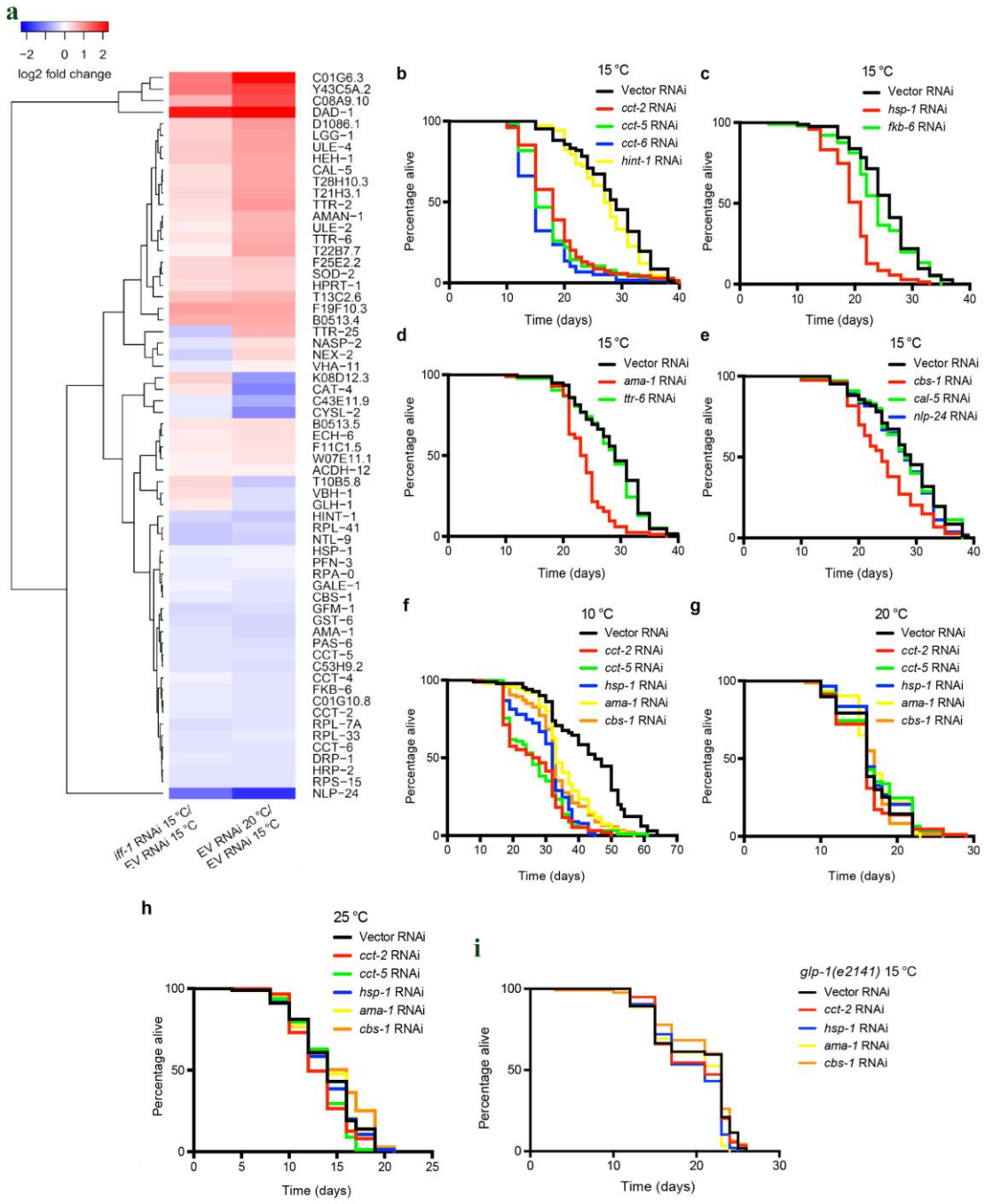


Figure 17. Heatmap with the full list of significantly changed proteins in both iff-1 RNAi treated worms and 20°C conditions. cbs-1 and other factors modulate cold-induced longevity induced by adult GSCs. Knockdown of cct-2, hsp-1, ama-1 or cbs-1 does not further decrease the short lifespan of germline-lacking worms at 15°C.

a, Heatmap depicting the $\log_2$-fold change of the differentially expressed proteins in both *iff-1* RNAi-treated worms at 15°C and empty vector (EV) RNAi-treated worms at 20°C when compared with EV RNAi-treated worms at 15°C. *fer-15(b26);fem-1(hc17)* control strain was raised at the restrictive temperature (25°C) during development to obtain sterile worms with a proliferating germline, which were then shifted to the indicated temperatures and RNAi treatment until day 6 of adulthood.. **b,**

CCT subunits are required for the lifespan extension induced by cold temperature (15°C) in N2 wild-type worms (Empty vector RNAi mean ± s.e.m: 28.26 ± 0.69; *cct-2* RNAi: 18.58 ± 0.71, P<0.0001; *cct-5* RNAi: 17.83 ± 0.68, P<0.0001; *cct-6* RNAi: 16.15 ± 0.66, P<0.0001; *hint-1* RNAi: 27.15 ± 0.69, P=0.1722). **c,** The *hsp-1* chaperone is required for the lifespan extension induced by cold temperature (15°C) (Empty vector RNAi mean ± s.e.m: 25.49 ± 0.62; *hsp-1* RNAi: 19.84 ± 0.49, P<0.0001; *fkb-6* RNAi: 24.10 ± 0.73, P=0.2535). **d,** The RNA polymerase II *ama-1* regulates longevity of wild-type worms at 15°C (Empty vector RNAi mean ± s.e.m: 28.54 ± 0.68; *ama-1* RNAi: 23.67 ± 0.42, P<0.0001; *ttr-6* RNAi: 27.95 ± 0.65 P=0.4734). **e,** Loss of *cbs-1* decreases lifespan of wild-type worms at 15°C (Empty vector RNAi mean ± s.e.m: 28.26 ± 0.69; *cbs-1* RNAi: 24.54 ± 0.69, P=0.0005; *cal-5* RNAi: 28.09 ± 0.78, P=0.8688; *nlp-24* RNAi: 27.16 ± 0.78, P=0.0664). **f,** Knockdown of *cct* subunits, *hsp-1*, *ama-1* or *cbs-1* decreases the longevity phenotype induced by 10°C in N2 wild-type worms (P<0.0001). Empty vector RNAi mean ± s.e.m: 43.31 ± 1.39; *cct-2* RNAi: 26.36 ± 0.02; *cct-5* RNAi: 26.76 ± 0.95; *hsp-1* RNAi: 30.17 ± 0.82; *ama-1* RNAi: 35.37 ± 0.87; *cbs-1* RNAi: 33.57 ± 0.93. **g,** Knockdown of *cct* subunits, *hsp-1*, *ama-1*, or *cbs-1* does not shorten lifespan at 20°C. Empty vector RNAi mean ± s.e.m: 16.34 ± 0.37; *cct-2* RNAi: median= 15.97 ± 0.41, P=0.5386; *cct-5* RNAi: 17.01 ± 0.48, P=0.1106; *hsp-1* RNAi: 17.10 ± 0.39, P=0.1571; *ama-1* RNAi: 17.02 ± 0.35, P=0.4403; *cbs-1* RNAi: 16.26 ± 0.43, P=0.9592. **h,** Knockdown of *cct* subunits, *hsp-1*, *ama-1*, or *cbs-1* does not shorten lifespan at 25°C. RNAi was initiated at day 1 of adulthood. Empty vector RNAi mean ± s.e.m: 13.95 ± 0.37; *cct-2* RNAi: 13.20 ± 0.31, P=0.0695; *cct-5* RNAi: 13.43 ± 0.29, P=0.0472; *hsp-1* RNAi: 13.83 ± 0.36, P=0.7389; *ama-1* RNAi: 14.07 ± 0.36, P=0.8221; *cbs-1* RNAi: 14.53 ± 0.44, P=0.1069. **i,** *cct-2*, *hsp-1*, *ama-1*, or *cbs-1* RNAi does not significantly shorten lifespan of *glp-1* germline-lacking worms at 15°C. Empty vector RNAi mean ± s.e.m: 20.00 ± 0.58; *cct-2* RNAi: 19.59 ± 0.52, P=0.5878; *hsp-1* RNAi: 19.28 ± 0.51, P=0.1008; *ama-1* RNAi: 19.57 ± 0.52, P=0.0570; *cbs-1* RNAi: 20.49 ± 0.59, P=0.7775. In all the lifespan experiments, RNAi was started at day 1 of adulthood (P-values: log-rank test, n= 96 worms/condition). Statistical comparisons were made by Student's t-test (n= 3, p-value <0.05 was considered significant).

Prompted by these findings, we asked how these factors influence the cold-induced longevity. For instance, one possibility could be modulating the cold-induced longevity by direct regulation of germ cells proliferation. Another intriguing possibility is that proliferating germ cells up-regulate specific factors in somatic tissues, resulting in longevity phenotype. To define the factors that fall into each category, we checked first their effects on germline proliferation (**Fig. 18a-c**). Loss of different *cct* subunits dramatically reduced the number of proliferating germ cells in wild-type worms (**Fig. 18a, b**). In these lines, tissue-specific knockdown of *cct* subunits in the germline alone reduced cold-induced longevity (**Fig. 19a**). Notably, their tissue-specific knockdown in either intestine or neurons also partially diminished the longevity phenotype (**Fig. 19b-e**). Thus,

cct subunits could fall in both categories by acting in both the germline and the soma to prolong lifespan at low temperature. Similar to *cct* subunits, knockdown of *ama-1* also strongly decreased germline proliferation (**Fig. 18a, b**). Tissue-specific knockdown of *ama-1* in the intestine alone did not alter the lifespan, but we found a reduction of cold-induced longevity when the *ama-1* RNAi was also efficient in the germline (**Fig. 20d**). In addition, tissue-specific knockdown of *ama-1* in neurons slightly decreased the cold-induced longevity (**Fig. 20b**), while we obsereved no effect when the RNAi targeted the muscle or the intestine (**Fig. 20a, c**). In comparison with *cct* subunits or *ama-1*, knockdown of *hsp-1* slightly decreased germline proliferation (**Fig. 18a, b**), indicating an important role in somatic tissues. Indeed, neuron-specific knockdown of *hsp-1* strongly shortened longevity at cold temperature, while its loss in either muscle or intestine did not significantly affect lifespan (**Fig. 21a-c**). However, knockdown of *hsp-1* in the germline also reduced cold-induced longevity (**Fig. 21d, e**). Thus, although *cct* subunits and *hsp-1* could act in soma to promote longevity, their direct effects in germ-cell proliferation also modulate organismal longevity. For this reason, we focused on *cbs-1*, the only regulator of cold-induced longevity that did not alter the number of proliferating germ cells (**Fig. 18a, b**). Whereas specific downregulation of *cbs-1* in neurons did not change lifespan, its knockdown in either the muscle or intestine significantly shortened cold-induce longevity (**Fig. 22a-c**). Notably, longevity was not further decreased when the germline also responded to *cbs-1* RNAi treatment (**Fig. 22d, e**). In addition, we found that downregulation of *cbs-1* in the germline alone does not impair cold-induced longevity (**Fig. 22f**).

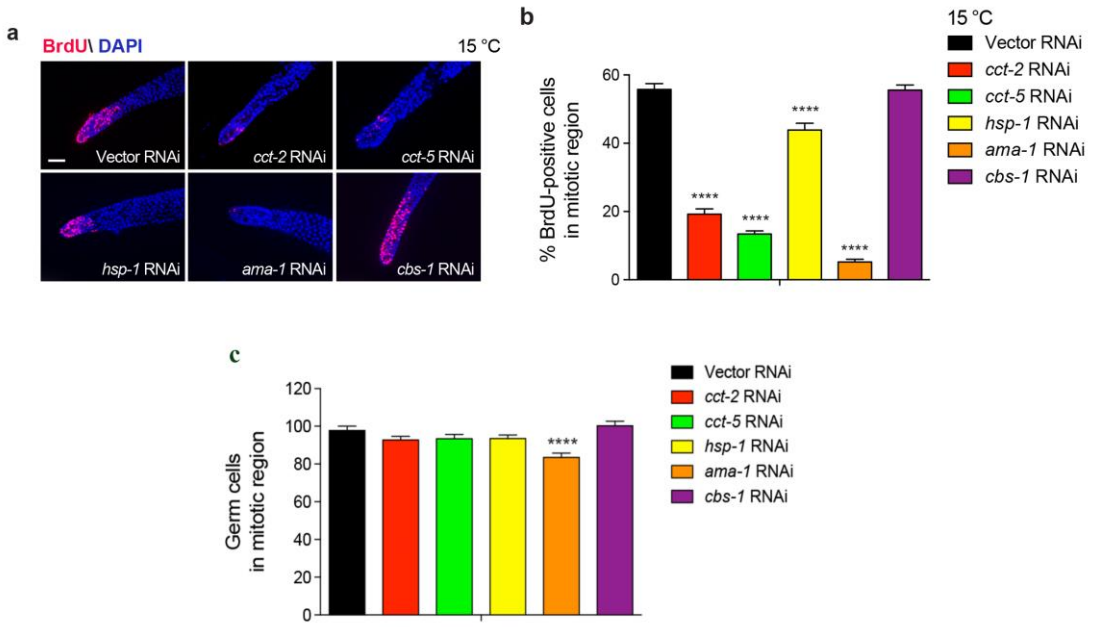


Figure 18. Knockdown of distinct regulators of cold-induced longevity diminished the proliferation of GSCs but total number of germ cells remained not affected.

a, BrdU staining of germline from wild-type *C. elegans* fed with the indicated RNAi from day 1 of adulthood. Worms were examined at day 5 of adulthood. Cell nuclei were stained with DAPI. Scale bar represents 20 μm. The images are representative of 3 independent experiments. **b,** Graph represents the percentage of BrdU-positive cells/total nuclei (mean ± s.e.m., 40-46 germlines scored per condition from 3 independent experiments). **c,** Graph represents the total number of germ cells in the mitotic region quantified by DAPI staining (mean ± s.e.m., 40-46 germlines scored per condition from 3 independent experiments). Wild-type worms treated with the indicated RNAis were examined at day 5 of adulthood. Statistical comparisons were made by Student's t-test for unpaired samples. P-value: ****(P<0.0001).

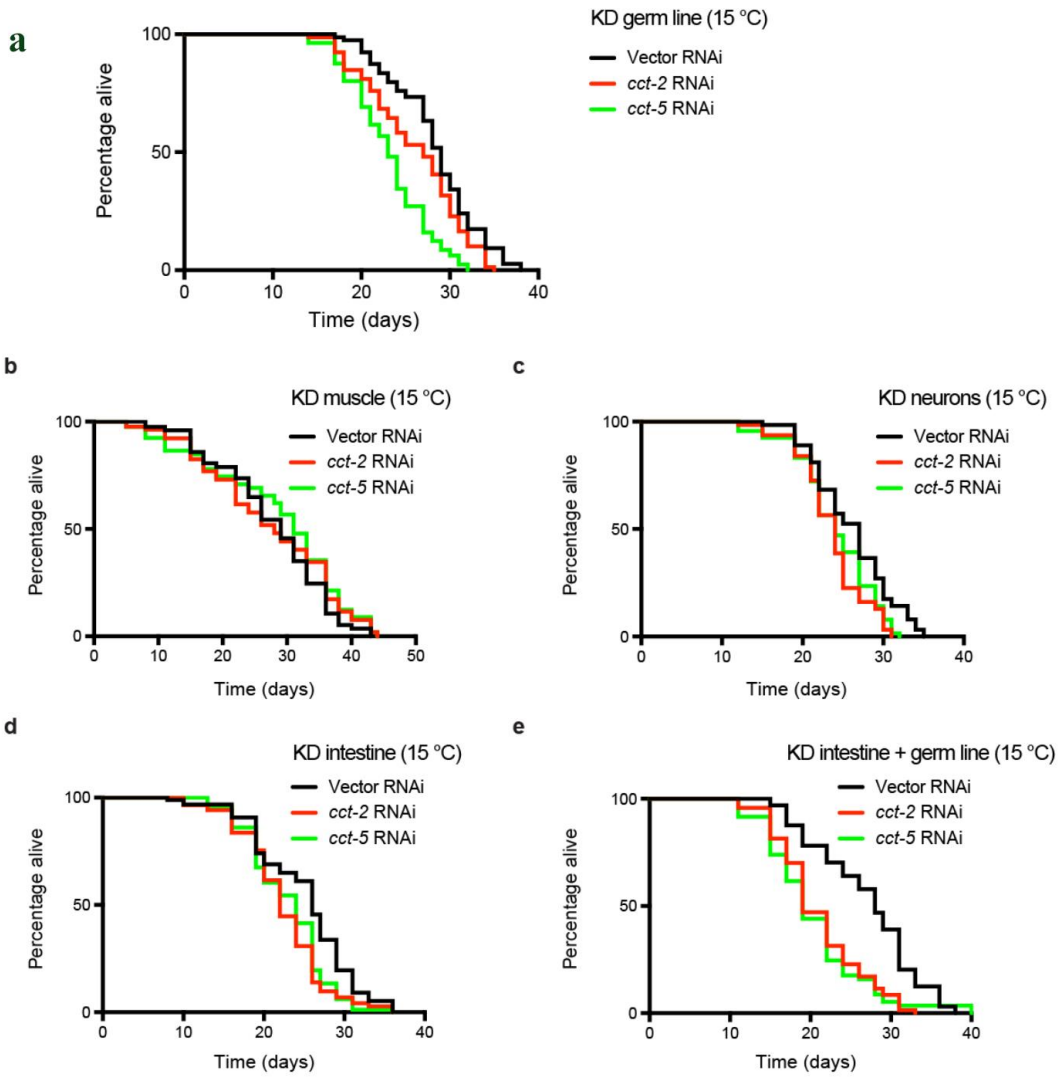


Figure 19. Both somatic and germline knockdown of CCT subunits diminishes cold-induced longevity.

a, Knockdown (KD) of *cct* subunits in the germline alone decreases cold-induced longevity at 15°C. RNAi rescued in the germline of RNAi-deficient worms (*rde-1(mkc36); sun-1p::rde-1::sun-1 3'UTR* strain). Empty vector RNAi mean ± s.e.m: 28.32 ± 0.55; *cct-2* RNAi: 25.94 ± 0.61, P=0.0119; *cct-5* RNAi 23.07 ± 0.49, P<0.0001. **b,** Muscle-specific knockdown of *cct* subunits does not affect longevity at 15°C. RNAi rescued in the muscle of RNAi-deficient worms (*rde-1(ne300)*; *myo-3p*::*rde-1* strain). Empty vector RNAi: 27.64 ± 1.08; *cct-2* RNAi: 27.36 ± 1.31, P= 0.5002; *cct-5* RNAi: 28.46 ± 1.31, P=0.2381. **c,** Neuronal-specific knockdown of *cct* subunits decreases longevity at 15°C. RNAi rescued in the neurons of RNAi-deficient worms (*sid-1(pk3321)*; *unc-119p*::*sid-1* strain). Empty vector RNAi: 26.09 ± 0.60; *cct-2* RNAi: 23.56 ± 0.52, P= 0.0010; *cct-5* RNAi: 24.05 ± 0.59, P=0.0088. **d,** Intestinal-specific knockdown of *cct* subunits decreases longevity at 15°C. RNAi rescued in the intestine of RNAi-deficient worms (*rde-1(ne219)*; *nhx-2p*::*rde-1* strain). Empty vector RNAi: 25.02 ± 0.65; *cct-2* RNAi: 22.37 ± 0.59, P= 0.0010; *cct-5* RNAi: 22.90 ± 0.53, P=0.0012. **e,** Intestinal and germline-knockdown of *cct* subunits induces a strong decrease in longevity at 15°C. RNAi rescued in both the intestine and

germline of RNAi-deficient worms (*rde-1(ne219)*; *mex-5p*::*rde-1* strain). Empty vector RNAi: 26.97 ± 0.79; *cct-2* RNAi: 21.01 ± 0.64, P<0.0001; *cct-5* RNAi 20.36 ± 0.82, P<0.0001. P-values: log-rank test, n= 96 worms/condition. In all the experiments, RNAi was started at day 1 of adulthood.

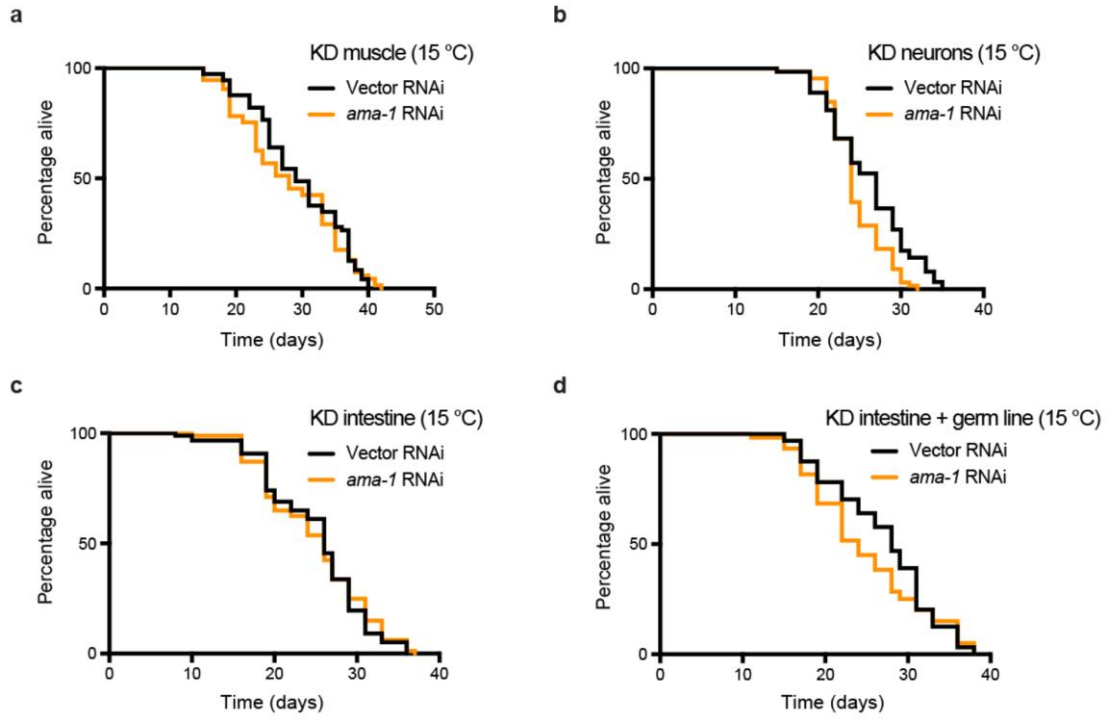


Figure 20. Knockdown of ama-1 in the intestine alone does not affect lifespan, whereas it reduces cold-induced longevity when the RNAi is also efficient in the germline.

a, Muscle-specific knockdown (KD) of *ama-1* does not decrease lifespan extension induced by cold temperature (15°C). RNAi rescued in the muscle of RNAi-deficient worms (*rde-1(ne300)*; *myo-3p*::*rde-1* strain). Empty vector RNAi mean ± s.e.m: 29.49 ± 0.81; *ama-1* RNAi: 28.04 ± 0.91, P=0.5692. **b,** Neuronal-specific KD of *ama-1* slightly decreases longevity at 15°C. RNAi rescued in the neurons of RNAi-deficient worms (*sid-1(pk3321)*; *unc-119p*::*sid-1* strain). Empty vector RNAi mean ± s.e.m: 26.09 ± 0.60; *ama-1* RNAi: 24.53 ± 0.40, P=0.0035. **c,** Intestinal-specific KD of *ama-1* does not affect cold-induced longevity at 15°C. RNAi rescued in the intestine of RNAi-deficient worms (*rde-1(ne219)*; *nhx-2p*::*rde-1* strain). Empty vector RNAi mean ± s.e.m: 25.02 ± 0.66; *ama-1* RNAi: 24.98 ± 0.68, P=0.7588. **d,** *ama-1* RNAi shortens cold-induced longevity when the knockdown is also efficient in the germline. RNAi rescued in both the intestine and germline of RNAi-deficient worms (*rde-1(ne219)*; *mex-5p*::*rde-1* strain). Empty vector RNAi mean ± s.e.m: 26.97 ± 0.79; *ama-1* RNAi: 24.92 ± 0.91, P=0.0302. P-values: log-rank test, n= 96 worms/condition. In all the experiments, RNAi was started at day 1 of adulthood.

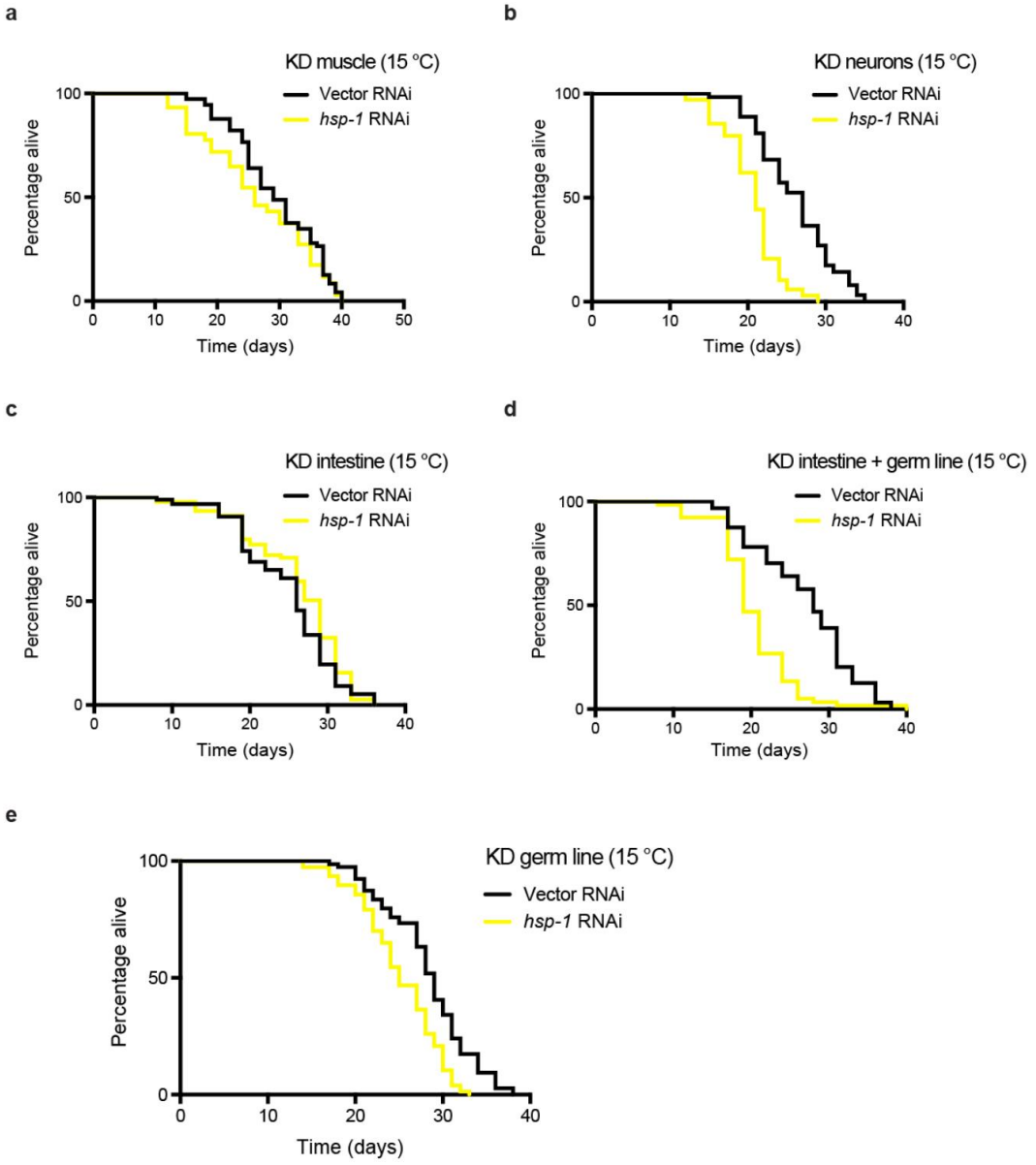


Figure 21. Knockdown of hsp-1 in either neurons or germline reduces cold-induce longevity.

a, Muscle-specific knockdown (KD) of *hsp-1* does not decrease lifespan extension induced by cold temperature (15°C). RNAi rescued in the muscle of RNAi-deficient worms (*rde-1(ne300)*; *myo-3p*::*rde-1* strain). Empty vector RNAi mean ± s.e.m: 29.49 ± 0.81; *hsp-1* RNAi: 26.56 ± 1.03, P=0.1574. **b,** Neuronal-specific KD of *hsp-1* decreases longevity at 15°C. RNAi rescued in the neurons of RNAi-deficient worms (*sid-1(pk3321)*; *unc-119p*::*sid-1* strain). Empty vector RNAi mean ± s.e.m: 26.09 ± 0.60; *hsp-1* RNAi: 20.60 ± 0.43, P<0.0001. **c,** Intestinal-specific KD of *hsp-1* does not affect cold-induced longevity at 15°C. RNAi rescued in the intestine of RNAi-deficient worms (*rde-1(ne219)*; *nhx-2p*::*rde-1* strain). Empty vector RNAi mean ± s.e.m: 25.02 ± 0.66; *hsp-1* RNAi: 26.29 ± 0.68, P=0.1258. **d,** *hsp-1* RNAi shortens cold-induced longevity when the knockdown is

also efficient in the germline. RNAi rescued in both the intestine and germline of RNAi-deficient worms (*rde-1(ne219)*; *mex-5p*::*rde-1* strain). Empty vector RNAi mean ± s.e.m: 26.97 ± 0.79; *hsp-1* RNAi: 20.33 ± 0.64, P<0.0001. **e,** Knockdown of *hsp-1* in the germline alone decreases cold-induced longevity at 15°C. RNAi rescued in the germline of RNAi-deficient worms (*rde-1(mkc36); sun-1p::rde-1::sun-1 3'UTR* strain). Empty vector RNAi mean ± s.e.m: 28.32 ± 0.55; *hsp-1* RNAi: 25.12 ± 0.51, P<0.0001. P-values: log-rank test, n= 96 worms/condition. In all the experiments, RNAi was started at day 1 of adulthood.

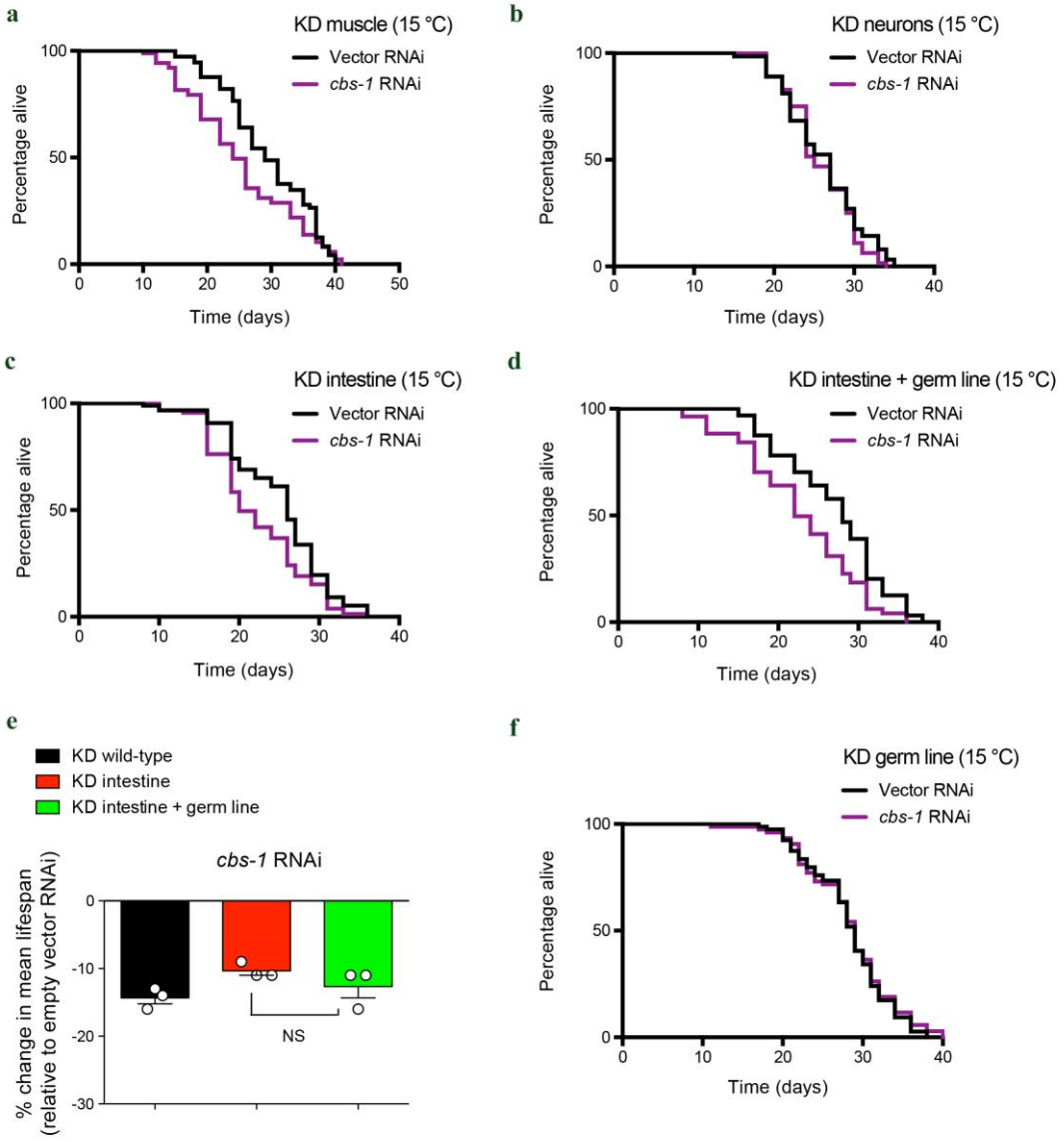


Figure 22. Tissue-specific knockdown of cbs-1 in the intestine or muscle reduces cold-induced longevity but not in germline.

a, Muscle-specific knockdown (KD) of *cbs-1* decreases lifespan extension induced by cold temperature (15°C). RNAi rescued in the muscle of RNAi-deficient worms (*rde-1(ne300)*; *myo-3p*::*rde-1* strain). Empty vector RNAi mean ± s.e.m: 29.49 ± 0.81; *cbs-1* RNAi: 25.33 ± 0.90, P=0.0048. **b,** Neuronal-specific KD of *cbs-1* does not affect longevity

at 15°C. RNAi rescued in the neurons of RNAi-deficient worms (*sid-1(pk3321)*; *unc-119p*::*sid-1* strain). Empty vector RNAi mean ± s.e.m: 26.09 ± 0.60; *cbs-1* RNAi: 25.78 ± 0.51, P=0.3367. **c,** Intestinal-specific KD of *cbs-1* decreases longevity at 15°C. RNAi rescued in the intestine of RNAi-deficient worms (*rde-1(ne219)*; *nhx-2p*::*rde-1* strain). Empty vector RNAi mean ± s.e.m: 25.02 ± 0.66; *cbs-1* RNAi: 22.25 ± 0.65, P<0.0001. **d,** RNAi rescued in both the intestine and germline of RNAi-deficient worms (*rde-1(ne219)*; *mex-5p*::*rde-1* strain). Empty vector RNAi mean ± s.e.m: 26.97 ± 0.79; *cbs-1* RNAi: 22.72 ± 1.00, P<0.0019. **e,** Percentage of mean lifespan change in different strains upon knockdown of *cbs-1* (relative to the respective empty vector RNAi control). Graph represents the mean ± s.e.m. from 3 independent lifespan experiments for each strain. Longevity was not further reduced when both the intestine and the germline responded to RNAi treatment when compared with knockdown in the intestine alone (KD intestine versus KD intestine + germline, P= 0.2634). Statistical comparisons were made by Student's t-test for unpaired samples. NS= no significant differences. **f,** Knockdown of *cbs-1* in the germline alone does not decrease cold-induced longevity at 15°C. RNAi rescued in the germline of RNAi-deficient worms (*rde-1(mkc36); sun-1p::rde-1::sun-1 3'UTR* strain). Empty vector RNAi mean ± s.e.m: 28.32 ± 0.55; *cbs-1* RNAi: 28.40 ± 0.64, P=0.5733. In all the lifespan experiments, P-values: log-rank test, n= 96 worms/condition. RNAi was started at day 1 of adulthood.

Altogether, our findings suggest that proliferating germ cells induce *cbs-1* in specific somatic tissues to extend lifespan at cold temperature. To assess this hypothesis, we generated a transcriptional reporter construct worm. At higher temperatures (20°C and 25°C), *cbs-1* was mostly expressed in the posterior intestine (**Fig. 23a, b**). At cold temperatures (15°C and 10°C), *cbs-1* expression increased especially in the intestine and also in the body muscles (**Fig. 23a, b**). Notably, treatment with FUdR diminished the high expression of *cbs-1* in somatic tissues induced by cold temperature (**Fig. 23a, b**). Similarly, knockdown of either *iff-1* or *mog-5* reduced somatic expression of *cbs-1* at cold temperature (**Fig. 23c, d**). Importantly, we found that somatic expression of *cbs-1* decreased when we knocked-down these factors specifically in the germline alone (**Fig. 23e, f**). Thus, our results show that proliferating germ cells regulate cold-associated expression of *cbs-1* in somatic tissues such as the intestine.

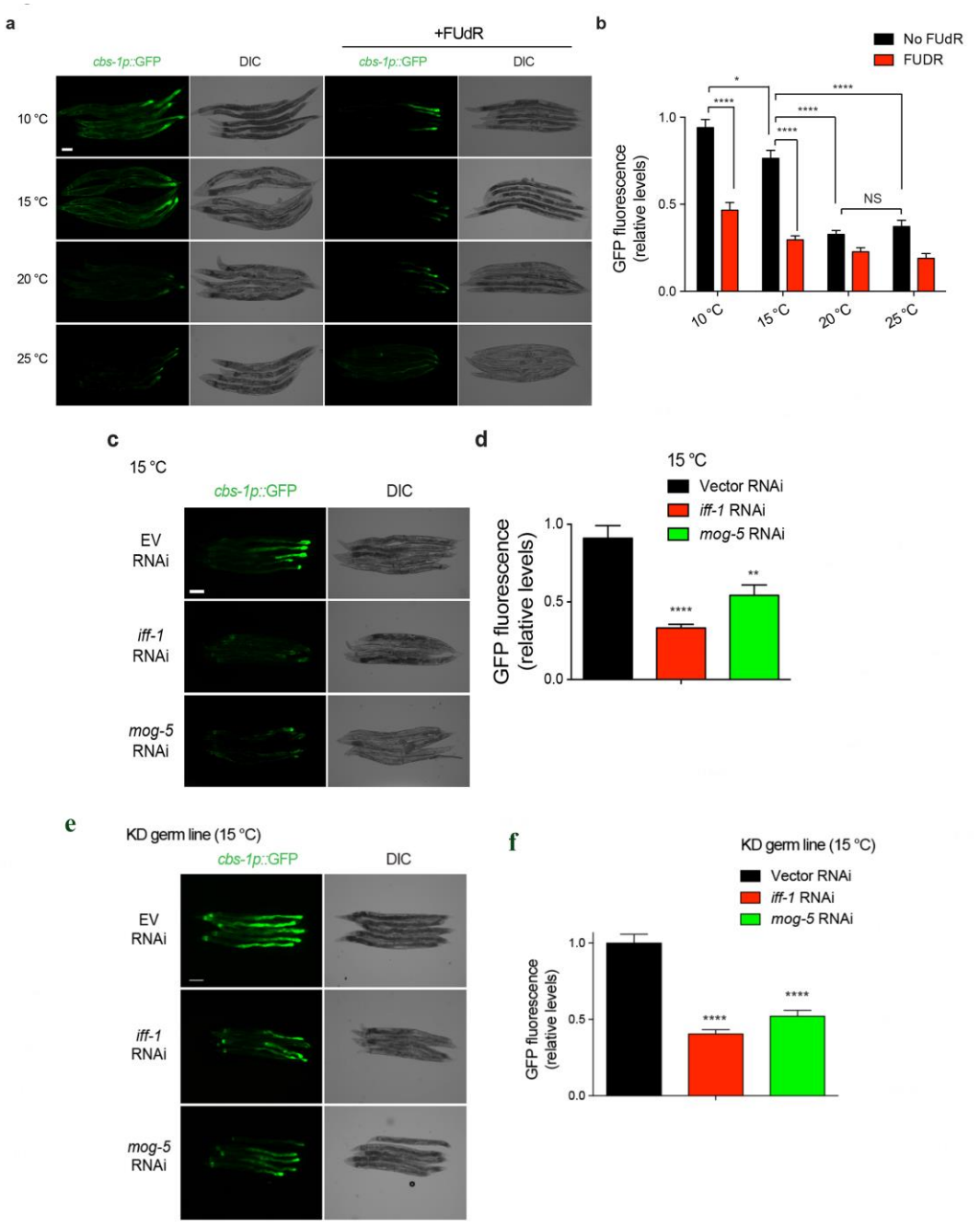


Figure 23. Robust proliferation of germ cells induces cbs-1 expression in somatic tissues at cold temperature. Ubiquitous or germline-specific knockdown of either iff-1 or mog-5 in the germline alone decreases somatic expression of cbs-1.

a, Representative images of GFP expressed under control of the *cbs-1* promoter in day 5 adult-worms. At 20°C and 25°C, *cbs-1* is mostly expressed in the posterior intestine. At cold temperatures, *cbs-1* expression increases in the intestine and muscle. FUdR treatment reduced the cold-induced expression of *cbs-1* in muscle and intestine. DIC, differential interference contrast microscopy. Scale bar, 100 µm. Images are representative of four independent experiments. **b,** Quantification of *cbs-1p::*GFP expression in animals grown at different temperatures during adulthood. Graph represents the mean ± s.e.m. relative to 10°C with no FUdR (40 worms per condition

from 4 independent experiments). **c,** *cbs-1p::GFP* expression in day 7 adult-worms at 15°C. Knockdown of either *iff-1* or *mog-5* during adulthood decreases *cbs-1* expression when compared with empty vector (EV) RNAi control. Scale bar, 50 µm. Images are representative of two independent experiments. **d,** Quantification of *cbs-1p::*GFP expression in animals fed with either *iff-1* or *mog-5* RNAi at 15°C. Graph represents the mean ± s.e.m. relative to EV RNAi (20 worms per condition from 2 independent experiments). **e,** Representative images of GFP expressed under control of the *cbs-1* promoter in germline-specific RNAi strain at day 9 of adulthood. RNAi rescued in the germline of RNAi-deficient worms (*rde-1(mkc36); sun-1p::rde-1::sun-1 3'UTR* strain). Knockdown of either *iff-1* or *mog-5* during adulthood in the germline alone decreases *cbs-1* expression in somatic tissues such as the intestine when compared with empty vector (EV) RNAi control. Scale bar, 50 µm. Images are representative of two independent experiments. **f,** Quantification of *cbs-1p::*GFP expression in germline-specific RNAi strain fed with either *iff-1* or *mog-5* RNAi at 15°C. Graph represents the mean ± s.e.m. relative to EV RNAi (18-20 worms per condition from 2 independent experiments). Statistical comparisons were made by Student's t-test for unpaired samples. P-value: *(P<0.05), **(P<0.01), ****(P<0.0001).

With the strong correlation between germline, cold-induced longevity and up-regulation of somatic expression of *cbs-1*, we asked whether intestinal overexpression of *cbs-1* rescues the short lifespan of germline-lacking worms at cold temperature. Whereas tissue-specific ectopic expression of *cbs-1* in the intestine of wild-type worms did not further increase cold-induced longevity, it extended the short lifespan of *glp-1* germline-lacking mutants (**Fig. 24a**). Additionally, intestinal overexpression of *cbs-1* also partially rescued the short lifespan of worms with impairments in adult germline proliferation induced by FUdR treatment at low temperature (**Fig. 24b**). Altogether, our data suggest that intestinal up-regulation of *cbs-1* modulated by GSC proliferation contributes to the cold-induced longevity.

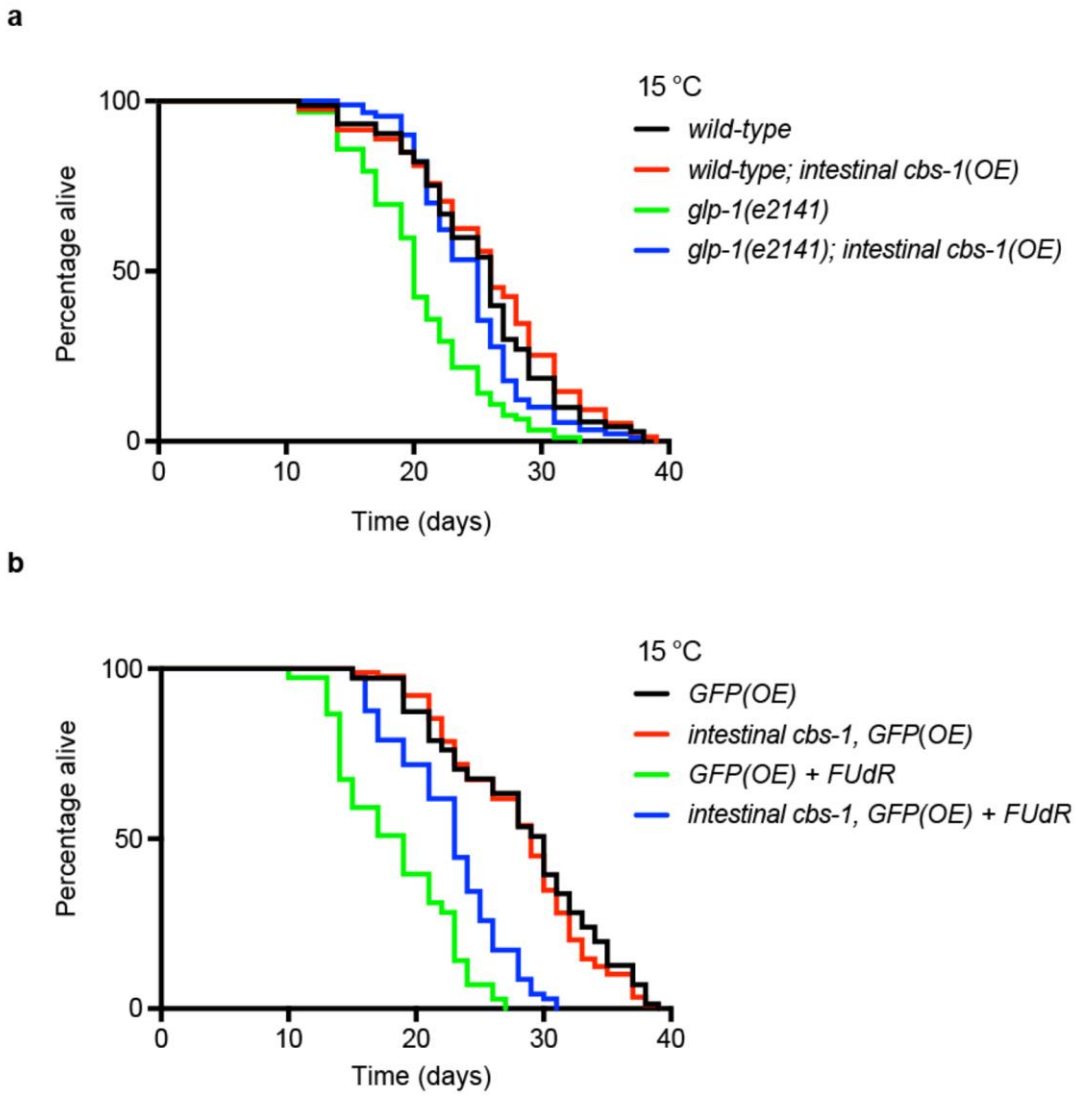


Figure 24. Intestinal overexpression of cbs-1 extends the short lifespan of germline-defective animals at cold temperature.

a, Tissue-specific overexpression (OE) of *cbs-1* in the intestine does not further increase cold-induced longevity of wild-type worms (wild-type versus wild type + intestinal *cbs-1(OE)*, P=0.3734), but it extends the short lifespan of *glp-1(e2141)* germline-lacking mutants (*glp-1* versus *glp-1* + intestinal *cbs-1(OE)*, P<0.0001). Wild-type mean ± s.e.m: 25.17 ± 0.67; wild type + intestinal *cbs-1(OE)*: 25.74 ± 0.71; *glp-1*: 20.39 ± 0.49, *glp-1* + intestinal *cbs-1(OE)*: 24.33 ± 0.46. **b,** Intestinal overexpression of *cbs-1* does not further increase cold-induced longevity of worms with an intact adult proliferating germline (*GFP(OE)* versus intestinal *cbs-1, GFP(OE)*, P=0.3964). However, it partially rescues the short lifespan of worms treated with 100 µg ml^{-1} FUdR during adulthood at cold temperature (*GFP(OE)* + FUdR versus intestinal *cbs-1, GFP(OE)* + FUdR, P<0.0001). *GFP(OE)* mean ± s.e.m: 28.34 ± 0.35; intestinal *cbs-1,GFP(OE)*: 28.06 ± 0.59; *GFP(OE)* + FUdR: 18.36 ± 0.53; intestinal *cbs-1,GFP(OE)* + FUdR: 22.66 ± 0.51. P-values: log-rank test, n= 96 worms/condition.

3.5: Up-regulated levels of *cb-1* in somatic tissues mimic low temperature conditions and extend lifespan.

CBS is a key enzyme of the transsulfuration pathway that mediates the inter-conversion of cysteine and homocysteine and its activity produces cystathionine and hydrogen sulfide (H_2S) (Hine et al., 2015; Vozdek et al., 2012). H_2S acts as a gaseous signaling molecule that decreases blood pressure (G. Yang et al., 2008) and prevents neurodegeneration in mammals (Paul & Snyder, 2012). In addition, dietary restriction results in high endogenous levels of H_2S that contribute to lifespan extension in distinct animals such as yeast, worm, fruit fly and rodents (Hine et al., 2015). Notably, exogenous addition of H_2S is sufficient to promote lifespan in *C. elegans* (Miller & Roth, 2007). Since our results indicated that up-regulated levels of *cbs-1* contribute to cold-induced longevity, we examined H_2S amounts as the product of *cbs-1* activity. Notably, low temperatures resulted in an increase in H_2S gas levels (**Fig. 25a**). Knockdown of *cbs-1* diminished the H_2S levels while its overexpression increased H_2S production at cold temperature (**Fig. 25b, c**). Inhibition of germline proliferation by knockdown of either *iff-1* or *mog-5* was also sufficient to reduce H_2S production levels at 15°C, suggesting a link between H_2S levels and cold-induced longevity modulated by adult GSCs (**Fig. 25d**).

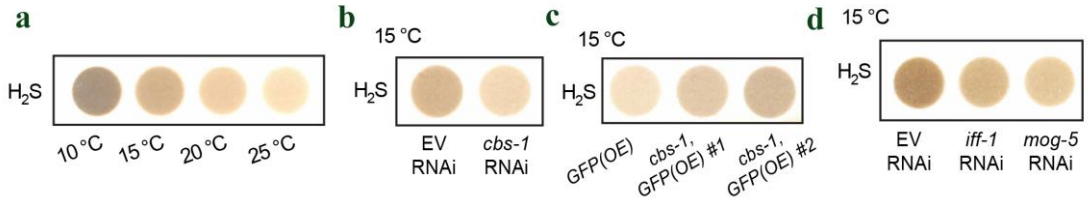


Figure 25. Low temperatures results in an increase in H2S gas levels. Inhibition of germline proliferation by knockdown of either iff-1 or mog-5 is sufficient to reduce H2S production levels at cold temperatures.

a, H_2S gas production in control sterile strain at day 6 of adulthood (*fer-15(b26);fem-1(hc17)*). Worms were raised at the restrictive temperature (25°C) and then shifted to the indicated temperatures during adulthood. H_2S production was detected by the black precipitate, lead sulfide, resulting from the specific reaction between H_2S and lead acetate. The images are representative of four independent experiments. **b,** H_2S

production in control sterile worms at day 6 of adulthood. Loss of *cbs-1* during adulthood decreases H_2S production. The images are representative of four independent experiments. **c,** Somatic overexpression (OE) of *cbs-1* increases H_2S production. Two independent *cbs-1(OE*) lines were tested. The images are representative of two independent experiments. **d,** Loss of either *iff-1* or *mog-5* during adulthood decreases H_2S production. Control sterile worms at day 6 of adulthood were examined. The images are representative of three independent experiments.

As shown in previous experiments, intestinal overexpression of *cbs-1* at cold temperature did not further extend longevity of worms with already high endogenous levels of this protein (**Fig. 24 and 26a**). Similarly, ubiquitous somatic overexpression of *cbs-1* did not significantly increase cold-induced longevity (**Fig. 26a**). Thus, we asked how ectopic somatic expression of *cbs-1* affects lifespan at higher temperatures when worms have lower endogenous *cbs-1* levels. Ubiquitous somatic overexpression of *cbs-1* extends lifespan at 20°C (Hine et al., 2015) (**Fig. 26b**). Similarly, we found that ubiquitous somatic up-regulation of *cbs-1* induced longevity even at warm temperature (25°C) (**Fig. 26c**). Although tissue-specific overexpression of *cbs-1* in the muscle and neurons extended longevity, we observed the strongest effects upon intestinal overexpression at both 20°C and 25°C (**Fig. 26d, e**). Thus, up-regulated levels of *cbs-1* in somatic tissues such as the intestine could mimic low temperature condition and prolong lifespan. Collectively, our results show that adult proliferating germ cells induce *cbs-1* expression in somatic tissues such as the intestine, a process that contributes to cold-induced longevity.

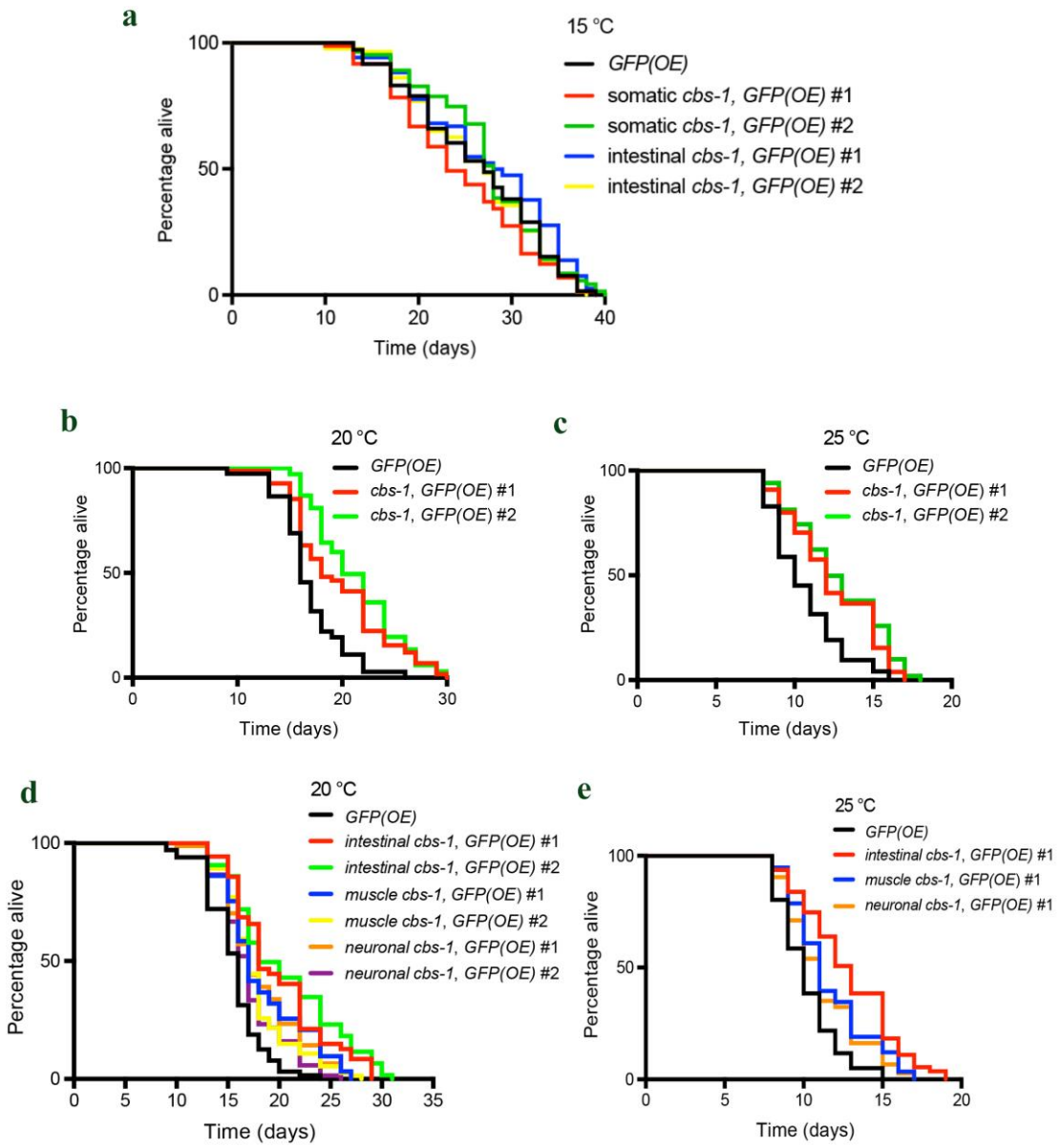


Figure 26. Neither ubiquitous somatic overexpression nor intestinal overexpression of cbs-1 further extends cold-induced longevity. Up-regulated levels of cbs-1 in somatic tissues such as the intestine mimics low temperature condition and prolongs lifespan at warm temperatures.

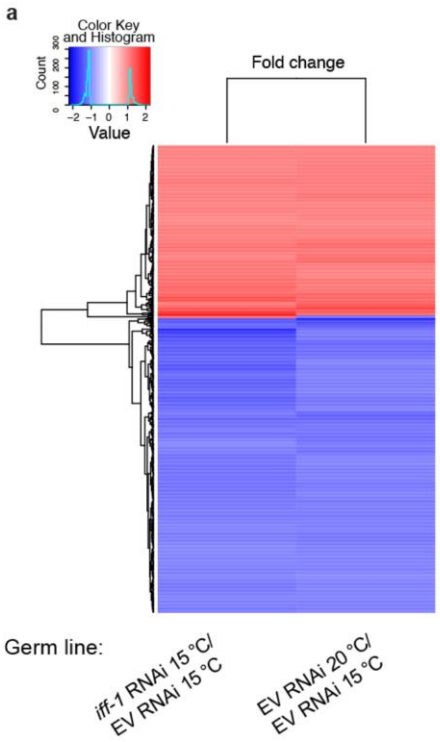
a, Ectopic ubiquitous overexpression (OE) of *cbs-1* under *sur-5* promoter does not extend cold-induced longevity. Likewise, *cbs-1* overexpression in the intestine alone under *gly-19* promoter does not increase longevity at cold temperature. *GFP(OE)* mean ± s.e.m: 26.29 ± 0.85; *cbs-1, GFP(OE)* #1: 24.57 ± 0.81, P=0.1982; *cbs-1, GFP(OE)* #2: 27.37 ± 0.75, P=0.5257; intestinal *cbs-1(OE),GFP(OE)* #1: 27.54 ± 0.83, P= 0.1330; intestinal *cbs-1(OE),GFP(OE)* #2: 26.17 ± 0.74, P=0.7372. P-values: log-rank test, n= 96 worms/condition. **b,** Ubiquitous somatic overexpression of *cbs-1* under *sur-5* promoter extends lifespan at 20°C (P<0.0001). Two independent *cbs-1(OE*) lines were tested. *GFP(OE)* mean ± s.e.m: 16.83 ± 0.36; *cbs-1, GFP(OE)* #1: 19.65 ± 0.59; *cbs-1, GFP(OE)* #2: 21.28 ± 0.49. **c,** Ubiquitous somatic *cbs-1(OE)* extends lifespan at 25°C (P<0.0001). *GFP(OE)*: 10.61 ± 0.26; *cbs-1, GFP(OE)* #1: 12.33 ± 0.35; *cbs-1, GFP(OE)* #2: 12.75 ± 0.38. **d,** Tissue-specific overexpression of *cbs-1* in the intestine (*gly-19p*), muscle (*myo-3p*) or

neurons (*rgef-1p*) extends lifespan at 20°C (P<0.0001). Intestinal-specific OE induced longer lifespan when compared with muscle or neuronal overexpression (P<0.01). *GFP(OE)*: 15.56 ± 0.34; intestinal *cbs-1(OE),GFP(OE)* #1: 19.82 ± 0.59; intestinal *cbs-1(OE),GFP(OE)* #2: 20.47 ± 0.66; muscle *cbs-1(OE),GFP(OE)* #1: 18.31 ± 0.51; muscle *cbs-1(OE),GFP(OE)* #2: 17.73 ± 0.40; neuronal *cbs-1(OE),GFP(OE)* #1: 18.11 ± 0.44; neuronal *cbs-1(OE),GFP(OE)* #2: 17.17 ± 0.36. Two independent tissue-specific *cbs-1(OE*) lines were tested for each tissue. **e,** Tissue-specific overexpression of *cbs-1* in the intestine (P<0.0001), muscle (P=0.0002) or neurons (P=0.0034) extends lifespan at 25°C. Intestinal-specific OE induced longer lifespan when compared with muscle (P=0.0142) or neuronal (P=0.0008) overexpression. *GFP(OE)* mean ± s.e.m: 10.24 ± 0.24; intestinal *cbs-1(OE),GFP(OE)* #1: 12.82 ± 0.36; muscle *cbs-1(OE),GFP(OE)* #1: 11.62 ± 0.28; neuronal *cbs-1(OE),GFP(OE)* #1: 11.25 ± 0.28. In all lifespan experiments, statistical comparisons were made by log-rank test, n= 96 worms/condition.

3.6: Prostaglandin E2 released by GSCs induces lifespan extension at cold temperatures

To determine the mechanism that adult germ cells use to induce *cbs-1* expression in the intestine at cold temperature, we performed a transcriptome analysis of extruded germlines from wild-type worms upon *iff-1* knockdown or temperature increase. When we compared the germline of worms cultured at 15°C with the germline of both *iff-1* RNAi-treated worms and 20°C-cultured worms, we found 248 up-regulated transcripts and 433 down-regulated transcripts upon RNAi treatment or temperature increase (**Fig. 27a**). Among the common down-regulated transcripts, we did not find a decrease in expression of signaling peptides (*i.e.*, nlp, flp or insulin-like peptides) or genes involved in the synthesis of sex steroid hormones (*e.g.*, pregnenolone). However, the expression of *pges-2*, the worm orthologue of human *PTGES2*, was decreased in the germline by either *iff-1* knockdown or temperature increase (**Fig. 27b**). *PTGES2* encodes a membrane-associated prostaglandin E synthase, which catalyzes the conversion of prostaglandin H2 into the more stable prostaglandin E2 (PGE2) (Kudo & Murakami, 2005). Notably, we found that levels of PGE2 increase upon temperature decrease (**Fig. 28a**). Similarly we confirmed by western blotting that worms exhibit higher protein levels of PGES-2 at cold temperature (**Fig. 28b**). Our immunohistochemistry experiments showed that PGES-2 is highly expressed in the germline compared with the intestine (**Fig. 28c**). Surprisingly, low temperature promoted the enrichment of PGES-2 in the plasma membrane of germ cells whereas at higher temperature PGES-2 was basically concentrated

in the nuclei (**Fig. 28c**). Prompted by these findings, we examined a potential link between *pges-2* and longevity at cold temperature. Notably, at cold temperature *pges-2* mutant animals lived significantly shorter when compared with wild-type worms (**Fig. 28d, e**). However, they exhibit no lifespan decrease at 20°C or 25°C (**Fig. 28f, g**). Moreover, we found that somatic expression of *cbs-1* at cold temperature significantly decline at *pges-2* mutant worms compared with wild-type animals (**Fig. 29a, b**). In contrast, *pges-2* mutation did not further decrease the low levels of *cbs-1* expression at 20°C (**Fig. 29a, b**). To further check whether the prostaglandin E synthase activity of *pges-2* is essential for cold-induced longevity, we added exogenous PGE2 hormone. Indeed, exogenous PGE2 rescued the low somatic *cbs-1* expression in *pges-2* mutants and extended their short lifespan at cold temperature (**Fig. 29 c-e**). A recent study reported that the co-chaperone *daf-41*/p23 shortens lifespan at warm temperature, while it is necessary for the longevity phenotype induced by cold temperature (Horikawa et al., 2015). Importantly, epistasis experiments showed that the co-chaperone activity of *daf-41*/p23 is involved in lifespan regulation at warm temperature, but not at cold temperature (Horikawa et al., 2015). Thus, *daf-41*/p23 may regulate cold-induced longevity via a different pathway, although the detailed mechanism is not entirely understood. Besides the co-chaperone activity, p23 exhibits prostaglandin E2 synthase activity *in vitro* (Tanioka et al., 2003). Although experiments in mice failed to support a function of p23 in PGE2 biosynthesis (Lovgren, Kovarova, & Koller, 2007a), we checked the potential PGE2 synthase activity of *daf-41*/p23 that might regulate the pro-longevity effects of the germline at cold temperature. We found that ubiquitous or germline-specific knockdown of *daf-41*/p23 did not reduce the somatic up-regulation of *cbs-1* induced by cold temperature (**Fig. 30 a-d**). Besides, germline specific knockdown of *daf-41*/p23 did not shorten longevity at cold temperature (**Fig. 30 e**). Altogether, our results support an essential role of *pges-2* in the germline to modulate cold-induced longevity, while *daf-41*/p23 is dispensable for the longevity phenotype induced by GSCs.



b

	EV-RNAi 20°C/EV-RNAi 15°C		*iff-1*-RNAi 15°C/EV-RNAi 15°C	
	Fold change	P-value	Fold change	P-value
pges-2	-1.23	<0.0001	-1.17	<0.0001

Figure 27. The transcript levels of pges-2 decrease in the germline upon iff-1 knockdown at 15°C or temperature increase.

a, The heat map presents the 681 transcripts changed in the same direction in the germline of both *iff-1* RNAi-treated worms at 15°C and empty vector (EV) RNAi-treated worms at 20°C when compared with the germline of EV RNAi-treated worms at 15°C. Among them, 248 transcripts were up-regulated whereas 433 were down-regulated. n= 3 (each replicate contains 150 extruded germlines from N2 wild-type worms), P-value <0.01 was considered significant. Germlines were extruded at day 6 of adulthood. **b,** Fold-change of *pges-2* expression from the RNA-sequencing data. *pges-2* transcript levels decrease in the germline upon either *iff-1* knockdown or temperature increase when compared with the germline of control worms at 15°C.

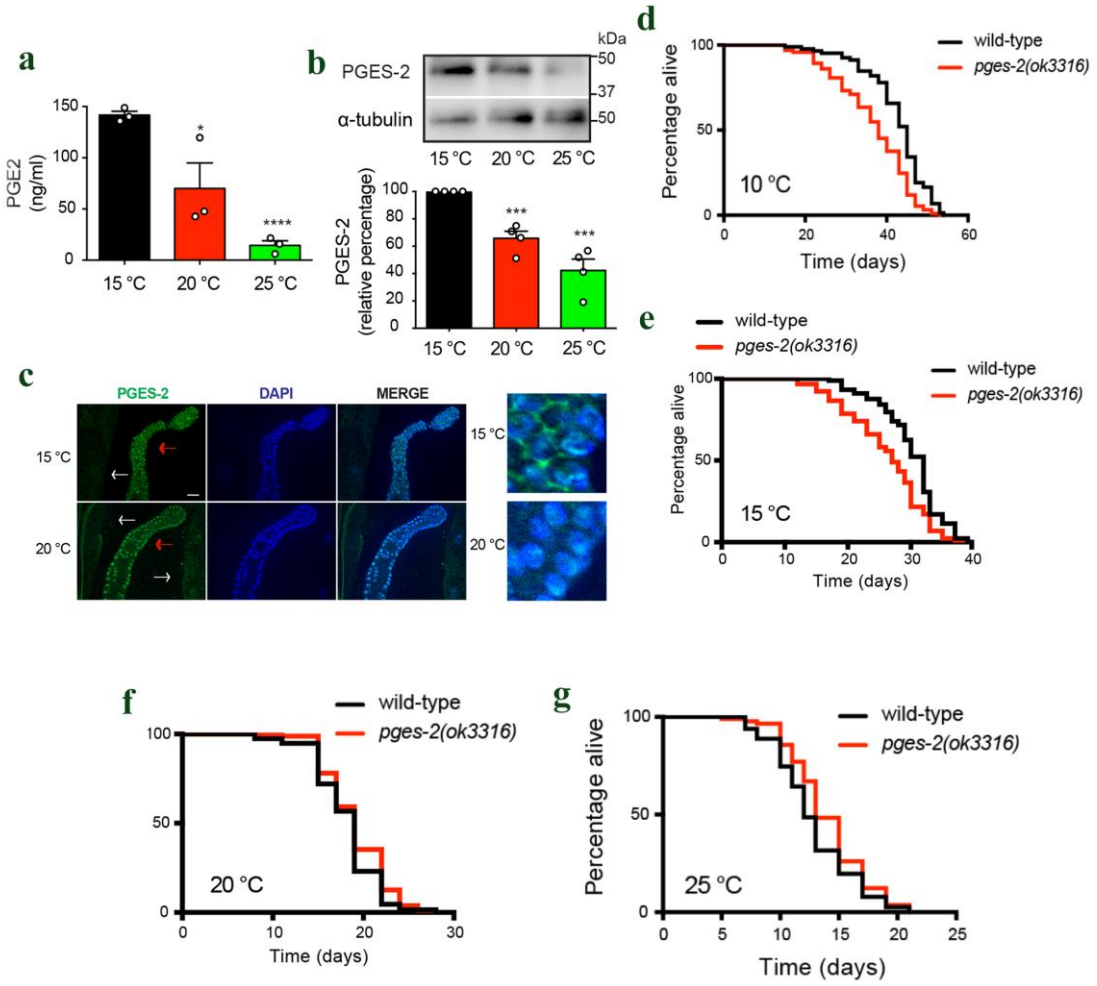


Figure 28. Release of prostaglandin E2 by GSCs promotes longevity at cold temperatures. pges-2(ok3316) are short lived at cold temperatures, but not at standard and warm temperatures.

a, Quantification of prostaglandin E2 (PGE2) levels in *fer-15*;*fem-1* control sterile worms at day 6 of adulthood (8.4 mg worms/ml). Graph represents the mean ± s.e.m. from 3 independent experiments. **b,** Western blot analysis of control sterile worms at day 6 of adulthood with antibodies to PGES-2 and α-tubulin loading control. The graph represents the PGES-2 relative percentage values (corrected for α-tubulin loading control) to 15°C (mean ± s.e.m. of four independent experiments). **c,** Immunostaining with PGES-2 antibody of extruded germline and intestine from day 6-adult N2 wild-type worms at 15°C and 20°C. Cell nuclei were stained with DAPI. White arrows and red arrows indicate intestine and germline, respectively. On the right, a higher magnification of germ cells is presented. The images are representative of three independent experiments. Scale bar represents 20 μm. **d,** *pges-2(ok3316)* are short lived at cold temperature (10°C) compared with N2 wild-type animals (*pges-2* 10°C mean ± s.e.m: 42.55 ± 0.87 versus *N2* 10°C mean ± s.e.m: 36.50 ± 0.94, P<0.0001). **e,** *pges-2(ok3316)* are short lived at cold temperature (15°C) compared with wild-type animals (*pges-2* 15°C mean ± s.e.m: 30.10 ± 0.52 versus *N2* 15°C: 26.09 ± 0.66, P<0.0001). **f,** *pges-2* mutant worms do not live shorter at 20°C (*pges-2* 20°C: 18.13 ± 0.41 versus *N2* 20°C

19.11 ± 0.37, P=0.1303). **g,** *pges-2* mutant worms do not live shorter at warm temperatures (25°C) (*pges-2* 25°C: 12.83 ± 0.38 versus *N2* 25°C: 13.99 ± 0.36, P=0.0491). Student's t-test for unpaired samples (P-value: * (P<0.05), *** (P<0.001), ****(P<0.0001)).

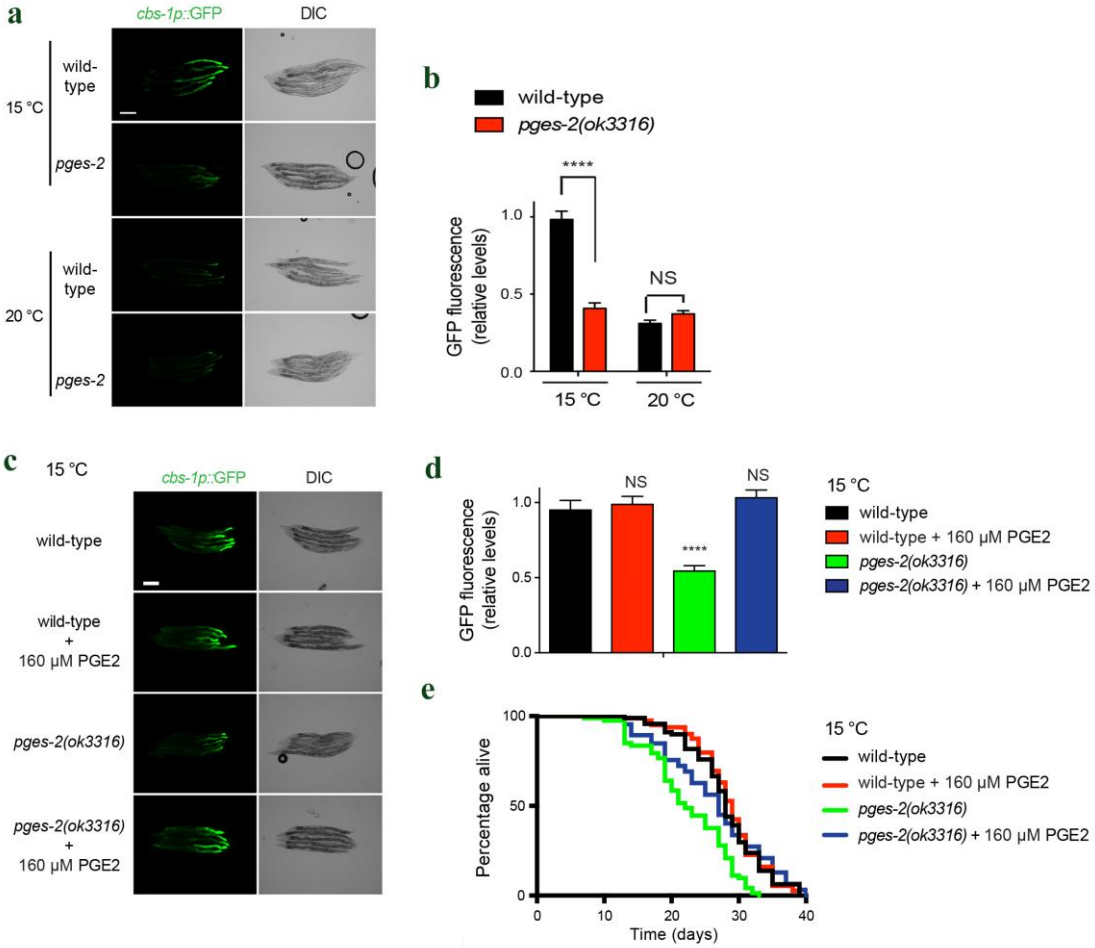


Figure 29. Exogenous PGE2 hormone rescues the low somatic expression of cbs-1 in pges-2 mutants and extends their short lifespan at cold temperature.

a, Representative images of GFP expressed under control of the *cbs-1* promoter in day 5 adult wild-type and *pges-2(ok3316)* mutant worms. DIC, differential interference contrast microscopy. Scale bar represents 1000 µm. Images are representative of three independent experiments. **b,** Quantification of *cbs-1p::*GFP expression in day 5-adult animals. Graph represents the mean ± s.e.m. relative to 15°C wild-type N2 worms (30 worms per condition from 3 independent experiments **c,** Representative images of GFP expressed under control of the *cbs-1* promoter in day 8 adult-worms at 15°C. Application of exogenous PGE2 rescues low expression of *cbs-1* in *pges-2(ok3316)* mutants. DIC, differential interference contrast microscopy. Scale bar represents 200 µm. Images are representative of two independent experiments. **d,** Quantification of *cbs-1p::*GFP expression in day 8-adult animals at 15°C. Graph represents the mean ±

s.e.m. relative to non-treated wild-type worms (12-15 worms per condition from 2 independent experiments). **e,** Application of exogenous PGE2 extends the short lifespan of *pges-2(ok3316)* mutants at cold temperature (*pges-2* versus *pges-2* +PGE2 hormone, P<0.0001). N2 mean ± s.e.m: 28.07 ± 0.59; N2 + PGE2: 28.60 ± 0.58; *pges-2*: 22.43 ± 0.73; *pges-2* + PGE2: 26.54 ± 0.95. P-values: log-rank test, n= 96 worms/condition. Statistical comparisons were made by Student's t-test for unpaired samples. P-value: ****(P<0.0001), NS (not significant).

Since *pges-2* mutants had standard number of proliferating germ cells (**Fig. 31 a, b**), our results show that *pges-2* activity in the germline modulates cold-induced longevity by affecting somatic tissues rather than directly influencing proliferation of germ cells. The specific transporter ABCC4 (ATP binding cassette subfamily C member 4), mediates the release of prostaglandin from cells (Kochel & Fulton, 2015). Knockdown of *mrp-6*, the worm orthologue of ABCC4, reduced cold-induced longevity (**Fig. 31c left**). Conversely, down-regulation of *mrp-6* did not shorten lifespan of worms at 20°C (**Fig. 31c right**). Whereas exogenous PGE2 hormone did not further extend cold-induced longevity of control worms, it rescued the short lifespan of worms with *mrp-6* RNAi treatment at cold temperature (**Fig. 31d**). Tissue-specific knockdown of *mrp-6* in either the intestine or neurons did not affect lifespan at cold temperature (**Fig. 31 e, f**), but importantly we found a decrease in cold-induced longevity when the RNAi was efficient in the germline (**Fig. 31g, h**). Thus, our results suggest that PGE2 released by the germline modulates cold-induced longevity. To test this hypothesis, we treated the adult GSC with FUdR to inhibit the proliferation and examined intestinal *cbs-1* expression upon application of exogenous PGE2 hormone. Remarkably, exogenous PGE2 application was sufficient to rescue the reduced expression of intestinal *cbs-1* induced by FUdR treatment (**Fig. 32 a, b**). Similarly, exogenous PGE2 rescued the decrease in cold-induced longevity induced by loss of GSC proliferation (**Fig. 32c**). Also, exogenous PGE2 extended the short lifespan of *glp-1* germline-lacking worms at cold temperature (**Fig. 32d**). Altogether, our data show that the germline communicates with somatic tissues via PGE2 signals to extend longevity at cold temperature (**Fig. 33**).

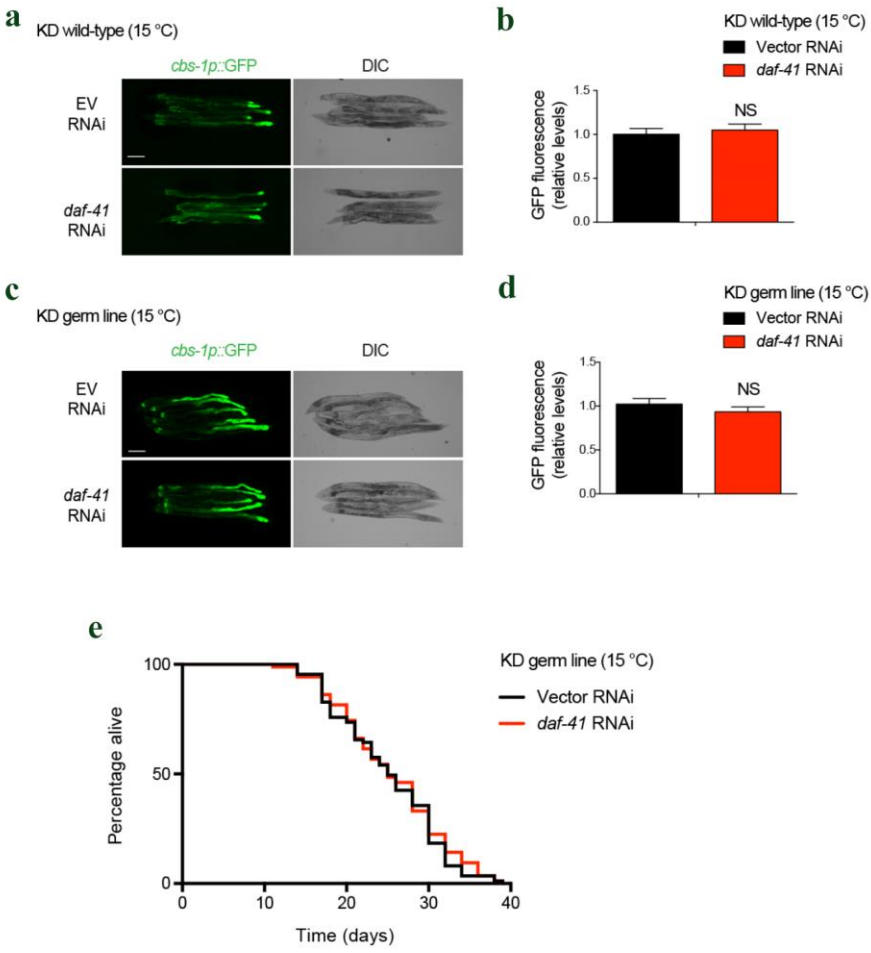


Figure 30. Ubiquitous or tissue-specific knockdown of daf-41 in the germline alone does not impair cold-induced cbs-1 somatic expression and longevity.

a, Representative images of GFP expressed under control of the *cbs-1* promoter in N2 wild-type worms at day 9 of adulthood. Knockdown (KD) of *daf-41* during adulthood does not decrease *cbs-1* expression in somatic tissues compared with empty vector (EV) RNAi control. Scale bar, 50 µm. Images are representative of two independent experiments. **b,** Quantification of *cbs-1p::*GFP expression in N2 worms treated with *daf-41* RNAi at 15°C. Graph represents the mean ± s.e.m. relative to EV RNAi (15 worms per condition from 2 independent experiments). NS= no significant differences (P=0.6723, Student's t-test for unpaired samples). **c,** Representative images of *cbs-1p::*GFP in germline-specific RNAi strain at day 6 of adulthood. RNAi rescued in the germline of RNAi-deficient worms (*rde-1(mkc36); sun-1p::rde-1::sun-1 3'UTR* strain). Scale bar, 50 µm. Images are representative of two independent experiments. **d,** Quantification of *cbs-1p::*GFP expression in germline-specific RNAi strain fed with *daf-41* RNAi at 15°C. Graph represents the mean ± s.e.m. relative to EV RNAi (15 worms per condition from 2 independent experiments). NS= no significant differences (P=0.3179). **e,** Germline-specific knockdown of *daf-41* does not affect lifespan at 15°C (Empty vector RNAi: 25.10 ± 0.67; *daf-41* RNAi: 25.49 ± 0.72, P=0.5938). P-value: log-rank test, n= 96 worms/condition.

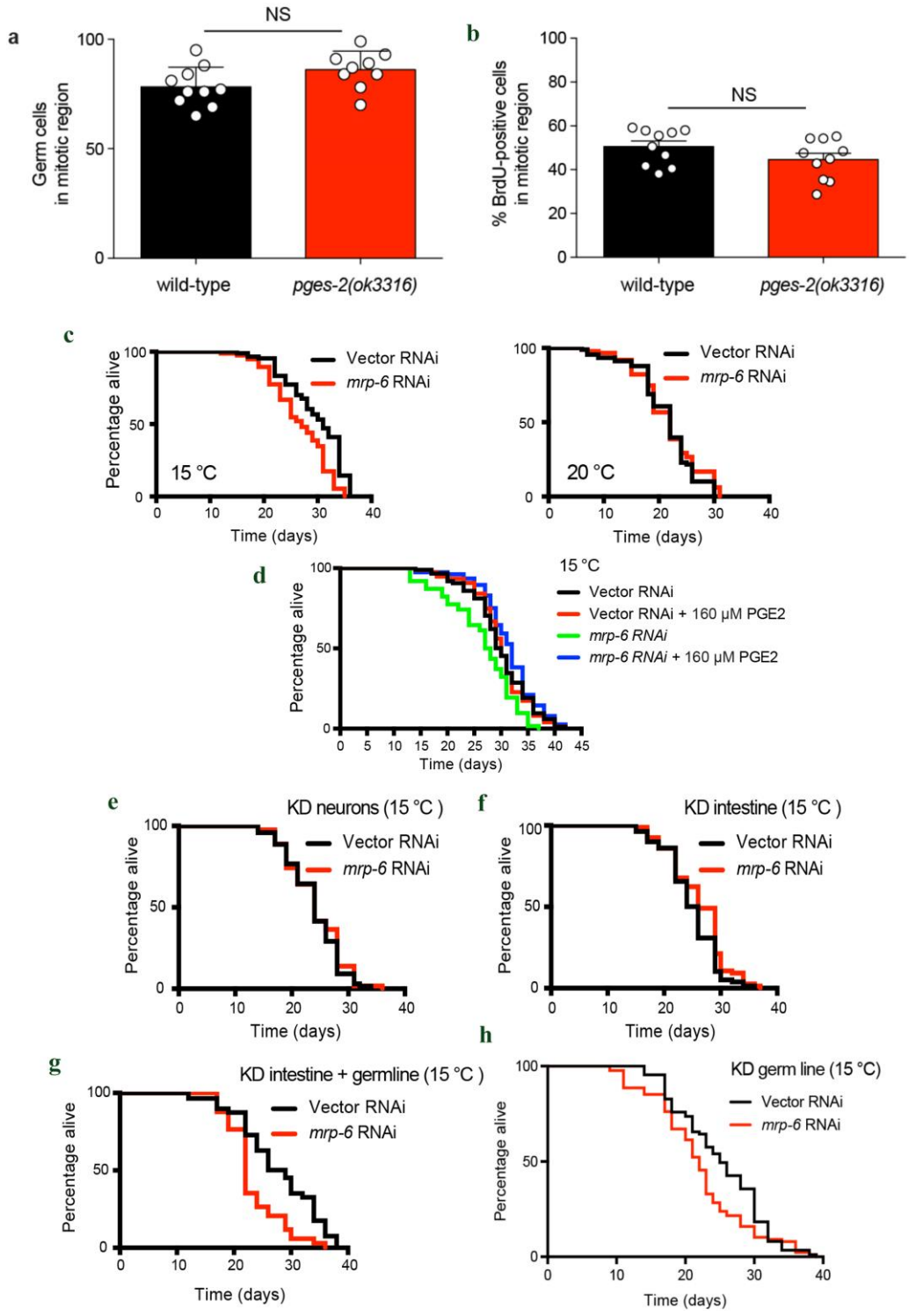


Figure 31. pges-2 mutants have normal numbers of proliferating germ cells within the mitotic region. Exogenous PGE2 does not further extend cold-induced longevity of control worms, but rescues the short lifespan phenotype of mrp-6

RNAi-treated worms at cold temperature. Tissue-specific knockdown of mrp-6 in the germline alone decreases cold-induced longevity.

a, Graph represents the total number of germ cells in the mitotic region quantified by DAPI staining (mean ± s.e.m., 10 germlines scored per condition from 2 independent experiments). Student's t-test for unpaired samples, P= 0.0582. **b,** Graph represents the percentage of BrdU-positive cells/total nuclei (mean ± s.e.m., 10 germlines scored per condition from 2 independent experiments). Student's t-test for unpaired samples, P= 0.1483. Worms were examined at day 6 of adulthood. NS= no significant differences. **c,** Loss of *mrp-6* decreases lifespan of wild-type worms at 15°C (left) (Empty vector RNAi mean ± s.e.m: 29.64 ± 0.59; *mrp-6* RNAi: 26.66 ± 0.61, P<0.0001). In contrast, *mrp-6* RNAi does not decrease lifespan 20°C (right) (Empty vector RNAi: 21.23 ± 0.58; *mrp-6* RNAi: 21.58 ± 0.64, P=0.3707). RNAi was initiated during adulthood. **d,** Application of exogenous PGE2 does not further increase cold-induced longevity of control worms (Vector RNAi mean ± s.e.m: 29.85 ± 0.61 versus Vector RNAi + PGE2: 29.98 ± 0.59, P=0.9106), but it extends the short lifespan of *mrp-6* RNAi-treated worms (*mrp-6* RNAi: 26.37 ± 0.82 versus *mrp-6* RNAi + PGE2: 31.43 ± 0.60, P<0.0001). RNAi was initiated at day 1 of adulthood. P-values: log-rank test, n= 96 worms/condition. **e,** Neuronal-specific KD of *mrp-6* does not affect longevity at 15°C (Empty vector RNAi: 23.87 ± 0.56; *mrp-6* RNAi: 24.18 ± 0.61, P=0.5119). **f,** Intestinal-specific KD of *mrp-6* does not affect longevity at 15°C (Empty vector RNAi: 24.86 ± 0.51; *mrp-6* RNAi: 26.16 ± 0.57, P=0.0531). **g,** When the germline is sensitive to RNAi treatment, knockdown of *mrp-6* reduces longevity at 15°C (Empty vector RNAi: 27.77 ± 1.03; *mrp-6* RNAi: 23.32 ± 0.78, P=0.0007). **h,** Germline-specific knockdown (KD) of *mrp-6* shortens lifespan at 15°C (Empty vector RNAi: 25.10 ± 0.67; *mrp-6* RNAi: 22.07 ± 0.74, P=0.0184). P-value: log-rank test, n= 96 worms/condition.

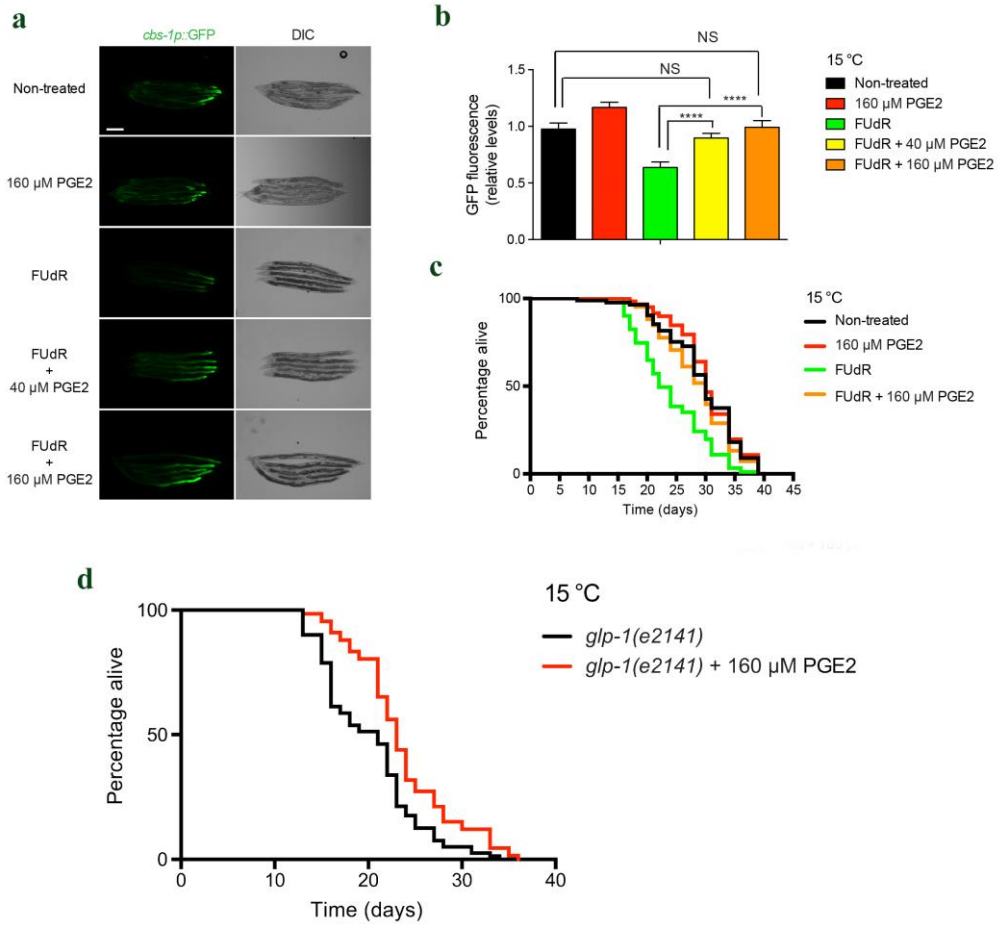


Figure 32. Application of exogenous PGE2 rescues proliferation of GSCs and extends lifespan after loss of GSC proliferation. Exogenous PGE2 extends lifespan of glp-1 germline-lacking worms at cold temperature.

a, Representative images of GFP expressed under control of the *cbs-1* promoter in day 8 adult-worms at 15°C. Application of exogenous PGE2 rescues low expression of *cbs-1* caused by FUdR treatment (100 µg ml^{-1}). PGE2 was added from day 5 of adulthood. DIC, differential interference contrast microscopy. Scale bar represents 200 µm. Images are representative of three independent experiments. **b,** Quantification of *cbs-1p::*GFP expression in day 8-adult animals at 15°C. Graph represents the mean ± s.e.m. relative to non-treated worms (50-65 worms per condition from 3 independent experiments). Statistical comparisons were made by Student's t-test for unpaired samples. P-value: ****(P<0.0001), NS (not significant). **c,** FUdR treatment reduces lifespan of wild-type worms at cold temperature (Non-treated versus FUdR, P<0.0001), a phenotype rescued by application of exogenous PGE2 (PGE2 + FUdR versus FUdR, P<0.0001). Non-treated mean ± s.e.m: 29.30 ± 0.69; PGE2: 30.39 ± 0.70, FUdR: 22.98 ± 0.63, FUdR + PGE2: 28.38 ± 0.64. In all lifespan experiments, P-values: log-rank test, n= 96 worms/condition. **d,** Lifespan analysis of *glp-1(e2141)* germline-lacking mutants treated with PGE2 hormone at 15°C (*glp-1* mean ± s.e.m: 20.08 ± 0.57; *glp-1* + PGE2: 23.66 ± 0.64, P=0.0004). P-value: log-rank test, n= 96 worms/condition.

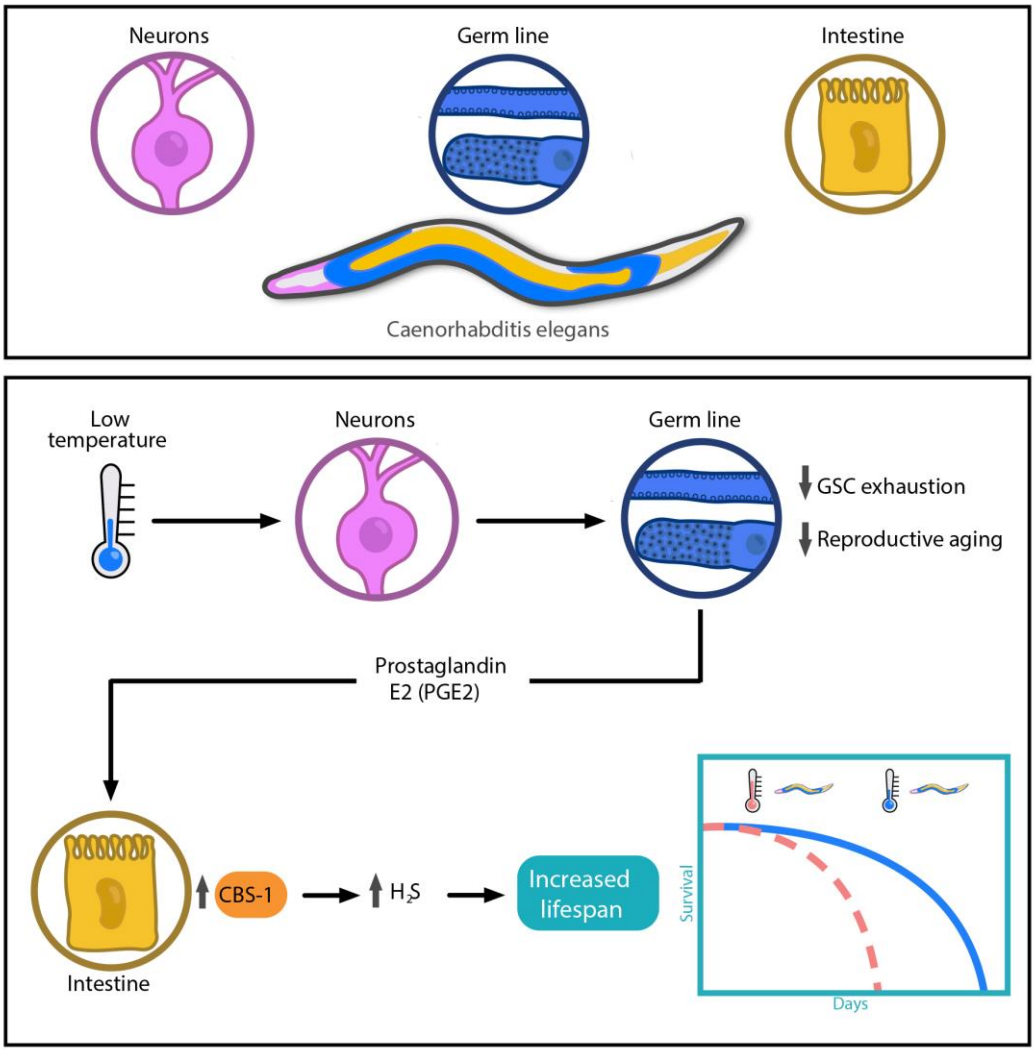


Figure 33. Model of cell-non-autonomous communication between somatic tissues and germline at cold temperature.

Somatic tissues such as IL1 and AFD neurons detect low temperature and ameliorate adult GSC exhaustion, which in turns delays reproductive aging. Adult GSCs release PGE2 hormone to induce *cbs-1* expression in the intestine, resulting in somatic production of pro-longevity H_2S gas. The rewiring of somatic tissues by GSCs extends organismal lifespan. This process coordinates extended reproductive capacity with long lifespan under advantageous conditions.

4: Discussion

In any living organism, a complex network of interconnected cell non-autonomous events regulates the process of aging, as distinct tissues can influence the aging of distal organs (Vozdek et al., 2012). A critical point in studying the biology of aging is understand how cells and tissues receive internal or external stimuli and that how they transfer this information to the neighboring tissues or tissues beyond direct contact (Rosello-Diez et al., 2018)

In this intricate biological system, the germline has a principal role in the regulation of somatic aging (G. Yang et al., 2008). In these lines, previous studies showed that the germline actively promotes the deterioration of the soma during the aging process (Liu & Walford, 1966; G. Yang et al., 2008). The integrity of tissues is constantly challenged by environmental or metabolic conditions and food sources can be also largely unpredictable and insufficient in nature. Thus, biological systems may facilitate a compromised distribution of limited resources to protect the germline ensuring the fitness of the progeny in line with the disposable soma theory of aging. In other words, given limited resources, the more the animals invest on body maintenance and its repair, the less the resources remaining for reproduction and sustaining healthy progeny, and vice versa (Ljubuncic & Reznick, 2009).

However, in our studies we found an unexpected role of the germline in the long lifespan phenotype induced by cold temperature, challenging the existing theories about germline and soma trade-off. Here we showed that cold temperature does not extend lifespan in germline lacking mutants, providing us the first indication that this tissue is essential for cold-induced longevity. Ablation of the germline from development might induce numerous changes, but our results indicate that this tissue is particularly required during adulthood for cold-induced longevity. Intrigued by these results, we examined whether temperature-associated changes determine the fitness of the adult germline and how these changes impinge upon cold-induced longevity. We found that besides its pro-longevity effects, low temperature also slows down reproductive aging, so that the organism has a temporally extended reproductive period. This process arises from a delay in the age-associated exhaustion of adult GSCs.

Notably, maintenance of GSC function is determined by the TRPA-1 channel, which can be directly activated by temperature reduction. TRPA-1 activation in

IL1 neurons is enough to detect low temperature, but still besides IL1 neurons, we find that dysregulation of thermosensory AFD neurons also shortens lifespan and germline proliferation at cold temperatures. Impairment of AFD neurons at standard or warm temperatures does not decrease lifespan. Since AFD neurons do not express TRPA-1 channel (Morita & Tilly, 1999), it will be fascinating to examine how these cells sense and process low temperature to communicate with the germline. It is known that TAX-2/TAX-4 channel in AFD neurons need intercellular signaling for activation and these are not temperature-gated per se (Ramot et al., 2008). Thus we speculate that AFD neurons constitutively release pro-longevity signal(s) when they are inactive or at a low activity state under cold temperature conditions. Conversely, AFD activation by temperature increase may prevent the release of this pro-longevity factor. Besides their effects at cold temperature, AFD neurons also induce heat shock response under heat stress conditions (>30°C) (Paul & Snyder, 2012). Although we did not observe effects of AFD neurons in lifespan at moderately warm temperature (25°C), other studies reported that ablation of these neurons can reduce survival at 25°C (Hsu, Murphy, & Kenyon, 2003). In our experiments, worms were maintained and raised at 20°C during development before shifting them to distinct temperatures to assess adult lifespan. In contrast, worms were cultured in the aforementioned study at a given experimental temperature for at least two generations before adult lifespan analysis at this specific temperature. Moreover, they performed lifespan experiments on FUdR to inhibit progeny, while we avoided this treatment given its effects on germline proliferation. Although it is difficult to directly compare both studies because of the divergent experimental conditions, these differences raise the intriguing possibility that adaptation to temperature and other conditions can also influence how AFD neurons process temperature signals to modulate lifespan.

Whereas our data indicate that somatic tissues such as IL1 and AFD neurons detect and process temperature reduction to delay GSC exhaustion and extend reproductive capacity, we find that GSCs, in turn, promote somatic fitness of distal tissues and organismal longevity at low temperatures. To assess the requirement for adult GSCs in cold-induced longevity, we first treated adult worms with FUdR, which is an inhibitor of DNA synthesis and targets the only

pool of mitotic cells in the adult worms, i.e. GSCs. FUdR is extensively used to inhibit progeny at standard temperatures because it does not affect longevity of wild-type worms at these temperatures (Arantes-Oliveira et al., 2002; Kudo & Murakami, 2005; Lovgren, Kovarova, & Koller, 2007b). We found that FUdR inhibits GSC proliferation at both 20°C and 15°C, but this demise of adult GSCs shortens lifespan only at cold temperature. However, caution is needed to interpret these results. Besides its effects on germline proliferation, FUdR treatment also improves proteostasis (Kochel & Fulton, 2015). In addition, FUdR affects bacterial ribonucleotide metabolism and has different effects depending on the *E. coli* strain used to feed the worms, modulating host-microbiome co-metabolism in *C. elegans* (Taylor, Berendzen, & Dillin, 2014). To avoid these potential side effects of FUdR, we further validated our results by knocking down the modulators of GSC proliferation during adulthood. For instance, we knocked down the expression of *iff-1*, a regulator of germline proliferation, which is only expressed in this tissue (Puoti & Kimble, 1999). Loss of *iff-1* during adulthood blocks cold-induced longevity, without altering lifespan at warmer temperatures. Importantly, knockdown of *iff-1* does not affect fertility in early adult stages while it has a strong effect in GSC proliferation at later stages, supporting a specific signaling role of adult GSCs in cold-induced longevity.

We then identified that robust proliferation of GSCs up-regulates proteostasis components (e.g., *cct* subunits and *hsp-1*) as well factors such as gaseous H2S. Different studies support this idea that H_2S has direct effect on aging and age-related diseases (Zhan et al., 2018). This signaling gaseous molecule is produced by *cbs-1*, and its expression is induced in somatic tissues such as the intestine by adult GSCs at low temperatures. Notably, intestinal *cbs-1* overexpression is sufficient to extend longevity even at warm temperatures, mimicking cold-temperature conditions. It is important to note that intestinal *cbs-1* overexpression does not completely rescue the pronounced short-lived phenotype of worms with defects in adult germline proliferation. This partial rescue upon intestinal *cbs-1* overexpression could be explained by different possibilities. Besides its effects in the intestine, cold temperature also triggers *cbs-1* expression in the muscle. In these lines, muscle-specific knockdown of *cbs-1* decreases cold-induced longevity. Therefore, up-regulation of *cbs-1* in the

muscle could also contribute to cold-induced longevity. In addition, our results indicate that *cbs-1* and its gaseous product (H2S) are not the only factors involved in cold-induced longevity and upregulation of other factors can also contribute to this phenotype. For instance, loss of *cct* subunits in the intestine and neurons as well as knockdown of *hsp-1* in neurons diminishes cold-induced longevity.

Interestingly, it has been reported that *glp-1* germline-lacking mutant worms also exhibit increased levels of *cct* subunits and H2S production in somatic tissues at standard and warm temperatures, contributing to lifespan extension under these conditions (Horikawa et al., 2015; Loeb & Northrop, 1916). Thus, a fascinating possibility is that germ cells can either promote or inhibit the same downstream pathways or elements in somatic tissues depending on the environmental and physiological conditions. In addition, depletion of the germline from development could induce additional physiological changes when compared with inhibition of GSC proliferation after the adult germline is formed. Our experiments in germline-lacking models were a first indication of a potential role of the germline in cold-induced longevity, as we further validated by rescue experiments overexpressing *cbs-1* in the intestine of these worms. However, absence of the germline cannot be directly equated with reduced adult GSC proliferation. In these lines, we observe that either *iff-1* or *glp-1* germline-lacking mutants are long-lived at 20°C, whereas *iff-1* RNAi in adult wild-type worms does not extend lifespan at this temperature.

Little is known about the specific signaling molecules by which the germline communicates with somatic tissues. At cold temperature, our findings establish a role of PGE2 release by adult GSCs in extended longevity. In worms PGE2 is produced by *pges-2*, which is a membrane-associated prostaglandin E synthase (Kudo & Murakami, 2005). Thus, this hormone could be an important determinant of long lifespan under specific conditions such as low temperature. Prostaglandins are lipid compounds derived from arachidonic acid. Interestingly, administration of arachidonic acid increases resistance of *C. elegans* to starvation and extends life span in favorable conditions of food abundance (Ossewaarde et al., 2005). It is important to note that alterations in the synthesis or release of PGE2 do not completely block cold-induced longevity. Therefore, we speculate

that other signaling pathways might also contribute to modulation of somatic tissues by germ cells at cold temperature.

Taken together, our results suggest a cell non-autonomous mechanism (i.e., neurons-germline-intestine axis) that coordinates extended reproductive capacity with long lifespan under cold temperature, without the need of sacrificing either the germline or the soma. Since it is generally considered that the germline and fertility induce somatic aging, this unexpected pro-longevity role of adult GSCs have important implications for the understanding of the aging process. Indeed, our results raise the intriguing possibility that germ cells activate distinct signals to differentially modulate somatic tissues depending on the physiological and environmental conditions. This could be of particular interest for our ever-aging society, because several studies indicate that women who have later menopause tend to live longer as well as reduced risk of cardiovascular disease and less loss of bone density (Brenner, 1974; Shadyab et al., 2017; Wei & Kenyon, 2016). An intriguing question is why the germline promotes longevity at cold temperature. In contrast to high temperatures, low temperature reduces the rate of chemical reactions and molecular entropy, a process that may decrease the damage and deterioration of distinct tissues triggered by cellular metabolism. Concomitantly, this process could ameliorate the pressure to prevent and repair damage to tissues. We speculate that these conditions facilitate the distribution of resources, reducing the need to compromise on either the soma or the germline. Cold temperatures are detected by thermosensory neurons that delay exhaustion of GSCs, resulting in the ability to reproduce at older ages. Since this process may also require a healthy soma, we speculate that GSCs actively communicate with other tissues such as the intestine to ensure the fitness of the organism and the ability to reproduce for extended periods of time.

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Erklärung

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Alireza Noormohammadi

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Alireza Noormohammadi

05.02.1988, Mashhad, Iran
Address: Bernkasteler Str. 52, Wg 2.1, 50969, Cologne, Germany
anoormoh@uni-koeln.de, Tel: +49-17623677296

Studies

05.2015	PhD student at CECAD institute of ageing research, university of Cologne, Germany
09.2010 - 09.2013	M.Sc. in Plant Systematics and Ecology, Tehran University, Iran, GPA: **17.35** / **20** (Top student)
09.2006 - 06.2010	B.Sc. in Biology, Faculty of Biological Sciences, Shahid Beheshti University, Iran, GPA: **17.66** / **20** (Top student)

Research Activities

05.2015 - present	PhD thesis: Investigating the role of Prostaglandin signals from adult germ stem cells on somatic aging delay of *Caenorhabditis elegans* at cold temperatures
05.2011 - 09.2013	M.Sc. Thesis: Anatomical studies in Tamaricales and their phylogenetical and ecological significance, score: **19.75** / **20**

Publications

2019	Hyun Ju Lee, **Noormohammadi, A.,** Koyuncu, S., Calculli, G., Simic, M., Herholz, M., Trifunovic, A., Vilchez, D. Prostaglandin signals from adult germline stem cells delay somatic aging of *Caenorhabditis elegans*. **Nature Metabolism**
2019	Akhani, H., Samadi, N., **Noormohammadi, A**., Borsch, T. A new species of *Tamarix* (*Tamaricaceae*) from Hormozgan Province, S Iran, supported by morphology and molecular phylogenetics. **Willdenowia**

2018 **Noormohammadi, A.**, et al. Mechanisms of protein homeostasis (proteostasis) maintain stem cell identity in mammalian pluripotent stem cells. **Cellular and Molecular Life Sciences**

2016 **Noormohammadi, A**., et al. Somatic increase of CCT8 mimics proteostasis of human pluripotent stem cells and extends *Caenorhabditis elegans* lifespan. **Nature Communications**

2012 Minai-Tehrani D, Tavakoli Tameh A, Rashidfarokhi A, **Noormohammadi A**, Khodakarami A, Talebi M. 2012. The Effect of light crude oil contaminated soil on the growth and germination of *Sorghum bicolor*. The **European Journal of Plant Science and Biotechnology**

Attended Conferences

09.2019 Oral and poster presentation: Prostaglandin signals from adult germline stem cells delay somatic aging of *Caenorhabditis elegans*. FEBS conference of Aging and Regeneration, Innsbruck, Austria

09.2016 Poster presentation: Somatic increase of CCT8 mimics proteostasis of embryonic stem cells and extends organismal longevity in *Caenorhabditis elegans*. 4th Annual Conference of the German Stem Cell Network (GSCN), Hannover, Germany

09.2012 Oral presentation on “Anatomical studies of leaves and young branches in Tamaricales” in the International Symposium of Caryophyllales, Moscow, Russia