HEINRICH HEINE
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Partial frataxin suppression in *Caenorhabditis elegans* reduces tumor formation by dampening the RAS/MAPk signaling

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“Science without religion is lame; religion without science is blind.”

— Albert Einstein

Table of Contents

Abbreviations

µM	Micrometer
AAK-2	AMP-Activated Kinase
ADP	Adenosine diphosphate
Afd-1	Actin filament binding protein
Alh-6	Aldehyde dehydrogenase
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BNIP3	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3
C. elegans	*Caenorhabditis elegans*
Cacl2	Calcium chloride
Cdk2	Cyclin-dependent kinase 2
cDNA	Complementary DNA
Cep-1	*C. elegans* P-53-like protein
CerS1	Ceramide Synthase 1
CI	Chemotaxis index
CR	Caloric restriction
CSCs	Cancer stem cells
DAG	Diacylglycerol
DAPI	4, 6-Diamidino-2-phenylindole
DDR	DNA damage response
DIC	Differential interferal contrast
DNA	Deoxyribonucleic acid
DNA	Deoxyribonucleic acid
DSL	Delta/Serrate/lag-2
DTCs	Distal tip cells
EDU	5-ethynyl-2'-deoxyuridine
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
egl-17	Egg Laying defective
EMS	Mesenchymal paradigm shift

eNOS	Endothelial nitric oxide synthase
ERK_S	Extracellular signal–regulated kinases
EtBr	Ethidium bromide
ETC	Electron transport chain
FBF	Fem-3 mRNA Binding Factor
Fe-S	Iron-sulphur
FGF	Fibroblast growth factor
FGF	Fibroblast growth factor
FRDA	Friedriech's Ataxia
Frh-1	Frataxin
GDP	Guanosine diphosphate
GEF	Guanine Nucleotide Exchange Factor
GEF	Guanine Nucleotide Exchange Factor
Gst-4	Glutathione S-Transferase
GTP	Guanosine triphosphate
Gy	Gray
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF-1	Hypoxia inducible factor
H_2O_2	Hydrogen peroxide
IGF-1	Insulin-like growth factor 1
iNOS	Inducible nitric oxide synthase
IP3	Inositol trisphosphate
Isp-1	Iron-Sulfur Protein
KCL	Potassium chloride
KSR-1	Kinase Suppressor of activated Ras
MFRTA	Mitochondrial free radical theory of aging
Mgcl2	Magnesium chloride
mM	Millimeter
mpk-1	mitogen-activated protein kinase
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
mtROS	Mitochondrial reactive oxygen species
MRC	Mitochondrial respiratory chain
MuV	Multivulva

NAC	N-acetylcysteine
NaCl	Sodium chloride
NAD^+	Nicotinamide adenine dinucleotide oxidized form
NADH	Nicotinamide adenine dinucleotide reduced form
NaN3	Sodium azide
NEX	Nexrutine
NGM	Nematode growth media
NO	Nitric oxide
•OH	Hydroxy radical
pH3	phosphoHistone-H3
Pi	Inorganic phosphate
PI3 kinase	Phosphoinositide 3-kinase
PLCε	Phospholipase C epsilon
PUF-8	PUF (Pumilio/FBF) domain-containing
Raf	Rapidly Accelerated Fibro sarcoma
RC	Respiratory chain
RNA	Ribonucleic acid
RNAi	RNA interference
Rog-1	Ras activating factor in development Of Germline
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription polymerase chain reaction
SEM	Standard error of mean
Sesn-1	SEStriN (peroxiredoxin reductase) homolog
SKN-1	SKiNhead
SOD	Superoxide dismutase
TOR	Target of rapamycin
TZ	Transition zone
UPRmt	Mitochondrial unfolded protein response
VPCs	Vulval precursor cells
WT	Wild type

Abstract

Frataxin is a nuclear encoded mitochondrial protein entailed in the assembly and repair of iron-sulphur (Fe-S) clusters and in cellular iron homeostasis (Bencze et al., 2006). Several components of mitochondrial electron transport chain (ETC) contain Fe-S clusters, and hence frataxin is vital for mitochondrial functionality. Severe frataxin deficiency leads, as it would be expected by severe mitochondrial dysfunction, to detrimental effects including arrested development and shortened lifespan in *Caenorhabditis elegans* and a life-threatening neurodegenerative disease in humans, Friedriech's Ataxia (FRDA). On the other hand, intriguingly, convincing studies from our lab reported that partial frataxin inactivation via RNA interference (RNAi) substantially extends the lifespan of *C. elegans* within a defined window of mitochondrial ETC impairment (Ventura et al., 2005, Rea et al., 2007, Schiavi et al., 2013). Further studies from our lab have shown that the opposite biological consequences in response to the different degree of mitochondrial alteration, are part of a hormetic stress response (a phenomenon by which beneficial processes are triggered upon mild doses of a stress which is otherwise toxic at higher doses). Namely, mild mitochondrial stress results in the activation of cellular compensatory processes, such as *cep-1/*p53-regulated autophagy, DNA repair pathways and mitophagy which concur to provide protection against moderate mitochondrial stress, eventually increasing animal resistance to stress and healthy lifespan (Ventura et al., 2009, Schiavi et al., 2013, Torgovnick et al., 2010, Schiavi et al., 2015).

Aging and cancer are intrinsically coupled. It is widely established that cellular damage accumulation is the major perpetrator that drives both aging and cancer. This notion highlights the fact that those mechanisms which are prompted to combat aging, are in many cases the same that provide an anti-cancer effect (Anisimov, 2001, Serrano and Blasco, 2007). Thus, the aim of my Ph.D. project was to investigate if the anti-aging effect elicited by partial frataxin inactivation might also confer tumor suppressor activity using *C. elegans* as a model to study cancer.

We found that moderate levels of frataxin inactivation via RNAi attenuates the deleterious effects, conceivably in a tissue-specific manner, and prevents premature organismal death caused by tumorigenic mutations in *C. elegans.* These observations suggest that making mitochondria work at the optimal level through partial frataxin depletion concurrently confers anti-aging and tumor suppressor activity.

Introduction

1. Mitochondria

1.1 General features

Mitochondria (singular – mitochondrion) are rod-shaped, double membrane-bound organelles, ranging in size from 1 - 2 μM. They are present in the cytoplasm of most eukaryotic cells except red blood cells and, as Karnkowska and colleagues recently discovered, gut microbe *Monocercomonoides* (Karnkowska et al., 2016). Depending on the cellular metabolic activity their number varies from cell to cell and tissue to tissue. Mitochondria are named as "powerhouses of the cells" since they play a critical role in the generation of adenosine triphosphate (ATP), which mediates energy transfer within the cell. Autogenous and endosymbiotic theories have been set forth to explain the origin of mitochondria. According to autogenous theory, all organelles shaped up from the compartmentalization of functions brought through the invagination of the plasma membrane of a prokaryote and as a consequence, mitochondria evolved separating the vesicles or plasmids of the DNA within the prokaryotic cell (Baum, 2015). Conversely, the widely accepted endosymbiotic theory argues that mitochondria evolved from alpha-proteobacteria taken up inside the eukaryotic cell as an endosymbiont (Gray, 2012).

A mitochondrion is composed of an outer membrane, intermembrane space, inner membrane, cristae and the matrix **(Figure 1)**. As the name suggests the outer membrane covers the entire surface of the organelle and is composed primarily of an integral membrane protein called porin. Spanning the outer membrane, porin proteins are 60 – 75 Å thick and forms channel that allows free diffusion of molecules 5000 Da or less in size across the membrane (Walther and Rapaport, 2009). The mitochondrial outer membrane serves as a potential site for the synthesis of multiple enzymes required for essential cellular reactions like epinephrine oxidation, fatty acids elongation, and tryptophan degradation. The space between the outer and inner membrane constitutes an inter-mitochondrial space and facilitates the exchange of metal ions, proteins, and lipids across the mitochondrial matrix and cytoplasm.

The inner membrane is more intricately organized than the outer one and is composed of 151 different polypeptides. Around one-fifth of total mitochondrial proteins, mainly phospholipids and cardiolipins are concentrated in the inner membrane. The inner membrane is highly impermeable to molecules and ions due to the absence of porins. Thus, all molecules and ions enter and exit through the matrix via special membrane transporters. The inner membrane serves as a primary site for proteins that are implicated in the redox reactions of oxidative phosphorylation, ATP synthesis, mitochondrial fission and fusion proteins and specific protein transporters. Invaginations of the inner mitochondrial membrane form the so-called cristae, structures that significantly increase the surface of the membrane, resulting in the generation of more ATP. The cristae are embedded by F1 particles or oxysomes (Mannella, 2006). The viscous space within the inner membrane constitutes the mitochondrial matrix. Its role is to facilitate various activities that include ATP synthesis, oxidative phosphorylation, Krebs cycle, pyruvate oxidation, fatty acid β-oxidation. The mitochondrial (mt)DNA, ribosomes, soluble enzymes, small organic molecules, nucleotide cofactors, and inorganic ions reside in the matrix.

Mitochondria are considered as semi-autonomous organelles due to the presence of their own short, circular, covalently closed double-stranded DNA (mtDNA) that can undergo independent replication. In humans, the mtDNA comprises of 16,569 bps and encodes approximately 37 genes (Anderson et al., 1981). Mitochondria also possess ribosomal factories from which they synthesize some of the essential proteins (e.g. succinate dehydrogenase) required for their function. Even though mitochondria contain their DNA and ribosomal factories, predominantly their structural and functional protein synthesis is controlled by the nucleus and the availability of the material from the cytoplasm. mtDNA is preferentially inherited from the maternal parent across the divergent phyla (Chen and Butow, 2005).

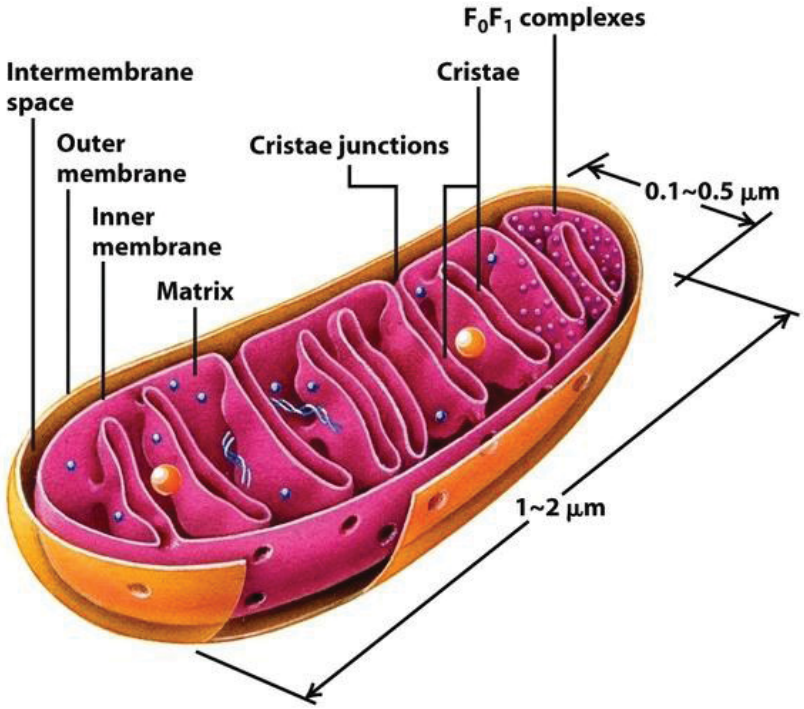

Figure 1: Typifies a mitochondrion structure. Figure from W.H. Freeman and company © 2008.

Mitochondria are the sites of aerobic (or cellular) respiration, a process that facilitates the generation of fuel (ATP), eventually required to carry out all the various cellular activities. Hence, as previously mentioned, they are named as "powerhouses of the cells." Cellular respiration demands the oxidation of pyruvate and nicotinamide adenine dinucleotide reduced form (NADH) (which are produced in the cytoplasm), via three major processes: glycolysis, Krebs cycle, and the oxidative phosphorylation (OXPHOS) through the electron transport chain (ETC). Mitochondria preferentially utilize the ETC machinery for the generation of ATP. The ETC is embedded in the inner mitochondrial membrane and is made up of five complexes (complex I-V) **(Figure 2)**. The electrons captured from the donor molecules (i.e. NADH) are transferred through a series of five complexes, coupling this transfer to the release of hydrogen ions, which ultimately creates a proton gradient used by the ATP synthase (complex V) complex to synthesize ATP from adenosine diphosphate (ADP) and inorganic phosphate (Pi) (Dudkina et al., 2010) .

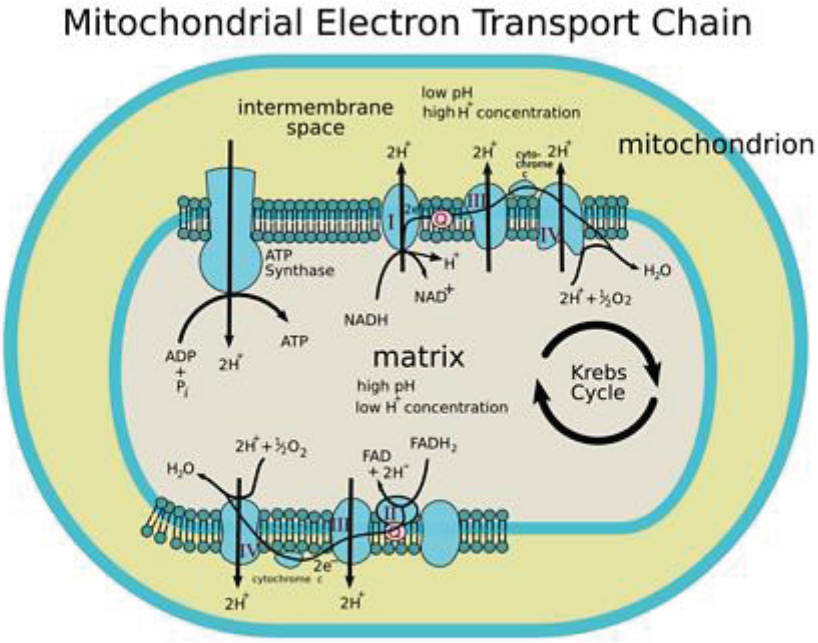

Figure 2: Represents the structure of mitochondrial electron transport chain. Figure from (www.wikidoc.org).

In addition, mitochondria plays a vital role in many other biological functions such as storage of calcium ions, regulation of apoptosis (programmed cell death), regulation of cellular metabolism, steroid and heme synthesis and signaling via mitochondrial reactive oxygen species (ROS) (Hajnoczky et al., 2006, Green, 1998, McBride et al., 2006, Rossier, 2006, Hou et al., 2014).

1.2 Mitochondria and Aging

Why do we age? This question is lingering scientists since decades. Although the exact mechanisms and factors that contribute to aging remained largely elusive, and are actively investigated by many researchers, accumulating evidence has identified mitochondrial impairment as a primary culprit that drives the aging process. Even though the word “aging’’ belongs to our everyday life vocabulary, defining it is not easy and straight forward. Organisms experience aging because of cellular damage accumulation, thus resulting in tissue failure and ultimately death. Various aging theories have been put forth, out of which

mitochondrial free radical theory of aging (MFRTA) was widely accepted by the scientific community for several decades. According to MFRTA, the mitochondrial (mt)ROS are considered to be toxic because of their ability to oxidize diverse spectrum of cellular macromolecules (Harman, 1956). The ETC, situated in the inner mitochondrial membrane, serves as a primary site for the generation of superoxide anions which are further converted to hydrogen peroxide (H_2O_2) by *superoxide dismutase* (SOD) (Harman, 1972). Although, by nature, H_2O_2 is not highly reactive, via Fenton reaction it gets further transduced to hydroxyl radical (•OH), an ultimate damaging form of ROS. The •OH if not properly neutralized can cause oxidative damage to all cellular molecules, thus compromising the cell integrity (Cheeseman and Slater, 1993).

Although widely accepted, the MFRTA has been recently questioned because of contradictive evidence. For instance, according to the MFRTA, long-lived mutants or species should generate reduced free radicals and lower levels of oxidative damage in their tissues. However, contradicting the MFRTA prediction, the longest-living rodent, the naked mole-rat *Heterocephalus glaber* suffer from excessive oxidative damage due to the generation of high levels of ROS (Andziak et al., 2006). Also, the conventional MFRTA argues that free radicals promote mtDNA damage resulting in mitochondrial impairment thereby provoking progressive aging. If this is true, then oxidative damage to mtDNA should negatively modulate the lifespan of the organism. However, in contrast, increased oxidative damage to mtDNA was found to not reduce the lifespan in mice and in *C. elegans* (Vermulst et al., 2007, Yang et al., 2007).

Moreover, several lines of evidence have demonstrated that caloric restriction (CR) extends the lifespan across the taxa (Fontana et al., 2010). According to MFRTA, caloric restricted animals generate reduced levels of mtROS (Beckman and Ames, 1998). However, apart from lowering mtROS, CR alters nutrient sensing pathways including the target of rapamycin (TOR) pathway, and insulin/insulin-like growth factor (IGF-1) signaling (Fontana et al., 2010, Vellai et al., 2003, Masoro, 2000). Hence, CR-mediated lifespan extension cannot be solely ascribed to lower levels of mtROS generation. Furthermore, studies from Schulz and colleagues has revealed a positive correlation between mtROS and life expectancy in *C. elegans*, they have demonstrated that in fact, CR promotes increased ROS production and thus hormetically extends the lifespan of *C. elegans* (Schulz et al., 2007). Finally, various studies in the nematode *C. elegans* indicated that different pro-longevity pathways (see next

chapter) in fact extends lifespan via ROS-increase modulated signaling and reduction of different antioxidant systems do not shorten animal lifespan (Van Raamsdonk and Hekimi, 2010, Hekimi et al., 2011). In summary, although clearly, ROS participate in the aging process, compelling evidence also indicate a positive correlation between oxygen free radicals and aging delay thus contradicting the MFRTA. Oxidized molecules produced in biological tissues alone cannot foster the aging process (Ristow and Schmeisser, 2011, Ristow and Zarse, 2010) and instead may elicit anti-aging effects, a concept that has been lately named mitohormesis. Hence, more efforts should be put forth to understand the role of mitochondria in the aging process, which is most likely not only ascribed to its activity in ROS production.

1.3 Mitochondria and Cancer

Aging and cancer are mechanistically coupled. In the society, we often come across the older people being diagnosed with cancer relative to younger individuals (Yancik, 2005). Hence, aging is considered as a major risk factor for cancer prevalence. This scenario suggests that the factors (e.g. cellular damage accumulation) that promote aging also contribute to cancer development and hence, preventing aging might also provide protection against cancer development. In line with this possibility, a substantial number of studies reported that the interventions that provide an anti-aging effect also exhibit tumor suppressor properties (Arlia-Ciommo et al., 2014, Pinkston et al., 2006, Anisimov, 2001).

In the next section, regarding the mitochondria-mediated anti-aging effect and how mitochondria can be further exploited in anti-cancer therapy is mainly described.

Severe mitochondrial dysfunction is linked to a wide variety of age-associated diseases, including cancer and neurodegeneration (Hung et al., 2012, Hroudova et al., 2014). Nonetheless, perhaps surprisingly landmark studies over the last decade have convinced the scientific community that modest inhibition of mitochondrial functionality substantially extends the lifespan of C. *elegans*, flies and mice (Ventura et al., 2005, Copeland et al., 2009, Lapointe and Hekimi, 2008). Mitochondria are intimately involved in cell division by rendering molecules and energy requisite for the *de novo* synthesis of the different cellular component. Therefore, mitochondria could represent an attractive target for anti-cancer

therapy. Accordingly, compelling evidence indicates that targeting mitochondrial functionality could be a powerful strategy in the war against cancer (Fulda et al., 2010). The governing molecular mechanisms are currently being investigated and several mechanisms that are activated in response to compromised mitochondrial functionality and which could also serve as anticancer strategy has been elucidated using *C. elegans* as a model organism *(e.g.* genes required for detoxification, mitophagy, iron starvation, and genomic stability (Liu et al., 2014, Schiavi et al., 2015, Torgovnick et al., *manuscript in preparation*).

Out of the above-listed mechanisms, I will mainly emphasize the role of mitophagy and anti-oxidant defenses, which are of relevance for our research.

Mitophagy is a form of macroautophagy, implicated in the selective degradation of old or damaged mitochondria. Mitophagy serves as a canonical mitochondrial quality control pathway from yeast to mammals (Hamacher-Brady and Brady, 2016). While transient activation of mitophagy may play a double faceted role either in cancer progression or cancer regression (Lu et al., 2013, Gargini et al., 2011), persistent activation of mitophagy enables the degradation of essential cell survival components and eventually leads to cell death. Moreover, observational studies reported that a wide variety of age-associated diseases such as myopathies, cancer, and neurodegeneration often experience a defect in mitophagy execution (Chourasia et al., 2015, Santos et al., 2011), and BNIP3, a mitophagy-specific regulator, was shown to be mutated in various cancers (Hamacher-Brady and Brady, 2016). Accordingly, during the past few years' multiple approaches involving mitophagy activation to suppress malignancies have been developed. For example, the linamarin, linamarase, and glucose oxidase system effectively eradicate the progression of human cancer cells both *in vitro* and *in vivo* through the persistent activation of mitophagy (Gargini et al., 2011). Linamarase, a β-glucosidase enzyme, is implicated in the hydrolysis of the cyanogenic glucoside substrate linamarin into acetone, glucose, and cyanide. Cyanide inhibits the activity of *cytochrome c oxidase* of the mitochondrial respiratory chain consequently resulting in the activation of mitophagy and ultimately autophagic cell death (Gargini et al., 2011). Other studies indicated that ceramide a bioactive sphingolipid is intimately involved in the induction of cell death, growth relapse, and senescence in various human cancer cells via mitophagy (Dany and Ogretmen, 2015). Overexpression of CerS1 or exogenous supply of C18-pyridinium ceramide facilitates the induction of mitophagy via binding of ceramide to the outer mitochondrial membrane, which in turn acts as a receptor for LC3-II-containing

autophagosomes and eventually recruits autophagolysosomes to damaged mitochondria (Sentelle et al., 2012). Indeed the *Phellodendron* extract has been used as an anti-diarrheal, anti-inflammatory and astringent agent in China. A research from (Wu et al., 2015) has demonstrated that the exogenous supply of Nexrutine (NEX - an herbal extract isolated from the bark of *Phellodendron amurense* also known as Amur cork tree) to various cancer cell lines such as U2OS, HCT116, and HELA, fosters lactate production instead of glucose most likely due to mitochondrial damage. This lead to inhibition of cell proliferation associated with an elevation of p21/p27 proteins (both implicated in cell cycle inhibition). In addition, they have demonstrated that NEX inhibits cell proliferation in a mitophagy dependent manner (Wu et al., 2015). Therefore, increasing the number of strategies to activate mitophagy may help developing novel anti-cancer strategies.

Cellular **antioxidant defense systems** are continuously challenged by the production of ROS. Antioxidant defenses are crucial in maintaining cellular homeostasis and organismal health (Birben et al., 2012). In principle, human body ‘‘mop up’’ excess ROS by the collective action of antioxidant enzymes which includes *catalase*, *superoxide dismutase*, *monoamine oxidase*, *thioredoxin*, α tocopherol, *glutathione*, *glutathione peroxidase* and *glutathione reductase* (Birben et al., 2012). Under hostile environmental conditions, more ROS is generated due to the overwhelmed antioxidant network. For example, observational studies showed an enhanced ROS production during the transition of a normal cell to an invasive carcinoma (Valko et al., 2006). Furthermore, free radical levels increased with decreased antioxidant levels in leukemia (Valko et al., 2006). Exogenous supply of antioxidants has proved to exert both anti and pro-genic effects on tumorigenesis (Galluzzo et al., 2009, Sayin et al., 2014). Why would an anti-oxidant which exert beneficial effect under normal conditions suddenly evoke an adverse effect in a cancerous cell is still a matter of debate.

Exogenous supply of antioxidants N-acetylcysteine (NAC) and Vitamin E remarkably enhanced tumor progression and reduced survival in B-RAF- and K-RAS-induced lung cancer mouse models (Sayin et al., 2014). Conversely, the exogenous supply of the three well-known antioxidants such as quercetin, curcumin, and canthaxanthin is known to induce apoptosis in various cancer cells without affecting the normal cells (Galluzzo et al., 2009, Kuo et al., 1996, Palozza et al., 1998). This conflicting data certainly deserves a more scientific explanation. However, ‘‘prevention is better than cure,’’ therefore activating the antioxidant defense system from the nascent stage of an organism might help to maintain the

genome integrity and confer tumor suppressor activity. For example, the *C. elegans* long-lived mitochondrial mutants express elevated levels of endogenous antioxidant enzymes including *sod-3*, *alh-6*, *gst-4*, *sesn-1* compared to their control counterparts (Torgovnick et al., 2010). Nevertheless, other studies showed no positive correlation between increased SOD levels and lifespan (Yang et al., 2007).

2. *Caenorhabditis elegans*

2.1 General features

C. elegans is a small transparent, free-living (non-parasitic), soil nematode. The generation time of *C. elegans* is around 3 days, and it grows to 1 mm length under nutrient-rich conditions (Wood, 1988). *C. elegans* body is covered by a sturdy cuticle which contains four epidermal cords and a body cavity (fluid filled pseudo coelom). It's basic anatomy includes a mouth, pharynx, intestine, gonad and a collagenous cuticle. Like all nematodes, it lacks a circulatory and respiratory system. *C. elegans* has a small yet intricate nervous system comprising of 302 neurons (White et al., 1986). *C. elegans* genome is completely sequenced and annotated. It's genome size is about 100 million bps long and contains 20,389 protein-coding genes (Consortium, 1998). *C. elegans* has two sexual forms: the self-fertilizing hermaphrodites (XX) **(Figure 3B)** and males (XO) **(Figure 3A)**, which are occasionally generated at a frequency of 0.1 - 0.2% (Hodgkin, 1987). *C. elegans* is a eutelic organism, with hermaphrodite and males having 959 and 1031 cells respectively whose number does not change after reaching the adulthood (Wood, 1988).

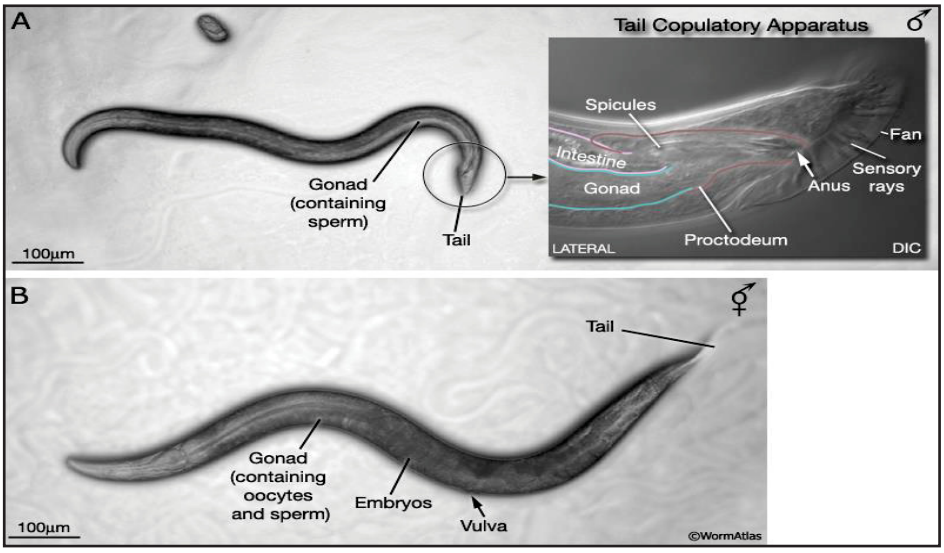

Figure 3: Depicts male and hermaphrodite anatomy. **(A)** The left side of the panel represents *C. elegans* male sexual form and right side of the panel, illustrates its tail region using Normaski DIC. **(B)** *C. elegans* hermaphrodite. Image from Wormatlas.

The embryogenesis in *C. elegans* takes around 14 hrs at 22°C. After fertilization, a virtually impermeable eggshell is produced, which can undergo further development independently. Nevertheless, the embryos are held within the uterus of the hermaphrodite up to 24-cell stage (egg lay time point). An embryo hatches with 558 nuclei (some cells are multinucleated hence lower cell count) and transmutes into a first larval stage (referred to as L1). After hatching, animal starts feeding and it metamorphoses through four larval stages (referred to as L1 to L4). The L1 larval stage is around 16 hrs long; while each of the other larval stages spends around 12 hrs **(Figure 4)**.

The end of each larval molt is accomplished by a brief lethargus like state (Raizen et al., 2008) during which pharyngeal pumping ceases and the new outer cuticle is built. Each lethargus-quiescence state is terminated with the molting of the old cuticle. Approximately 12 hrs after the L4 larval stage, a newly matured hermaphrodite enters reproductive phase and produce 250 - 350 self-fertilised offspring. When a hermaphrodite is instead mated with a male, it produces up to four times more number of offspring, suggesting the sperm production as the limiting factor for progeny production (Hodgkin and Barnes, 1991). Self-fertilization of hermaphrodites generates genetically identical progeny. Whereas mating with male facilitates the exchange of genetic material between two mutant strains. Under suitable living conditions, the average mean lifespan of *C. elegans* is around three weeks.

When an L2 larva encounter hostile environmental conditions such as lack of food, high population density or temperature, it enters into an alternative L3 larval form called "dauer stage''(Riddle and Albert, 1997). During the dauer stage, feeding and locomotion are markedly reduced. The dauer larvae are surrounded by a thick impermeable cuticle. Hence they are highly resistant to environmental stressors and chemical agents. In the presence of food, dauer enters into a slightly different L4 larval stage and resumes normal development (Riddle and Albert, 1997).

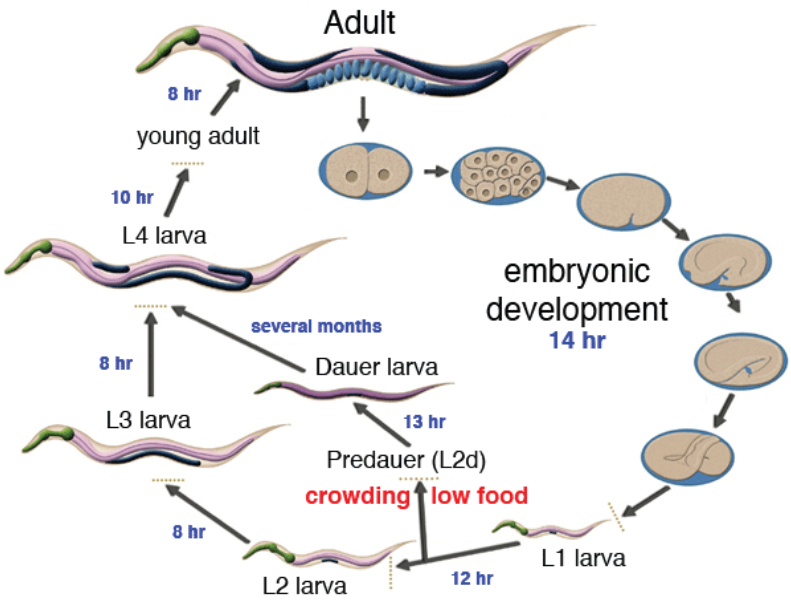

Figure 4: A closer look at the *C. elegans* life cycle at 22ºC. Along the arrows, numbers in blue indicate hrs of the time animal spends at each specific stage. Figure from WormAtlas.

2.2 *C. elegans* as a model for aging studies

Sydney Brenner introduced *C. elegans* in 1974 with its studies on developmental neurobiology, and within a short time, this organism has emerged as a leading model system for aging research mainly because *C. elegans* display a number of age-related changes like those observed in higher vertebrates. Indeed, with progressive aging worms show the following age-related phenotypes:

C. elegans pharynx is orthologous to mammalian heart (Mango, 2007) and like mammals, pharyngeal pumping capacity declines with age (Chow et al., 2006). Furthermore, like humans, during aging *C. elegans* experience a progressive decline in muscle integrity and

coordination (sarcopenia) **(Figure 5A-B),** decline in sensory abilities (chemotaxis), defecation cycle and accumulation of lipofuscin (age pigment) **(Figure 5C)** (Herndon et al., 2002, Glenn et al., 2004, Felkai et al., 1999, Gerstbrein et al., 2005).

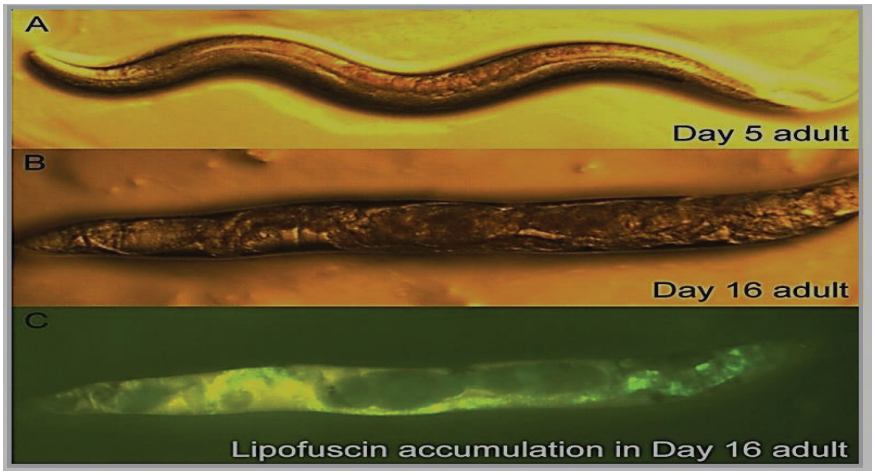

Figure 5: Deciphers the sign of aging in *C. elegans*. **(A)** Image exemplifies the morphological difference of 5-day old adult compared to **(B)** 16-day old worm; suffers from the deterioration of locomotion and wrinkled skin. **(C)** The image illustrates the lipofuscin accumulation in the 16-day old adult. As the cells age, lipofuscin (age pigment) compiles up and serves as an autofluorescence aging biomarker in *C. elegans* as well as in humans. Figure from (Kaletsky and Murphy, 2010).

A unique advantage of using *C. elegans* for aging research is its ability to grow in massive quantities within a short period and its cost-effectiveness. *C. elegans* has a short (mean of three weeks at 20°C) and largely invariant lifespan. Its propagation and recovery process is relatively fast and straightforward. Predominantly, the aging studies in *C. elegans* were established by the discovery of gene mutations that dramatically affect the lifespan.

In the next section, the three major canonical pathways that modulate the lifespan of C. *elegans* are emphasized **(Figure 6)**.

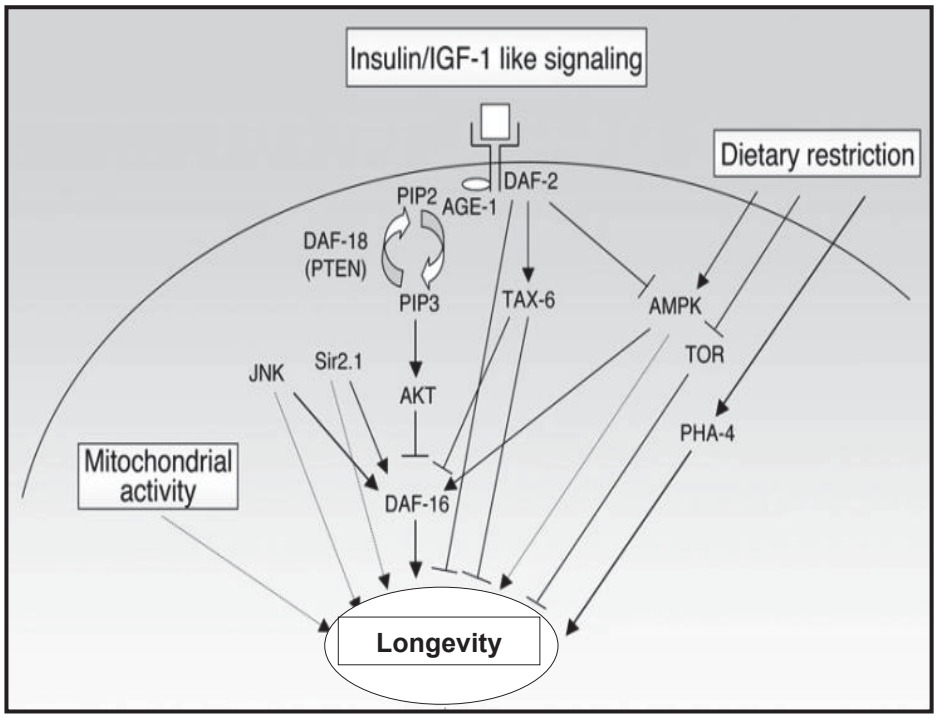

Figure 6: Illustrates the three canonical pro-longevity pathways. Figure adapted from (Jia and Levine, 2010).

2.2.1 Insulin/IGF-1 signaling

Insulin and IGF-1 are anabolic hormones that stimulate growth process and food storage in an evolutionarily conserved manner (Laron, 2001, Laviola et al., 2008). The genetic mutations that decrement the activity of *C. elegans daf-2* (encodes for the IGF-1) doubles the lifespan across the divergent phyla (Kenyon, 2005). Consistently, lowering the downstream PI(3)K/AKT/PDK cascade also proved to exert a beneficial effect on *C. elegans* lifespan (Altintas et al., 2016). Dampening the insulin/IGF-1 signaling alters lifespan by changing the expression of a wide variety of transcription factors such as DAF-16 (an FOXO transcription factor), HSF-1 (heat shock transcription factor), and SKN-1 (xenobiotic response factor) (Altintas et al., 2016). This handful of transcription factors, in turn, alters the expression of a set of genes that act concomitantly to induce significant effects on *C. elegans* lifespan. *daf-2* dependent extension of lifespan also requires AAK-2 (catalytic subunit of the AMP-activated protein kinase) (Apfeld et al., 2004). In mammals, AMP-kinase regulates energy metabolism through the phosphorylation of various substrates that include transcription factors and metabolic enzymes (Hardie, 2011). However, the mechanism by which AAK-2 kinase

extends lifespan remains ambiguous. Insulin\IGF-1 knockdown mutants of *Drosophila* live 50% longer than wild type (Hwangbo et al., 2004). Unlike worms, mice retain distinct insulin and IGF-1 receptor and knocking down the IGF-1 receptor has shown to extend the lifespan of mice by ~ 30% (Selman et al., 2008). The beauty of this long-lived mutant (and many other long-lived mutants) is that even the older worms (~ 22 day's age) retain the youthful qualities compared to their wild-type counterparts.

2.2.2 Calorie Restriction

Caloric restriction (CR) refers to a dietary regime accomplished by a low-calorie intake without reducing the consumption of vital nutrients. *C. elegans* can be subjected to CR by employing various approaches such as decreasing the concentration of *Escherichia coli* (food source), complete absence of bacteria as food source (axenic media), or by using mutants (e.g. *eat-2*) with a defect in feeding (Houthoofd et al., 2003, Kaeberlein et al., 2006, Lakowski and Hekimi, 1998). *eat-2* mutant is acknowledged as an excellent model for CR, it flaunts postsynaptic activity and regulates the pharyngeal pumping rate. Thus, mutations in *eat-2* gene reduce the partial consumption of food intake. Various isolated *eat-2* mutants like *eat-1(ad427)*, *eat-2(ad1116)* and *eat-6(ad467)* have shown to extend the *C. elegans* lifespan by 33%, 57%, and 37%, respectively (Lakowski and Hekimi, 1998). In *C. elegans*, chronic dietary restriction prolongs the lifespan by the down-regulation of the nutrient sensor, TOR (Vellai et al., 2003). Studies have shown that inhibition of TOR kinase activity elicits a pro-longevity beneficial effect in an autophagy-dependent manner (Madeo et al., 2015). Furthermore, CR prolongs lifespan through the activation of PHA-4 mediated autophagy, induction of SKN-1 and HSF-1 transcription factors (Hansen et al., 2008, Bishop and Guarente, 2007, Steinkraus et al., 2008). Sirtuins (NAD^+-dependent protein deacetylases) also mediates the extension of chronic dietary restriction-induced lifespan via DAF-16/FOXO dependent manner (Kenyon, 2005).

2.2.3 Mitochondrial activity

Mitochondrial electron transport chain through OXPHOS provides the major source of ATP production. Therefore, severe mitochondrial dysfunction often leads to various lethal

consequences such as arrested development and premature organismal death in *C. elegans* and a life-threatening neurodegenerative disease in humans, Friedriech's Ataxia (FRDA). However, perhaps surprisingly, a substantial number of evidence across the phyla revealed that modest inhibition of mitochondrial ETC functionality does not lead to lethal consequences or organismal death, rather results in the extension of adult lifespan (Copeland et al., 2009, Ventura et al., 2005). In *C. elegans*, such long-lived mutants are defined as Mitochondrial mutants. Depending on how the lifespan extension is achieved via various means of gene inactivation the Mit mutants are classified into three categories: RNAi-mediated gene inactivation, genetic mutations or external interventions. Inactivation of genes via RNAi led to the identification of several potential mitochondrial longevity genes (Hamilton et al., 2005, Lee et al., 2003), including frataxin (Ventura et al., 2005), a nuclear-encoded mitochondrial protein involved in iron-sulphur cluster assembly and cellular iron homeostasis (Bencze et al., 2006). Downregulation of *frh-1* (the *C. elegans* frataxin homolog) mRNA levels to 70% of normal, which can be accomplished by feeding worms with *frh-1* RNAi for three successive generations has shown to invoke a robust longevity response (Ventura et al., 2005). In addition, RNAi-mediated of *nuo-2* (encodes the NDUFS3 subunit of NADH-ubiquinone oxidoreductase), *atp-3* (encodes the ATP5O/OSCP subunit of ATPase complex) and of other ETC regulatory proteins substantially extends the *C. elegans* lifespan (Rea et al., 2007). Inhibition of mitochondrial functionality via RNAi invoke pro-longevity beneficial effects within a discrete window of mitochondrial functionality impairment, beyond or below this threshold concentration lead to deleterious or negligible effects (Rea et al., 2007). Studies revealed that specific signal instigated during the larval stages of *C.elegans* is crucial for triggering a lifespan extension response (Rea et al., 2007, Dillin et al., 2002).

Genetic mutants belong to a small class of long-lived Mit mutants, including *clk-1(qm30)* and *isp-1(qm150)*, which are the most studied genetic mutants. *clk-1(qm30)* is a fist isolated mitochondrial genetic mutant; it has a mutation in an ortholog of COQ9 (mitochondrial *hydroxylase*), which is required for the generation of ubiquinone (Wong et al., 1995). *clk-1(qm30)* mutant display reduced respiration rate due to a defect in the transfer of electrons from complex I to complex III of ETC. Heterozygous knockout of *mClk-1* (mouse ortholog of *clk-1*) also extends lifespan in mice with various genetic backgrounds (Lapointe and Hekimi, 2008). The *isp-1* genetic mutant has a missense point mutation in the *qm150* allele

that leads to reduced protein function (Feng et al., 2001). *isp-1* is involved in making the Rieske iron-sulfur protein subunits which belong to complex III of mitochondrial ETC.

Lastly, lifespan extension through the down-regulation of mitochondrial ETC functionality can be accomplished by using appropriate concentration of chemicals such as ethidium bromide (EtBr), antimycin (complex III inhibitors), and a DNA crosslinker (Dillin et al., 2002, Tsang and Lemire, 2002). Mit mutants display distinct phenotypic features including smaller body and brood size, reduced ATP levels and oxygen consumption rate, altered mitochondrial morphology, reduced behavioral rates (Ventura et al., 2006). Lifespan extension in this class of longevity mutants is achieved thanks to the activation of different protective, mitochondrial stress response compensatory pathways, such as different transcription factors (Munkacsy and Rea, 2014, Schiavi and Ventura, 2017), a p53-regulated autophagy (Ventura et al., 2009, Schiavi et al., 2013), mitophagy (Schiavi et al., 2015) and metabolic reprogramming (Mishur et al., 2016).

2.3 *C. elegans* as a model for cancer studies

Core hallmarks of a malignant transformation include resistance to apoptosis, genomic instability, uncontrolled proliferation, alteration in the growth factor signaling components, angiogenesis induction, immortalization, metastasis, and invasion (Hanahan and Weinberg, 2000). Although cancerous cells experience partly or most of the above-listed features, a particular set of mutations provokes changes that can vary among different types of cancer thus increasing the complexity of cancer biology. Therefore, dissection of the oncogenic signaling demands the use of diverse complementary approaches and different model organisms. In this context, *C. elegans* could serve as a streamlined *in vivo* model system for cancer research because of the well conserved oncogenic signaling events with higher eukaryotes and due to the presence of less isoforms or less redundant genes in the *C.elegans* gene families. For example, p53 and pRb are the tumor suppressors which are widely mutated in most of the human cancers (Sherr and McCormick, 2002). In mammals p53 and pRb exists in three isoforms (Chen, 1999, Mulligan and Jacks, 1998). While in *C. elegans* CEP-1 (mammalian p53 ortholog) and LIN-35 (mammalian pRb ortholog) exist only in one isoform (Derry et al., 2001, Lu and Horvitz, 1998). Finally, the *C. elegans* transparent body

throughout its development facilitates the direct visualization of cellular processes, including abnormal or ectopic cell proliferation.

In the next section, the scenarios of using two well established *C. elegans* cancer models related to our research are mainly described.

***(i) C. elegans* germline tumor:** The *C. elegans* germline serves as a primary site for the generation of pluripotent cells and in fact, it is the only tissue primed to undergo proliferation throughout the life of the organism (Crittenden et al., 2003). The above feature of the *C.eleagns* germline allows investigating the molecular mechanisms involved in the maintenance of stem cell population, an acquired feature of cancerous cells. Hence, *C.elegans* germline could serve as a powerful model system to study various governing molecular mechanisms of malignant transformation. Mutations in the notch signaling cascade have been largely implicated in cancer formation and progression. In *C.elegans*, GLP-1/notch signaling (see 2.3.1) is involved in the regulation of germ cell proliferation in development and maintenance of germline stem cells in the adult (Crittenden et al., 2003). The gain of function mutation in *glp-1* results in the development of a germline tumor, in which mitotic cells failed to differentiate and never enter meiosis thus accumulating (a tumor like a state) (Berry et al., 1997) **(Figure 7B)**. Loss of function mutation in *gld-1* (a downstream target of *glp-1*) also contributes to the mitotic-meiotic imbalance, thereby resulting in the development of hyperproliferating germ cells in a tumorous fashion. Nevertheless, in this mutant; the tumor-like state is prompted from germ cells that failed to complete gametogenesis (Francis et al., 1995) **(Figure 7C)**. Both mutants for tumorous germline with over-proliferative mitotic cells which eventually break the gonad and diffuse through the entire body thus prematurely killing the animals (Berry et al., 1997, Francis et al., 1995).

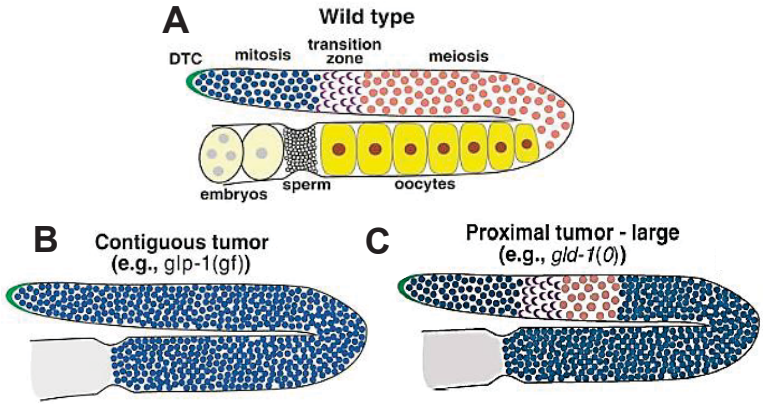

Figure 7: Denotes the gonad arm of **(A)** wild type **(B)** *glp-1(ar202)* mutant **(C)** *gld-1(qm485)*. Modified figure from (Kirienko et al., 2010).

(ii) *C. elegans* ectopic cell growth: EGFR>RAS signaling regulates various biological processes including cell proliferation and differentiation across the metazoans (Schlessinger, 2000) and hyperactivating mutations in its regulatory genes are causally involved in cancer (Fernandez-Medarde and Santos, 2011). In *C.elegans, let-23* (mammalian EGFR ortholog) dependent *let-60* (mammalian *ras* ortholog) signaling (see 2.3.2) plays a critical role in the development of animal vulva (Sternberg, 2005). Constitutive activation of LET-23(EGFR) or its downstream cascade components induces the formation of multi vulva phenotype (ectopic cell growth) **(Figure 8B)** (Sternberg, 2005). The EGFR signaling is highly conserved between *C.elegans* and mammals **(Figure 11)**. Therefore, the multi vulva formation of *C. elegans* could serve as another powerful model system for cell proliferation and differentiation studies.

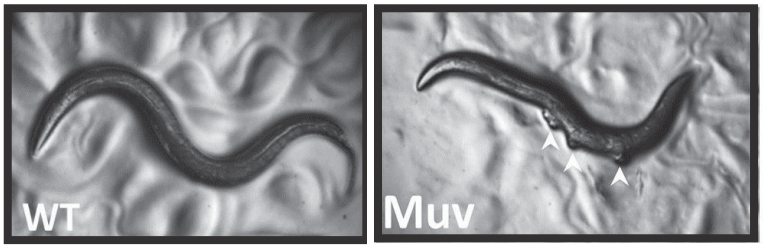

Figure 8: (A) Represents wild type. **(B)** Elucidates multi vulva phenotype and the ectopic vulval protrusions marked with white arrows. Figure from Wormbook.

In the next subchapters, the molecular mechanisms and biological roles of notch and RTK/Ras signaling are discussed in detail.

2.3.1 The notch signaling (germline tumor formation)

The notch signaling regulates essential biological processes, and it is very well conserved across the taxa. It is intimately involved in the regulation of a diverse range of cell fate decisions throughout the animal development, and therefore faulty notch leads to cancer and to various deleterious effects (Aster, 2014).

2.3.1.1 Signal transduction and core components

Notch family comprises of a signaling ligand (LAG-2), transmembrane receptors (GLP-1/LIN-12) and transcription factors such as LAG-1, LAG-3/SEL-8. Notch receptor belongs to type I transmembrane protein family (Greenwald, 2005). Their structure is composed of multiple EGF-like motifs after the signal sequence, followed by the presence of three copies of LNR (LIN-12/Notch repeat) motif. In *C. elegans*, LIN-12 and GLP-1 receptors are activated by ligands which belong to (Delta/Serrate/LAG-2) DSL family (Greenwald, 2005). The structure of these ligands also comprises of one or more EGF motifs, followed by amino-terminal ‘‘DSL motif”. Binding of the DSL ligands to LIN-12 facilitates shedding of the extracellular ectodomain. Consequently, the short residual transmembrane protein undergoes further cleavage via ‘‘*γ-secretase*’’ thereby releasing intracellular domain, which translocates to the nucleus (Greenwald, 2005). The intracellular domain comprises of a repetitive CDC10/SWI6/Ankyrin motif, which helps the intracellular domain to bind to LAG-1 (a transcription factor) and facilities gene expression **(Figure 9)** (Greenwald, 2005).

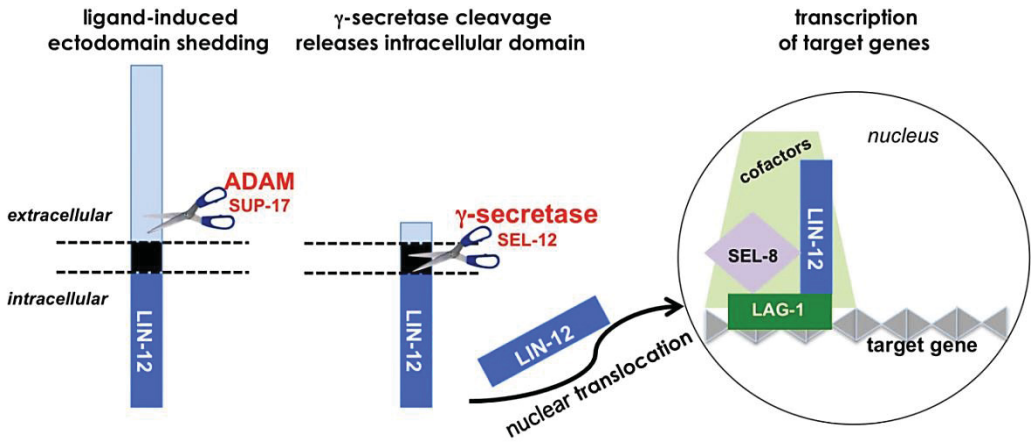

Figure 9: Molecular mechanism of LIN-12/Notch signal transduction. Figure from Greenwald homepage.

2.3.1.2 Biological functions

In *C. elegans* the notch signaling controls numerous biological processes. However, in this section, its vital role in the maintenance of germline **mitotic and meiotic compartment** are mainly emphasized **(Figure 10)**.

In *C.elegans* hermaphrodite germline, the distal tip cell (serves as a stem cells niche) signals via notch and controls mitosis and meiosis (Kimble and White, 1981). Upon binding to the ligand protein (LAG-2), the activated GLP-1 receptor together with other RNA-binding proteins, FBF-1 and FBF-2 promote endless mitotic proliferation (Crittenden et al., 2002). On the other hand adjacent to the mitotic zone the increased expression of GLD-1 and GLD-2-GLD-3 complex facilitates the inhibition of mitotic proliferation and promote entry into meiosis via transition zone (Crittenden et al., 2003). In the transition zone, cells go through various stages of meiosis, from which damaged cells are removed via apoptosis (Hubbard, 2005), and the healthy cells complete meiotic differentiation and form the oocytes. Mature oocytes are fertilized as they move through the spermatheca into the uterus, and the embryos are released out through the animal vulva.

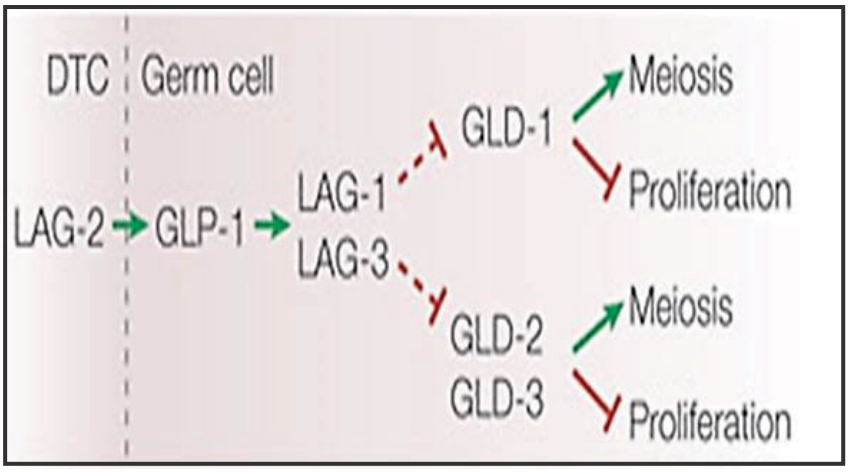

Figure 10: Elucidates the LAG-2 mediated GLP-1 pathway. Figure from (Kipreos, 2005).

In addition, LIN-12, and GLP-1 significantly contributes to many other cell fate paradigms. For instance, LIN-12 is implicated in the regulation of lateral signaling among a various group of cells such as vulval precursor cells (VPCs) and also facilitates the initiation of certain ventral uterine fates (Sundaram, 2005, Newman et al., 1995).

2.3.2 The RTK/RAS signaling (ectopic cell growth)

Receptor Tyrosine Kinase (RTK) mediated signaling network is intimately involved in the regulation of multiple biological processes throughout the metazoan development (Schlessinger, 2000). Thus, mutation's deregulating this finely controlled process leads to many human syndrome and diseases, including cancer (Fernandez-Medarde and Santos, 2011). In *C. elegans* genome, approximately 33 RTKs has been characterized. Of the depicted RTks half of the families are nematode-specific (Popovici et al., 1999). Out of all, only LET-23 homologous to mammalian EGFR (epidermal growth factor receptor) and EGL-15 homologous to mammalian FGFR (fibroblast growth factor receptor) signals widely through the core LET-60(RAS) pathway (Aroian et al., 1990, DeVore et al., 1995). LIN-3 homolog of mammalian EGF is the sole ligand known to activate LET-23(EGFR) (Hill and Sternberg, 1992). Similarly, depending on the cellular context EGL-17 and LET-756 homologs of mammalian FGF (Popovici et al., 2004, Burdine et al., 1997). have been shown to activate EGL-15 (Burdine et al., 1997, Roubin et al., 1999).

RTK is an intricate regulatory network comprising of a various number of receptors and signaling cascades, out of which, the conserved LET-23(EGFR)>LET-60(RAS) dependent and independent receptors and signal transduction are further emphasized for my studies.

2.3.2.1 Signal transduction and core components

Upon binding of the corresponding extracellular ligand, the LET-23(EGFR) undergoes dimerization and autophosphorylation (Ferguson, 2008). The activated receptor aids as a docking site and recruits an adaptor protein, SEM-5(Grb2). that together with Guanine Nucleotide Exchange Factor(GEF) SOS-1 activates small GTPase, LET-60(RAS) (Karnoub and Weinberg, 2008). Activated GTP-bound LET-60(RAS) assists in stabilizing and activating LIN-45(RAF) by recruiting it to the plasma membrane. The scaffold protein KSR-1 is implicated in modulating LIN-45(RAF) activation (Udell et al., 2011) and also permits the transmission of a further signal by bringing together MPK-1(ERK) kinase cascade (Morrison and Davis, 2003). LIN-45(RAF) then activates MEK-2(MEK) via phosphorylation, which, in turn, activates MPK-1(ERK) by phosphorylation. Finally, MPK-1(ERK) depending on the biological context either inactivates or activates various

downstream substrates, such as nuclear transcription factors Ets domain protein, LIN-1, SUR-2 (Yoon and Seger, 2006) (**Figure 11**).

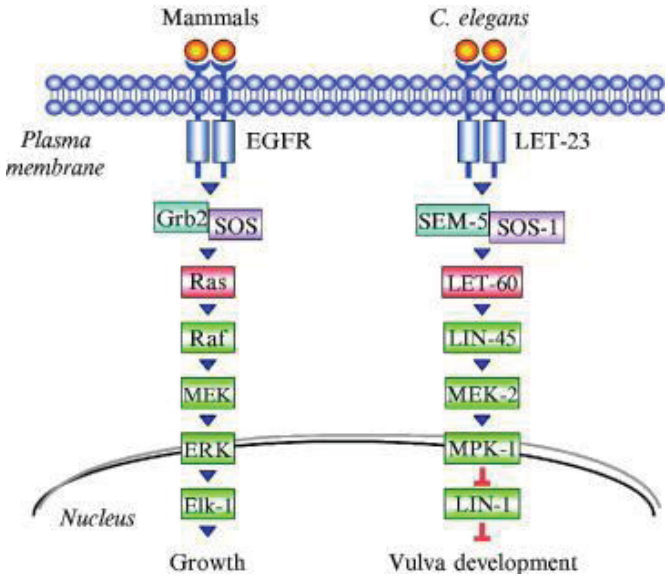

Figure 11: Represents a well-conserved LET-23>LET-60>LIN-45>MPK-1 signaling cascade specifies vulva development in *C. elegans* and to growth in mammals. Figure from (Reiner et al., 2008).

2.3.2.2 Biological functions

In this section, LET-60(RAS) biological roles dependent and independent of LET-23(EGFR) are majorly emphasized. As indicated in **(Figure 14)** emerging consensus revealed the participation of the entire LET-60(RAS)>MPK-1(ERK) cascade in various biological processes, but the clear molecular mechanisms modulating this signaling cascade are still largely being investigated. For instance, the receptor implicated in the activation of LET-60(RAS) in the germline is unclear, although data from (Matsubara et al., 2007) shed light on the requirement of ROG-1As (an adapter protein), suggesting the partly involvement of RTks. Conversely, a new line of research from (Lopez et al., 2013) reported that DAF-2 insulin-signaling in the presence of food drives the meiotic progression via LET-60(RAS)>MPK-1 (ERK) cascade activation in the germline of the *C. elegans* hermaphrodite. Here I will mainly focus on the role of LET-60(RAS)>MPK-1(ERK) cascade in **germline (Figure 12) (and vulva) development (Figure 13).**

In the germline, MPK-1(ERK) acts cell autonomously and exerts multiple roles (Lee et al., 2007), above all it facilitates the smooth transition from mitotic to meiotic germ cells. Indeed, various studies demonstrated that partial suppression of *mpk-1* via RNAi displayed a germline with expanded mitotic zone and reduced early meiotic transition compartment, thus indicating that MPK-1(ERK) signaling promote meiotic entry (Lee et al., 2007). Lee and colleagues also reported the partly requirement of MPK-1(ERK) for mitotic germ cells proliferation (Lee et al., 2007).

LET-60(RAS) cascade is intimately involved in pachytene cellular organization (Lee et al., 2007) indeed null mutants display germ cells with arrested meiotic pachytene nuclei and disorganized membrane pattern. LET-60(RAS)>MPK-1(ERK) signaling also facilitates the regulation of germline apoptosis in multiple aspects. The RAS-ERK signaling null mutants, display defect in the physiological-induced germline apoptosis (Kritikou et al., 2006), as their germline lack a region primed to undergo apoptosis (Lee et al., 2007). Indeed, studies showed that by partially reducing or enhancing the MPK-1(ERK) activation aids both in inhibiting (Arur et al., 2009) or promoting the (Kritikou et al., 2006) physiological apoptosis. Lately, research work from (Rutkowski et al., 2011) revealed its possible direct role in DNA-damage-induced apoptosis. Finally, in the germline, MPK-1(ERK) signaling facilitates the transition from diakinesis to metaphase of meiosis I (i.e. sperm-dependent oocyte maturation (Lee et al., 2007). The maturation process occurs before ovulation. The MPK-1(ERK) signaling mutants suffer from maturation and ovulation defect, or they display standard ovulation time and delay in the maturation rate (Lee et al., 2007).

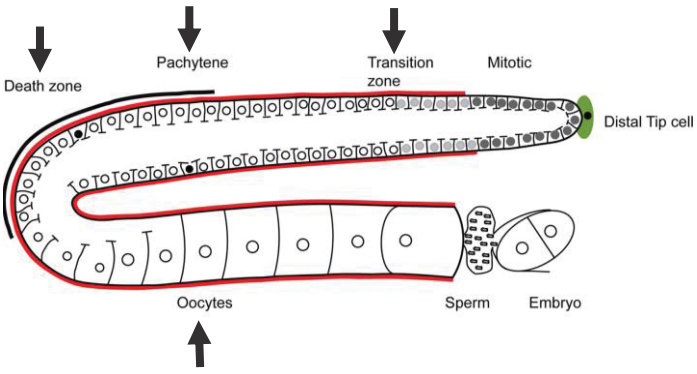

Figure 12: Depicts various RAS>ERK cascade mediated (arrows in black) germline-specific events of. *C. elegans* hermaphrodite. Figure from Wormbook.

Moreover, LIN-3(EGF)>LET-23(EGFR) dependent LET-60(RAS) pathway interact with LIN-12(notch) and Wnt signaling to promote **vulva development** of *C. elegans* hermaphrodite **(Figure 13)** (Sternberg, 2005, Greenwald, 1997).

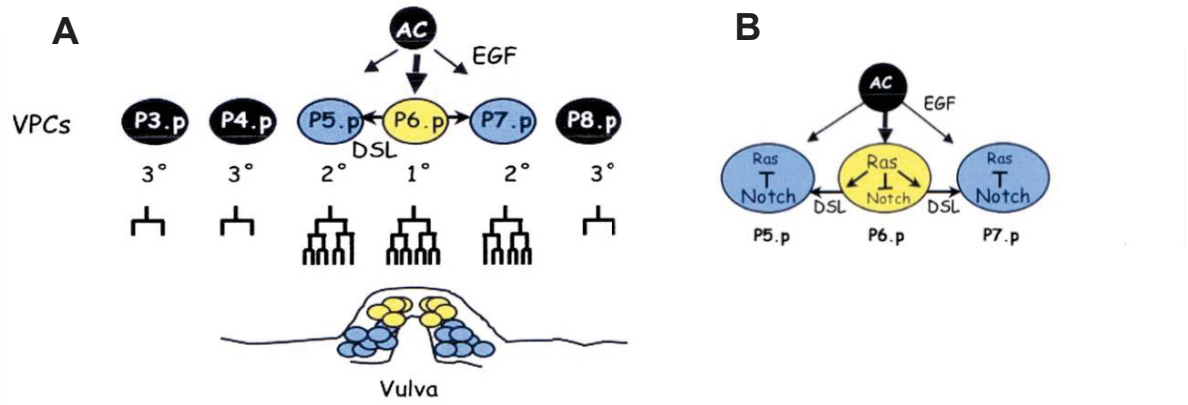

Figure 13: Cartoon representation of *C. elegans* vulval development. **(A)** Six vulval precursor cells, adapts 1º, 2º, and 3º vulval cell fates in a graded EGF and notch-mediated lateral signaling manner. **(B)** EGF and LIN-12/Notch like receptor antagonize to generate 1º and 2º vulval cell fates. Figure adapted from (Sundaram, 2005).

Finally, novel insights from (Hirotsu et al., 2000) demonstrated that LET-60(RAS)>MPK-1(ERK) signaling is intimately involved in the regulation of **olfaction specialized neurons** (i.e. AWC, AWA neurons). They showed that compared to wild-type animals, mutants with reduced or over-activated LET-60(RAS) signaling are less attracted towards volatile compounds such as isoamyl alcohol and diacetyl, which are specifically sensed by AWC and AWA neurons. The receptor mediating this effect remains unclear, but a novel emerging scenario is that LET-60(RAS) might regulate olfaction via a G-protein coupled receptor (Chen et al., 2011).

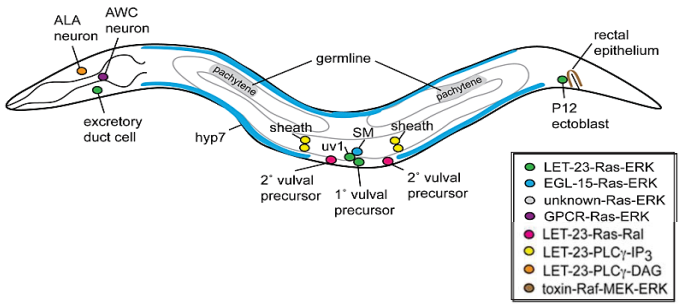

Figure 14: Depicts various biological roles arbitrated via canonical RTK-RAS-ERK signal transduction. The signaling cascade biological functions are colored per cell type. Please note, although many of the signaling events occur during the larval development, for convenience the location of the pertinent cells is shown in the adult. Figure from (Sundaram, 2013)

Materials and methods

3.1 *C. elegans* strains and maintenance

Standard methods were employed for *C. elegans* maintenance (Stiernagle, 2006). All strains were grown on nematode growth media (NGM) seeded with either OP50 or HT115 transformed bacteria. Worms were maintained at 15ºC, 20ºC, and 25ºC. Strains used in this study were: N2 (wild type, Bristol), MT4698 (*let60(n1700))*, MT2124 (*let-60(n1046)),* JG324 *(jgIs6*=(LET-23::hEGFR-TK(L858R)), GC833 *(glp-1(ar202)),* FX00395 *(pdr-1(tm395)),* FX00598 *(pdr-1(tm598)),* ZG31 *(hif-1(ia4)),* SS104 *(glp-4(bn7)),* CB4037 *(glp-1(e2141)), ced-1::gfp.* (*ced-1,* homologous to mammals CD91, encodes a transmembrane protein, and belongs to apoptotic machinery; *ced-1* piles up around the engulfed apoptotic cells and thus facilitates the visualization of the apoptotic corpse) (Zhou et al., 2001).

3.2 RNAi feeding

RNAi feeding technique followed as previously described in (Ventura et al. 2005; Rea et al. 2007). *frh-1* RNAi construct was generated as previously described in (Ventura et al., 2005, Schiavi et al., 2013). *isp-1* (F42G8.12) RNAi-transformed HT115 bacterial clone was derived from ab initio as previously described in (Rea et al., 2007). Animals were allowed to grow on *frh-1* RNAi-transformed HT115 bacteria for three successive generations as previously described in (Ventura et al., 2005). *isp-1* RNAi-transformed HT115 bacteria was grown to a concentration of 0.9 OD and diluted to 1:15 with HT115 bacteria transformed with an empty vector as described in (Rea et al., 2007).

3.3 Quantification of the apoptotic corpse

The synchronized L4 staged animals were treated using ionizing radiations (IR) (NORDION International INC) device and UVB germicidal bulb (254 nm) with the indicated doses. Twenty-four hrs after the L4 stage, worms were anesthetized using 15 mM sodium azide in M9 buffer and mounted on 5% agarose pads. The apoptotic corpses were scored in the late pachytene stage of the meiotic compartment (i.e. loop region) of the adult hermaphrodite germline using Zeiss differential interference contrast (DIC) microscopy and fluorescence

microscopy. Apoptotic corpses were identified as previously described in (Gumienny et al., 1999, Zhou et al., 2001).

3.4 Fertility and fecundity assay

Twenty-four hrs after the L4 stage or after treatment (i.e. either UVB or ionizing radiations), animals were transferred to a freshly seeded NGM plates and allowed to lay eggs for 4 hrs. After 4 hrs of eggs lay, adult worms were removed, and the number of eggs and small eggs laid were counted (i.e. Fertility or egg lay rate). After 48 hrs, the number of larvae hatched from the eggs on each plate was counted (i.e. Fecundity or survival rate). In each experiment, for each condition four plates with three adults were used.

3.5 Quantification of mitotic germ cells

Twenty-four hrs after L4 stage, germlines extracted from control and *frh-1* RNAi animals were stained with the DAPI (4',6-diamidino-2-phenylindole). DAPI a fluorescent DNA intercalating dye enables cell nuclei morphology visualization. Images were acquired using Zeiss fluorescence microscopy. The mitotic compartment extends from the distal part of the germline until the transition zone (TZ) (distinct with the crescent half-moon-shaped nuclei) (Francis et al., 1995, Dernburg et al., 1998), all the germ cell that exists in the mitotic compartment were quantified. Around five germlines were analyzed per condition.

3.6 Measurement of gonad size

Age-synchronized gravid adults were transferred to NGM agar plates and allowed to lay eggs. After obtaining an appropriate number of eggs, adult worms were picked off the plates. NGM plates with age-synchronized eggs were moved to the strain-specific temperature (i.e. 20ºC and 25ºC). Three-day, Five-day age-synchronized worms were washed off the plates and stained with DAPI (Vectashield). Images of the intact worms were acquired using Zeiss fluorescence microscopy, and the germline tumor size was estimated by measuring the distance between the anterior mouth region (M) and the anterior gonadal loop region (GL). The measurement was performed using ImageJ software.

3.7 Immunofluorescence of the dissected germlines

Worms were dissected on a poly-lysine coated slides and germlines were extracted into the dissection buffer as previously described in (Rutkowski et al., 2011). Germlines were fixed with 4% formaldehyde for 5 mins (for pH3 and dpMPK-1). Post-fixation was carried out in 50:50 acetone:methanol (for pH3) and in 100% methanol (for dpMPK-1) for 10 mins at -20°C and permeabilized in 0.2% Triton X-100 for 10 mins (for pH3 and dpMPK). pH3 antibody from (Abcam catalog number#ab5176, used at 1:400 dilution) and anit-rabbit AlexFluor-488 from (Abcam catalog number#ab150077, used at 1:1000 dilution). dpMpk-1 antibody (used at 1:100 dilution) and anti-mouse AlexaFluor-488 (used at 1:500 dilution) was a kind gift from (Prof. Gartner). The pH3 positive stained cells were manually counted, and the pictures were acquired using 40x magnification with Zeiss fluorescence microscopy. While the images of the dpMPK-1 stained germlines were taken with 10x magnification with the same settings using Axioskop (Zeiss) microscope fitted with RTke camera and spot analysis software (diagnostic instrument). Brightness and contrast of the respective dpMPK-1 stained germline images were modified for clear staining pattern visualization. The fluorescence intensity of the dpMPk-1 stained germlines was measured using the following formula; Corrected total cell fluorescence (CTCF) = Integrated Density - (Area of selected region X Mean fluorescence of background readings). Image J software was used to extract the values of integrated density, the area of the cell and background mean fluorescence.

3.8 Behavioral assays

Behavioral assays such as chemotaxis index was quantified in four-days old adults whereas the body bend assay and pharyngeal pumping rate was performed in four-days old and seven-days old gravid adults. **Pharyngeal pumping rate:** Pharyngeal pumping rate was assessed by counting the number of pharyngeal contractions per minute in approximately 20 animals. One pump was counted every time the pharynx undergoes a rhythmic contraction. **Chemotaxis assay:** Chemotaxis assay was performed to compute chemosensory ability of the animals towards a specific chemical compound. The experiment was carried out in the following way: To immobilize the worms, sodium azide (NaN3) was added to the unspotted NGM agar plate, 180º opposite on a 10 cm dish; then the attractant was added to NaN3 spot. On the other hand, ethanol was placed on the other NaN3 spot, in this case, ethanol was used as a neutral

odor (control) since the attractant was diluted in ethanol. Approximately 100 age-synchronized worms were placed in the middle of the 10 cm NGM plate. The assay was performed for 4 hrs in which a number of worms reached towards the attractant and control were counted for every 15 mins until 1 hr and for every 60 mins until 4 hrs (i.e. end point). From the obtained values chemotaxis index was calculated (CI) using the following formula: CI = (A-B)/(A+B+C), where "a'' represents the of animals reached the attractant, "B'' is number of animals reached control, "C'' represents total undecided population (i.e. total number of animals which does not reach either attractant or control spot, this point was counted at the end of the experiment). In principle, three-day old wild-type animals exhibit CI of around 0.8 for attractant and -0.8 for repellent after 2 hrs (CI=0, signifies no attraction, whereas CI=1 or -1 denotes maximum attraction or repulsion. In this assay, benzaldehyde and ethanol compounds used were purchased from Sigma-Aldrich. **Locomotor performance assay:** Locomotor performance was monitored by counting the number of body bends during a 60-min interval. The assay was performed on NGM agar plate without bacteria. One body bend was counted whenever the mid-body of the animal displays a maximum bend. Body bends were monitored approximately in 20 age-synchronized worms in three independent biological replicates.

3.9 Quantification of multivulva

Multivulva count was performed on four-day, six-day and eight-day age-synchronized population of worms per condition at 20ºC. Four-day and six-day and eight-day old correspond to their age from the egg. In each experiment, approximately 90 synchronized worms per condition were used for quantification. Same worms were used to assess the multivulva count on different days. Multivulva was identified and scored by simply counting the number of ectopic vulval protrusions (pseudovulva) (**Figure 8B**) in age-synchronized worms using a dissecting microscope.

3.10 Lifespan and statistical analysis

Lifespan assay was performed from hatching of the eggs using approximately 60-80 synchronized population of worms per condition at 20ºC. Mean and standard deviation of the animals was calculated by using a log-rank test from Kaplan-Meier survival analysis. Kaplan-

Meier survival analysis shows the difference in the survival curves between the pooled populations as previously described in (Yang et al., 2011).

3.11 Quantitative Real-Time PCR

For RT-PCR, mRNA extraction and cDNA synthesis were carried out using Qiagen RNA extraction kit (catalog number#74104) and Omniscript reverse transcriptase kit (catalog number#205113). All primers were designed using Primer-Blast software and purchased from Sigma-Aldrich. At least two housekeeping genes were used for gene expression quantification and normalization. RT-PCR was carried out using SyBrGreen master mix and Bio-Rad iQTM5 multicolor real-time PCR detection system. The fold change in gene expression was assessed using the 2-ΔΔCt method. In this assay, *aco-1*, *aco-2*, and *dct-1* transcripts were evaluated, and *pmp-3* and F23B2.13 were used as housekeepers. For every primer pair, a no template control was used, and three independent biological replicates were performed.

3.12 Additional statistical analysis

All the data points represented as mean ± SEM calculated from at least two independent biological replicates unless otherwise stated. Depending on the type of the data various statistical test such as ANOVA or Students t-test was used to calculate the significance: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ in comparison to the respective internal control counterparts and between other interested groups. All the data and statistics were calculated using GraphPad Prism software and Microsoft excel.

Aim of the project

Cellular damage accumulation with consequent tissues failure induced by genetic alterations and environmental factors is considered as a common determinant that fuels both cancer and aging (Hoeijmakers, 2009). This notion, highlight the fact that, those mechanisms that safeguard the cells from cellular damage concurrently provide the anti-aging effect, and may be the same which confer protection against cancer.

Frataxin is a mitochondrial protein that plays a crucial role in the biogenesis of iron-sulfur cluster proteins and thus in mitochondrial ETC functionality (Bencze et al., 2006). Substantial evidence from our lab reported that partial frataxin suppression through RNAi elicits distinct metabolic changes, which contribute to lifespan extension of *C. elegans* (Ventura et al., 2005), such as activation of p53*/cep-1*-dependent autophagy, anti-oxidant defenses, mitophagy, and protective neuronal pathways (Schiavi et al., 2013, Ventura et al., 2009, Torgovnick et al., 2010, Schiavi et al., 2015, Maglioni et al., 2014). Moreover, a more recent study from our lab demonstrated that partial suppression of frataxin prompts germline resistance to genotoxic stress (Torgovnick et al., *manuscript in preparation*).

Interventions that enhance p53*/cep-1* activity, anti-oxidant defenses, autophagy and genomic stability converge and incite an anti-aging and anti-cancer effect (Serrano and Blasco, 2007, Pinkston et al., 2006). Notably, many canonical signaling and genes pertinent to aging and cancer are remarkably conserved between humans and *C. elegans.* In line with these observations, the aim of my project was to investigate if the anti-aging effect of partial frataxin depletion might also confer tumor suppressor activity using *C. elegans* as a cancer model.

During the almost four years of my Ph.D. studies, I had the opportunity not only to work on my specific project but also to contribute to other ongoing research in the lab. As a result, some of my findings are included in my thesis and also in a manuscript currently in preparation entitled "Pro-longevity mitochondrial disturbance promotes BRC-1/BRD-1 dependent and independent resistance to genotoxic stress" (Torgovnick et al., *manuscript in preparation*). I also collected data which are included in two publications entitled (1) "Mitochondrial stress extends lifespan in *C. elegans* through neuronal hormesis" (Maglioni et al., 2014) and (2) "Iron-Starvation-Induced Mitophagy Mediates Lifespan Extension upon Mitochondrial Stress in *C. elegans*.'' (Schiavi et al., 2015). Fortunately, I also got the

opportunity to write a short comment emphasizing our work in a publication entitled "Mitochondrial autophagy promotes healthy aging." (Shaik et al., 2016). Finally, enclosed data of my Ph.D. thesis is included in a manuscript currently in preparation entitled "Partial frataxin suppression in *Caenorhabditis elegans* reduces tumor formation by dampening the RAS/MAPk signaling.'' (Refer page 61, for enclosed list of my publications).

Results

4.1 Partial suppression of frataxin mediates germline resistance to DNA damage-induced apoptosis and reduces germ cell proliferation

Aging is often associated with reduced resistance to various infections and stressors (i.e. DNA damaging agents), and consistently, manipulations that promote pro-longevity beneficial effects enable enhanced resistance to different stressors (Larsen, 1993, Murakami and Johnson, 1996, Ventura et al., 2005). A classical example of this paradigm has been expansively surveyed in the nematode *C. elegans* where long-lived mutants have often been identified with their enhanced resistance to various stressors (Kim and Sun, 2007, Johnson et al., 2002). Frataxin (*frh-1*) RNAi-mediated animals belong to the class of long-lived *C. elegans* mitochondrial mutants (Ventura et al., 2005, Ventura et al., 2006). Frataxin is a mitochondrial protein implicated in synthesizing iron-sulfur clusters (Bencze et al., 2006). Observational studies from our lab showed that partial *frh-1* inactivation via RNAi at the lifespan extending conditions (which I will refer to as *frh-1* RNAi through the rest of my thesis) displayed enhanced germline resistance to DNA damage induced apoptosis (Torgovnick et al., *manuscript in preparation*). To strengthen our previous findings, wild type, and *ced-1::gfp* expressing animals fed control or *frh-1* RNAi were treated with DNA damage-inducing agents such as gamma radiations and UVB at the L4 stage. CED-1 is a transmembrane protein involved in cell corpse engulfment and therefore its transgenic GFP expression facilitates the detection of the apoptotic corpse (Zhou et al., 2001). Apoptotic germ cells were revealed 24 hrs after stress through DIC microscopy in the wild type strain, and with fluorescence microscopy in the *ced-1::gfp* strain. The obtained data revealed that *frh-1* RNAi provides resistance to germline apoptosis compared to control counterparts in response to both gamma radiations **(Figure 15A)** and UVB **(Figure 15B)**.

Furthermore, in *C. elegans*, DNA damage in response to various genotoxic insults can be indirectly assessed by monitoring the fertility rate (the number of eggs laid in a specific time interval after stress). The fertility rate is usually inversely proportional to DNA damage (i.e. more damage less number of eggs laid and vice versa). In agreement with the protection against DNA-damage-induced germline apoptosis, fertility rate performed 24 hrs after UVB treatment (when the apoptotic corpse were also assessed) revealed that *frh-1* RNAi-depleted

animals laid significantly increased number of eggs **(Figure 15C-D)** compared to treated control animals. Together with our previous findings indicating that *frh-1* silencing does not alter the core apoptotic machinery (Torgovnick et al., *manuscript in preparation*), these observations reinforce the notion that *frh-1* RNAi-depleted animals at the lifespan extending conditions display an improved cell defense against DNA damage.

A typical phenotype associated with the long-lived Mit mutants is that they lay eggs at a slower rate (in the first three fertile days) but for more days compared to wild type animals. Accordingly, also *frh-1* RNAi animals display a reduced daily fertility in both wild type and *ced-1::gfp* strain **(Figure 15E-F)** (Ventura et al., 2005). Normal egg laid rate is the result of balanced germ cells mitotic proliferation and apoptosis. The intact apoptotic machinery and improved germ cell's DNA-damage resistance suggest that the altered fertility rate observed in *frh-1* RNAi-depleted animals could be ascribed to slower mitotic germ cell proliferation. Consistent with this possibility in basal, unstressed conditions *frh-1* RNAi-depleted animals displayed reduced number of mitotic germ cells relative to control, assessed following *in vitro* germline staining with the fluorescent DNA intercalating dye DAPI (4',6-diamidino-2-phenylindole) **(Figure 15G-I)**. This is in agreement with our previous observation (Torgovnick et al., *manuscript in preparation*) showing that *frh-1* RNAi-depleted animals displayed reduced incorporation of EDU, an *in vivo* marker for DNA synthesis and suggests that reduced number of germ cells in the mitotic compartment in response to *frh-1* silencing is due to reduced mitotic cells **(see also Figure 15G-I).**

Thus, long-lived *frh-1* RNAi animals display improved DNA damage repair pathways activation and slower (germ cells) proliferation rate, features almost often affected in cancer. These findings encouraged us to question whether the anti-aging effect of partial frataxin depletion might also confer tumor suppressor activity.

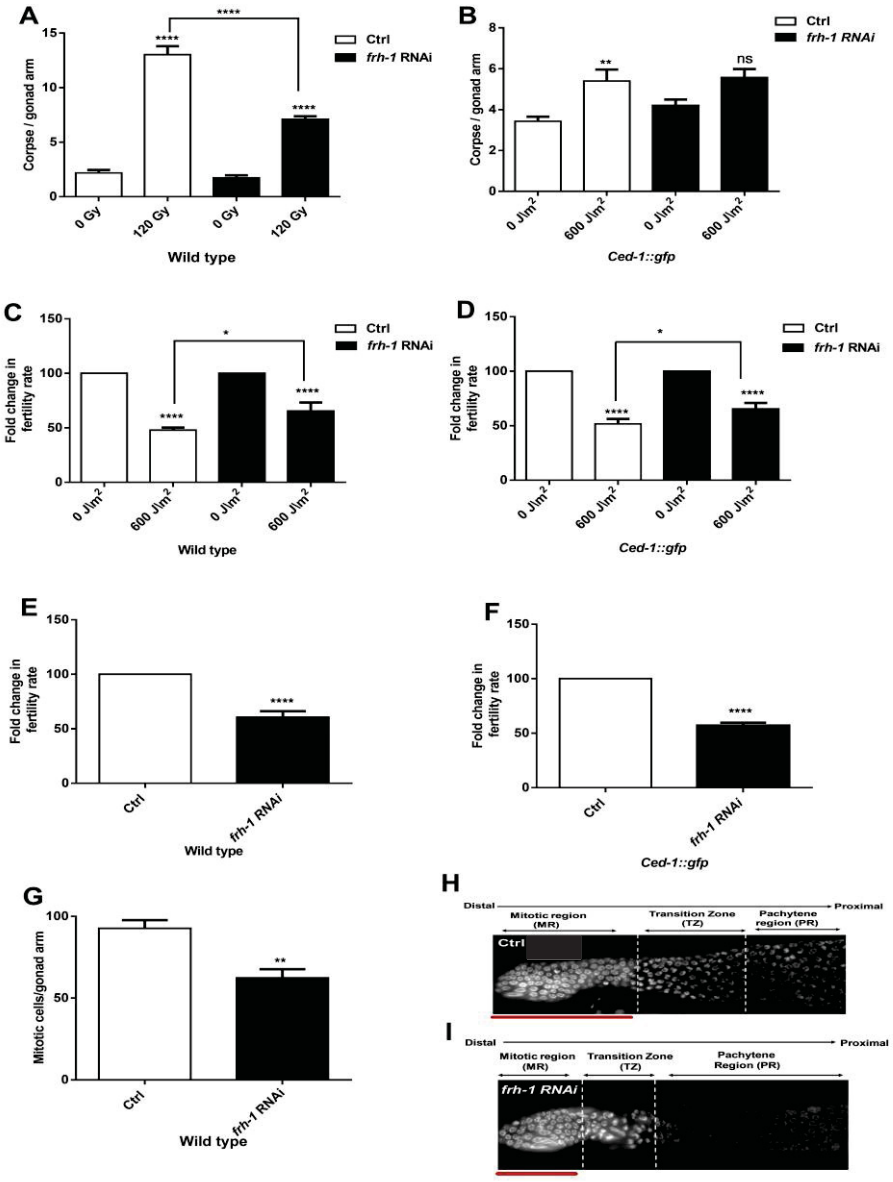

Figure 15: Partial suppression of frataxin mediates germline resistance to DNA damage-induced apoptosis and reduces germ cell proliferation: (A-B) Quantification of the apoptotic corpse: L4 staged animals were treated with gamma radiation and UVB at the indicated doses. Apoptosis was quantified 24 hrs after the treatment in control and *frh-1* RNAi fed animals. Bars and errors represent mean ± SEM of three independent biological replicates with at least ten germlines scored in each experiment and the asterisks the p-value for ANOVA (**p < 0.01, ****p < 0.0001, 'n.s.' stands for not significant). **(C-F) Fertility assay: (C-D)** Control and *frh-1* RNAi stressed (UVB), and unstressed animals **(E-F)** Control and *frh-1* RNAi fed, unstressed animals, were allowed to lay eggs for 4 hrs. After 4hrs the number of eggs laid were counted. Bars and errors indicate mean ± SEM of three independent experiments and the asterisks the p-value for **(C-D)** ANOVA and **(E-F)** for an unpaired t-test (*p < 0.05, ****p < 0.0001). **(G) Quantification of mitotic germ cells:** Mitotic germ cells were quantified in control, and *frh-1* RNAi fed animals following *in vitro* staining of isolated germline with the fluorescent DNA intercalating dye DAPI. Bars and errors indicate mean ± SEM of two independent experiments with at least three germlines scored in each experiment and the asterisk indicates p-value for an unpaired t-test (**p < 0.01). **(H-I)** Showing the representative DAPI staining of *C. elegans* hermaphrodite dissected germline from control and *frh-1* RNAi animal. Mitotic compartment extends from the distal tip cell until the transition zone (which can be recognized by the presence of crescent half-moon-shaped nuclei). In all bar graphs, if not connected by lines, the p-value is always to the respective internal control.

4.2 Partial suppression of frataxin reduces germline tumor growth

Given that partial frataxin depletion reduces mitotic germ cell proliferation **(Figure 15G-I)** we then asked whether it could also reduce or prevent the over proliferation caused by lethal germline tumorigenic mutations. Indeed, although *C. elegans* is a post-mitotic organism that does not naturally develop cancers, the existence of different strains carrying mutations in highly conserved oncogenic signaling pathways (e.g. *glp-1*/Notch and *let-60*/Ras regulated pathways) allows mimicking the tumorigenic process. Namely, germ cells at the distal region of the gonad proliferate and enter meiosis in a *glp-1* signaling-dependent manner and the temperature sensitive gain of function mutation in *glp-1(ar202)* mutant blocks mitotic – meiotic spatiotemporal progression resulting in the persistence of mitotic cells at the proximal terminus (a tumor like a state) (Berry et al., 1997) **(Figure 7B)**. Thus, at 25ºC *glp-1(ar202)* mutant animals lack oocyte due to the tumorous germline, which subsequently spread throughout the animal's body leading to its premature death. To address our question, we thus took advantage of the *glp-1(ar202)* germline tumor model and measured the tumor size (an indirect parameter to quantify germ cell proliferation) in three- and five-days old-synchronized *glp-1(ar202)* control and *frh-1* RNAi animals grown at 25ºC. We compare the tumorous growth with the gonad size of age-synchronized wild type animals fed control and *frh-1* RNAi. Tumor size was assessed by measuring the length between mouth (M) and the gonad loop region (GL) (i.e. the length between the mouth and the gonad loop region decreases with increased tumor growth and vice versa) **(Figure 16A – representative gonad picture)**. Perhaps surprisingly, the obtained results showed that when compared to three-days old wild type animals, the length between the mouth and the loop region was in fact slightly increased in *glp-1(ar202)* mutant animals fed either control or *frh-1* RNAi, suggesting the germ cells tumor still didn't develop in three days old mutants. Instead, the length between the mouth and loop region was drastically decreased in five-day old *glp-1(ar202)* mutant animals fed control compared to wild type control animals, indicating the presence of a massive tumorous growth **(Figure 16A)**. Of note, *frh-1* silencing completely suppressed the massive tumorous growth in five-day old *glp-1(ar202)* mutant animals which indeed displayed similar length between the mouth and gonad loop region of *frh-1* RNAi fed wild type animals **(Figure 16A)**.

Next, we asked if the reduction in the tumor size of *glp-1(ar202)* animals fed *frh-1* RNAi could be directly ascribed to the inhibition of mitotic germ cell over-proliferation rate.

To assess this possibility, we quantified mitotic germ cell proliferation, following an *in vitro* immunostaining of the isolated germlines with an antibody against phospho-Histone H3 (pH3). The pH3 is a mitotic marker which specifically stains the G2/M phase transition (i.e. pH3 positive cells number is directly proportional to mitotic cell proliferation) (Hendzel et al., 1997). We observed that three-day old *glp-1(ar202)* fed control animals show a drastic increase in the pH3 marked cells relative to age-synchronized wild type animals fed control, clearly suggesting the persistence of mitotic germ cell over-proliferation **(Figure 16B - representative pH3 gonad staining)**. Of note, *frh-1* silencing significantly reduced the number of proliferative cells in the G2/M phase in three-day old *glp-1(ar202)* animals relative to their control counterparts. While a similar trend was observed in wild type animals, *frh-1* RNAi did not significantly reduced the number of G2/M cells compared to control fed animals. This is in contrast to our previous results showing a significant reduction in the mitotic germ cells in wild type animals in response to *frh-1* silencing relative to control **(Figure 15G-1)**. This apparent conflicting observation could be explained by the difference in the experimental conditions (i.e. temperature at which worms were grown, the sensitivity of the assay). Perhaps surprisingly, in five-day old *glp-1(ar202)* animals fed control displayed a drastic reduction in the number of pH3 marked cells relative to three-day old *glp-1(ar202)* fed control animals, in fact, pH3 positive cells were similar to five-day old wild type fed control animals **(Figure 16B)**. This effect could be due to the pH3 specificity that only marks the G2/M phase transition, with cells at later time points which could have already pass through the G2/M transition phase (hence lower pH3 positive cell count). Nonetheless, a similar tendency to reduce pH3 positive cells (although not significant) was also observed in five-day old *glp-1(ar202),* and wild type animals fed *frh-1* RNAi compared to control fed animals **(Figure 16B).**

Altogether, the combination of gonad length measurement (which allowed us to determine the tumor size in later stages of the animal life) and the pH3 immunostaining (which allowed us to determine the actual germ cell in G2/M phase) reinforce the notion that partial suppression of frataxin reduces tumor size by inhibiting germ cell over-proliferation rather than by inducing their death (as we showed *frh-1* RNAi does not affect the apoptotic machinery and actually confers resistance to DNA-damage-induced germ cell apoptosis).

The block in mitotic to meiotic progression in the *glp-1(ar202)* mutant resulting in the tumor formation also leads to animal sterility. We then asked whether *frh-1* silencing not only

reduced germ cell proliferation but also unblocks mitotic to meiotic spatiotemporal progression thus restoring oocyte production.

To test this, we assessed the egg lay rate (fertility) of age-synchronized wild type and *glp-1(ar202)* mutant fed control or *frh-1* RNAi. As expected wild type *frh-1* RNAi animals laid a reduced (although not significant) number of eggs at 25ºC **(Figure 16C)** – again indicating a difference in the penetrance of the phenotype at different temperature (*frh-1* RNAi animals at 25ºC also don't look as smaller and paler than control fed animals as we normally notice at 20ºC – data not shown). Contrary to our expectation partial suppression of frataxin did not restore oocyte (egg) production of *glp-1(ar202)* mutant animals at 25ºC **(Figure 16D**), although the tendency to produce some eggs was noticed. This observation indicates that *frh-1* RNAi specifically affects (reduces) germ cell mitosis rather than the ability of mitotic cells to proceed into meiosis.

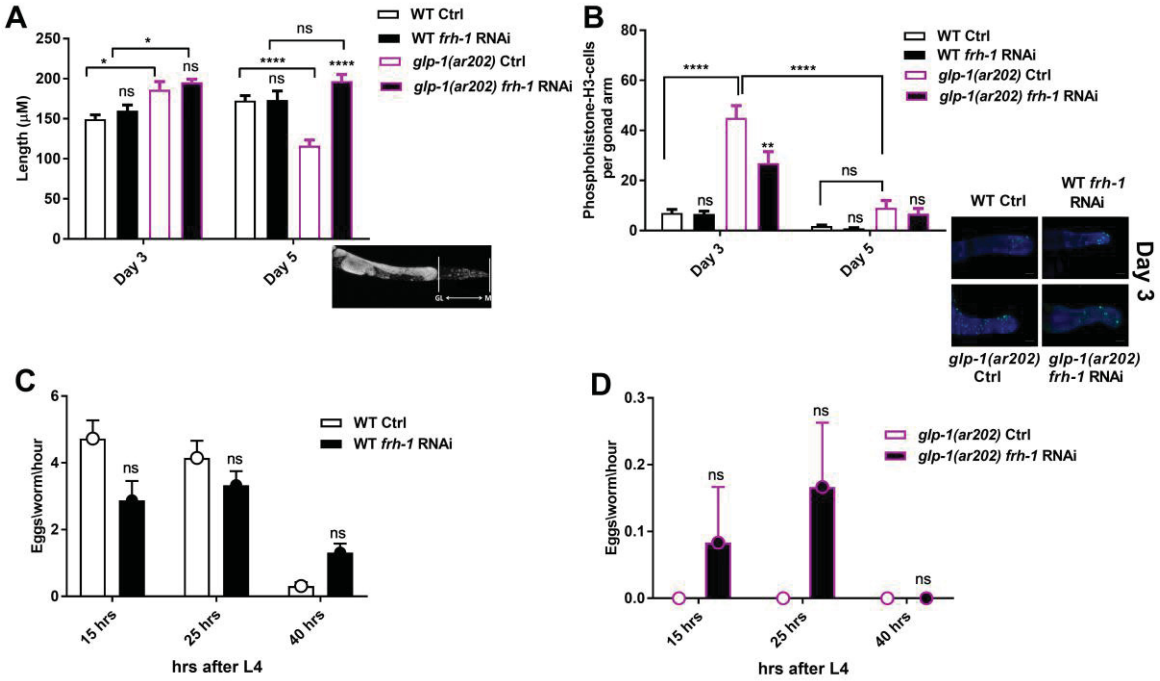

Figure 16: **Partial suppression of frataxin affects mitotic compartment rather mitotic-meiotic progression: (A) Measurement of gonad size:** Image from the graph is the representative picture of the DAPI stained intact worm. The gonad size was quantified by measuring the distance between the mouth region (M) and the gonad loop region (GL). The tumor size is inversely proportional to the length between the mouth and good loop. Three-day and five-day age-synchronized wild type and *glp-1(ar202)* mutant animals fed control, or *frh-1* RNAi were stained with DAPI for DNA visualization. In the graph, bars and errors represent mean ± SEM from at least twelve intact worm's gonad size scored per condition and the asterisks indicate the p-value for ANOVA (*p < 0.05, ****p < 0.0001, 'n.s' stands for not significant). One representative plot is shown; two additional ones were performed with comparable results. **(B) Immunofluorescence:** Images from the graph are the representative pictures of the dissected control and *frh-1* RNAi *glp-1(ar202)* and wild type three-day old adult germlines stained against phospho-Histone H3 (green) and for DNA (DAPI, Blue). Germlines were treated identically, and the images were taken with the same magnification (40x), Scale bar 20 µm. Graph showing the number of pH3 positive cells per gonad arm in three-day and five-day age-synchronized *glp-1(ar202)* and wild type animals fed control and *frh-1* RNAi. One representative plot is shown; two additional ones were performed with reproducible results. Bars and errors values are mean ± SEM with at least nine germlines quantified per condition and the asterisk the p-value for ANOVA (**p < 0.01, ****p < 0.0001, 'n.s' stands for not significant). **(C-D) Egg lay rate (fertility):** Graph showing the number of eggs laid by **(C)** wild type and **(D)** *glp-1(ar202)* mutant animals fed control or *frh-1* RNAi. The egg lay (fertility) assay was performed at the indicated time points. One representative plot is shown; two additional ones were carried out with similar results. Egg lay values are mean ± SEM from twelve animals and the asterisks indicate the p-value for ANOVA ('n.s' stands for not significant). In all bar graphs, if not connected by lines, p-value is always to the respective internal control.

4.3 Partial suppression of frataxin attenuates germline LET-60(RAS) signaling

We then sought to investigate the possible molecular mechanism underlying frataxin control of cell proliferation. In mammalian cells, in particular, in malignant cells, proliferation is stimulated by growth factor-induced RAF-MEK-ERK signal transduction (Roberts and Der, 2007), whereas activation of stress-induced MAPKs, such as p38 and JNK, may counteract mitogenic stimuli (Xia et al., 1995). In *C. elegans*, as in mammals, MPK-1(ERK) activation is promoted through the conserved LET-60(RAS)>LIN-45(RAF)>MEK-2(MEK) signaling cascade and it acts cell autonomously eliciting multiple roles in the worm germline (Lee et al., 2007). We thus set to examine MPK-1(ERK) activation pattern following *in vitro* germline immunostaining with an antibody against diphosohorylated MPK-1. Clearly, MPK-1(ERK) activation was significantly reduced in the wild type *frh-1* RNAi germlines compared to their control counterparts **(Figure 17A)**. Thus, the reduced proliferation rate in response to partial frataxin suppression in the wild type *C. elegans* germline could be ascribed to attenuated MPK-1(ERK) activation.

Around 30% of all tumor types and ~ 90% of pancreatic cancer occur due to the dominant mutations in human *ras* alleles (Fernandez-Medarde and Santos, 2011). Since we observed, dampen MPK-1(ERK) (a downstream target of LET-60(RAS)) activation in response to *frh-1* silencing we then asked whether partial suppression of frataxin could also inflict the same effect on sustained LET-60(RAS) signaling. To address this question, we turned to a *C. elegans* mutant strain expressing a dominant *let-60* gain of function (*gf*) mutation. *let-60 (n1046) gf* mutant possess a dominant mutation which attenuates the conversion rate of GTP-bound active form to GDP-bound inactive form and thus results in the constitutive activation of the encoded LET-60(RAS) protein (Beitel et al., 1990). LET-60(RAS)>MPK-1(ERK) kinase pathway is implicated in the regulation of germline apoptosis and elevated MPK-1(ERK) activation in this *let-60(n1046) gf* aids in promoting physiological (Kritikou et al., 2006) as well as DNA-damage induced apoptosis (Rutkowski et al., 2011). The *C. elegans* hermaphrodite germline consists of two U-shaped gonad arms, in which the mitotic cells at the distal tip of each gonad enter meiosis via transition zone. The germ cell death (apoptosis) occurs only in the late pachytene stage (loop region) of the meiotic compartment (Hubbard, 2005). We quantified the number of apoptotic cells in the *let-60(n1046) gf* mutant fed either control or *frh-1* RNAi.

Consistent with a previous report (Rutkowski et al., 2011), we found that while elevated levels of LET-60(RAS) in basal condition is not enough to elicit an effect on apoptotic machinery, in response to gamma radiation (120 Gy) *let-60(n1046) gf* mutant fed control vector displayed high levels of apoptosis compared to wild type animals fed control vector **(Figure 17B)**. Of note, following gamma radiation, both wild type and *let-60(n1046) gf* mutant fed *frh-1* RNAi showed markedly reduced apoptotic levels compared to their control counterparts and importantly, *frh-1* suppression reduced the number of apoptotic cells in *let-60(n1046) gf* mutant to the level observed in the wild type animal fed with control vector **(Figure 17B)**.

In summary, partial suppression of frataxin rescues *let-60 gf*-mediated DNA damage-induced apoptosis, and this effect is at least in part ascribed to reduced LET-60(RAS) signaling adaptive protective pathways.

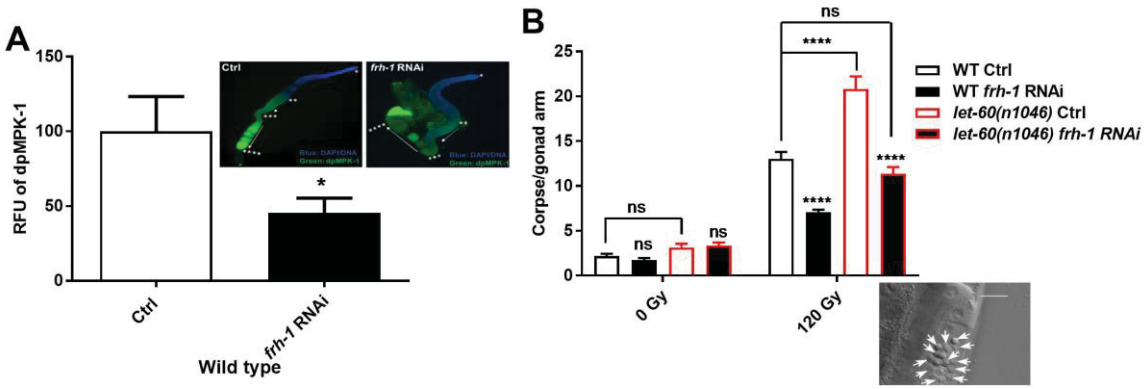

Figure 17: Partial suppression of frataxin attenuates MPK-1(ERK) signaling and mediates *let-60 gf*-mediated DNA damage-induced apoptosis: (A) Immunofluorescence: Images from the graph are the representative pictures of the dissected control and *frh-1* RNAi wild type hermaphrodite germlines stained with diphosphorylated MPK-1 (green) and for DNA (DAPI, Blue). Germlines were treated identically, and the images were taken at the same magnification (10x) and the same settings. In each picture, the germline extends from the mitotic zone (indicated by a * asterisk), the meiotic compartment (indicated by a ** asterisk) and the diakinesis (indicated by a **** asterisk). MPK-1 active regions in the germline are marked with white arrows. The graph represents the relative fluorescence intensity of the active MPK-1 in wild type control and *frh-1* RNAi germlines. Bars and errors indicate mean ± SEM of three independent experiments with at least five germlines analyzed per condition from each experiment. The asterisk in the graph indicates p-value for an unpaired t-test (*p < 0.05). **(B) Quantification of the apoptotic corpse:** Synchronized L4 staged animals were treated using gamma radiations (IR) with the indicated dose. Apoptotic corpses were scored in the wild type, *let-60(n1046) gf* mutant fed control and *frh-1 RNAi* adult hermaphrodite germlines, 24 hrs after the treatment. In the graph, Bars and errors represent mean ± SEM of three independent biological replicates with at least eight germlines scored per condition from each experiment and the asterisks indicate the p-value for ANOVA (****p < 0.0001, 'n.s' stands for not significant). In the graph **(B)** the representative picture of the germline taken using 40x magnification with DIC microscopy, white arrows in the pictures denotes the apoptotic corpse. In all graphs, if not connected by lines, p-value is always to the respective internal control.

We then went on to investigate the effect of partial frataxin depletion on other germline-specific events regulated by LET-60(RAS) signaling such as oocyte maturation and development (Lee et al., 2007). To this end, we decided to monitor fertility and fecundity rate (process tightly linked to oogenesis) in response to *frh-1* silencing. The fertility rate was determined as the number of eggs laid during a 4 hrs time interval while fecundity is the number of larvae hatched from the laid eggs. As expected by the fact that, in basal, unstressed conditions, *let-60(n1046) gf* mutant worms fed control vector did not show overt germ cells apoptosis compared to control fed wild type animals, they also laid a similar number of eggs **(Figure 18A)**. Moreover, consistent with our previous data, partial frataxin suppression significantly reduced egg lay rate in both wild type and *let-60(n1046) gf* mutant worms relative to their control counterparts **(Figure 18A)**. *let-60(n1046) gf* mutant also lay a small percentage of small eggs, which can be identified by their size (half the size of the normal eggs) and shape (round instead of oval). We found that this percentage is not significantly increased relative to wild type animals fed control vector, but it was instead increased by partial frataxin suppression **(Figure 18B)**. Finally, also the survival rate of progeny laid by *let-60(n1046) gf* and wild type animals fed control and *frh-1* RNAi is not significantly different **(Figure 18C)**. Overall, data described so far indicate that *frh-1* RNAi affects specific RAS/MAPk-regulated processes, such as germ cell proliferation and apoptosis, but not others, like oocyte maturation and development. Although the reasons for this specific effect remain to be elucidated, these findings are consistent with above data showing that *frh-1* RNAi does not affect mitosis to meiosis progression.

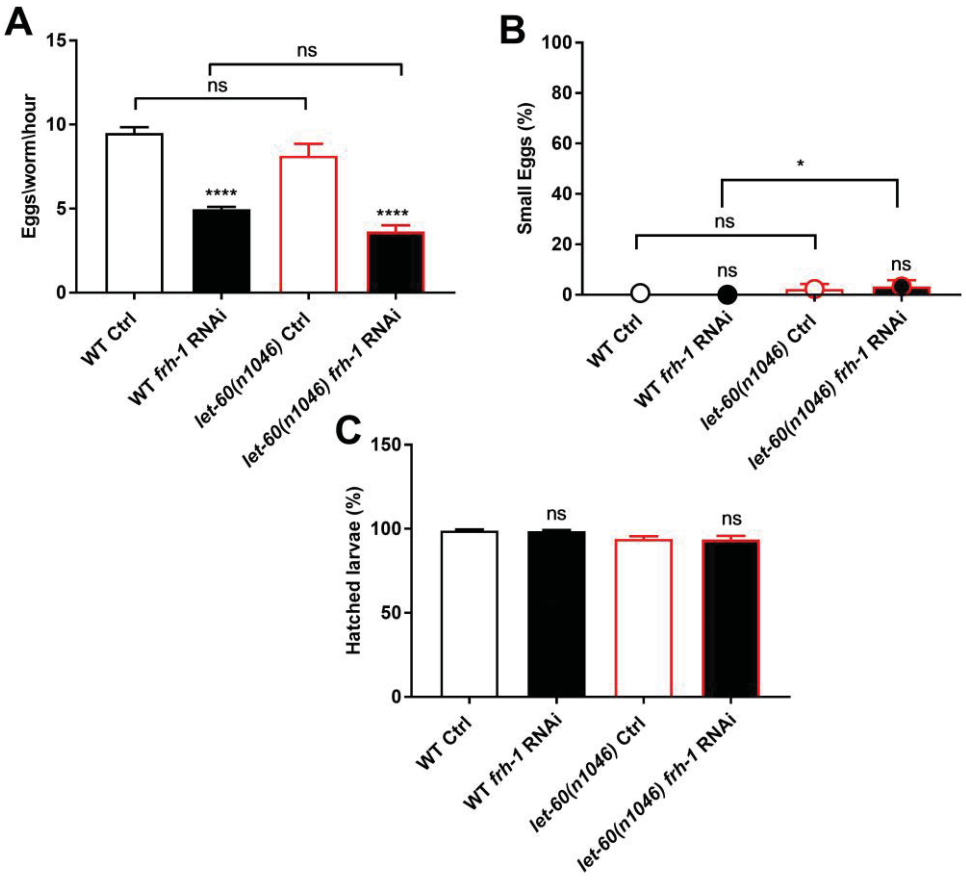

Figure 18: Partial suppression of frataxin does not affect oocyte maturation and development: (**A) Egg lay rate (Fertility):** Graph showing the average number of eggs laid by wild type and *let-60(n1046) gf* mutant animals fed control and *frh-1* RNAi. Bars and errors represent mean ± SEM of two independent biological replicates and the asterisks indicate the p-value for ANOVA (****p < 0.0001, 'n.s' stands for not significant). **(B) Egg lay rate (Fertility):** Graph indicating small eggs percentage laid by wild type and *let-60(n1046) gf* mutant animals fed control and *frh-1* RNAi. Bars and errors represent mean ± SEM of two independent biological replicates and the asterisks indicate the p-value for ANOVA (*p < 0.05, 'n.s' stands for not significant). **(C) Fecundity rate:** Graph showing the percentage of the larvae hatched from the eggs laid by wild type and *let-60(n1046) gf* mutant animals fed control and *frh-1* RNAi. Bars and errors represent mean ± SEM of two independent biological replicates and the asterisks indicate the p-value for ANOVA ('n.s' stands for not significant). In all bar graphs, if not connected by lines, p-value is always to the respective internal control.

4.4 Partial suppression of frataxin attenuates LET-60(RAS) signaling conceivably in a tissue-specific manner

To further assess the interaction between frataxin and specific Ras-regulated processes we decided to investigate the effect of partial frataxin suppression on LET-60(RAS) signaling in different *C. elegans* tissues. A landmark study from (Hirotsu et al., 2000) has demonstrated a dynamic regulatory role of LET-60(RAS)>MPK-1(ERK) kinase in perception and

transmission of sensory signals in a specific subset of olfactory neurons. They showed that normal activation of LET-60(RAS)>MPK-1(ERK) is required for the maturation of AWA and AWC subset of olfactory neurons and either overactivation or inactivating mutations of this pathway leads to olfactory neuronal dysfunction and subsequently olfaction deficit. To reveal the effect of partial suppression of frataxin on sustained LET-60(RAS) signaling in the sensory neurons, chemotaxis assay was performed to compare the sensing ability of wild type and *let-60(n1046) gf* mutant worms fed control or *frh-1* RNAi in response to AWC-sensed chemoattractant benzaldehyde. Consistent with a previous report (Hirotsu et al., 2000) the obtained data demonstrates that *let-60(n1046) gf* mutants fed control vector displayed statistically significant defect relative to wild type animals fed control vector in the attraction to AWC-sensed benzaldehyde. Interestingly, the chemotaxis index in response to partial frataxin suppression in *let-60(n1046) gf* and wild type animals is not significantly different from their control counterparts **(Figure 19A-B).**

To exclude the fact that altered ability of the worms to reach the attractant (chemotaxis index) was explicitly ascribed to specific sensory rather than locomotor defect, we monitored animal's ability to move by counting the number of body bends during a 60-sec interval. *let-60(n1046) gf frh-1* RNAi animals speed was significantly reduced relative to *let-60(n1046) gf* control and a similar trend was observed in the wild type animals **(Figure 19C)**. Overall, compared to wild type, *let-60(n1046) gf* mutant animals speed was dramatically reduced in response to both control and *frh-1* RNAi. This result suggests that AWC-sensed olfaction deficit observed in the *let-60(n1046) gf* mutant is mainly due to dramatically reduced animals speed. Nonetheless, a marginal difference of chemotaxis index between 1 hr and 4 hrs **(Figure 19A-B)** indicates that reduced olfactory defect observed in *let-60(n1046) gf* mutant animals was not simply ascribed to their reduced ability to move but also in part to a specific AWC neuronal deficit. Consistent with our idea, prior work from our lab (Maglioni et al., 2014) reported that sensory and locomotor ability of the worms is not always mechanistically correlated. In line with our data, observational studies from (Hirotsu et al., 2000) showed that despite their abnormality in locomotion *let-60(n1046) gf* mutants normally respond in chemotaxis to the ASE-sensed water-soluble attractant sodium chloride (NaCl). In summary, partial frataxin suppression does not influence the AWC-sensed olfaction deficit of the *let-60(n1046) gf* mutant animals again suggesting frataxin is affecting unique Ras-regulated processes conceivably in a tissue-specific manner.

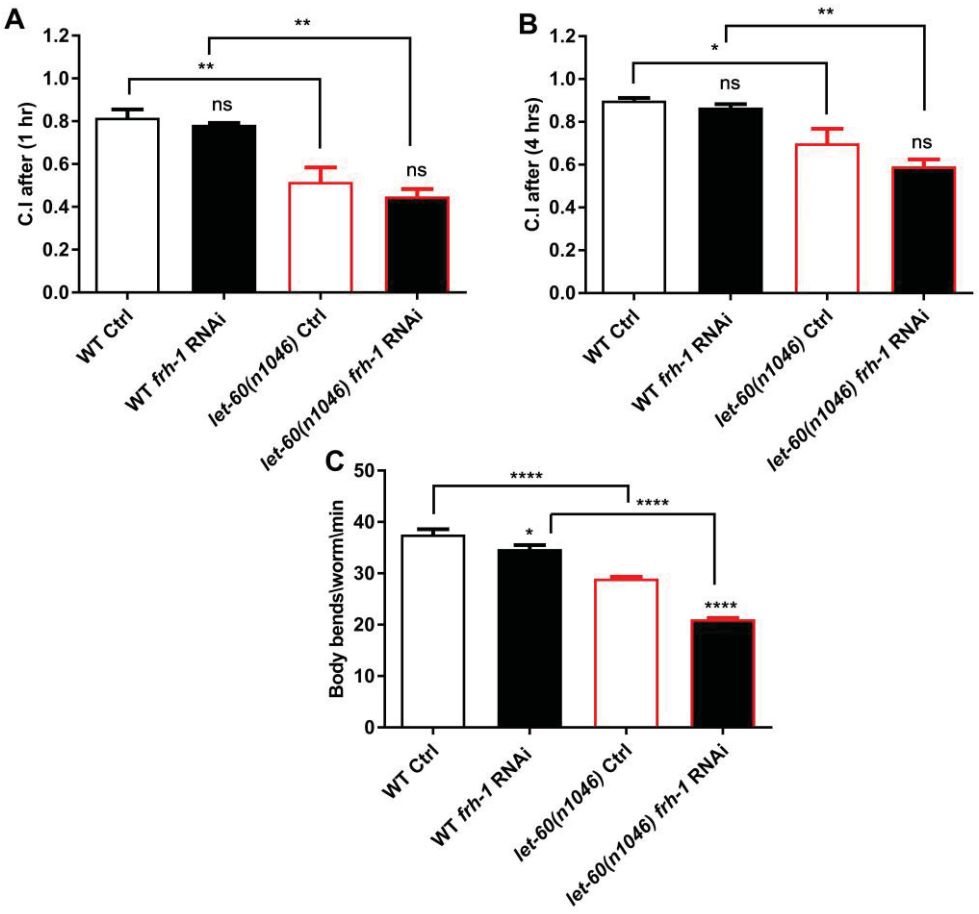

Figure 19: Partial suppression of frataxin does not affect the *let-60 gf* mediated AWC olfactory signaling: (A-B) Chemotaxis index (C.I) to AWC-sensed chemoattractant benzaldehyde, of wild type and *let-60(n1046) gf* mutant worms fed control and *frh-1* RNAi. Chemotaxis index calculated one hr **(A)** and **(B)** four hr after beginning the assay. Bars and errors indicate mean ± SEM of three independent biological replicates and the asterisks indicate the p-value for ANOVA (*p < 0.05, **p < 0.01, 'n.s' stands for not significant). **(C) Locomotor performance assessment:** The graph indicates the number of body bends (Locomotor performance) displayed by wild type and *let-60(n1046) gf* mutant worms fed control and *frh-1* RNAi. Bars and errors indicate mean ± SEM of three independent biological replicates and the asterisks indicate the p-value for ANOVA (*p < 0.05, ****p < 0.0001). Both chemotaxis and body bend assay was performed in day-4 old gravid adults. In all bar graphs, if not connected by lines, p-value is always to the respective internal control.

In an attempt, to further elucidate the effect of partial frataxin suppression on sustained LET-60(RAS) signaling in various tissues, we then took advantage of *C. elegans* multivulva (MuV) mutants. The *C. elegans* hermaphrodite vulval development is a well-established process for studying LET-60(RAS) signaling network. Indeed the LIN-3(EGF)>LET-23(EGFR)>LET-60(RAS)>LIN-45(Raf)>MEK-2(MEK)>MPK-1(ERK) cascade in combination with LIN-12 notch signaling dictates cell fate decisions required for the proper development of the vulva, an epithelial opening through which embryos are released out from

the mother (Greenwald, 1997). Persistent activation of LET-23(EGFR)>LET-60(RAS) kinase pathway results in the formation of a single functional vulva and ectopic protruding non-functional vulva-like structures called pseudovulva thus resulting in a multivulva (MuV) phenotype **(Figure 20B – representative picture)**. We used *let-60(n1700) gf* mutant allele, which, similar to the other *let-60 gf* mutant, possess a G13E point mutation in the *let-60* gene leading to reduced conversion rate of GTP-bound active form to GDP-bound inactive form and subsequent increased activation of the encoded LET-60(RAS) protein (Beitel et al., 1990). Excessive LET-60 activation in the vulva precursor cells leads to the MuV phenotype, which can be easily scored by counting the ectopic vulval protrusions under a dissecting microscope. Intriguingly, the obtained data showed that *let-60(n1700) gf* mutant animals fed *frh-1* RNAi displayed significantly reduced number of ectopic vulval protrusions relative to control animals **(Figure 20A)**. This effect was even more evident when ectopic vulval protrusions were scored at later days of the animal life.

Next, we aimed to map the specific site of action of partial frataxin suppression on the EGFR dependent LET-60(RAS) signaling. To this extent, we scored the ectopic vulval protrusions caused by a dominant mutation in *C. elegans* EGFR ortholog, LET-23, which is upstream to LET-60(RAS). *jgIs6* transgenic strain consists of a chimeric construct (LET-23::hEGFR-TK(L858R), expressing C. *elegans* EGFR ortholog LET-23 N-terminal domain attached to human EGFR-TK domain, and to LET-23 C-terminal domain. *jgIs6* mutant display a single point mutation in L858R of human EGFR-TK domain, found in lung cancer in human, resulting in sustained EGFR signaling and subsequently a multivulva phenotype (Bae et al., 2012). As expected, an ectopic number of vulval protrusions were scored in dominant *jgIs6* mutants, but this number did not change between animals fed control or *frh-1* RNAi **(Figure 20B)**. The obtained data so far suggests that partial frataxin depletion reduces the *let-60* gain of function induced Muv phenotype but not by acting on the upstream LET-23(EGFR) receptor.

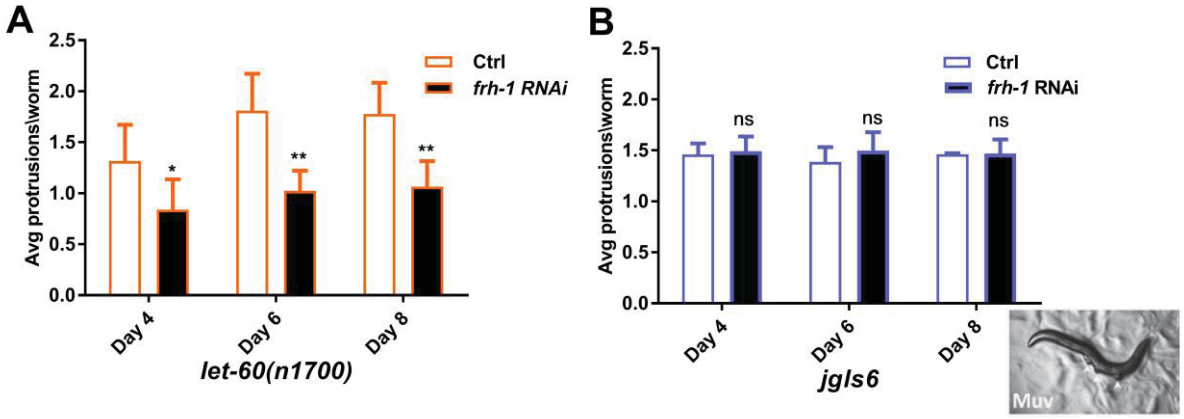

Figure 20: Partial frataxin suppression reduces the *let-60* gain of function induced Muv phenotype but not by acting on the upstream LET-23(EGFR) receptor: (A-B) Quantification of ectopic vulval protrusions: **(A)** In *let-60(n1700) gf* mutant and **(B)** *jgIs6 gf* mutant animals fed control and *frh-1* RNAi. Multivulva count was assessed on day 4, day 6 and day 8 age-synchronized population of worms per condition at 20ºC. Bars and errors indicate mean ± SEM of three independent biological replicates with at least 90 age-synchronized worms ectopic vulval protrusions scored per condition from each experiment, and the asterisks indicate the p-value for ANOVA (*p < 0.05, **p < 0.01, 'n.s' stands for not significant). The image from the graph **(B)** indicates the representative picture of the multivulva phenotype. White arrowheads indicate the ectopic vulval protrusions. Image from Wormbook. In all bar graphs, the p-value is always to the respective internal control.

4.5 Pro-longevity frataxin suppression prevents premature organismal death caused by *let-60*/*ras* tumor mutations

In *C. elegans*, genetic interventions that promote pro-longevity beneficial effects prevent the premature organismal death caused by germline tumor mutations (Pinkston et al., 2006). Compelling evidence from our lab showed that partial suppression of frataxin through RNAi prolongs the lifespan of *C. elegans* (Ventura et al., 2005). We, therefore, asked whether reduced frataxin expression could also prevent the premature death caused by *let-60 gf* tumor mutations. To test this possibility, we assessed the lifespan of *let-60(n1700) gf* worms fed control vector or *frh-1* RNAi. Of note, *frh-1* RNAi significantly extended the lifespan compared to control animals **(Figure 21A)**. The mean lifespan of *let-60(n1700) gf* fed control vector is short-lived when compared to the lifespan of wild type control animals (20.03 ± 0.31). Of note, the mean lifespan of *let-60(n1700) gf* fed *frh-1* RNAi animals is about 21 days **(Figure 21A)**, indicating that partial frataxin depletion extends the lifespan of short-lived *let-60(n1700) gf* mutant animals up to the wild type level.

We next asked whether partial frataxin suppression extends the lifespan of the short-lived *let-60(n1700) gf* mutant animals in a healthy manner. Like humans, *C. elegans* experience a progressive decline in muscle integrity and coordination (sarcopenia) during aging. Hence, we aimed to assess the locomotor performance, a parameter which serves as a reliable behavioral readout of healthspan in *C. elegans* (Huang et al., 2004). To this extent, we monitored animal's ability to move (locomotion) by counting the number of body bends during a 60-sec interval. Locomotion assay was performed on day 4 and day 7 age-synchronized wild type and *let-60(n1700) gf* mutant animals fed control or *frh-1* RNAi. At day 4, both wild type and *let-60(n1700) gf* mutant control and *frh-1* RNAi animals displayed similar locomotor performance **(Figure 21B)**. Whereas, at day 7, wild type and *let-60(n1700) gf* long-lived *frh-1* RNAi animal's locomotor performance was significantly improved compared to their control counterparts **(Figure 21B)**. Our data, in agreement with a previous report (Huang et al., 2004) suggests that lifespan and locomotor performance are positively correlated and increased by *frh-1* RNAi in both wild type and *let-60* mutants.

Next, we also assessed the functionality of another muscle, the pharynx, a neuromuscular organ, which undergoes rhythmic contractions and facilitates feeding. Pharyngeal pumping rate is another parameter which serves as a marker of healthspan in *C. elegans* and indeed declines with progressive aging (Huang et al., 2004). Pharyngeal pumping was assessed by counting the number of pharyngeal contractions during a 60-sec interval. At day 4, wild type and *let-60(n1700) gf* control and long-lived *frh-1* RNAi animals displayed similar pharyngeal pumping rate **(Figure 21C)**. Whereas, at day 7, wild type long-lived *frh-1* RNAi animals displayed significantly increased pharyngeal pumping rate relative to wild type control animals. On the other hand, pharyngeal pumping rate was not significantly different between *let-60(n1700) gf* fed control or *frh-1* RNAi animals **(Figure 21C)**.

Although most of the research groups consider pharyngeal pumping as a reliable parameter of healthspan in *C. elegans*, this is not always the case. For instance, *daf-2(e1370), clk-1 (qm30), ife-2(ok306)* well established *C. elegans* long-lived mutants, do not display improved pharyngeal pumping capacity as the animals age, suggesting that healthspan and pharyngeal pumping are not always positively correlated (Bansal et al., 2015). Taken together, data shown so far indicate that reduced frataxin expression extends the lifespan of the short-lived *let-60(n1700) gf* animals in a healthier manner.

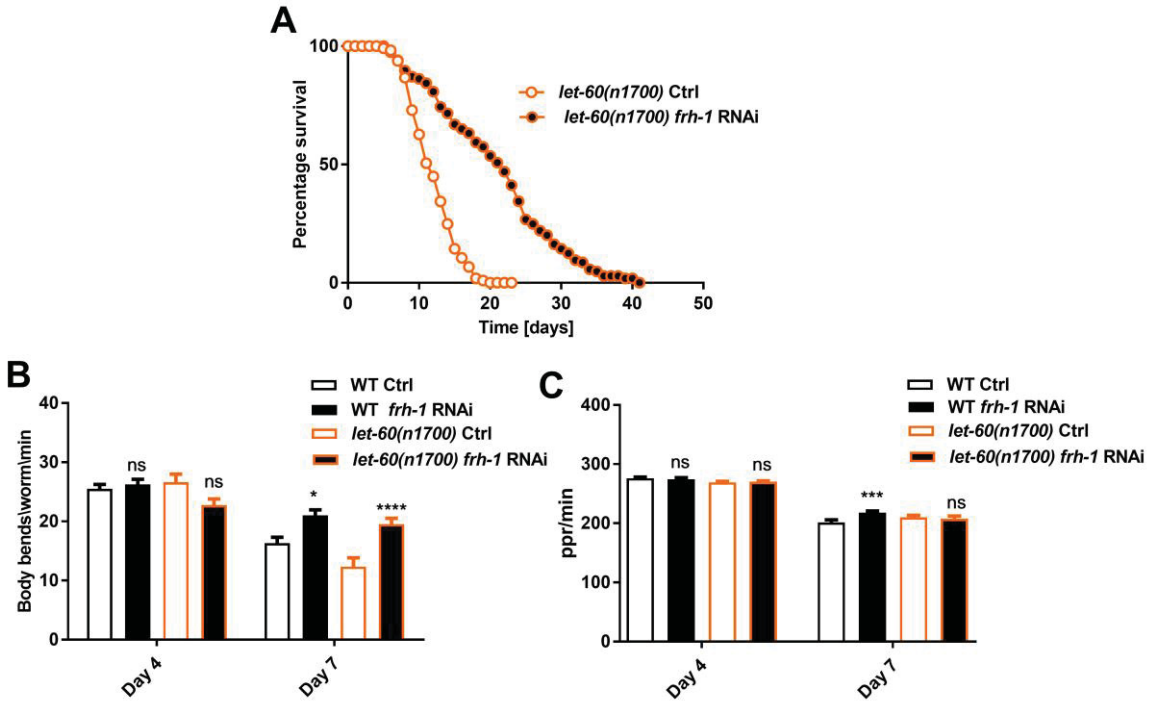

Figure 21: Partial suppression of frataxin extends lifespan of the short-lived *let-60(n1700) gf* mutant in a healthier manner: (A) Lifespan survival plot: Mean lifespan of *let-60(n1700) gf* control is 12.03 ± 0.31 (120 animals) vs 20.83 ± 0.82 (120 animals) for *let-60(n1700) gf frh-1* RNAi. Mean pooled data of two biological replicates calculated by using a log-rank test from Kaplan-Meier survival analysis (***p < 0.001). **(B) Locomotor performance assessment:** Graph showing the number of body bends (Locomotor performance) displayed by wild type and *let-60(n1700) gf* mutant worms fed control and *frh-1* RNAi. Locomotion assay was performed on day 4 and day 7 age-synchronized wildtype and *let-60(n1700) gf* mutant animals. Bars and errors indicate mean ± SEM of two independent biological replicates with at least ten age-synchronized worms scored per condition from each experiment and the asterisks indicate the p-value for ANOVA (*p < 0.05, ****p < 0.0001, 'n.s' stands for not significant). **(C) Pharyngeal pumping rate:** Graph showing the pharyngeal pumping rate, assessed by observing the number of pharyngeal contractions per minute. Bars and errors indicate mean ± SEM of two independent biological replicates with at least ten age-synchronized worms scored per condition from each experiment and the asterisks indicate the p-value for ANOVA (***p < 0.001, 'n.s' stands for not significant). In all bar graphs, the p-value is always to the respective internal control.

4.6 Partial suppression of frataxin modulates longevity and anti-tumor effect within a defined window of mitochondrial impairment

Pioneering evidence from our lab showed that by using an RNAi-based methodology, inactivation of *frh-1* and of other mitochondrial respiratory chain (MRC) regulatory protein extends the lifespan of wild type *C. elegans* only within a discrete window of gene suppression and thus of MRC disruption (Rea et al., 2007, Ventura et al., 2005). On the other hand, inactivation of *frh-1* below or beyond a given threshold concentration had no effect or

detrimental effect on *C. elegans* lifespan (Rea et al., 2007). We then asked whether *frh-1* silencing also reduces ectopic vulval protrusions and prevents premature organismal death caused by *let-60* tumorigenic mutations within a defined window of mitochondrial disruption. To this extent, we assessed lifespan and ectopic vulval protrusions of *let-60(n1700) gf* mutant animals fed *frh-1* RNAi for three consecutive generations (designated as F1, F2, F3). Indeed, *frh-1* RNAi was shown to have its most effective pro-longevity activity after three generation of continuous feeding (Ventura et al., 2005), the generation analyzed in all of the above experiments. Feeding worms with *frh-1* RNAi over several generations incrementally reduce *frh-1* mRNA levels from 30% (F1 generation) to 70% (F3 generation) (Ventura et al., 2005). As expected, the obtained data showed that gradual reduction of *frh-1* expression levels (F1, F2, F3 generations) monotonically increased the lifespan and reduced ectopic vulval protrusions of *let-60(n1700) gf* mutant animals compared to *let-60(n1700) gf* control animals **(Figure 22A-F)**. In summary, our data indicate that *frh-1* inactivation modulates longevity and attenuates *let-60 ras*-mediated tumor effects only when a certain threshold of mitochondrial stress is achieved.

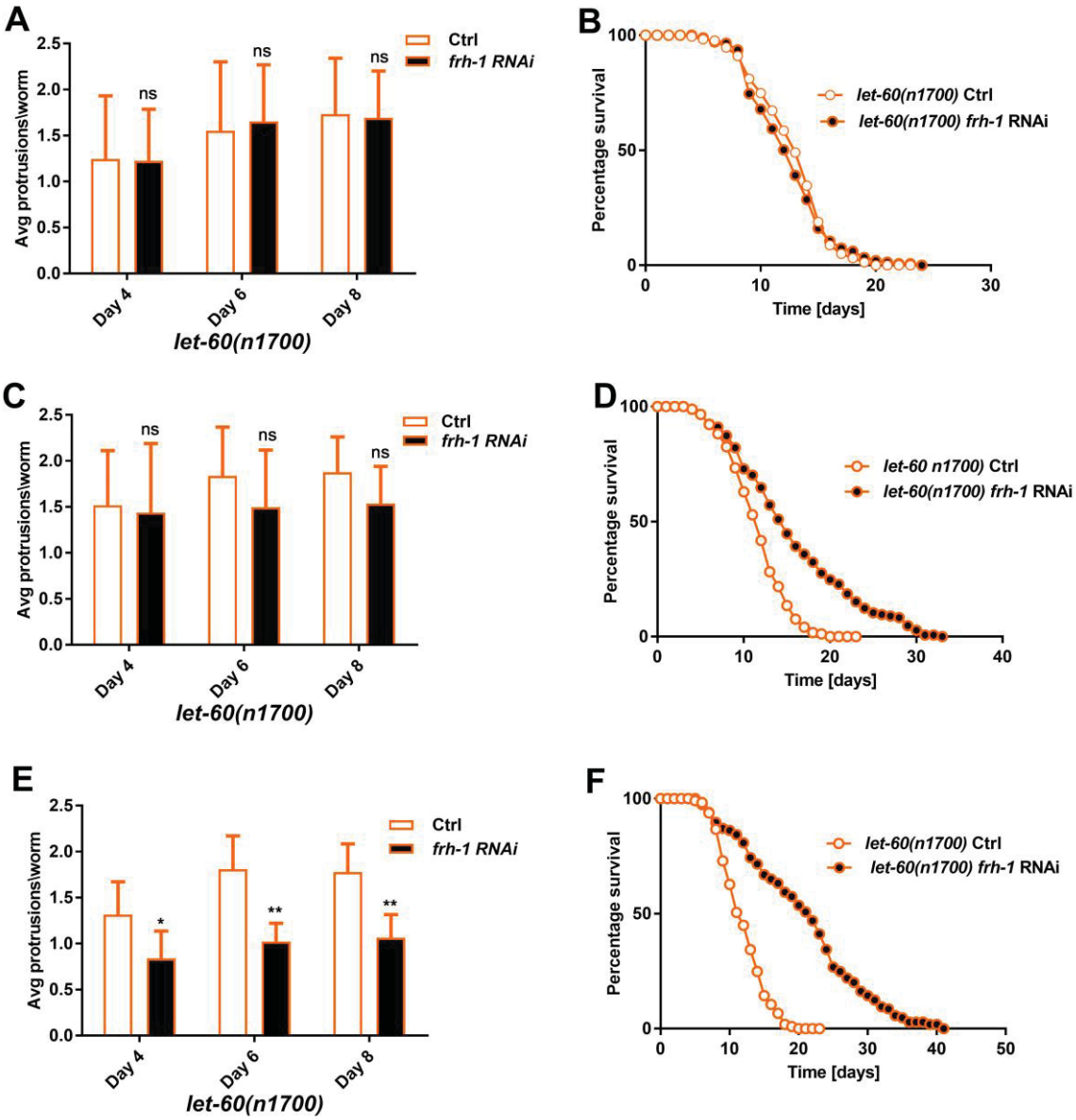

Figure 22: Partial suppression of frataxin extends lifespan and reduces Muv in a defined window of mitochondrial impairment: (A-C-E) Quantification of ectopic vulval protrusions: (A) F1 generation **(C)** F2 generation **(E)** F3 generation of *let-60(n1700) gf* mutant fed control and *frh-1* RNAi animals. Multivulva count was assessed in day 4, day 6 and day 8 age-synchronized population of worms at 20ºC. Bars and errors indicate mean ± SEM of three independent biological replicates with at least 90 age-synchronized worms ectopic vulval protrusions were scored per condition from each experiment and the asterisks indicate the p-value for ANOVA (*p < 0.05, **p < 0.01, 'n.s' stands for not significant). In all bar graphs, the p-value is always to the respective internal control. **(B-D-F) Lifespan survival plots: (B)** F1 generation; mean lifespan of *let-60(n1700) gf* control is 12.84 ± 0.25 (180 animals) vs 12.54 ± 0.27 (180 animals) for *let-60(n1700) gf frh-1* RNAi, (p < 0.64; ''ns'' not significant). **(D)** F2 generation; mean lifespan of *let-60(n1700) gf* control is 11.68 ± 0.26 (180 animals) vs 15.85 ± 0.56 (180 animals) for *let-60(n1700) gf frh-1* RNAi, (***p < 0.001). **(F)** F3 generation mean lifespan of *let-60(n1700) gf* control is 12.03 ± 0.31 (120 animals) vs 20.83 ± 0.82 (120 animals) for *let-60(n1700) gf frh-1* RNAi, (***p < 0.001). Mean pooled data calculated using a log-rank test from Kaplan-Meier survival analysis.

Discussion

Frataxin is a nuclear encoded mitochondrial protein implicated in the biogenesis of iron-sulphur cluster proteins (Bencze et al., 2006). Compelling evidence from our lab showed that, using an RNAi-based methodology, inactivation of frataxin extended the lifespan of wild type *C. elegans* within a defined window of mitochondrial impairment which is required to trigger protective, pro-longevity pathways (Ventura et al., 2005, Rea et al., 2007). Further investigations from our lab demonstrated that the long-lived *frh-1* RNAi-depleted animal's germline displayed an improved cell defense against DNA damage and dampened mitotic-meiotic proliferation rate (Torgovnick et al., *manuscript in preparation*) features almost often affected in cancer. Aging and cancer are intrinsically coupled. For instance, people aged above 50 are often vulnerable to cancer relative to younger individuals and a similar correlation persists across the divergent phyla. In line with this possibility, we aimed to exploit the anti-aging effect of partial frataxin suppression as a tumor suppressor strategy using *C. elegans* as a cancer model.

We found that pro-longevity partial frataxin suppression reduces basal germ cells proliferation in the mitotic compartment, and more importantly it reduced germline tumor size in the *glp-1(ar202*) mutants, as the animals age. *glp-1(ar202*) tumors are ascribed to a block in germ cells mitotic-to-meiotic progression with concomitant germ cell mitotic over-proliferation. Reduced tumor size could result either from reduced germ cells proliferation or from increased apoptosis. So, there are no cells which could undergo apoptosis. However, only meiotic cells are prompted to undergo apoptosis, and we have shown that *frh-1* RNAi does not restore oocyte production by overcoming the mitotic-to-meiotic block suggesting that *frh-1* silencing is most likely dampening *glp-1(ar202)* germ cell proliferation rather than increasing apoptosis. This is supported by our previous findings showing that partial frataxin suppression induces cellular protective pathways resulting in germline cell-cycle stalling and consequently slower cell cycle progression (Torgovnick et al., *manuscript in preparation*).

Interestingly, according to a specific role of frataxin depletion in reducing germ cell proliferation, rather than in favoring mitotic to meiotic progression, we found that in basal conditions, wild type *frh-1* RNAi-depleted animal's displayed reduced germ cells also in the meiotic compartment (Torgovnick et al., *manuscript in preparation*) and reduced activation of MPK-1(ERK), whose activation is in fact required for meiotic progression) . We then

asked whether *frh-1* RNAi could also attenuate the sustained activation of MPK-1(ERK) prompted by a dominant mutation in *let-60/ras* (an upstream activator of MPK-1). Consistent with this possibility we showed that the increased sensitivity of the *let-60(n1046)* gain of function mutants to gamma radiation-induced germline apoptosis was reduced in response to *frh-1* silencing up to the level of wild type control. Frataxin silencing could reduce genotoxic stress induced germline apoptosis in the *let-60* mutants either by direct attenuation of sustained LET-60(RAS) activation or through activation of BRC/BRD complex, which we have recently found to provide resistance to germline apoptosis in response to partial frataxin suppression (Torgovnick et al., *manuscript in preparation*). BRC and BRD are the orthologs of human BRCA-1, and BRDA-1 (tumor suppressor genes) play a critical role in double standard DNA break repair (Boulton et al., 2004). Another interesting possibility would be that frataxin depletion reduces radiation-induced germ cells apoptosis through a cross-talk between RAS and the BRD/BRC complex.

To further investigate the specific role of frataxin depletion in dampening the Ras signaling, we looked at other Ras-regulated processes. Sustained activation of LET-60(RAS) in the germline provokes deleterious effects on the oogenic process (a process that occurs after meiosis), resulting in reduced egg lay rate, increased small eggs production, reduced progeny survival rate. The above listed germline-specific phenotypes were not prominent in the *let-60(n1046)* mutant animals most likely because this is a vulval specific gain of function mutant, which display frail sustained LET-60(RAS) activation in the germline, whose strength is not enough to incite an effect on oogenic process, and is indeed more sensitivity to genotoxic stress only in response to high doses of radiations such as those used in this study. However, the tendency of increased small egg production was noticed suggesting that partial *frh-1* depletion does not influence the oocyte development and maturation processes.

We also investigated the effect of partial frataxin suppression in a specific subset of olfactory neurons, whose sensing ability is negatively regulated by sustained LET-60(RAS) activation (Hirotsu et al., 2000). Also, in this case, we could not find any effect of *frh-1* silencing on the AWC-sensed olfaction deficit of the dominant *let-60(n1046)* mutants. Although in *C. elegans*, studies have shown that sensitivity to the RNAi in neuronal cells varies depending on the specific neuronal properties and neuron subtype (Asikainen et al., 2005), it is unlikely that *frh-1* RNAi is not working in these neurons since it is in fact reducing their functions in the wild type animals. However, we cannot exclude that *let-60* mutants display a general

reduced sensitivity to RNAi (as silencing efficacy can vary between genotypes). Interestingly, we also found that frataxin silencing reduces multivulva phenotype (ectopic vulva protrusions), and extends the lifespan of the *let-60* gain of function mutants, and this effect was not present in mutants with overactivating mutations in genes upstream to *let-60*. Partial frataxin depletion reduces ectopic vulval protrusions and extends the lifespan of the dominant *let-60* gain of function mutants within a discrete window of mitochondrial ETC disruption, suggesting that similar to its effect on lifespan, making mitochondrial work at the optimal level through partial frataxin inactivation also confer tumor suppressor activity. Taken together our findings suggest that frataxin silencing is inhibiting unique Ras tissue-specific signaling, and this, at least in the case of the vulva specific effects, directly correlate with the ability of *frh-1* RNAi to extend animal lifespan. Whether or not reduced Ras signaling in the germline is also causally involved in lifespan extension remain to be evaluated. However, data collected for the other manuscript *(*Torgovnick et al*., manuscript in preparation*) indicate that moderate mitochondrial stress, through partial *frh-1* or *isp-1* silencing, elicits protective germline pathways, which are not causally involved in somatic resistance to stress. Namely, we found that mild mitochondrial stress confers germline resistance to genotoxic insults **(Figure 15A-D)** but perhaps surprisingly do not provide longevity and somatic resistance (Torgovnick et al., *manuscript in preparation*). Namely, following UVB (genotoxic stress) the long-lived *frh-1* RNAi and *isp-1* RNAi (another long-lived mitochondrial mutant) do not provide resistance but in fact affected the lifespan similar to control animals in a dose-dependent manner **(Figure S1).** Furthermore, we showed that the germline is not required for mitochondrial stress mediated lifespan extension **(Figure S2)**. The requirement of the germline-specific Ras signaling in modulating lifespan along with the exact molecular mechanisms linking frataxin suppression to diminished Ras signaling will require further studies.

In wild type worms, moderate levels of frataxin inactivation extend the lifespan by invoking several compensatory pathways in response to compromised mitochondrial functionality, explicitly genes involved in detoxification, autophagy, and genomic stability (Liu et al., 2014, Schiavi et al., 2013, Torgovnick et al., *manuscript in preparation*). Lately, for the first time, novel insights from our lab also showed that partial frataxin suppression mediated mild mitochondrial stress extends lifespan through the activation of mitophagy, which is promoted in response to iron deprivation **(Figure S3)** (Schiavi et al., 2015). It will be interesting to investigate whether some of the same genes or pathways (most important defenders against

cancer development and aging) could interact with the Let-60(Ras) signaling thus being responsible for reducing germline tumor formation and MuV phenotype and for preventing organismal death caused by *let-60* tumor mutations.

Conclusion and future research directions

We have shown that making mitochondria work at the optimal level through mild frataxin depletion reduces the deleterious effects, conceivably in a tissue-specific manner and hinder premature organismal death caused by tumor mutations. These findings, in line with previous reports (Pinkston et al., 2006), reveals that those mechanisms that promote anti-aging effect concurrently provide anti-cancer protection. Consistent with this notion, we propose that the compensatory pathways such as iron depletion-mediated mitophagy and DNA damage repair pathways evoked in response to moderate levels of frataxin inactivation might act as a brake on tumor development and aging **(Figure 23)** (Shaik et al., 2016). Persistence of moderate iron deprivation, faithful mitophagy, and DNA damage response machinery safeguard against cancer development and aging in an evolutionarily conserved manner (Zhang and Zhang, 2015, Gargini et al., 2011, Broustas and Lieberman, 2014). Based on findings presented in my Ph.D. thesis it will be thus interesting to assess the possible crosstalk between those pathways and the Ras signaling in providing an anti-cancer effect and concurrently extend lifespan. A deeper investigation of how mild mitochondrial stress (through frataxin depletion or other genetic or pharmacological interventions) may trigger anti-aging mechanisms ultimately promoting resistance to cancer might provide new insights into cancer biology and indicate novel possible targeted strategy to prevent or suppress tumor formation.

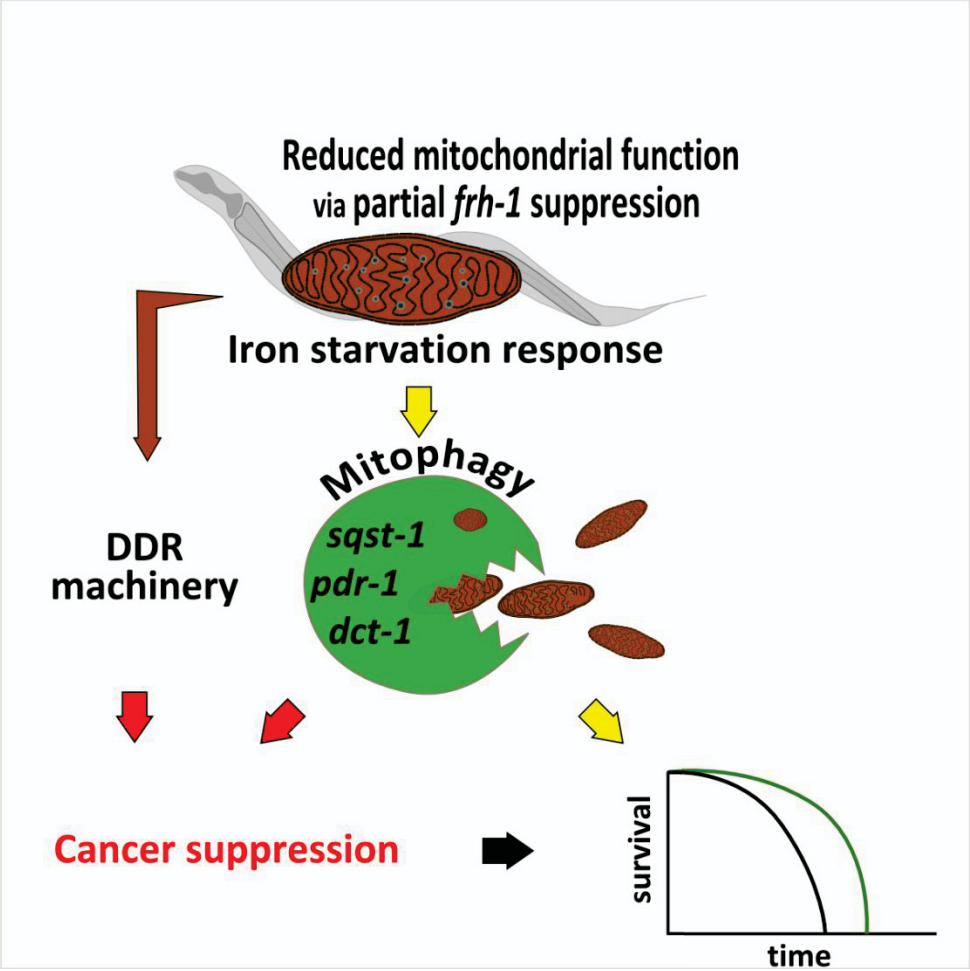

Figure 23: A recent elegant study from our lab reported (yellow arrows) that in *C. elegans* lowering mitochondrial functionality via partial suppression of *frh-1* elicit pro-longevity beneficial effect in a *pdr-1*/ Parkin-, *pink-1*/ Pink-, and *dct-1*/ Bnip3- mediated mitophagy dependent manner, which is promoted through mild iron depletion (Schiavi et al., 2015). On the other hand, DNA damage response (DDR) machinery components (e.g. BRC-1, BRD-1, CEP-1), are activated to cope up with mild mitochondrial stress ultimately resulting in efficient maintenance of genomic integrity (brown arrow) (Torgovnick et al., *manuscript in preparation*). DNA damage signaling, moderate iron deprivation and mitophagy are the most important defenders against cancer development (red arrows) (Zhang and Zhang, 2015, Gargini et al., 2011, Broustas and Lieberman, 2014). Therefore, it will be interesting to study whether constitutive activation of iron depletion mediated mitophagy and existence of faithful DNA damage signaling are implicating in providing an anti-cancer effect. Figure with modifications from (Shaik et al., 2016).

List of publications

Publications

Shaik, A., Schiavi, A. and Ventura, N. (2016) 'Mitochondrial autophagy promotes healthy aging,' *Cell Cycle,* 15(14), pp. 1805-6.

Schiavi, A., Maglioni, S., Palikaras, K., **Shaik, A.**, Strappazzon, F., Brinkmann, V., Torgovnick, A., Castelein, N., De Henau, S., Braeckman, B. P., Cecconi, F., Tavernarakis, N. and Ventura, N. (2015) 'Iron-Starvation-Induced Mitophagy Mediates Lifespan Extension upon Mitochondrial Stress in C. elegans', *Curr Biol,* 25(14), pp. 1810-1822.

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In preparation

Torgovnick, A., Schiavi, A., Kassahun, H., **Shaik, A**., Maglioni, S., Schumacher, B., Nilsen, H. and Ventura, N. (*manuscript in preparation*) 'Pro-longevity mitochondrial disturbance promotes BRC-1/BRD-1 dependent and independent germline resistance to genotoxic stress'

Shaik, A., Runchi, A. and Ventura, N. (*manuscript in preparation*) 'Partial frataxin suppression in *Caenorhabditis elegans* reduces tumor formation by dampening the RAS/MAPk signaling'.

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Appendix

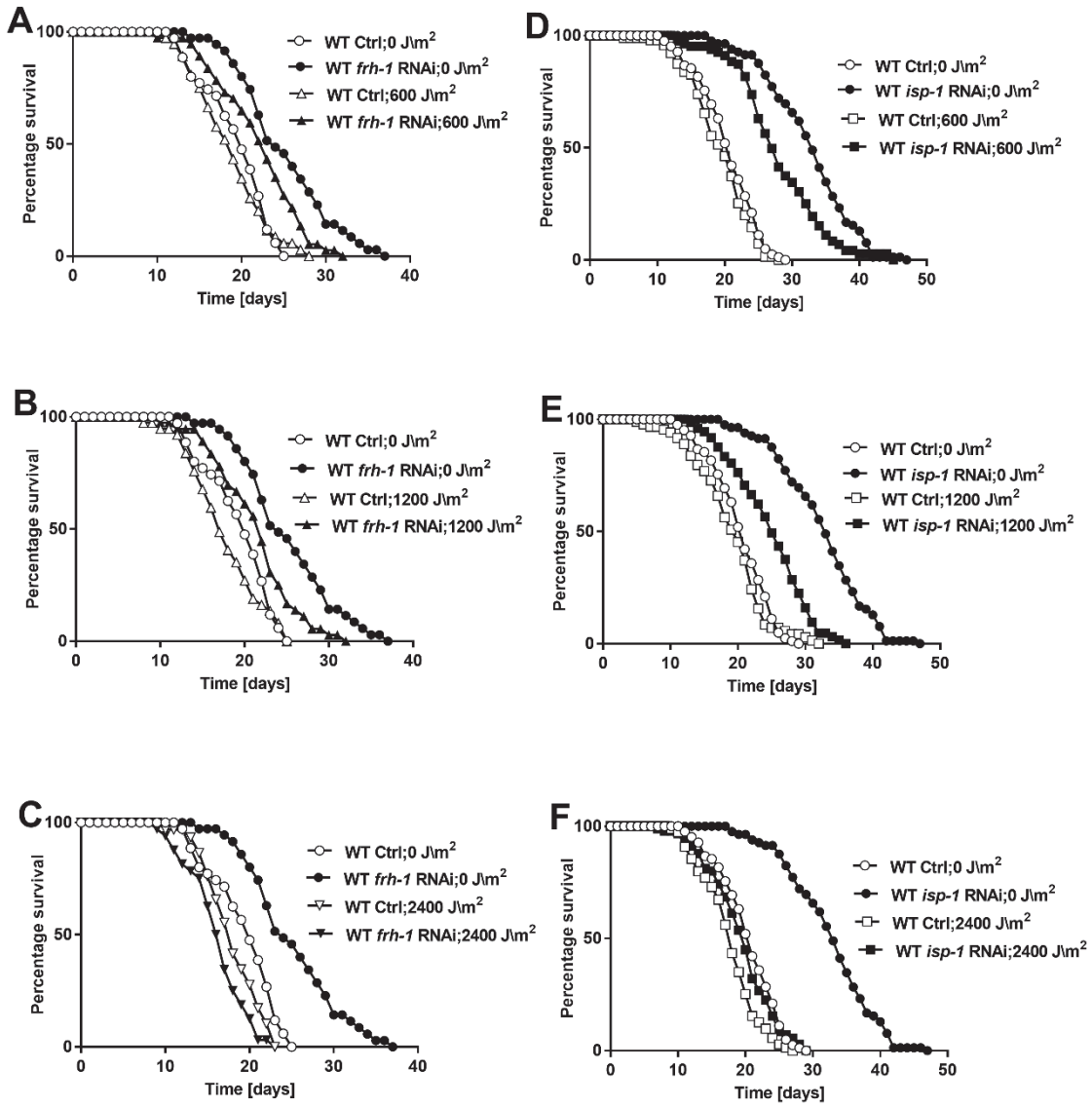

Figure S1: UVB affects the lifespan of *frh-1* RNAi and *isp-1* RNAi similar to control in a dose-dependent manner: (A-C) Lifespan Survival plots: Of wild type control vs wild type *frh-1* RNAi animals; **(A)** Gravid adults treated with 600 J\m^2 UVB dose **(B)** Gravid adults treated with 1200 J\m^2 **(C)** Gravid adults treated with 2400 J\m^2 UVB dose. Data of one biological replicate (~ 40 gravid adults used per condition) calculated by using a log-rank test from Kaplan-Meier survival analysis. **(D-F) Lifespan Survival plots:** Of wild type control vs. wild type *isp-1* RNAi animals; **(D)** Gravid adults treated with 600 J\m^2 UVB dose **(E)** Gravid adults treated with 1200 J\m^2 **(F)** Gravid adults treated with 2400 J\m^2 UVB dose. Mean pooled data of two biological replicates (~ 95 gravid adults used per condition) calculated by using a log-rank test from Kaplan-Meier survival analysis.

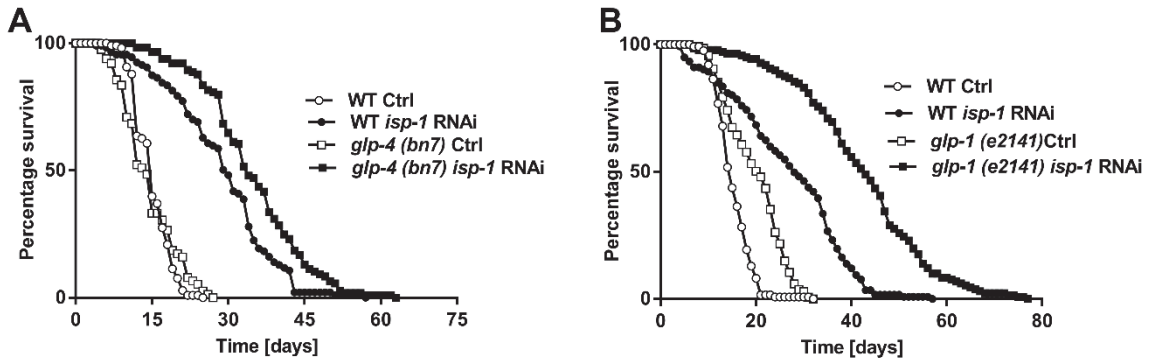

Figure S2: Partial suppression of *isp-1* via RNAi extends lifespan in a germline independent manner. (A-B) Survival plots of wild type (WT) and **(A)** *glp-4(bn7), **(B)** glp-1(e2141)* mutant control and *isp-1* RNAi animals. Mean pooled data of two biological replicates (~ 120 gravid adults used per condition) calculated by using a log-rank test from Kaplan-Meier survival analysis.

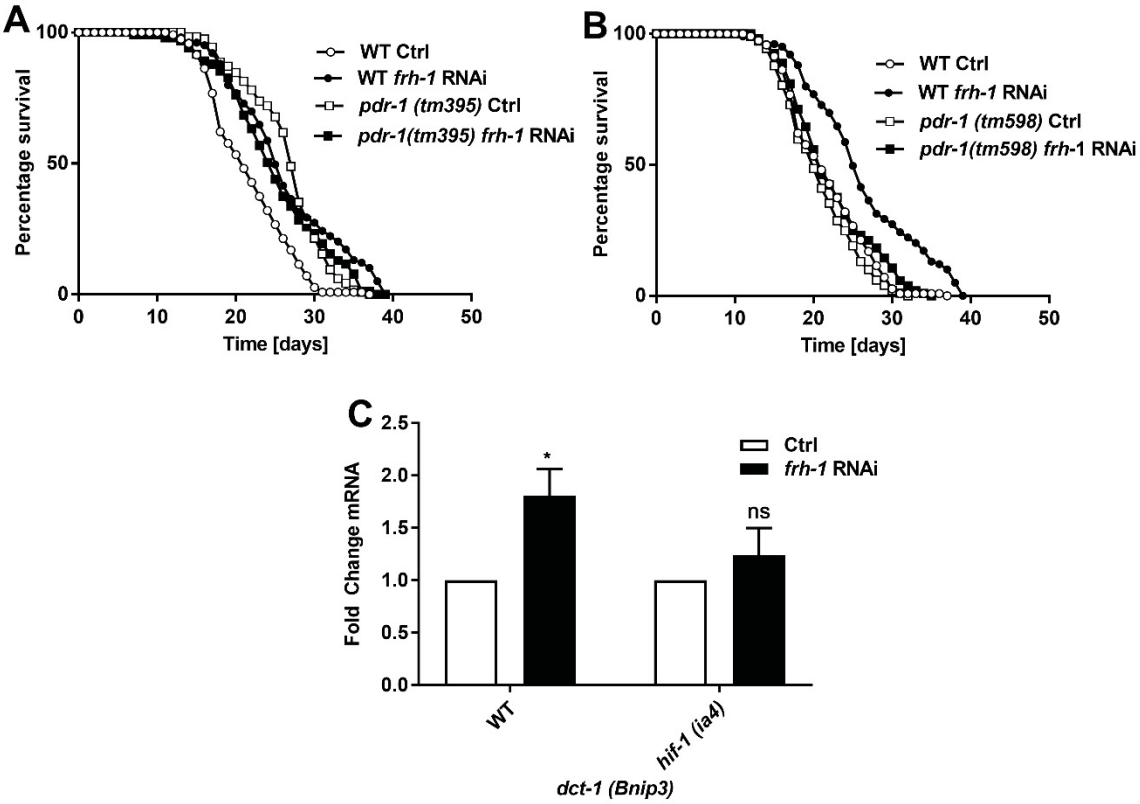

Figure S3: Partial frataxin depletion mediated mitophagy activation is required for the extension of lifespan. (A-B) Survival plots of wild type (WT) and **(A)** *pdr(tm395), **(B)** pdr-1(tm598)* mutant control and *frh-1* RNAi animals. Mean pooled data of two biological replicates (~ 135 gravid adults used per condition) calculated by using a log-rank test from Kaplan-Meier survival analysis. **(C)** Quantification of *dct-1/Bnip3* gene expression in wild type and *hif-1(ia4)* mutant control and *frh-1* RNAi animals. The fold change in gene expression was calculated using the 2-ΔΔCt method. Bars and errors represent mean ± SEM of three independent biological replicates; *p < 0.05, ns = not significant in an unpaired t-test.

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