CHARACTERIZATION OF INTESTINALLY EXPRESSED *shc-3* (K11E4.2) IN *CAENORHABDITIS ELEGANS*

CHARACTERIZATION OF INTESTINALLY EXPRESSED *shc-3* (K11E4.2) IN *CAENORHABDITIS ELEGANS*

By VICTORIA L. LEÓN-GUERRERO, B.Sc

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TITLE: Characterization of intestinally expressed *shc-3* (K11E4.2) in *Caenorhabditis elegans*

AUTHOR: Victoria L. León Guerrero, BSc. (ITESM, Mexico City)

SUPERVISOR: Dr. Lesley T. MacNeil

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Abstract

SHC proteins are a family of adaptor proteins that play an important role in signal transduction, they are characterized by three crucial domains: the phosphotyrosine binding (PTB) domain, a Src2 homology (SH2) domain and a less conserved collagen homolog (CH1) domain. Two *Caenorhabditis elegans* SHC proteins have been described: SHC-1 and SHC-2. We have identified a third SHC protein, K11E4.2, that is intestinally expressed. Our analysis revealed that K11E4.2 null mutant animals suffer from a diet-dependent change in fat accumulation and increased sensitivity to starvation and oxidative stress. *C. elegans shc-1* plays a role in stress response and lifespan regulation through the insulin signaling pathway. Our data suggest that *shc-1* and K11E4.2 do not act redundantly to regulate stress or starvation response, but rather each plays a distinct role in these processes. This project proposes a model where K11E4.2 could have a role as positive insulin signaling regulator in *C. elegans*.

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Content

List of Figures

List of Tables

List of all abbreviations and symbols

Shc: Src and Collagen homology

PTB: Phosphotyrosine binding

SH2: Src2 homology

CH1: Collagen homology 1

Sos: Son of senvenless

Grb2: growth factor receptor-bound protein 2

RTK: Receptor Tyrosine Kinase

TOR: Torso

DER: Drosophila EGF receptor

ILPs: insulin like peptides

PDGF: Platelet derived growth factor

GNRF: Guanine nucleotide releasing factor

UV: Ultraviolet

L1: Larval stage 1

L2: Larval stage 2

L3: Larval stage 3

L4: Larval stage 4

NGM: Nematode growth medium

IPTG: Isopropyl ß-D-1-thiogalactopyranoside

FUDR: Floxuridine

RNAi: RNA interference

PQ: Paraquat

ROS: Reactive oxygen species

Chapter 1

1 Introduction

1.1 Introduction

Signaling cascades are crucial for intracellular communication and homeostasis. Adaptor proteins are key components of signaling cascades that allow external stimuli to be transduced into internal cues, culminating in the appropriate biological responses. Adaptors are proteins lacking enzymatic activity that contain protein-binding domains that mediate protein-protein interactions (Flynn 2001; Schechtman and Mochly-rosen 2001). The Src homology and collagen homology (Shc) family of adaptor proteins is characterized by three key domains: the N-terminal phosphotyrosine binding (PTB) domain, the central collagen homology domain (CH1) and the C-terminal Src2 Homology domain (SH2) (Pelicci et al. 1992, 1996). While PTB and SH2 domains are found in a number of proteins individually, the presence of both an N-terminal PTB and a C-terminal SH2 domain is a defining characteristic of the Shc family (Luzi et al. 2000). These domains interact with specific phosphotyrosine motifs and therefore play an important role in mediating interactions that are dependent on phosphotyrosine signaling. An additional collagen homology region (CH2) is present in some isoforms of mammalian ShcA (p66), ShcB (p68), ShcC (p67) and ShcD (p69) (Ravichandran 2001).

In mammals, there are four Shc genes and multiple Shc isoforms: ShcA (p66, p52, p46), ShcB (p68), ShcC (p67, p52) and ShcD (p69, p59, p49) (Luzi et al., 2000; G Pelicci et al., 1996, Jones et al., 2007). ShcA, also known as Shc1, is the most studied as it is the

most broadly expressed in humans, as well as having significant roles in cell proliferation and migration (Pelicci et al. 1992). The ShcA isoforms play a role in oxidative stress responses and cell survival (Pelicci et al. 1992; Pellegrini, Pacini, and Baldari 2005; Purdom and Chen 2003). The ShcB (p68), ShcC (p67, p52) and ShcD (p69) proteins are expressed in the brain and regulate neuronal cell development and survival by interacting with the receptor tyrosine kinase (RTK) TkrA (Pelicci et al. 1996; Sakai et al. 2000).

Shc proteins are required for many cellular processes including motility, proliferation, apoptosis and differentiation (Ahmed and Prigent 2017). Phosphorylation of Ser, Thr and Tyr residues in Shc, allow members of the Shc family of proteins, particularly ShcA(p66) to relocate to organelles and bind to other signaling proteins (Ahmed and Prigent 2017). While the Shc family has been thoroughly studied in mammals, homologs of Shc are also found in simpler organisms such as *Drosophila melanogaster* (Lai et al. 1995) and *Caenorhabditis elegans* (Luzi et al. 2000), suggesting that Shc proteins have an evolutionary conserved role. In *D. melanogaster*, the sole Shc homolog DSHC has a PTB and SH2 domain, but lacks the Grb2 binding region found in mammal Shc (Lai et al. 1995). DSHC is required for development and cell differentiation through TOR (Torso) and DER (Drosophila EGF receptor) signaling, as homozygous *dshc* mutant survivors display delayed development and defects in the eye, wing and ovary (Luschnig et al. 2000). These findings suggest that DSHC is most similar to mammal ShcA, as ShcA (particularly the p66 isoform) functions in the EGF receptor signaling pathway (Okada et al. 1997). *C. elegans* Shc protein homologs are present as SHC-1 and SHC-2. Based on sequence homology SHC-1 is most similar to mammal p52ShcA, and *shc-1* mutant phenotypes can

be rescued by expression of human p52ShcA (Neumann-haefelin et al. 2008). There are no available reports of the function of SHC-2. However, the PTB and SH2 SHC-2 domains are reported to have homology with the mammal ShcC PTB and SH2 domains (Luzi et al. 2000).

Interactions with the PTB, SH2 and CH1 domains of Shc, have been mostly studied for the ShcA protein, as it is ubiquitously expressed in human tissue and has been the most investigated member of the Shc family (Pelicci et al. 1992; Wills and Jones 2012). All three ShcA isoforms share the highly conserved PTB and SH2 domains and the less conserved CH1 domain. However, the p66ShcA isoform has an additional N-terminal region (Migliaccio et al. 1997), the CH2 domain, as a result of the activation of an alternative promoter (Ventura et al. 2002; Wright et al. 2019). While all the isoforms share a similar structure, different functions and intracellular localization patterns have been reported for each ShcA isoform (Migliaccio et al. 1997). Both the p46 and the p52 isoforms of ShcA are ubiquitously expressed (Migliaccio et al. 1997) and are required for the regulation of mitochondrial lipid oxidation (Tomilov et al. 2016) and Ras signaling (Geer et al. 1996; Isakoff, Marcantonio, and Giancotti 1996; Terada 2019) respectively. While the p46 isoform was shown to localize to the mitochondria (Tomilov et al. 2016; Ventura et al. 2004) and, in the case of cancerous hepatocytes (Yoshida et al. 2004) and gastric cells (Yukimasa et al. 2005) to the nucleus, the p52 isoform is mainly localized in the endoplasmic reticulum membrane and the cytoplasm (Lotti et al. 1996; Yoshida et al. 2004). In contrast, the presence of p66ShcA varies from cell type to cell type (Migliaccio et al. 1997). The p66 ShcA isoform is required for the regulation of oxidative stress

responses and lifespan in mammals (Migliaccio et al. 1999). ShcA p66 localizes to the cytoplasm, endoplasmic reticulum and mitochondria (Galimov 2010; Raker et al. 2004).

1.2 SHC domains

The PTB and SH2 domains in Shc bind phosphorylated tyrosines within activated receptors or receptor substrates (Sakaguchi et al. 1998), allowing Shc to associate with tyrosine-phosphorylated proteins (Migliaccio et al. 1997). In Shc proteins, the PTB domain is located in the amino-terminal region and binds to NPXpY-containing sequences (van der Geer et al. 1996; Zhou, Ravichandran, et al. 1995). Structurally, the PTB domain of Shc is composed by two β sheets and three α helices and within this structure, it was shown that the Arg 175 residue plays a crucial role in phosphotyrosine interaction as well as being conserved in other proteins that contain PTB domains (Zhou, Ravichandran, et al. 1995). One novel feature of the ShcA PTB domain is the ability to bind acidic phospholipids, which aids in the localization of ShcA to the membrane (Ravichandran et al. 1997; Uhlik et al. 2005; Zhou, Ravichandran, et al. 1995). This interaction is hypothesized to facilitate the recruitment of ShcA to activated receptors.

The Shc SH2 domain is found in the C-terminus region of Shc proteins and binds to specific phosphotyrosine sequences contained in a pYXXΦ motif (where Φ represents a hydrophobic amino acid) (Zhou, Meadows, et al. 1995) in a variety of receptors. For example, ShcA SH2 binds to the phosphorylated epidermal growth factor (EGF) (Pelicci et al. 1992) and the platelet-derived growth factor (PDGF) (Yokote et al. 1994) receptors. Another domain present in all members of the mammalian Shc family is the collagen homology domain (CH1), named after its similarity to α1 collagen. CH1 is a proline-rich

central region in Shc and contains binding sites for Grb2: Tyr 317, 239 and 240 (Geer et al. 1996). The interaction of ShcA CH1 with Grb2, results in the formation of the Grb2-Sos complex, leading to the activation of the Ras/MAPK signaling pathway (Ravichandran et al. 1995). Absent from the shorter ShcA isoforms, the CH2 domain is a N-terminal region only present in the p66ShcA isoform (Migliaccio et al. 1997) that is serine phosphorylated (Migliaccio et al. 1999) by JNK1/2 (Le, Connors, and Maroney 2001) in response to oxidative stress (Ahmad et al. 2020; Le et al. 2001). This serine phosphorylation is required for the regulation of apoptotic responses upon oxidative stress (Migliaccio et al. 1999; Pellegrini et al. 2005).

1.3 SHC proteins are required for activating RAS signaling in response to insulin

One of the first descriptions of the role of Shc adaptor proteins in the insulin signaling pathway was reported in 1993 by Pronk et al. Pronk and coworkers demonstrated that the ShcA 46 kDa and 52 kDa isoforms become tyrosine-phosphorylated upon insulin treatment and bind to the Grb2 protein, leading to the initial hypothesis that both Shc and Grb2 are involved in the activation of Ras upon insulin stimulation (Pronk et al. 1993). This group later added another piece to the puzzle, when they demonstrated that the interaction between the guanine nucleotide exchange factor Son of Sevenless (Sos) and Shc is also required for insulin induced Ras activation, by showing the presence of Sos in Shc immunoprecipitates and *vice versa*, after treatment with insulin (Pronk et al. 1994). Shc proteins were later shown to be the major connection between the insulin receptor and the Grb2-Sos complex in the Ras signaling cascade by Sasaoka et al. with an experiment that

showed reduced guanine nucleotide releasing factor (GNRF) activity after treating insulin stimulated quiescent living cells with anti-Shc antibody (Draznins et al. 1994) microinjection. Since Sos promotes GNRF activity (Zon et al. 1993) and Grb2-Sos complexes can exist in cells without requiring stimulation (Chardin et al. 1993; Gale et al. 1993; Rozakis-adcock et al. 1993) the authors concluded that Shc is directly linked to Ras activation through the Grb2-Sos complex after insulin stimulation. Finally, it was shown by several groups that Shc interacts with the insulin receptor through the PTB domain (Craparo, Neill, and Gustafsont 1995; Dey et al. 1996; He et al. 1995; Isakoff et al. 1995). The main clue was provided by Gustafson et al. with a yeast two-hybrid assay that demonstrated that Shc interacts directly with the insulin receptor through a non-SH2 Shc domain (Gustafson et al. 1995), later termed the Shc PTB domain (Kavanaugh and Williams 1994). Together these findings led to the elucidation of the mechanism we know today: after insulin stimulation, the activated insulin receptor binds to the Shc PTB domain, Shc itself becomes phosphorylated and recruits the Grb2-Sos complex through the central Shc CH1 region, resulting in the activation of the Ras signaling pathway (**Fig 1.1**). Activation of Ras signaling through the insulin signaling pathway via Shc results in insulin-induced mitogenesis (Imamura et al. 1996; Sasaoka et al. 1994) which makes the Shc family of adaptor proteins an interesting candidate for the study of biological functions mediated by insulin stimulation.

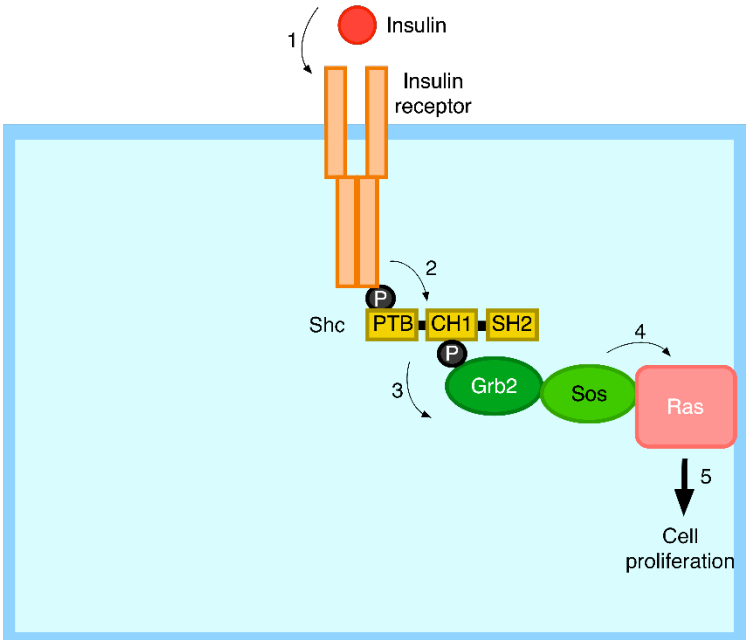


Figure 1.1: Shc is required for active Ras signaling via the insulin signaling pathway. Upon insulin stimulation (1), the Shc PTB domain binds to the insulin receptor (2), leading to the recruitment of the Grb2-Sos complex through the Shc CH1 central region (3), resulting in the activation of Ras (4), activating downstream signaling cascades for cell proliferation (5).

1.4 The intestine plays a key role in regulating insulin signaling in *C. elegans*

Insulin signaling is a crucial pathway regulating growth, reproduction, aging and metabolism in *C. elegans*, as insulin loss of function phenotypes (**Table 1.1**) include changes in development, behavior, lifespan, fat storage, stress responses and reproduction (Ashrafi et al. 2003; Baugh and Sternberg 2006; Gottlieb and Ruvkun 1994; Hughes et al. 2007; Kenyon, Chang, and Gensch 1993; Kimura et al. 1997; Lithgow et al. 1994; Ruaud, Katic, and Bessereau 2011).

The *C. elegans* insulin signaling pathway is regulated by insulin-like peptides (ILP) that activate the insulin receptor DAF-2, resulting in the recruitment and activation of the AGE-1/PI3K phosphoinositide 3-kinase, the FOXO transcription factor DAF-16 is then phosphorylated by the activated serine/threonine kinases PDK-1, AKT-1 and AKT-2. Phosphorylation of DAF-16 promotes interaction with 14-3-3 proteins PAR-5 and FTT-2, leading to cytoplasmic sequestration of DAF-16 (Murphy and Hu 2013).

Table 1.1: Phenotypes resulting from decreased insulin signaling

Mutant	Phenotype	Reference
daf-2/IGFR age-1/P13K	Increased lifespan	(Kimura et al. 1997; Morris, Tissenbaum, and Ruvkun 1996)
	Slow development	(Ayyadevara et al. 2008; Ruaud et al. 2011)
daf-2/IGFR age-1/P13K akt-1;akt-2	Dauer-constitutive	(Alam et al. 2010; Kimura et al. 1997; Morris et al. 1996; Riddle, Swanson, and Albert 1981)
daf-2/IGFR	Increased heat resistance	(Lithgow et al. 1994)
	Increased oxidative stress resistance	(Honda and Honda 1999; Vanfleteren 1993)
	Hypoxia resistance	(Mabon, Scott, and Crowder 2009; Scott, Avidan, and Crowder 2003)
	Osmotic stress resistance	(Lamitina and Strange 2005)
	Heavy metal stress resistance	(Barsyte, Lovejoy, and Lithgow 2001)
	UV radiation resistance	(Murakami and Johnson 1996)
	Proteotoxicity resistance	(Cohen et al. 2006; Morley and Morimoto 2004; Teixeira-Castro et al. 2011)
	Enhanced RNAi response	(Wang and Ruvkun 2004)
	Enhanced resistance to bacterial pathogens	(Garsin et al. 2003)
	Prolonged L1 survival	(Baugh and Sternberg 2006)
	Better morphology maintenance and mobility with age	(Garigan et al. 2002; Herndon et al. 2002; Kenyon et al. 1993)
	Increased fat content	(Ashrafi et al. 2003; Rourke, Soukas, and Carr 2010; Yen et al. 2010)

	Extended reproductive span	(Hughes et al. 2007; Luo et al. 2010)
	Reduced neural decline with aging	(Stein and Murphy 2012)
	Reduced brood size	(Tissenbaum and Ruvkun 1998)
	Reduced germline tumors in mutants of *daf-16* downstream targets	(Pinkston-gosse and Kenyon 2007)

The intestine is a key organ for insulin signaling as DAF-16/FOXO activity in this tissue is required for reproductive span extension and longevity (Libina et al. 2003; Luo et al. 2011; Shi, Booth, and Murphy 2019). Intestinal insulin signaling is also required for the control of responses to environmental stressors such as simulated gravity (Kong et al. 2019), nanoplastic particles (Liu, Tian, and Wang 2021; Qu et al. 2020; Shao et al. 2019) and carbon nanomaterials (Zhao et al. 2016) as well as for the regulation lipid metabolism (Clark et al. 2018) and infection responses (Engelmann, Ewbank, and Pujol 2018; Evans, Kawli, and Tan 2008; Mohri-shiomi and Garsin 2008). Because this organ is the main site for nutrient uptake, digestion and fat storage in *C. elegans* (Dimov and Maduro 2019; Rourke et al. 2010) the intestine remains an interesting target for the study of the relationship between energy uptake and expenditure and fundamental biological processes, as well as the signaling pathways that mediate them.

1.5 *C. elegans* SHC-1 is required for insulin signaling and JNK pathways

Two SHC proteins have been identified in *C. elegans*: SHC-1 and SHC-2 (Luschnig et al. 2000; Luzi et al. 2000). Both SHC-1 and SHC-2 proteins in *C. elegans* have PTB and SH2 domains characteristic of the Shc family of proteins, lacking however any of the collagen homology domains (Luzi et al. 2000; Neumann-haefelin et al. 2008). SHC-2 is exclusively

expressed in embryos and no phenotypes have been reported following knockdown of this gene in large-scale screens (Luzi et al. 2000). However, SHC-1 has been better characterized and plays a role in insulin signaling and JNK pathways. *shc-1* mutants display accelerated aging, shorter lifespan, increased sensitivity to oxidative and metal stress response and germline disruption (Hisamoto et al. 2016; Neumann-haefelin et al. 2008; Pastuhov et al. 2012) (**Fig 1.2**). SHC-1 is broadly expressed, with expression reported in head and tail neurons, vulval muscles, the intestine, the hypodermis and the pharynx (Neumann-haefelin et al. 2008).

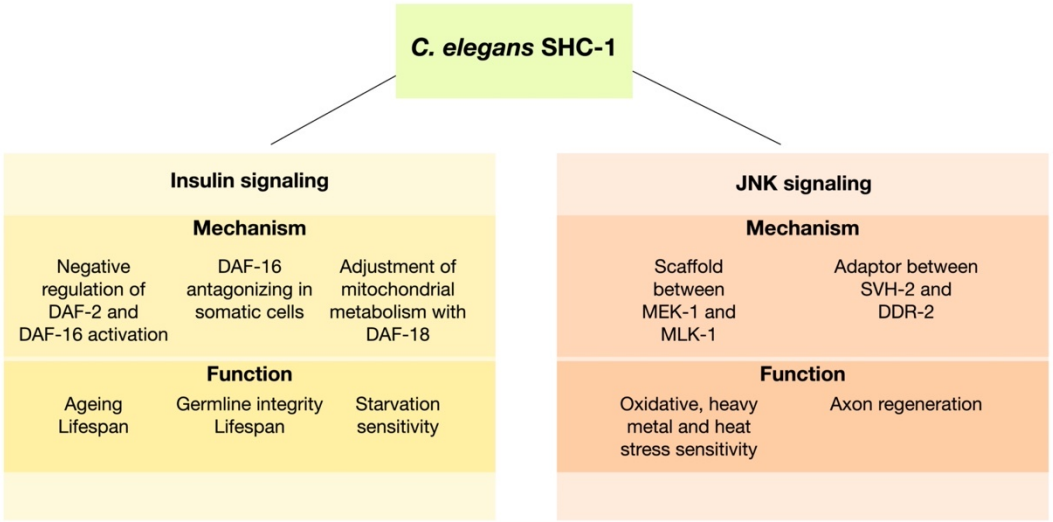


Figure 1.2 : SHC-1 mediates aging, stress response, lifespan and axon regeneration through the insulin and JNK/MAPK pathways. In the insulin signaling pathway, SHC-1 has been reported to play a role in negative regulation of DAF-2, activation of DAF-16 and adjusting mitochondrial metabolism with DAF-18. In the JNK pathway, SHC-1 acts as a scaffold between MEK-1 and MLK-1 and between SVH-2 and DDR-2.

1.6 SHC-1 interacting proteins in *C. elegans*

In mammals, SHC proteins interact with a wide range of receptors, including growth factor receptors, antigen receptors, cytokine receptors, G-protein coupled receptors and hormone receptors (Ravichandran 2001). These interactions take place through the different domains of Shc and result in protein complexes formation, for example, while the PTB domain of Shc interacts with the activated insulin receptor, the CH1 domain of Shc interacts with the SH2 region of Grb2, and Grb2 recruits SOS, activating the Ras/MAPK signaling pathway (Sasaoka and Kobayashi 2000).

SHC-1 interacts with SVH-2 (MERTK), DDR-2 (DDR-2), MLK-1 (MAP3K10,MAP3K9), MEK-1 (MAP2K7) and DAF-2 (IGF1R) (Hisamoto et al. 2016; Mizuno et al. 2008; Neumann-haefelin et al. 2008; Pastuhov et al. 2012) .The interaction of SHC-1 with SVH-2 and DDR-2 regulates the JNK/MAPK pathway for axon regeneration. Importantly, SHC-1 binding with SVH-2, was shown to be required for SVH-2 activity for axon regeneration (Hisamoto et al. 2016). SHC-1 connects MEK-1 with MLK-1 as a component of the KGB-1 (MAPK10) pathway that regulates the response to heavy metal and endoplasmic reticulum stress in *C. elegans* (Mizuno et al. 2008). In the KGB-1 pathway, *shc-1* loss of function prevents KGB-1 activation, resulting in enhanced sensitivity to metal stress (Mizuno et al. 2008). Also, SHC-1 acts as an inhibitor of the insulin receptor DAF-2, negatively regulating the insulin pathway (Neumann-haefelin et al. 2008). However, the precise mechanism of interaction of SHC-1 with the insulin receptor DAF-2 has not been elucidated. The group that characterized SHC-1 propose that SHC-1 negatively regulates DAF-2 by any of three possible mechanisms: 1) SHC-1

functions as a modulator of DAF-2 phosphorylation by recruiting a kinase, 2) SHC-1 regulates DAF-2 receptor internalization or 3) SHC-1acts more indirectly by removing a source of PIP2 from the AGE-1 (PIK3CA) complex (Neumann-haefelin et al. 2008). Additionally, by co-immunoprecipitation, they found that both the PTB and SH2 domains of SHC-1 interact with DAF-2. In mammals, Shc directly interacts with the activated insulin receptor through the PTB domain, resulting in the formation of the Shc-Grb2-Sos complex (Giorgetti et al. 1994; Sasaoka and Kobayashi 2000). However, the possibility of SHC-1 performing a similar function, and the biological significance of this mechanism remains to be explored.

SHC-1 is also required for modulating DAF-16 (FOXO) function in the hypodermis. This was shown in an experiment where DAF-16 (FOXO) is over-expressed in the hypodermis in combination with loss of function of SHC-1, resulting in enhanced gonadal basement disruption, a weakly penetrant phenotype in *shc-1* mutants, causing a tumor-like germline phenotype and further reducing the short lifespan of *shc-1*(Qi et al. 2012). Therefore, it was proposed that in the presence of insulin signaling, SHC-1 acts by antagonizing nuclear DAF-16 (FOXO) in somatic cells (Qi et al. 2012; Wolf et al. 2014).

SHC-1 is required to adjust mitochondrial metabolism to nutrient availability via the intestine. This was shown by exposing *daf-18*/PTEN and *shc-1* double mutants to prolonged starvation during the first larval stage (L1). *daf-18/*PTEN mutants show disrupted gonadal development after refeeding and this phenotype was enhanced, from 61% to 91% and 85% to 100% after two and three days of starvation, by the loss of function of *shc-1(ok198)* (Wolf et al. 2014), suggesting that both SHC-1 and DAF-18 act in

adaptation to starvation. Interestingly, single *shc-1(ok198)* mutants display this phenotype at lower penetrance after seven days of starvation (Wolf et al. 2014). By adding doxycycline, an antibiotic that targets mitochondrial translation, the gonadal disruption phenotype was suppressed in double *shc-1* and *daf-18* mutants, leading to the conclusion that both DAF-18 and SHC-1 regulate mitochondrial metabolism in response to starvation (Wolf et al. 2014).

Overall, the loss of SHC-1 results in reduced lifespan, accelerated ageing, germline disruption and increased sensitivity to oxidative and heavy metal stress and starvation.

1.7 The role of PTB, CH1 and SH2 domains in SHC-1 interactions

The SHC-1 PTB interaction mechanism for the regulation of heavy metal stress responses was described in a study by Mizuno et al (Mizuno et al. 2008). It was shown that the SHC-1 PTB domain interacts with MEK-1(MAP2K7) and MLK-1 (MAP3K10,MAP3K9), in the KGB-1 pathway, a pathway that regulates stress responses to heavy metals(Mizuno et al. 2004, 2008). The same study showed that the SHC-1 PTB domain binds to the NPXY motif in MLK-1. These interactions are important for stress sensitivity mediated by the KGB-1 pathway, as SHC-1 acts as a scaffold that links MEK-1 and MLK-1 and the loss of SHC-1 results in heavy metal hypersensitivity (Kim and Sieburth 2019; Pastuhov et al. 2012).

Unlike mammalian Shc proteins, SHC-1 does not have a central CH1 domain containing the 239, 240 and 317 tyrosines that are critical for binding to SEM-5/Grb2 , and to our knowledge *shc-1* mutants do not recapitulate all the phenotypes of Ras defective mutants (Mizuno et al. 2008), such as lethality (Yochem, Sundaram, and Han 1997), uterine defects (Chang, Newman, and Sternberg 1999), male spicule defects (Chamberlin and

Sternberg 1994), vulvaless phenotype (Sundaram 2006), sterility (Church, Guan, and Lambie 1995), olfaction defects (Hirotsu et al. 2000) and axon guidance defects (Bülow, Boulin, and Hobert 2004). However, SHC-1 mutants have been reported to recapitulate some LET-60/Ras gain of function phenotypes, including sluggishness and dysregulated Erk activation in the germline causing sterility (Arur et al. 2011). SHC-1 has therefore been suggested to negatively regulate Erk activation and phosphorylation (Suen et al. 2013). Currently, data implicating the LET-60/Ras pathway as a target of insulin signaling in *C. elegans* is not available.

Notably, SHC-1 is required for axon regeneration. In *C. elegans* axon regeneration is mediated through the JNK/MAPK pathway, one of the components of this pathway is SVH-2, a HGF-like receptor tyrosine kinase (Nix et al. 2011). SVH-2 interacts with SHC-1 through Shc SH2 domain, as it was demonstrated in an experiment with a mutation of Arg 234 to Lys resulting in the inability of SVH-2 to interact with SHC-1(Hisamoto et al. 2016). In this same study, it was shown that SHC-1 is able to interact with the cytoplasmic domain of DDR-2, one of the two discoidin domain receptors in *C. elegans,* highlighting the importance of the interaction of SHC-1 with the DDR-2 and SVH-2 receptor tyrosine kinases to promote axon regeneration. Overall, this study describes SHC-1 as a link of SVH-2 to the cytoplasmic domain of DDR2.

1.8 Concluding section

Since they were first identified in 1992, Shc proteins have been described as key adaptor proteins for signal transduction, and the best-known example of their role is in the formation of the Grb2-Sos complex upon insulin stimulation. Importantly, the

understanding of Shc signaling has led to innovation in obesity, diabetes and cancer research.

In *C. elegans*, two SHC homologs have been described: SHC-1 and SHC-2. The available experimental data shows that SHC-1 has a role in insulin and JNK pathways, mediating axon regeneration, ageing and stress responses in *C. elegans*. Interestingly, SHC-1 mutants do not display all phenotypes characteristic of insulin and Ras defective mutants, which could be due to the lack of a CH1 domain, that might interact with the Grb2 homolog, SEM-5. In *C. elegans*, SEM-5/Grb2 is required for vulval induction and sex myoblast migration (Clark, Stern, and Horvitz 1992). Mutant SEM-5/Grb 2 worms display lethality and vulvaless phenotypes (Clark et al. 1992). The loss of the characterized *C. elegans* SHC proteins, does not result in these phenotypes, which could indicate that: 1) There are additional Shc-like proteins not yet identified in *C. elegans* or 2) Shc-like proteins in *C. elegans* are not required for Ras signaling in the same manner as other organisms. Since interactions between Shc and Grb2 occur via the CH1 domain in mammals, it would be interesting to know if there are additional binding sites in Shc-like proteins that could interact with Grb2 and that have not yet been identified. Furthermore, if Shc-like proteins in *C.elegans* do not play the same role as mammalian Shc, it might suggest that the role of the Shc family in Ras signaling, was acquired later in evolution. Elucidating the role of Shc signaling in *C. elegans* remains an interesting subject for understanding intracellular communication. Because learning about Shc signaling has led to the discovery of potential therapies for cancer and diabetes (Dong et al. 2019; Hui et al. 2020; Lin et al. 2019; Liu et

al. 2019; Shih et al. 2012) exploring additional interactions and mechanisms of Shc, might lead to new clinical alternatives.

Chapter 2

2 Research problem

Since their identification in 1992, Shc proteins have been associated with diverse functions such as regulation of cell survival, proliferation and oxidative stress responses, making the family of Shc adaptor proteins a subject of study for more than 20 years (Wills and Jones 2012). While some therapeutic strategies targeting Shc functions for diabetes (Hui et al. 2020) and cancer (Ahn et al. 2017; Lin et al. 2019) have been explored, their ubiquitous expression and the similarity between isoforms stills poses a challenge for therapeutic agents (Wright, Staruschenko, and Sorokin 2018) as knockout of all three isoforms result in embryonic lethality in mice (Lai and Pawson 2000). Studying the mechanism of Shc proteins and their interactions might present an opportunity to target diseases such as cancer, obesity and diabetes, without directly targeting Shc proteins which could potentially result in detrimental consequences. Shc proteins are conserved through evolution, as their presence has been confirmed not only in mammals, but also in invertebrates such as *Drosophila melanogaster* and *Caenorhabditis elegans* (Luzi et al. 2000). While only one Shc protein has been identified in *D. melanogaster* (dSHC)(Luschnig et al. 2000), two SHC proteins have been identified in *C. elegans* (SHC-1, SHC-2)(Luzi et al. 2000; Neumann-haefelin et al. 2008). The knowledge on SHC-2 is limited, but SHC-1 has been better described and is now known to be required for oxidative stress responses, ageing and lifespan and has been reported to be most similar to the p52 isoform of human ShcA (Neumann-haefelin et al. 2008; Wolf et al. 2014). The MacNeil laboratory identified a third

Shc-like protein in *C. elegans*, expressed by the K11E4.2 gene. The K11E4.2 protein was predicted to contain both a PTB and SH2 domain. Because the already identified SHC-1 and SHC-2, do not recapitulate all phenotypes proper of insulin and Ras mutant animals (Luzi et al. 2000; Neumann-haefelin et al. 2008; Wolf et al. 2014), we hypothesize that K11E4.2 might recapitulate these phenotypes and that K11E4.2 might display a different role from already identified SHC proteins in *C. elegans.* This project aims to identify the phenotypes associated to K11E4.2 mutants and provide evidence of its function in *C. elegans,* The knowledge obtained from the characterization of K11E4.2 might shed some light into conserved Shc-like functions throughout evolution and what is learned from a third Shc-like protein and its interactions in *C. elegans* could potentially be extrapolated to more complex organisms such as humans, offering the possibility of identifying novel therapeutic targets for Shc dysregulated functions.

Chapter 3

3 Methodology

3.1 Strains used

N2 was used as the wild-type strain. All worm strains were maintained on nematode growth medium (NGM) at 20°C on *E. coli* OP50 standard laboratory diet (Brenner 1974). All mutant strains were outcrossed at least four times. Strains used: K11E4.2 (syb1634), *shc-1* (*ok198*), *shc-1*(*ok198*); K11E4.2(syb1634), K11E4.2 (gk890887) and TJ356 (zIs356 [daf-16p::daf-16a/b::GFP + rol-6(su1006)]).

3.2 L1 synchronization

To generate synchronous populations, eggs were collected by hypochlorite treatment from gravid adult plates and allowed to hatch from 18 to 24 hours in M9 buffer. When hatched in the absence of food, worms enter L1 arrest and resume development upon feeding (Baugh 2013).

3.3 Expression pattern

The LMN015 strain (K11E4.2p: GFP) was used to observe expression pattern during the animals' life cycle. A parent GFP positive animal was allowed to lay eggs and the offspring was observed to monitor the expression pattern of K11E4.2.

3.4 Developmental score

Wild-type or mutant strains were transferred to NGM 60mm plates seeded with 200 μL *E. coli* OP50 or *Comamonas aquatica* following L1 synchronization. At least 100 to 150 worms were transferred to each plate. Thirty worms were scored at L4 developmental stage

on three separate occasions for each condition and classified into L4 substages. Development delay in worms in the fourth larval stage is scored by looking at the position of the cells that fuse and migrate to form the vulva (Mok, Sternberg, and Inoue 2015). Animals were paralyzed with 1% sodium azide and mounted on 2% agarose pads for visualization on a compound microscope. Statistical analysis was performed using GraphPad Prism P-values were obtained by comparing observed (K11E4.2 mutants) adults to expected (wild-type) number of adults.

3.5 Oil Red O staining and ImageJ analysis for lipid accumulation

Oil red O staining was performed (Rourke et al. 2010) on day one adult worms (48 hours after L1 transfer to plates). Briefly, after exposure to a diet of *E. coli* OP50 or *C. aquatica*, 200-300 animals were collected and washed twice with 1X PBS. After washing, 500 µL of 2X MRWB and 125 µL of 16% paraformaldehyde were added to 400 µL of sample. After an incubation period of 30 minutes at room temperature, samples were washed twice with Tris-Cl (100 mM, pH 7.4). After the second wash, 900 µL of reduction buffer were added to 100 µL of sample and incubated for 30 minutes at room temperature. After the incubation period, a wash was performed with 1X PBS, and 700 µL of isopropanol were added to 300 µL of the sample. After 15 minutes, 1 mL of Oil Red O working solution was added and samples were washed once with 1X PBS on the next day before visualization. At least 300 worms were stained per condition. Up to 30 animals were imaged per condition on a compound microscope.

To analyze the staining, we used ImageJ. Briefly, using the green channel we selected the area of the intestine below the pharyngeal-intestinal valve to the start of the

gonad and measured the percentage of the area that was stained. In *C. elegans* anatomy, the gonad overlaps with the intestine, making the visualization of lipid vesicles less clear, therefore we selected the area of the intestine above the gonad tubes and manually singled out the lipid vesicles from the background. Aggregates were subtracted from the selection. Statistical analysis was performed using GraphPad Prism, P-values were obtained by an unpaired T-test.

3.6 Brood size

Single L4 hermaphrodites were plated on 35mm plates seeded with *E. coli* OP50 at 20ºC. Each worm was transferred to a new plate every 24 hours for three consecutive days. Embryos and newly hatched worms on each plate were counted. For every strain we used samples of at least 24 worms. Worms that died from internal hatching or gonad defects were censored. Worms that burrowed and laid eggs within the agar were censored. Statistical analysis was performed using GraphPad Prism, P-values were obtained by ordinary one-way ANOVA and multiple comparisons. This experiment was performed on three separate occasions.

3.7 L1 Starvation assay

Eggs were obtained by bleaching from gravid adult plates and allowed to hatch from 18 to 24 hours in M9 buffer. Tubes were kept on a rocker at 20ºC and on each time point samples were plated on *E. coli* OP50 and live worms were counted after two days (Hibshman et al. 2017; Mata-cabana et al. 2020). Because density of animals is known to be an environmental factor that influences L1 arrest survival (Mata-cabana et al. 2020), concentrations of animals per μL were controlled to be similar across strains (~1 to 2

worms/μL). Animals were scored for survival on days 1, 5, 11/12 and 15/16 after L1 arrest. The mean survival was calculated relative to the maximum number of worms counted per strain and this value was taken as 100%. Five plates were scored per strain, with at least 30 worms in each plate, and the mean survival was plotted for each time point. This experiment was performed on two different occasions. Statistical analysis was performed using GraphPad Prism, P-values were determined by using a Mantel-Cox log-rank test.

3.8 Lifespan

NGM plates were supplemented with 100μM FUDR to avoid the presence of progeny (Gandhi et al. 1980) . For each condition, a sample of at least 70 L4/young adult worms was used. The day the worms were transferred to the FUDR plates was taken as day zero. Animals were gently prodded with a platinum wire, if there was no response, they were scored as dead for the day, worms that burrowed were censored. This experiment was performed in two different occasions. Statistical analysis was performed using GraphPad Prism, P-values were determined by using a Mantel-Cox log-rank test.

3.9 Acute Paraquat Assay

NGM plates were supplemented with 100 mM paraquat. For each condition, a sample of at least 70 L4/young adult worms was used. Animals were scored for survival after 6, 12, 24 and 48 hours after transferring to paraquat plates. Animals were gently prodded with a platinum wire, if there was no response, they were scored as dead, worms that crawled off to the side of the plate were censored. Statistical analysis was performed using GraphPad Prism, P-values were determined by using a Mantel-Cox log-rank test. This experiment was performed in two different occasions.

3.10 Chronic Paraquat Assay

NGM plates were supplemented with 4 mM paraquat and 100 μM FUDR. For each condition, a sample of at least 80 L4/young adult worms was used (Senchuk, Dues, and Raamsdonk 2017). The day the worms were transferred to paraquat plates was taken as day zero. Animals were gently prodded with a platinum wire, if there was no response, they were scored as dead for the day, worms that crawled off to the side of the plate were censored. Statistical analysis was performed using GraphPad Prism, P-values were determined by using a Mantel-Cox log-rank test. This experiment was performed in two different occasions.

3.11 DAF-16 nuclear localization

NGM plates supplemented with 50 μg/mL carbenicillin and 5 mM IPTG were seeded with 200 μL of bacterial clones containing double stranded RNA for *daf-2*, KIIE4.2 or the empty vector (L4440) (Kamath and Ahringer 2003). Transgenic worms (TJ356) containing a GFP reporter to monitor DAF-16 localization were plated at L1 stage and scored for DAF-16 nuclear localization at the L4/young adult stage. At least 30 worms were scored per condition, animals were paralyzed with 12mM levamisole and mounted on a 2% agarose pad for visualization. Intracellular localization of DAF-16 was classified as nuclear if 50% or more intestinal cells had predominant nuclear localization, intermediate if nuclear localization was predominant in more than 10% but less than 50% of intestinal cells, and cytoplasmic if equal or less than 10% of cells had this characteristic (Ke et al. 2020). Statistical analysis was performed using GraphPad Prism, P-values were determined by using Two-way ANOVA. This experiment was repeated in three separate trials.

Chapter 4

4 Results

4.1 Expression of K11E4.2 is maintained during all larval stages and adults.

In order to characterize the function of K11E4.2 in *C. elegans*, its expression pattern was monitored using a translational GFP reporter. K11E4.2::GFP expression was observed in the intestine at all larval stages and in adults (**Fig. 4.1**). *C. elegans* intestinal cells have a circular structure with the basement membrane facing the outermost part of the intestine and the apical or inner most section surrounding the lumen (Dimov and Maduro 2019). Localization was found in the cytoplasm and at the apical membrane of intestinal cells. Notably, K11E4.2 is also found in nuclei and cytoplasm in some worms of the same population. Subcellular localization may be regulated in response to environmental stimuli, for example stress response, however this hypothesis requires further testing.

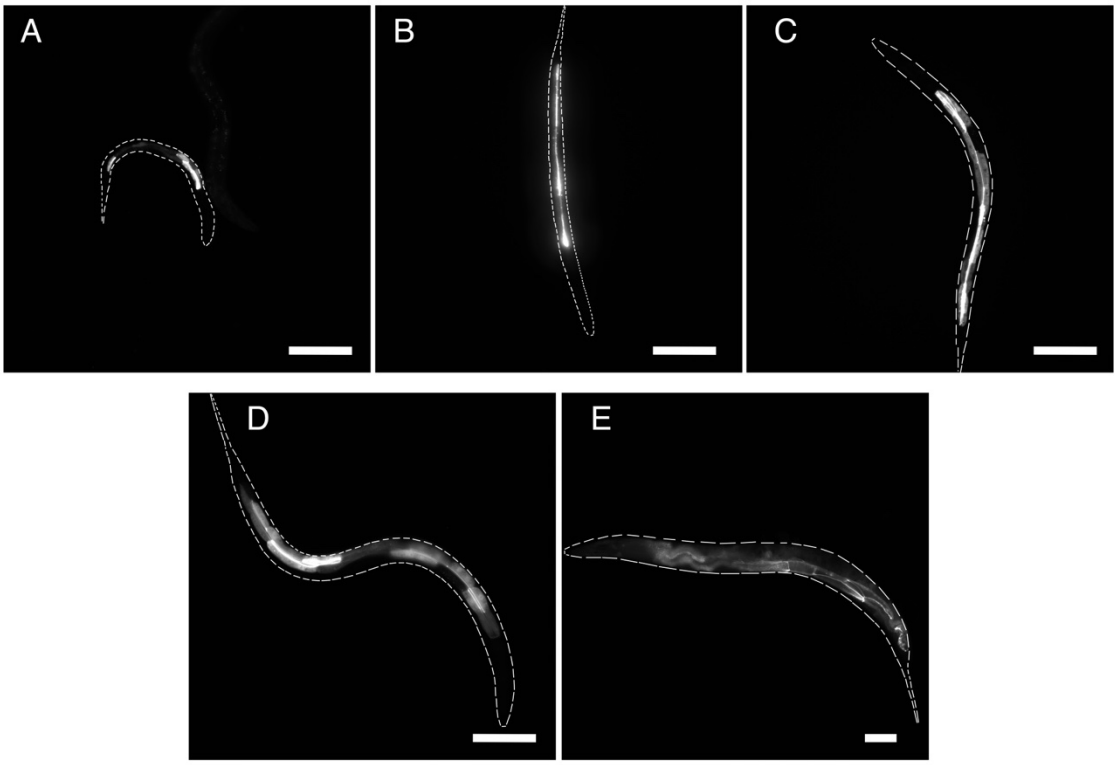


Figure 4.1 : K11E4.2 is intestinally expressed during all larval stages. **A**: Larval stage one (L1), **B**: Larval stage two (L2), **C**: Larval stage three (L3), **D**: Larval stage four (L4) and adulthood **(E)**. A translational reporter with GFP fused to the K11E4.2 gene was used to observe expression during the animals' life cycle, dotted lines outline the worms' body. Scale bar in all images is 100μm

4.2 Apical localization of K11E4.2 in intestinal cells is at least partially mediated by the PTB domain.

SHC adaptor proteins are characterized by the PTB and SH2 domains and the less conserved CH1 and CH2 domains (Ahmed and Prigent 2017). In *C. elegans*, K11E4.2 is predicted to have both PTB and SH2 domains and a CH1 domain. In order to determine if

the localization of K11E4.2 in the apical membrane was mediated by a specific domain, we compared wild-type worms to K11E4.2 mutants. To do this, the afore mentioned strain, K11E4.2::GFP, and a mutant strain with a GFP reporter fused to a K11E4.2 protein that contains a substitution of arginine to glutamine in the 145 position of the PTB domain, a residue conserved among the ShcA isoforms (Zhou, Ravichandran, et al. 1995) and essential for the interaction of ShcA with activated receptors (van der Geer et al. 1996), were monitored for expression. While GFP in worms expressing K11E4.2::GFP was observed at the apical intestinal membrane (**Fig. 4.2, A-B**), GFP in worms expressing K11E4.2(R145Q)::GFP was present in the cytoplasm an nuclei of intestinal cells (**Fig. 4.2 C**), suggesting that cellular localization might be at least partially mediated by the PTB domain.

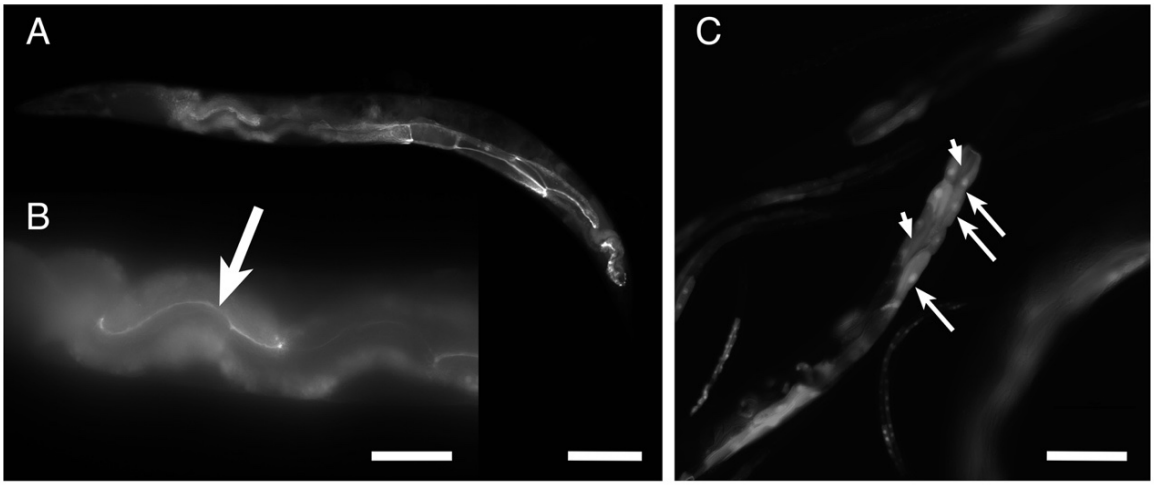


Figure 4.2: K11E4.2 localizes to the apical membrane in *C. elegans* intestinal cells. **A-B)** A GFP reporter fused to the K11E4.2 gene shows expression in intestinal cells, white arrow indicates apical localization. **C**) K11E4.2 R145Q mutants show K11E4.2 localization in

nuclei and cytoplasm, short arrows indicate cytoplasmic localization and long arrows indicate nuclear localization. Scale bars: **A)** and **C)**: 100 μm, **B)** 50 μm

4.3 K11E4.2 mutants develop normally on an *E. coli* OP50 diet but develop more slowly on a *Comamonas aquatica* diet as compared to wild-type animals

Slow development is a phenotype found in *daf-2/IGFR* mutants as DAF-16/FOXO is required to control developmental speed of the second larval stage (L2), a dauer-formation independent function (Ruaud et al. 2011). To test if K11E4.2 recapitulated this phenotype, we assessed developmental rate of K11E4.2 mutants compared to wild-type animals in a standard *E. coli* OP50 diet, an uracil auxotroph used across *C. elegans* laboratories for maintenance (Brenner 1974). When comparing the number of adult animals in the wild-type population compared to the K11E4.2 mutant population at 40 hours after plating synchronized L1 animals, there was no significant difference (**Fig. 4.3A**). Because K11E4.2 is expressed in the intestine, the main site for nutrient uptake and lipid storage (Dimov and Maduro 2019), animals were exposed to a *Comamonas aquatica* bacterial diet, known to accelerate developmental rate in wild-type animals in a TOR and insulin independent manner (MacNeil et al. 2013), to test if there would be a difference in developmental rate in response to this nutrimental change. Upon exposure to *C. aquatica*, a significant increase in the number of adults in the wild-type population compared to the K11E4.2 mutant population was observed (**Fig. 4.3B**). These findings suggest that K11E4.2 has a role in metabolism in response to different bacterial diets by modulating developmental rate.

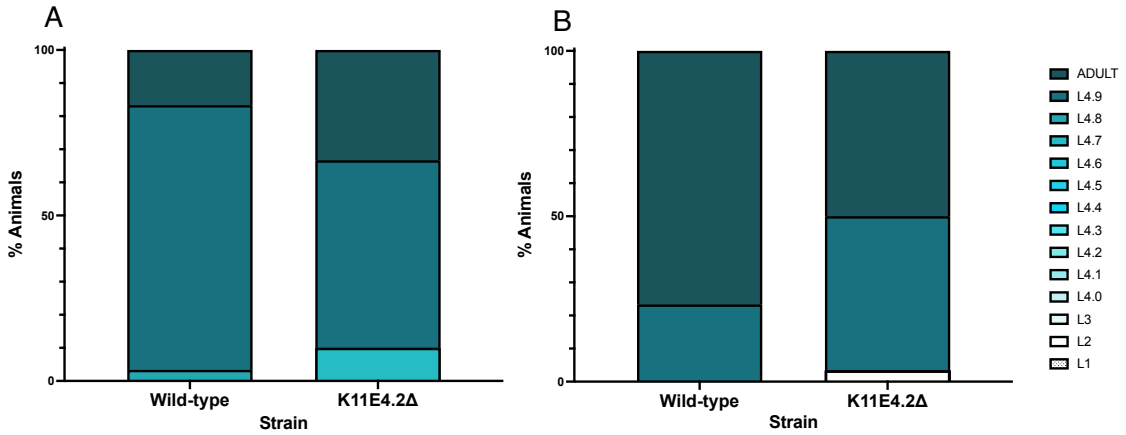


Figure 4.3: K11E4.2 mutant animals develop at a normal rate on an *E. coli* OP50 diet (left) but more slowly on a *C. aquatica* diet (right) compared to wild-type animals. Animals were scored at 40 hours after plating the synchronized L1 populations in plates seeded with the respective diets. Scoring was performed by assessing vulva morphology at the L4 stage. **A)** N2 on *E. coli* OP50 (n=30) and K11E4.2 mutants on *E. coli* OP50 (n=30) **B)** N2 on *Comamonas aquatica*, (n=30) and K11E4.2 mutants on *Comamonas aquatica*, (n=30). Expected number of adults (wild-type) vs observed (K11E4.2): *E. coli* OP50 (P=ns), *C. aquatica* (P = 0.0006)

4.4 K11E4.2 mutants have increased fat accumulation on an *E. coli* OP50 diet as assessed by Oil Red O staining

daf-2/Insulin receptor mutants display increased fat accumulation in a *daf-16*/FOXO dependent manner (Ashrafi et al. 2003; Perez and Van Gilst 2008); this phenotype is often seen in mutants with slow development (Srinivasan 2015). Because there were differences in developmental rate in response to different bacterial diets, fat accumulation was assessed

by performing Oil red O staining of neutral triglycerides (Rourke et al. 2010) after wild-type worms and K11E4.2 mutants were exposed to an *E. coli* OP50 diet or a *C. aquatica* diet. In wild-type *C. elegans*, fatty acids obtained from their bacterial diet are the main form of body fat and once they are turned into triglycerides, fatty acids are stored in droplets both in intestinal cells and hypodermal cells (Ashrafi 2007; Lemieux and Ashrafi 2015; Srinivasan 2015). When fed *E. coli* OP50, K11E4.2 mutant worms showed greater lipid accumulation than wild-type worms as assessed by ImageJ (**Fig. 4.4A**). Additionally, wild-type worms fed *C. aquatica* accumulate more fat than K11E4.2 mutants under the same conditions (**Fig. 4.4B**). These findings suggest that K11E4.2 might act as a regulator of energy expenditure and storage in the intestine, dependent on the bacterial diet.

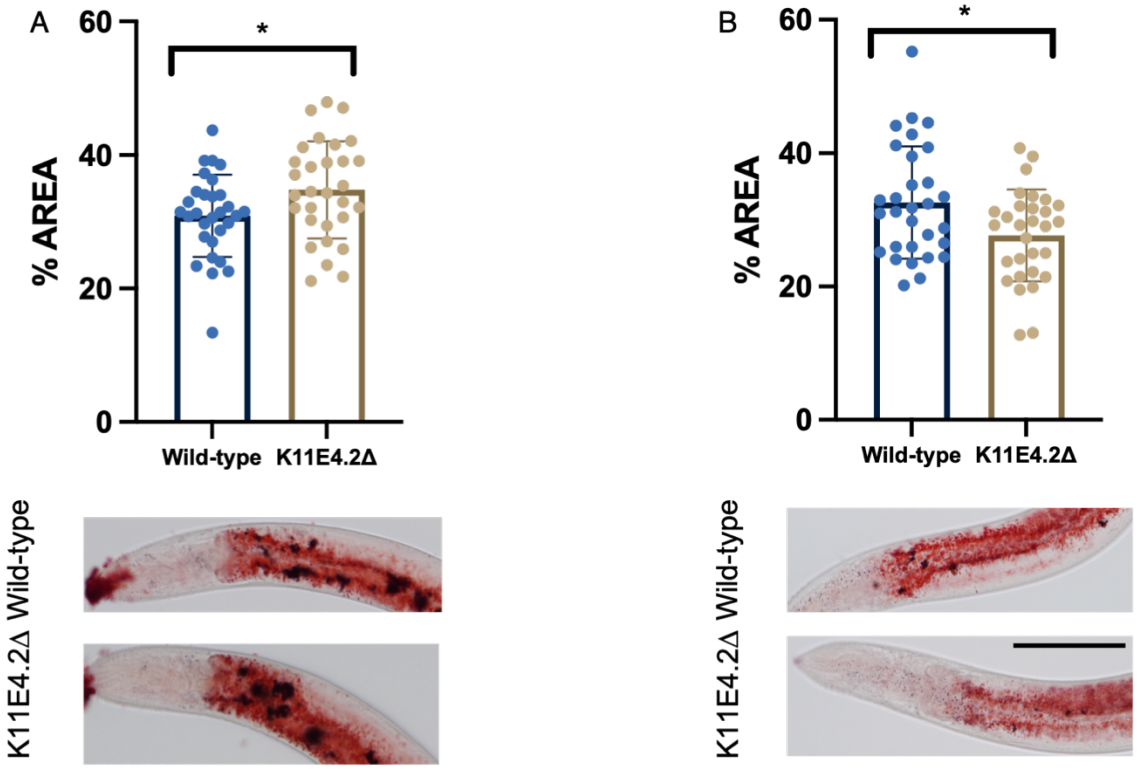


Figure 4.4: Fat accumulation in wild-type and in K11E4.2 mutant worms as assessed by ImageJ analysis after Oil red O staining. Oil red O staining is more intense in K11E4.2 mutants fed E. coli OP50, but fainter when fed Comamonas aquatica. Staining was performed in young adults at 48 hours after plating L1 synchronized populations. **A)** E. coli OP50, n=30 **B)** Comamonas aquatica, n=30. Each dot represents the stained area between the pharyngeal valve and the start of the gonadal arm of an individual worm. Unpaired T-test: *P<0.05. Scale bar: 100µm

4.5 K11E4.2 is not required for normal lifespan but K11E4.2 mutation rescues the short lifespan phenotype of *shc-1* mutants

In mammals and *C. elegans*, SHC proteins promote survival by regulating stress resistance through insulin and MAPK pathways (Neumann-haefelin et al. 2008; Pellegrini et al. 2005). The lifespan of *shc-1* mutants is 25-38% less than that of wild-type animals (Neumann-haefelin et al. 2008; Qi et al. 2012) principally due to defects in germline integrity. While K11E4.2 mutants have not been assessed for egg-laying defects, *shc-1* mutants die early on due to internal hatching. To avoid the internal hatching early death, FUDR is supplemented in the media. Importantly, FUDR extends the lifespan of *shc-1* mutants (Qi et al. 2012). However, even in the presence of FUDR, the lifespan of *shc-1* mutants is decreased relative to wild-type animals (**Fig. 4.5**). Double SHC mutants displayed a lifespan similar to wild-type animals, this suggests that not only does the loss of K11E4.2 not enhance the early death of *shc-1* mutants, but prevents it. Together these results suggest that K11E4.2 is not required for lifespan extension in a single K11E4.2 mutant background, but the loss of K11E4.2 in a *shc-1* mutant background results in the rescuing of the *shc-1* mutant short lifespan phenotype.

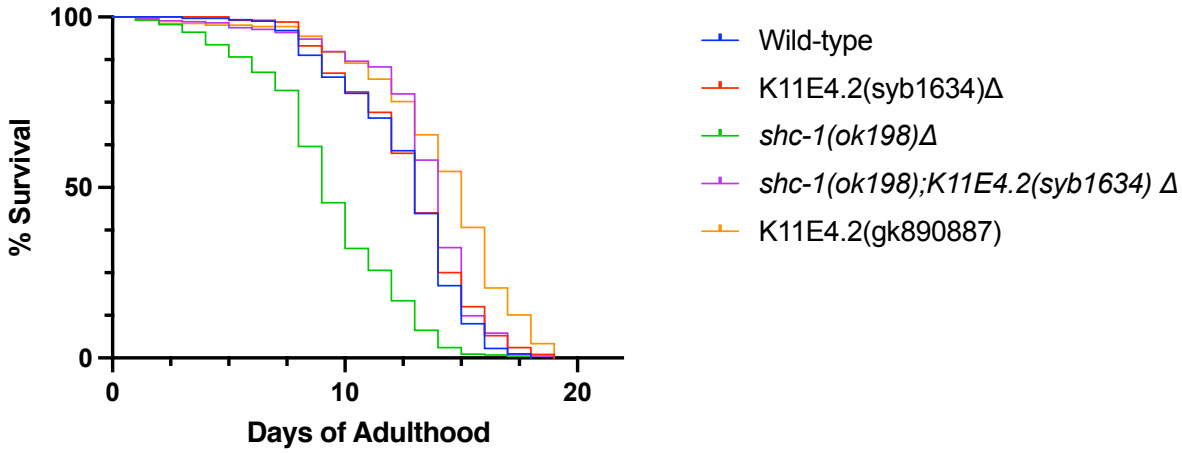


Figure 4.5: K11E4.2 mutants display normal lifespan. Survival over time is shown. There is no significant difference between the lifespan of wild-type animals and K11E4.2 (syb1634) mutants. While single *shc-1(ok198)* mutants display significantly reduced lifespan, double *shc-1(ok198);*K11E4.2(syb1634) mutants display increased lifespan. L4 animals were transferred to NGM plates with 100 μM FUDR to prevent the development of progeny. Day 0 indicates the day of transfer. For all strains: n ≥ 200. Mantel-Cox test: K11E4.2 (syb1634) (P = ns), *shc-1 (ok198)* (P<0.0001), *shc-1(ok198);*K11E4.2(syb1634) (P<0.0001), K11E4.2 (gk890887) (P<0.0001). This is a representative figure of two independent experiments.

4.6 K11E4.2 is not required for fertility

In *C. elegans*, insulin signaling is one of the main pathways that mediates reproduction and fertility. This is shown in mutants that have a significantly reduced brood size, such as *daf-2* and *age-1* (Tissenbaum and Ruvkun 1998). Interestingly, *daf-16*/FOXO mutants have a slightly decreased brood size as compared to N2 wild-type worms, but this same mutation

partially rescues reduced brood size in *daf-2* mutants, which suggests that *daf-16* downregulation is at least partially responsible for normal reproduction (Tissenbaum and Ruvkun 1998). When assessing brood size in *shc-1* and *shc-3* mutants, there was no statistical difference in the number of progeny between strains (**Fig. 4.6**). All strains, including K11E4.2 mutants, had the same number of offspring as wild-type animals which suggests that K11E4.2 is not required for normal fertility.

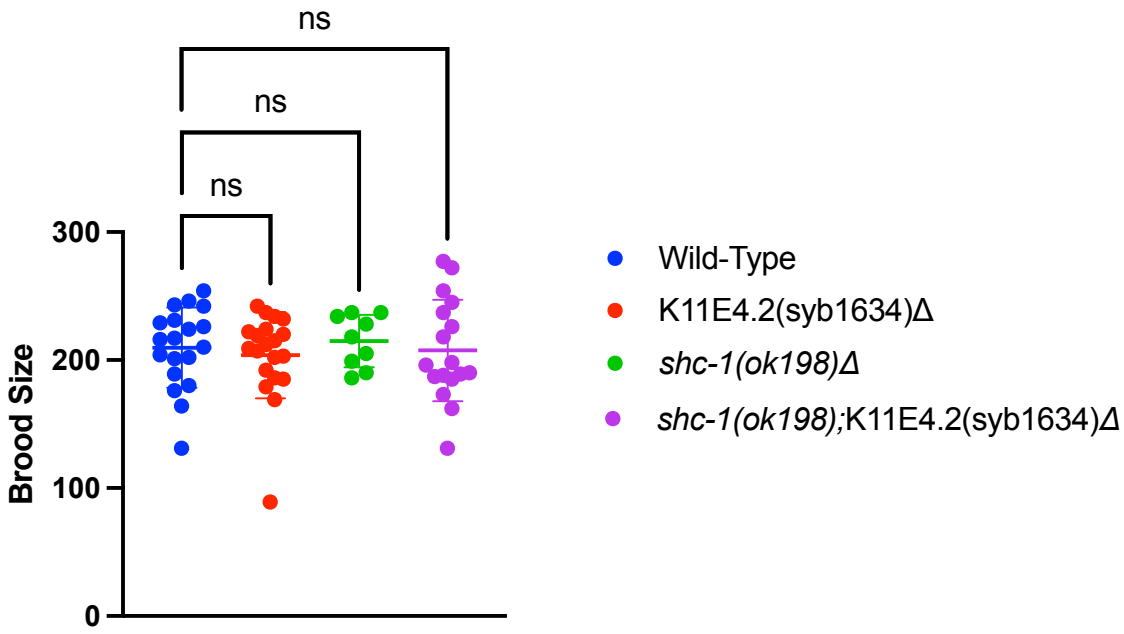


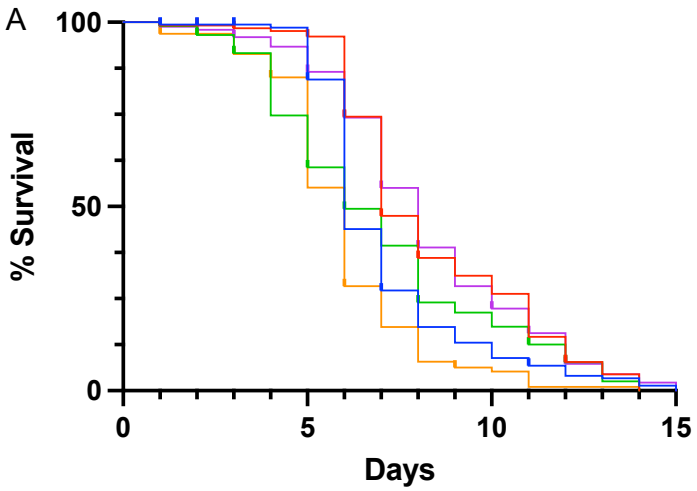
Figure 4.6:. Fertility is not affected in K11E4.2 mutants. All strains were scored for number of progeny for three consecutive days. Each point represents the total number of offspring for an individual animal. No significant differences were detected for any of the mutant strains. For all strains: n ≥ 9. One-way ANOVA and Dunnett's multiple comparisons: Mean and standard deviation are shown.

4.7 K11E4.2 knockout rescues enhanced *shc-1* oxidative stress sensitivity in double mutants. K11E4.2 are more sensitive to oxidative stress in an allele dependent manner in acute and chronic paraquat assays

In mammals, SHC proteins are reported to play a role in the regulation of oxidative stress response, specifically the 66kDa isoform of mammal Shc functions as a sensor of intracellular ROS concentrations and promotes apoptosis (Pellegrini et al. 2005). In *C. elegans,* SHC-1 is also required for oxidative stress survival through the JNK pathway (Neumann-haefelin et al. 2008), but it is known to be more similar to the 52 kDa isoform of human Shc an isoform less involved in oxidative stress responses (Mcglade et al. 1992; Wu et al. 2016). SHC-1 mutants display an increased sensitivity to ROS upon acute paraquat exposure as compared to wild-type animals.

To test if K11E4.2 is also required for oxidative stress regulation, animals were first exposed animals to 4mM paraquat and scored every day for survival. As previously reported, SHC-1 mutants were more sensitive to the treatment as compared to wild-type worms (Neumann-haefelin et al. 2008). Interestingly, while double *shc-1(ok198);*K11E4.2(syb1634*)* mutants were the most resistant to the treatment, the K11E4.2(gk890887) mutant strain was the most sensitive (**Fig. 4.7A**) . These results suggest that not only both SHC proteins act in oxidative stress survival mechanisms but also the loss of both SHC-1 and K11E4.2(syb1634) results in increased resistance rather than increased sensitivity, which leads to the hypothesis that both SHC proteins do not act redundantly in oxidative stress response mechanisms. Because survival significantly decreased during the first days of treatment, a higher concentration of paraquat (100mM)

was tested to analyze the effect of acute oxidative stress. Similarly to the first time points of the chronic assay, the *shc-1(ok198)* and K11E4.2 (gk890887) mutants were the most sensitive and the K11E4.2(syb1634) and double mutant *shc-1(ok198);*K11E4.2(syb1634*)* strains were less sensitive to the treatment (**Fig. 4.7B**). Increased stress sensitivity of SHC-1 mutants is reported to be mediated by the interaction of SHC-1 with the JNK pathway component MEK-1/MAP2K7 (Neumann-haefelin et al. 2008). Further experiments would be needed to understand the mechanism by which K11E4.2 regulates responses to oxidative stress and to determine why two different alleles result in opposite effects and to elucidate if K11E4.2 acts in the same pathway as SHC-1.



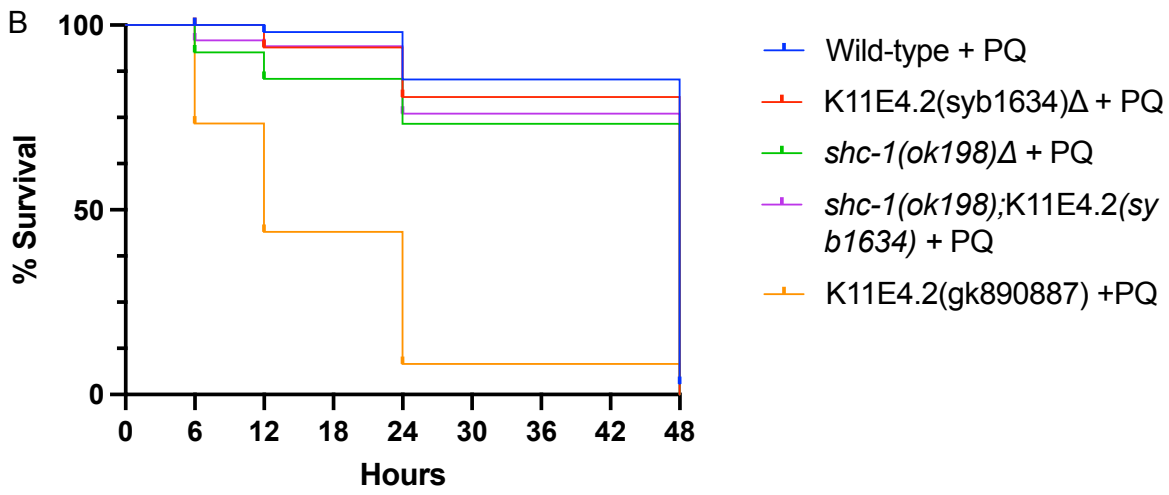


Figure 4.7: K11E4.2 mutants are more sensitive to chronic and acute oxidative stress in an allele-dependent manner. Upon exposure to 4mM and 100mM paraquat, K11E4.2(gk890887) mutants have a shorter lifespan compared to all strains. L4 and young adults were transferred to NGM plates added with 4mM or 100mM paraquat and scored for survival every day during the chronic assay (4mM) and after 6, 12, 24 and 48 hours during the acute assay (100mM). **A)** For all strains: n ≥ 200, Mantel Cox test: K11E4.2(syb1634) (P<0. 0001), *shc-1(ok198)* (P=ns), *shc-1(ok198);*K11E4.2(syb1634) (P<0. 0001), K11E4.2 (gk890887)(P<0. 0001) **B)** For all strains n ≥ 70, Mantel Cox test: K11E4.2(syb1634) (P=ns), *shc-1(ok198)* (P=0.0037), *shc-1(ok198);*K11E4.2(syb1634) (P=ns), K11E4.2 (gk890887)(P<0. 0001).

4.8 K11E4.2 mutants are more sensitive to prolonged starvation during L1 arrest than wild-type animals

L1 arrest is controlled by insulin signaling (Baugh 2013). While *daf-2*/insulin receptor mutants display an enhanced resistance to L1 arrest, *daf-16*/FOXO mutants show increased sensitivity and increased death rate during L1 arrest(Baugh and Sternberg 2006).

shc-1 mutants display an enhanced sensitivity to prolonged starvation during L1 arrest. SHC-1 and DAF-18/PTEN are required for L1 arrest resistance, by regulating mitochondrial metabolism (Wolf et al. 2014). To test if K11E4.2 recapitulates this phenotype seen in both *shc-1* and insulin signaling mutants, we tested the survival of K11E4.2 mutants upon L1 arrest. To explore this hypothesis, eggs of wild-type, *shc-1*, K11E4.2, and double *shc-1(ok198);K11E4.2(syb1634)* mutant backgrounds, were collected by hypochlorite treatment and allowed to develop to L1 larval stage in M9 buffer (Lee et al. 2012). Every day worms were scored for survival by extracting a fixed volume of each sample that was placed on *E. coli* OP50 seeded plates. Across all biological replicates, the least resistant worms were *shc-1* mutants, which is consistent with the available literature (Wolf et al. 2014). However, K11E4.2 mutants were also more sensitive to starvation than wild-type worms (**Fig. 4.8**). This suggests that both SHC-1 and K11E4.2 promote survival after prolonged starvation. Importantly, loss of both SHC-1 and K11E4.2 proteins did not enhance the effect of either individual mutant, this suggests that SHC-1 and K11E4.2 do not act redundantly to regulate L1 arrest survival. However, it does suggest that both proteins function in the same genetic pathway, which might lead to the hypothesis

that each SHC protein acts to control development in response to nutrient availability through different mechanisms.

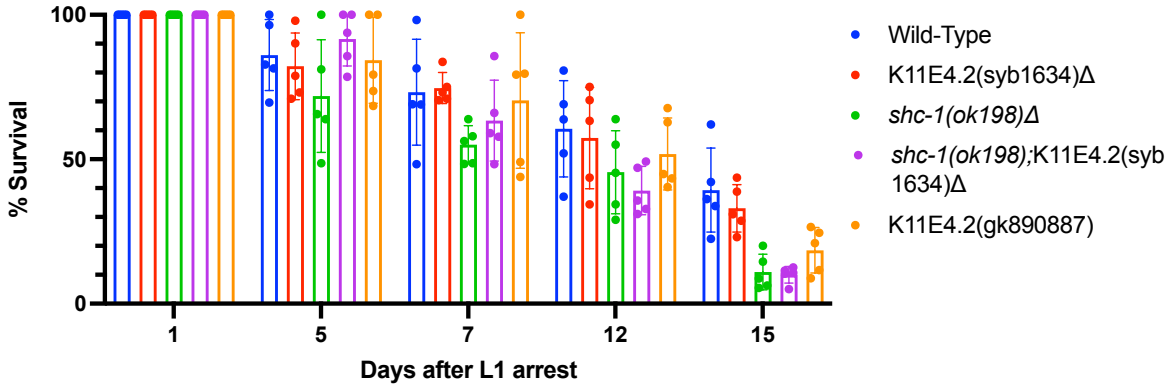


Figure 4.8: K11E4.2 mutants are more sensitive to prolonged starvation compared to wild-type animals, but more resistant compared to *shc-1* mutants. K11E4.2 mutants show reduced recovery from L1 arrest as compared to wild-type animals. Each time point represents the mean survival of five individual plates with at least 30 animals per plate. Recovery was assessed after 1, 5, 7, 12 and 15 days after L1 arrest. Each dot represents the survival proportion of an individual plate. Mantel Cox test: K11E4.2 (syb1634) (P=ns), *shc-1(ok198)* (P=0.0001), *shc-1(ok198);*K11E4.2(syb1634) (P=0.0011) K11E4.2 (sgk890887) (P=0.0191). This is a representative figure of two independent assays including all strains.

4.9 K11E4.2 knockdown induces cytoplasmic and intermediate DAF-16 intracellular localization

The DAF-16/FOXO transcription factor, is the major downstream target of the insulin signaling pathway in *C. elegans* (Lee, Hench, and Ruvkun 2001) and when DAF-16 is activated, upon insulin signaling downregulation, it migrates to the nucleus and regulates

transcription of target genes, promoting longevity and increased stress resistance (Lee et al. 2003).

In order to know if K11E4.2 acts in the insulin signaling pathway, we tested the effect of K11E4.2 knockdown and its effect in DAF-16 intracellular localization, assessing for cytoplasmic, intermediate or nuclear localization (**Fig. 4.9**). We hypothesized that if the K11E4.2 RNAi treatment induced similar DAF-16 nuclear localization compared to *daf-2* RNAi, then K11E4.2 is likely to be a positive regulator of insulin signaling in wild-type animals. To this end, we exposed a transgenic strain DAF-16::GFP to double stranded RNA for *daf-2*, K11E4.2 and the empty vector L4440 and analyzed DAF-16 intracellular localization at the fourth larval stage (L4). While animals exposed to *daf-2* RNAi displayed predominantly nuclear and intermediate localization (**Fig. 4.10A-B**) K11E4.2 RNAi treated worms displayed cytosolic and intermediate localization of DAF-16 (**Fig. 4.10A-B**). These findings suggest that K11E4.2 knockdown induced a mild decrease in insulin signaling as cytoplasmic localization was significantly decreased compared to the empty vector ($P=0.0393$) but significantly increased compared to *daf-2* RNAi ($P=0.0461$) (**Fig. 4.10A-B**). Collectively, these results lead to the hypothesis that K11E4.2 normal function aids in the positive regulation of insulin signaling, therefore promoting DAF-16 cytoplasmic sequestration.

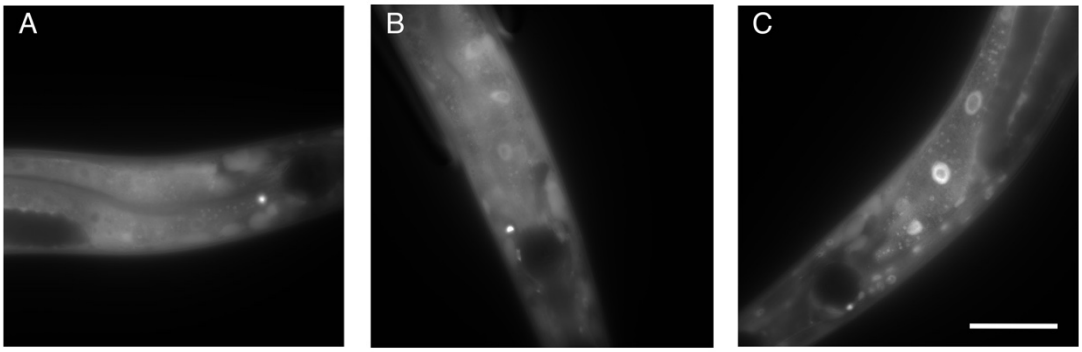
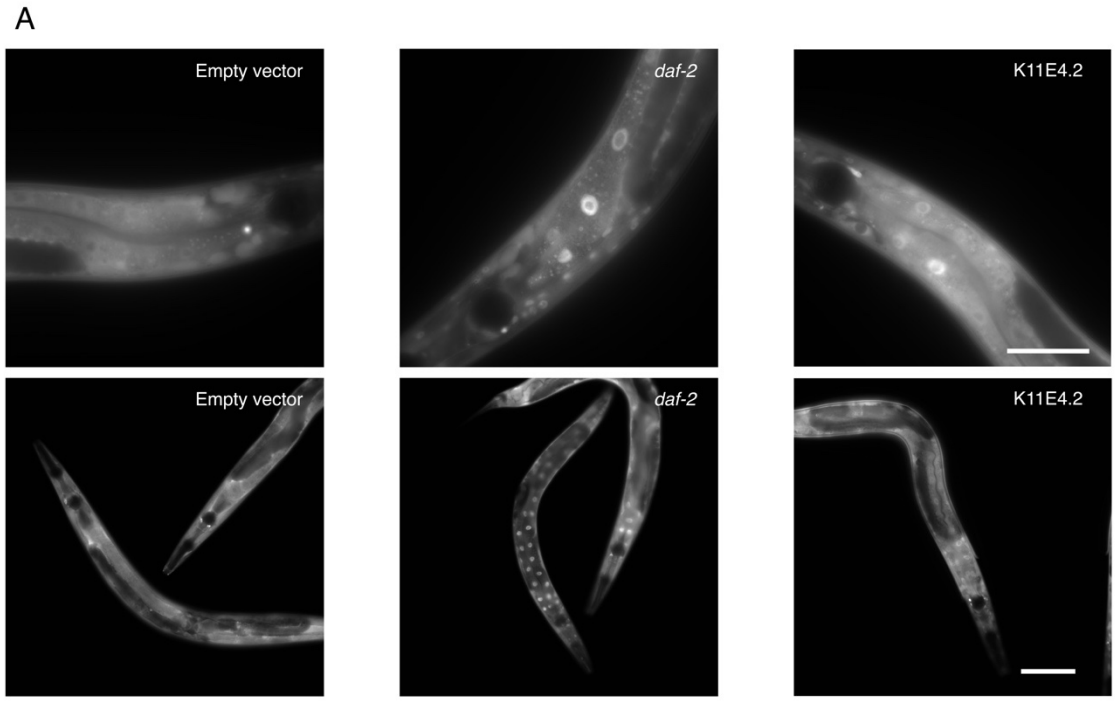


Figure 4.9: Animals were assessed for DAF-16 intracellular localization, and classified in cytoplasmic (**A**), intermediate (**B**) and nuclear (**C**) DAF-16 localization. Scale bar: 50 μm. Representative pictures of animals expressing a GFP reporter fused to *daf-16.*


A
Empty vector
daf-2
K11E4.2
Empty vector
daf-2
K11E4.2

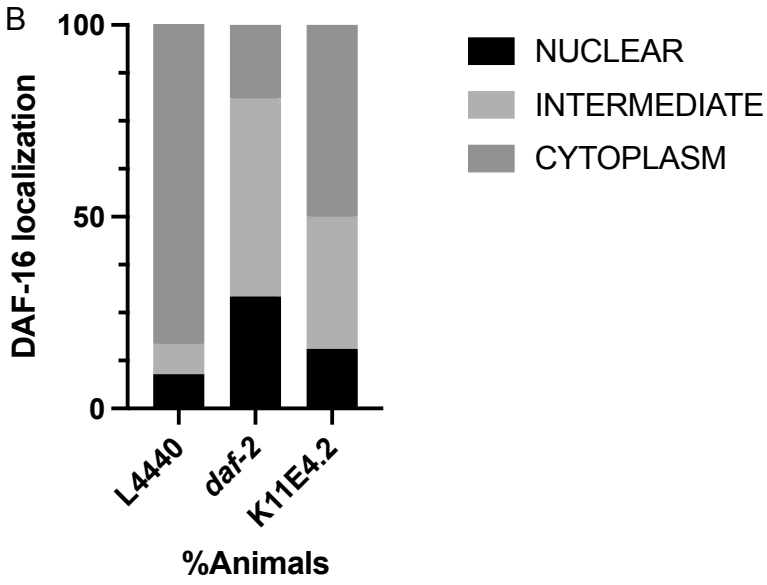

B
100
50
0
DAF-16 localization
L4440
daf-2
K11E4.2
%Animals
NUCLEAR
INTERMEDIATE
CYTOPLASM

Figure 4.10: K11E4.2 RNAi treated worms show significantly less cytoplasmic DAF-16 intracellular localization than animals fed with the empty vector. Animals treated with the empty vector show cytoplasmic DAF-16 localization (**A**). *daf-2* knockdown induces nuclear and intermediate localization of DAF-16 (**A**). K11E4.2 RNAi treated animals show cytoplasmic and intermediate DAF-16 localization (**A**). **A**: Pictures are representative of three independent experiments. Scale bar: 50μm and 100 μm. **B**: Empty vector (n=89), daf-2 (n=89) K11E4.2 (n=90). Two-way ANOVA, Cytoplasmic localization compared to the empty vector : K11E4.2 (P=0.0393), *daf-2* (P=0.0007*)*.

Chapter 5

5 Discussion and suggestions for further research

5.1 Discussion

This project aimed to characterize the function of a novel Shc protein, K11E4.2, based on the knowledge that 1) Shc proteins are evolutionarily conserved (Luzi et al. 2000), 2) Shc proteins are involved in various cellular processes such as oxidative stress responses and survival, through conserved signaling pathways such as insulin signaling (Ahmed and Prigent 2017; Ravichandran 2001) and 3) K11E4.2 is predicted to have the characteristic PTB and SH2 domains of Shc proteins, as well as a portion of the CH1 region.

Collectively, the data obtained suggest that K11E4.2 is likely to act as a metabolism regulator in response to different environmental conditions. While K11E4.2 mutants display a normal lifespan and brood size, developmental rate and fat accumulation change in response to different bacterial diets. These results suggest that K11E4.2 might have a role in regulating energy storage and expenditure. K11E4.2 mutants were more sensitive to oxidative stress and prolonged starvation.

Based on the finding that K11E4.2 knockdown increases DAF-16/FOXO cytoplasmic localization, K11E4.2 is likely to act as a positive regulator of insulin signaling. However, phenotypic analysis of K11E4.2 mutants did not provide a clear understanding of how this protein may function insulin signaling (**Table 5.1**). In fact, some phenotypic data would be consistent with K11E4.2 acting as a negative regulator of insulin signaling. Additional phenotypes shown by insulin signaling mutants include extended

lifespan and increased resistance to stress (Kimura et al. 1997; Tissenbaum and Ruvkun 1998). *shc-1* mutants suffer from an early death due to accelerated aging (Neumann-haefelin et al. 2008). When assessing lifespan, the short lifespan phenotype of *shc-1(ok198)* was rescued in double *shc-1(ok198);*K11E4.2(syb1634) mutants, which suggests that as a single mutation, the loss of K11E4.2 does not have an effect on lifespan, but in the context of double mutants, it extends the short-lived phenotype of the *shc-1(ok198)* mutant. The short lifespan of *shc-1(ok198)* mutants is reported to be *daf-16*-dependent (Neumann-haefelin et al. 2008), however whether the K11E4.2 rescue in the double mutants is also mediated by *daf-16* remains unclear.

In mammals and *C. elegans*, SHC proteins are implicated in oxidative stress response (Galimov 2010; Migliaccio et al. 1999; Neumann-haefelin et al. 2008). The results obtained show that the loss of both SHC-1 and K11E4.2 does not result in enhanced oxidative stress sensitivity, leading to the hypothesis that SHC-1 and K11E4.2 have different functions in regulating responses to oxidative stress. SHC-1 regulates oxidative stress responses by interaction with the insulin receptor DAF-2 in the insulin signaling pathway and positive regulation of the JNK-1 signaling pathway (Neumann-haefelin et al. 2008). Importantly, SHC-1 is reported to interact with DAF-2, through its PTB domain and to interact with MEK-1 through its SH2 domain (Neumann-haefelin et al. 2008). Since K11E4.2 is likely to function in the same genetic pathway as SHC-1, one possibility could be that K11E4.2 also interacts with DAF-2 through the K11E4.2 PTB domain but modulates DAF-2 activity by recruiting an additional protein through its CH1 or SH2 domain (**Fig 5.1B**). In this scenario, K11E4.2 could recruit additional proteins such as

kinases or protein complexes through the SH2 domain and increase DAF-2 activity, promoting decreased tolerance to environmental stressors in wild-type animals by DAF-16 cytoplasmic retention. Thus, explaining the less severe sensitivity to environmental aggressions seen in K11E4.2(syb1634) mutants. As opposed to SHC-1, a negative regulator of the insulin signaling pathway, whose function is hypothesized to promote survival in response to environmental changes.

Furthermore, since the loss of K11E4.2 results in consistent but not significant oxidative stress sensitivity, it could be possible that K11E4.2 does not negatively regulate DAF-2 in wild-type animals. Notably, while *shc-1* is broadly expressed in *C. elegans,* K11E4.2 is only expressed in the intestine, which also raises an important question as to what is the extent of the role of the intestine in modulating oxidative stress responses and how the loss of function of K11E4.2 in this tissue, is able to rescue *shc-1(ok198)* increased sensitivity in double mutants.

In *C. elegans*, recovery from L1 arrest is assessed as an indicator of nutritional control of development as it depends solely on nutrient availability (Chen and Baugh 2014). *daf-2* mutants show enhanced resistance to prolonged starvation during L1 arrest in a DAF-16 dependent manner (Baugh and Sternberg 2006). In contrast, decreased adaptation to prolonged starvation in *shc-1(ok198)* mutants, as assessed by the presence of germline tumors upon refeeding after prolonged starvation, is reported to be likely caused by increased insulin signaling in a DAF-16 independent manner (Wolf et al. 2014). Similarly to the oxidative stress assays double *shc-1(ok198);*K11E4.2(syb1634) mutants showed better recovery from L1 arrest than single *shc-1(ok198)* mutants. Further supporting the

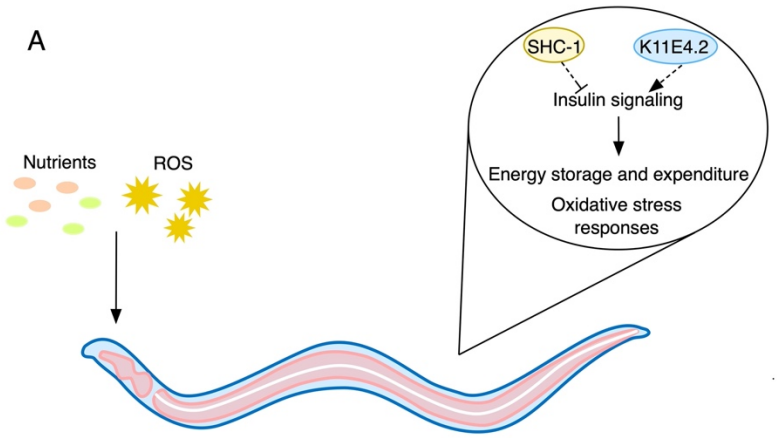
hypothesis that SHC-1 and K11E4.2 have different functions but act in the same genetic pathway. Whether K11E4.2 acts in a DAF-16 dependent manner to regulate L1 survival remains an interesting question for future research.

Because K11E4.2(syb1634) mutants showed significantly less severe phenotypes from *shc-1(ok198)* mutants in both oxidative stress and L1 recovery assays, an additional K11E4.2 mutant strain was included in these assays. Intriguingly, while both mutants are predicted null mutants, the K11E4.2(syb1634) and K11E4.2(gk890887) mutations resulted in different phenotype penetrance after chronic and acute paraquat exposure (**Fig. 4.7**). K11E4.2(syb1634) mutants did not show significantly decreased survival from wild-type animals during paraquat assays. However, K11E4.2(gk890887) mutants displayed significantly enhanced sensitivity in both assays, and their survival was significantly different from both wild-type animals and *shc-1(ok198)* mutants. The K11E4.2(syb1634) mutation is a deletion that removes the first and second exons and should result in no protein production and the K11E4.2(gk890887) mutation is a nucleotide substitution, resulting in the introduction of a stop codon at Q39. This raises the question of whether one of these alleles might retain function and thus, explain the difference in penetrance in the observed phenotypes. Although both strains were outcrossed, a linked mutation may also explain differences in the two strains. K11E2.4 RNAi knockdown in these strains could confirm loss of function and possibly offer an answer to this question in the future. Furthermore, rescue of the mutant strains with a wild type gene could also address this issue.

Overall, the data obtained from this project suggests that K11E4.2 could function as positive regulator of the insulin signaling pathway, likely competing with SHC-1 for

DAF-2 signal transduction in intestinal cells (**Fig. 5.1A**). Examining genetic interactions with *daf-2* and *daf-16* mutants could help to better understand the role of K11E4.2 in insulin signaling.

Table 5.1: Summary of phenotypes found in K11E4.2 mutants			
Phenotype	*daf-2* mutants	*shc-1* mutant	K11E4.2 mutant
Development	Slow	Not reported	Normal
Lifespan	Increased	Decreased	Normal
Brood size	Decreased	Normal	Normal
Fat accumulation	Increased	Not reported	Increased on *E. coli* OP50, decreased on *C. aquatica*
Oxidative stress resistance	Increased	Decreased	Decreased in allele dependent manner
Prolonged starvation resistance	Increased	Decreased	Decreased
DAF-16 activation	Increased	Decreased	Increased compared to empty vector



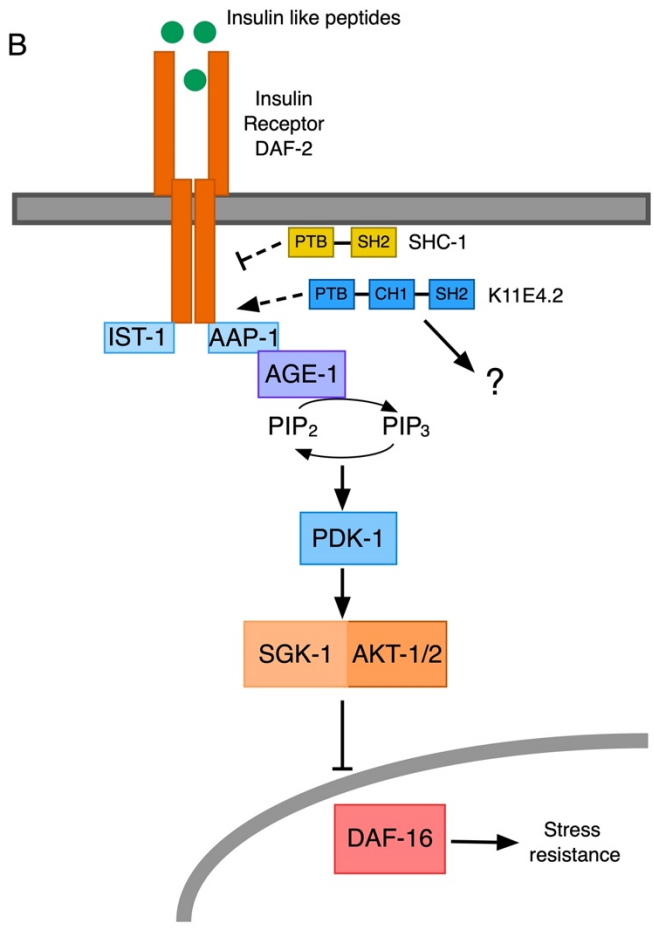


Figure 5.1 : Proposed model for K11E4.2 positive regulation of the insulin signaling pathway in *C. elegans*. **A)** Upon environmental stimuli, such as nutrient availability and composition or stressors, K11E4.2 might positively regulate insulin signaling in the intestine and compete with SHC-1, a negative regulator. **B)** Both K11E4.2 and SHC-1 are predicted to contain an N-terminal PTB domain. Since K11E4.2 is likely to have opposite functions from SHC-1, a negative insulin regulator, K11E4.2 could potentially interact with the DAF-2 insulin receptor, through the K11E4.2 PTB domain, and modulate DAF-2 activity by recruiting additional proteins through the predicted K11E4.2 CH1 or SH2 domain.

5.2 Future directions

The results obtained suggest that K11E4.2 might have an interesting regulating function in the *C. elegans* intestine. However, a number of questions remain. Firstly, what is the role of K11E4.2 in adapting to different bacterial diets? To answer this question, it would be useful to know if K11E4.2 has a role in regulating pathways such as lipid metabolism in response to dietary composition. While lipid storage was assessed with an ImageJ software after Oil red O staining, the code used is a very simple algorithm (Supplementary information), which might result in less accurate comparisons, therefore more sophisticated methods like mass spectrometry or thin layer chromatography (Lemieux and Ashrafi 2015) could be performed to assess precise differences in fat storage. To our knowledge, fat assessment has not been reported in *shc-1(ok198)* single mutants, therefore including both *shc-1(ok198)* and double mutant strains in lipid assessment could also provide more clues about the role of *C. elegans* SHC proteins in lipid metabolism. Additionally, diet-dependent phenotypes are mainly attributed to the composition of the bacterial diet (MacNeil et al. 2013; Rashid et al. 2020; Zhang et al. 2010). Therefore, if the difference in lipid storage is indeed significant, identification of the components of the diet that cause the difference in phenotypes could also aid in the identification of additional metabolic pathways that K11E4.2 could act in to regulate nutrient uptake and storage. Because K11E4.2 is found to be intestinally expressed, this knowledge could also elucidate the mechanism whereby tissue intercommunication is able to regulate processes such as development and energy expenditure.

As discussed above, the data obtained suggest a possible competition with SHC-1 for DAF-2 signal transduction in the insulin signaling pathway. However, whether K11E4.2 could also act in other metabolic pathways remains unclear. Furthermore, Shc proteins are adaptor proteins which by definition mediate protein-protein interactions, therefore it is likely that K11E4.2 interacts with additional molecules aside from DAF-2. To this end, examination of protein interactions will prove essential in order to fully characterize the function of K11E4.2. Methods such as proximity labeling could provide a clue as to which proteins could potentially form complexes with K11E4.2 and which metabolic pathways could require K11E4.2 for signal propagation.

5.3 Limitations

Experimental procedures can result in variability for a number of reasons, and experiments with *C. elegans* are no exception (Pho and MacNeil 2019). For assessing L1 survival, population density has to be taken into account as larvae density influences survival (Mata-cabana et al. 2020). Therefore, accuracy in the comparison of survival between populations can be affected if the density of animals in each sample varies, as animals in a more dense population show increased survival (Mata-cabana et al. 2020). Another factor to take into account is contamination as our protocol has a duration of 21 days. As a fixed volume is extracted for each time point, it is important to maintain sterility for the duration of the experiment. Thus, special care has to be taken in both handling the samples and comparing similar population densities.

C. elegans is known to display avoidance behaviors after being exposed to environmental stressors, such as paraquat (Gourgou and Chronis 2016). During the initial

time points of both chronic and acute exposure to paraquat, a percentage of the populations will crawl to the side of the plates and will have to be censored. This was a limitation for initial experiments as the majority of the animals in the samples had to be censored, interfering with the interpretation of the effect of paraquat on survival. Therefore after repeating the experiments, we found that for this set of assays it is important to have an adequate number of worms: at least 50 per plate during chronic exposure and at least 30 for the acute assay. With larger numbers of animals, the effect of paraquat on survival can be more accurately assessed.

Finally, an important limitation was the assessment of fat accumulation after Oil Red O staining. For the purpose of this project, we focused on a representative area of the intestine, as K11E4.2 is expressed in this tissue. However, the image analysis was performed with a very simple ImageJ algorithm and each image was assessed independently, which could result in variability between samples and/or experiments. Additionally, we did not take intensity of the dye into account, as we found black aggregates in some of our samples, we aimed to avoid the interference this may cause. To our knowledge, there is no intestine-specific image analysis protocol for the assessment of Oil red O staining. Additionally, Oil red O is an indirect measure of lipids, therefore more sophisticated methods, like liquid chromatography or mass spectrometry, should be employed to measure changes in specific lipids (Lemieux and Ashrafi 2015).

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Appendix A

A. Supplementary Information

A.1 ImageJ Macro for Oil red O assessment in ImageJ.

The scale of the images is adjusted from pixels to micrometers. TIFF files are processed by splitting the images into RGB stacks and using the green channel to manually adjust the threshold to highlight the area stained between the end of the pharyngeal valve and the start of the gonadal arm in young adults. The code used was run on ImageJ as follows:

```
//setTool("line");
run("In [+]");
run("In [+]");
//setTool("hand");
makeRectangle(1182, 1409, 489, 315);
//setTool("line");
makeLine(2300, 1947, 2796, 1949);
run("Set Scale...", "distance=496.0018 known=100 pixel=1 unit=µm");
run("Out [-]");
run("Out [-]");
//setTool("rectangle");
run("RGB Stack");
setAutoThreshold("Default dark");
//run("Threshold...");
//setTool("rectangle");
makeRectangle(1179, 1403, 465, 318);
makeRectangle(1179, 1427, 459, 294);
makeRectangle(1170, 1451, 468, 270);
makeRectangle(1170, 1451, 456, 243);
makeRectangle(1173, 1457, 453, 237);
run("Measure");
```

A.2 Chapter 4: Experimental replicates

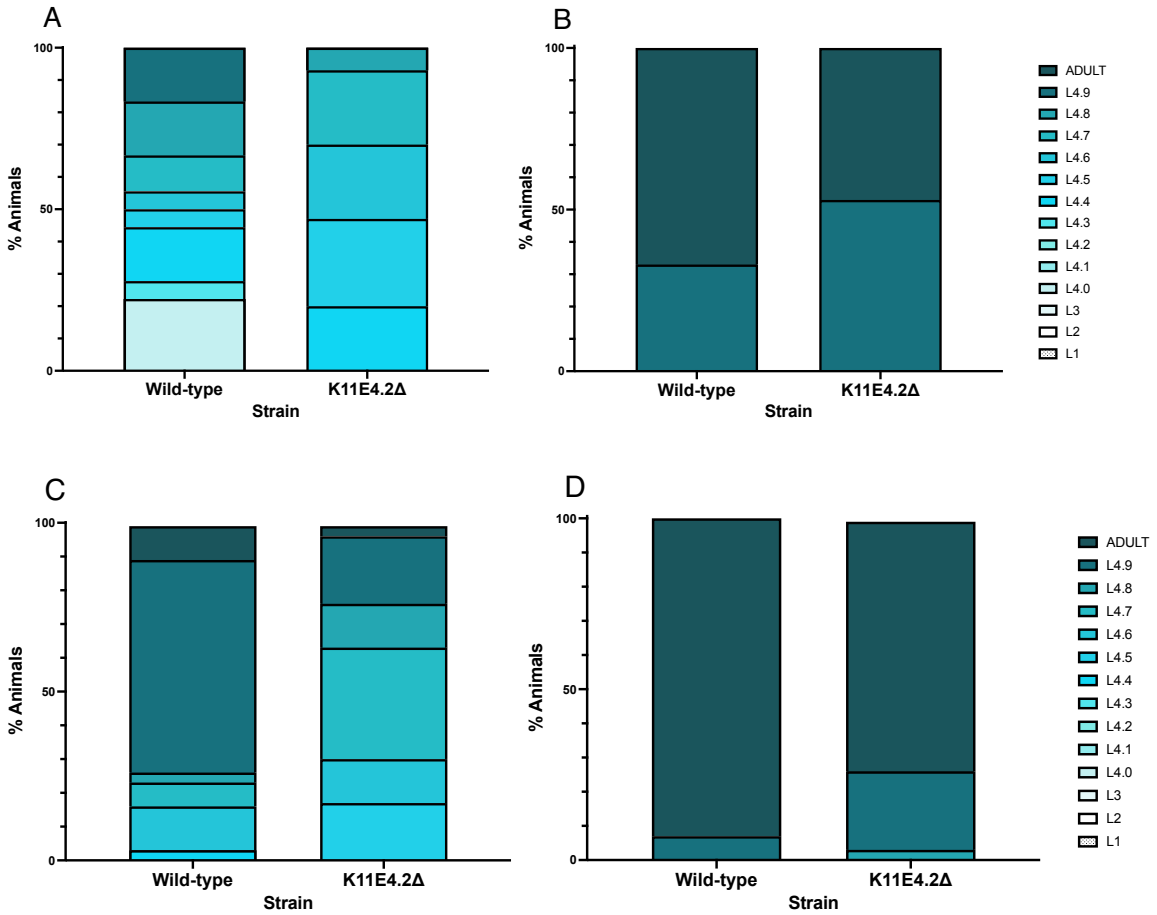

Figure A.2. 1: K11E4.2 mutant animals develop at a normal rate on an *E. coli* OP50 diet (**A,C**) but more slowly on *a C. aquatica* diet (**B,D**) compared to wild-type animals in two separate experiments. Animals were scored at 40 hours after plating the synchronized L1 populations in plates seeded with the respective diets. Scoring was performed by assessing vulva morphology at the L4 stage. **A)** N2 on *E. coli* OP50 (n=18) and K11E4.2 mutants on *E. coli* OP50 (n=30). Expected number of L4.9 (wild-type) vs observed (K11E4.2): *E. coli* OP50 ($P<0.05$) **B)** N2 on *Comamonas aquatica*, (n=30) and K11E4.2 mutants on *Comamonas aquatica*, (n=30). Expected number of adults (wild-type) vs observed

(K11E4.2): *C. aquatica* (P =ns) **C)** N2 on *E. coli* OP50 (n=30) and K11E4.2 mutants on *E. coli* OP50 (n=30). Expected number of adults (wild-type) vs observed (K11E4.2): *E. coli* OP50 (P=ns **D)** N2 on *Comamonas aquatica*, (n=30) and K11E4.2 mutants on *Comamonas aquatica*, (n=30). Expected number of adults (wild-type) vs observed (K11E4.2): *C. aquatica* (P =ns).

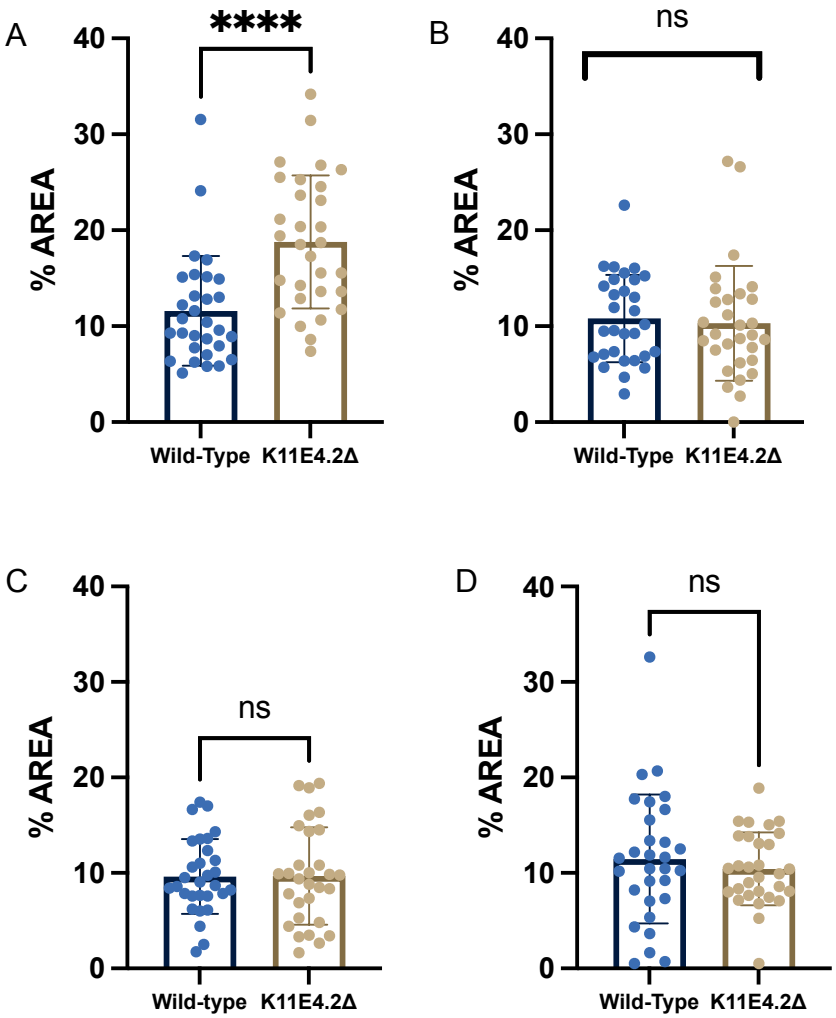

Figure A.2. 2: Fat accumulation in wild-type and in K11E4.2 mutant worms as assessed by ImageJ analysis after Oil red O staining. Oil red O staining is more intense in K11E4.2

mutants fed *E. coli* OP50, but fainter when fed *Comamonas aquatica*. Staining was performed in young adults at 48 hours after plating L1 synchronized populations. **A,C)** *E. coli* OP50, n=30 **B,D)** *Comamonas aquatica*, n=30. Each dot represents the stained area between the pharyngeal valve and the start of the gonadal arm of an individual worm. Unpaired T-test: ****P<0.0001.

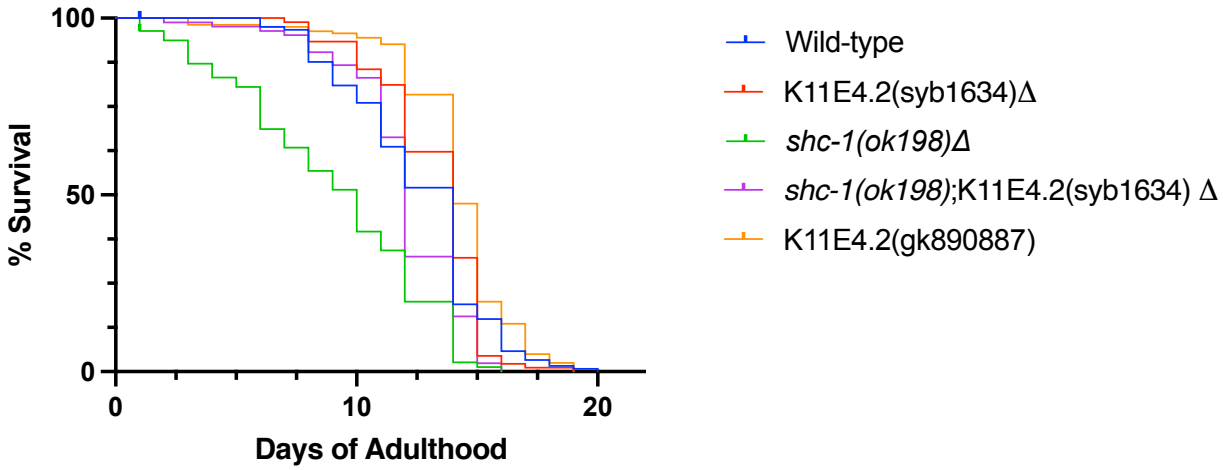

Figure A.2. 3: K11E4.2 mutants display normal lifespan. Survival over time is shown. There is no significant difference between the lifespan of wild-type animals and K11E4.2 (syb1634) mutants. While single *shc-1(ok198)* mutants display significantly reduced lifespan, double *shc-1(ok198);*K11E4.2(syb1634) mutants display increased lifespan. L4 animals were transferred to NGM plates with 100 μM FUDR to prevent the development of progeny. Day 0 indicates the day of transfer. For all strains: n ≥ 78. Mantel-Cox test: K11E4.2 (syb1634) (P = ns), *shc-1 (ok198)* (P<0.0001), *shc-1(ok198)*;K11E4.2(syb1634) (P=0.0262), K11E4.2 (gk890887) (P<0.0001).

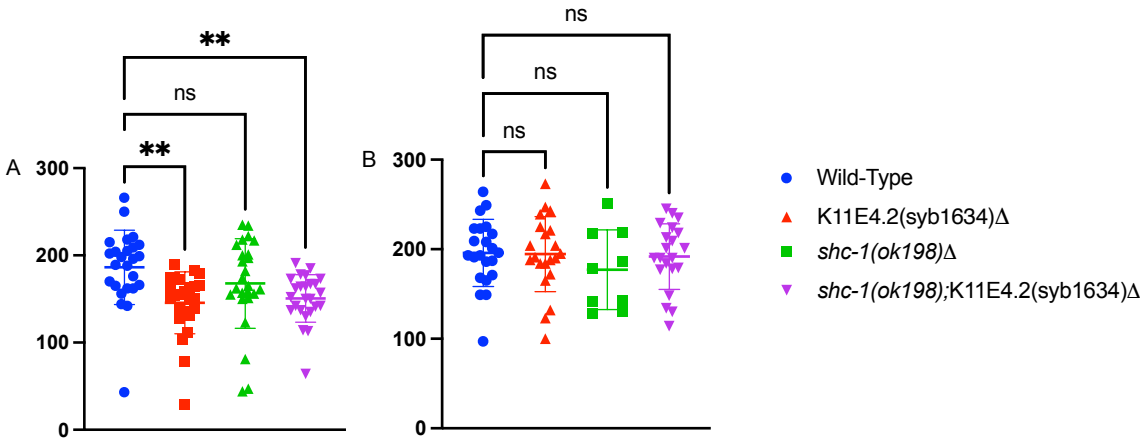

Figure A.2. 4: Fertility is not affected in K11E4.2 mutants. All strains were scored for number of progeny for three consecutive days. Each point represents the total number of offspring for an individual animal. **A)** K11E4.2(syb1634) and double mutants displayed reduced brood size compared to wild-type animals in a first trial (**P<0.05). For all strains: n=25. **B)** No significant differences were detected for any of the mutant strains in a second trial. For all strains: n ≥ 9. One-way ANOVA and Dunnett's multiple comparisons: Mean and standard deviation are shown.

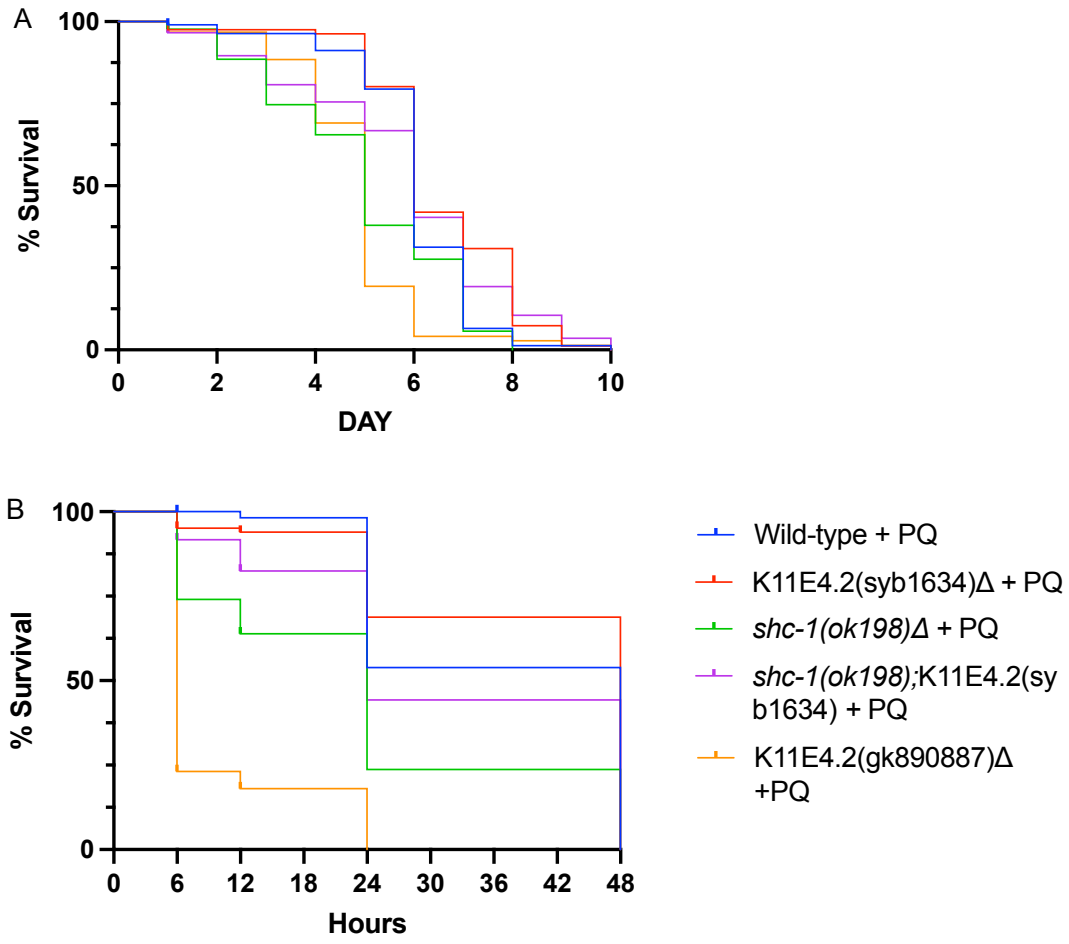

Figure A.2. 5: K11E4.2 mutants are more sensitive to chronic and acute oxidative stress in an allele-dependent manner. Upon exposure to 4mM and 100mM paraquat, K11E4.2(gk890887) mutants have a shorter lifespan compared to all strains. L4 and young adults were transferred to NGM plates added with 4mM or 100mM paraquat and scored for survival every day during the chronic assay (4mM) and after 6, 12, 24 and 48 hours during the acute assay (100mM). **A)** For all strains: n ≥ 80, Mantel Cox test: K11E4.2(syb1634) (P=ns), *shc-1(ok198)* (P=0.0004), *shc-1(ok198);*K11E4.2(syb1634) (P=ns), K11E4.2 (gk890887)(P<0. 0001) **B)** For all strains n ≥ 100, Mantel Cox test: K11E4.2(syb1634)

(P=ns), *shc-1(ok198)* (P<0. 0001), *shc-1(ok198);*K11E4.2(syb1634) (P=0.0079), K11E4.2 (gk890887)(P<0. 0001).

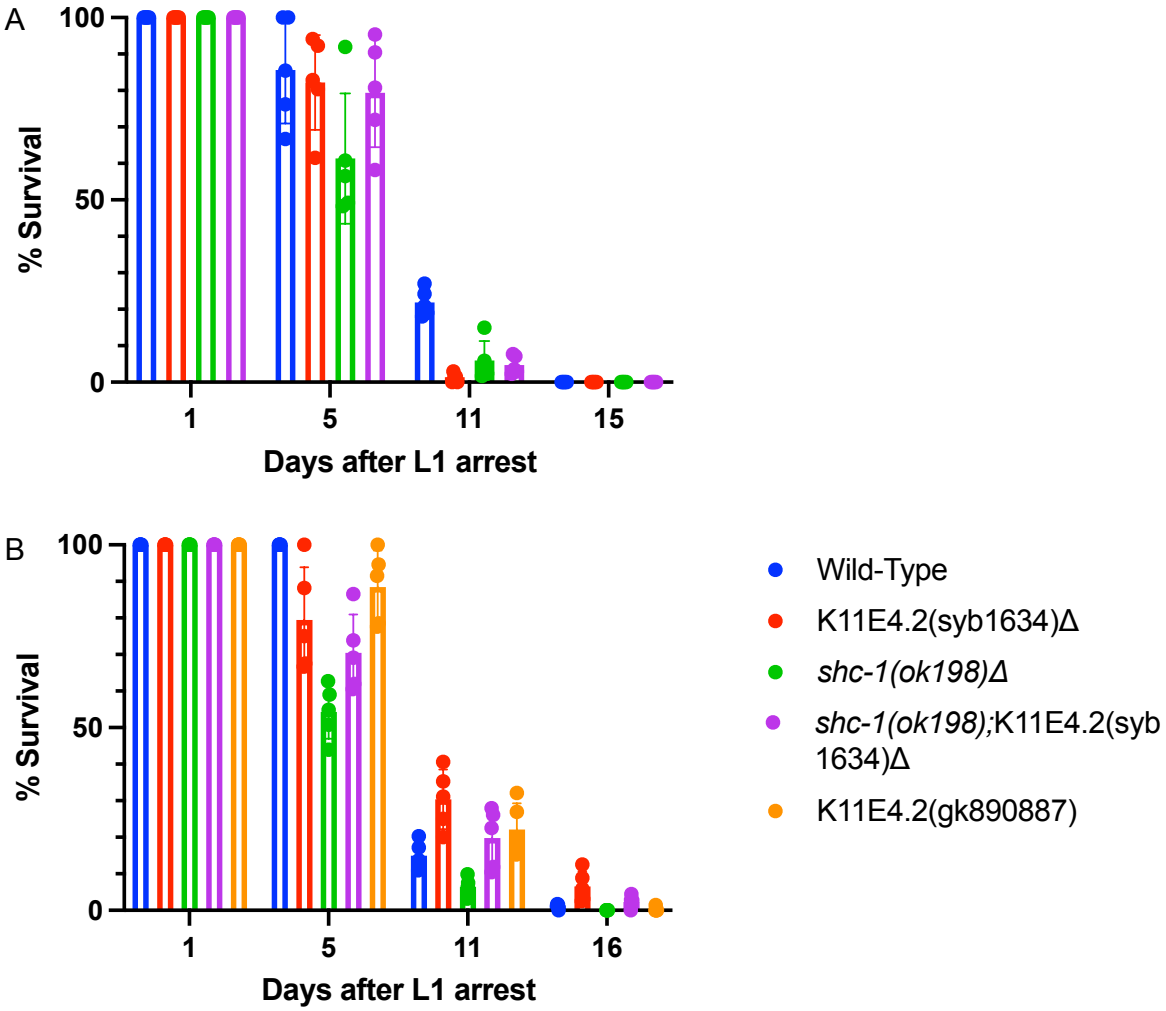

Figure A.2. 6 : K11E4.2 mutants are more sensitive to prolonged starvation than wild-type animals, but more resistant than *shc-1* mutants. Percent survival is shown over time. K11E4.2 mutants show reduced recovery from L1 arrest as compared to wild-type animals. Each time point represents the mean survival of five individual plates with at least 30 animals per plate. Each dot represents survival proportion of an individual plate. **A)**

Recovery was assessed after 1, 5, 11 and 15 days after L1 arrest. Mantel Cox test: K11E4.2 (syb1634) (P=0.0003), *shc-1(ok198)* (P<0.0001), *shc-1(ok198);*K11E4.2(syb1634) (P=0.0006) **B)** Recovery was assessed after 1, 5, 11 and 16 days after L1 arrest. Mantel Cox test: K11E4.2 (syb1634) (P=ns), *shc-1(ok198)* (P<0.0001), *shc-1(ok198);*K11E4.2(syb1634) (P=0.0177) K11E4.2 (sgk890887) (P=ns).

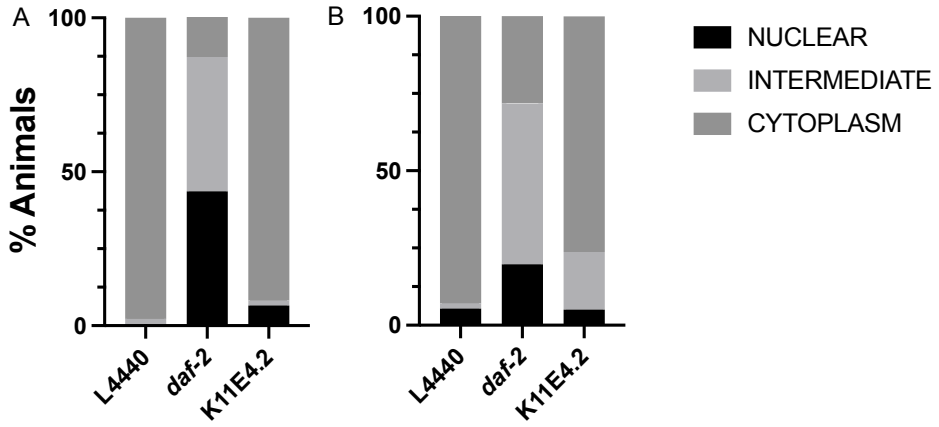

Figure A.2. 7: K11E4.2 RNAi treated worms show significantly less cytoplasmic DAF-16 intracellular localization than animals fed with the empty vector. Animals treated with the empty vector show predominantly cytoplasmic DAF-16 localization. *daf-2* knockdown induces nuclear and intermediate localization of DAF-16. K11E4.2 RNAi treated animals show cytoplasmic and intermediate DAF-16 localization. **A)** Empty vector (n=46), daf-2 (n=55) K11E4.2 (n=61). Two-way ANOVA, Cytoplasmic localization compared to the empty vector: K11E4.2 (P=ns*), daf-2* (P=0.0211). **B)** Empty vector (n=56), daf-2 (n=71) K11E4.2 (n=59). Two-way ANOVA, Cytoplasmic localization compared to the empty vector: K11E4.2 (P=ns), *daf-2* (P=0.0358).