Investigating the Role of the Small Heat Shock Protein, HSP-12.6, in Longevity in *Caenorhabditis elegans*

by

Laura Fay Ramsay

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Name: **Laura Fay Ramsay**

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Examining Committee:

Chair: **Dr. William Davidson**, Professor

Dr. Eve G. Stringham
Co-Senior Supervisor
Professor

Dr. Michel R. Leroux
Co-Senior Supervisor
Professor

Dr. David L. Baillie
Supervisor
Professor

Dr. Harald Hutter
Internal Examiner
Professor
Biological Sciences

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Abstract

The insulin-like/IGF-1 signaling (IIS) pathway is evolutionarily conserved from yeast to humans (Barbieri et al., 2003). Mutations in the insulin receptor homologue in worms, *daf-2*, extend lifespan in *Caenorhabditis elegans* and this phenotype is dependent on the activity of the forkhead transcription factor, DAF-16/FOXO (*f*orkhead b*ox*, subgroup *O*) (Murphy et al., 2003). The small heat shock protein (smHSP) HSP-12.6 is a target of DAF-16/FOXO and is implicated to influence lifespan (Murphy et al., 2003). To monitor HSP-12.6 expression in vivo, a transgenic strain carrying a translation fusion of *phsp-12.6::HSP-12.6::DSRED2* was constructed. Using this transgenic mutant, longevity assays were performed and we found that overexpression of HSP-12.6 extends lifespan of the animal while reduction of function of *hsp-12.6* by RNAi decreases lifespan. Longevity assay data also suggest that HSP-12.6 requires the transcription factor DAF-16 but not necessarily HSF-1 for its function.

Keywords: *Caenorhabditis elegans*; heat shock proteins; insulin; transgenics; ageing; HSP-12.6

This work is dedicated to the memory of

Leone Audrey Ramsay

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List of Acronyms

AAP-1	*C. elegans* P13K homologue, part of complex with AGE-1
AFX	*a*cute-lymphocytic-leukaemia-1 *f*used gene from chromosome *X*
AKT	serine/threonine PI3K homologues
AGE-1	*C. elegans* PI3K homologue
aip-1	*a*rsenite *i*nducible *p*rotein-1
A. caninum	*Ancylostome caninum*
A. ceylanicum	*Ancylostome ceylanicum*
B. malayi	*Brugia malayi*
BmHSP-12.6	*Brugia malayi* HSP-12.6
C. elegans	*Caenorhabditis elegans*
cbl	*c*asitas *B*-lineage *l*ymphoma
chico	*Drosophila* IRS
CS	citrate synthase
daf-2	abnormal *da*uer *f*ormation-2
daf-16	*da*uer de*f*ective, *C. elegans* FOXO homologue
DAF-18	*C. elegans* PTEN homologue
DBD	DNA binding domain
dilp	*D*rosophila *i*nsulin-*l*ike *p*eptides
Drosophila	*Drosophila melanogaster*
eIF4e	eukaryotic initiation factor 4E
FKHR	*f*or*kh*ead in *r*habdomyosarcoma
FKHRL1	FKHR-like 1
FOXO	*f*orkhead b*ox*, subgroup *O*
FTF	forkhead transcription factor
FUDR	5-fluoro-2'deoxyuridine
GFP	green fluorescent protein
HS	heat shock
huIL10	human IL-10
huIL10R	human interleukin-10 receptor
HR-A/B	heptad repeat A/B

HSE	heat shock element
hsf-1	heat shock factor-1
HSP	heat shock protein
hsp-12.1	heat shock protein-12.1
hsp-12.2	heat shock protein-12.2
hsp-12.3	heat shock protein-12.3
hsp-12.6	heat shock protein-12.6
hsp-16.1	heat shock protein-16.1
hsp-16.2	heat shock protein-16.2
hsp-16.11	heat shock protein-16.11
hsp-16.48	heat shock protein-16.48
hsp-16.49	heat shock protein-16.49
hsp-70	heat shock protein-70
IGF	insulin/insulin-like growth factor
IGF-1	insulin/insulin-like growth factor-1
IGF-2	insulin/insulin-like growth factor-2
IGF-1R	insulin/insulin-like growth factor-1 receptor
IIS	insulin-like/IGF-1 signaling
INR	Insulin-like receptor (in *Drosophila*)
INS	Insulin-like
ins-1	insulin-like gene 1
IR	insulin receptor
IRa	insulin receptor a-isoform
IRa::IGF-1R	insulin receptor a-isoform hybrid with IGF-1R
IRb	insulin receptor b-isoform
IRb::IGF-1R	insulin receptor b-isoform hybrid with IGF-1R
IRS	insulin receptor substrate
IST-1	insulin receptor substrate-1
JNK-1	c-*J*un *N* terminal *k*inase
lnk	*Drosophila* IRS
MNC	median neurosecretory cell
mtl-1	metallothionein gene
N. brasiliensis	*Nippostrongylus brasiliensis*

PDK	phosphoinositide-dependent kinase
PH	pleckstrin homology
PI3K	phosphoinositide 3-kinase or AGE-1 in worms
PIP2	phosphatidylinositol (4,5)-biphosphate
PIP3	phosphatidylinositol (1,4,5)-triphosphate
PK	protein kinase
PKA	protein kinase A
PKB	protein kinase B, resembles properties of PKA and PKC
PKC	protein kinase C
poly-Q	polyglutamine-repeat protein aggregates
PTB	phosphotyrosine-binding
PTEN	phosphatase and tensin homologue deleted on chromosome 10
RNAi	RNA interference
RT-PCR	reverse transcription polymerase chain reaction
S. cerevisiae	*Saccharomyces cerevisiae*
SAGE	serial analysis of gene expression
SGK-1	*s*erum and *g*lucocorticoid inducible *k*inase-1
SH2	*S*rc-*H*omology *2*
sip-1	*s*tress *i*nduced *p*rotein
smHSP	small heat shock protein
sod-3	*s*uper-*o*xide *d*ismutase gene
swPCR	single worm PCR
UPR^{mt}	unfolded protein response in mitochondria
4E-BP	4E-binding protein

1. Introduction

The regulation of ageing has been studied for many years, but it was not until recently that breakthroughs into the genetics of ageing have been uncovered. There are many ageing theories that attempt to describe and uncover why and how organisms age and as new discoveries in molecular biology occur, new theories of ageing are produced and older theories are revised (Medvedev, 1990). An early record of evolutionary theory is attributed to August Weismann who argued that ageing evolved to the advantage of the species and not the individual, in that older members die of old age so that competition for food and resources with younger members would lessen (Weissmann, 1882). Moreover, Weissman proposed that somatic cells undergo a finite number of cell divisions and this number is determined in the embryonic cell (Weismann, 1882). Hayflick and Moorhead experimentally confirmed this hypothesis, showing that human fibroblast cells undergo 50 cell divisions and then they 'age' and 'die' (Hayflick and Moorhead, 1961). Many other laboratories have confirmed this same process in other cell types and it is now known as the 'Hayflick limit' (Hayflick, 1965). General thoughts on ageing are that the force of natural selection declines with age (Kirkwood, 2002). Evolutionary theory argues against programmed ageing; that longevity comes at the cost of investments in growth, reproduction and activities that might enhance fitness (Kirkwood, 2002). Another similar theory of ageing is that it is a result of wear and tear on the body and not developmentally or genetically regulated (Medvedev, 1990). However, it is now known that distinct signaling pathways exist and directly contribute to ageing in both invertebrates and vertebrates (Kenyon, 2011; Partridge, 2011; Ziv and Hu, 2011). The first pathway to be identified to directly affect longevity was the insulin/insulin-like growth factor-1 (IGF-1) signaling (IIS) pathway and it was first identified in the free-living soil nematode, *Caenorhabditis elegans* (*C. elegans*) (Kenyon et al., 1993; Dorman et al., 1995; Kimura et al., 1997; Lin et al., 1997). By studying this pathway and the genes that affect ageing, the process by which ageing is genetically regulated can be further uncovered. Insights into how and why species age could lead to possible treatments to both extend lifespan and the quality of life of the elderly.

1.1. *C. elegans* as a Powerful Model Organism

C. elegans has been used as a model organism since it was developed by Sydney Brenner in the 1970's (Brenner, 1974). *C. elegans* is a powerful model system for studying ageing and age-related diseases as it shares similar physiological ageing characteristics and similar pathological processes at the molecular level to humans (Morcos and Hutter, 2009). It also shares conserved ageing pathways with mammals and humans but is easier to manipulate (Kaletsky and Murphy, 2010). In contrast to mammals, *C. elegans* has a relatively short life cycle of approximately 2-3 weeks and there are many genetic resources available to identify genome-wide changes associated with disease and ageing. These include: hundreds of mutant strains, gene deletion strains which are being generated for each of the ~19,000 genes, over 2000 *promoter::gfp* (*g*reen *f*luorescent *p*rotein) strains in order to characterize cellular and sub-cellular gene expression patterns and full-genome RNA interference (RNAi) libraries (Kaletsky and Murphy, 2010). While the worm is a self-fertilizing hermaphrodite, male strains can be generated and they make genetic crosses fairly easy to perform (Kaletsky and Murphy, 2010). In addition, *C. elegans* is relatively inexpensive and simple to care for and propagate, the genome has been sequenced and the 959 cells of invariable lineage has been mapped. Finally, the clear cuticle allows visualization of the cells and manipulation of the worm, for example, the injection of recombinant DNA or RNA into the animal (Kaletsky and Murphy, 2010). Ageing can be studied directly in *C. elegans* by performing lifespan assays using mutant strains or by RNAi that knocks down gene expression by simply feeding the worms with bacteria expressing the desired double stranded RNAs (Kaletsky and Murphy, 2010).

1.2. Normal Lifespan and Dauer Diapause of *C. elegans*

Under conditions of low food, elevated temperature, or crowding, *C. elegans* can regulate lifespan by entering into a dauer phase, where the animal arrests development as a reproductively immature dauer larva (Figure 1; Golden and Riddle, 1984; Klass and Hirsh, 1976). Once food is re-introduced, the worm re-enters the life cycle to develop normally and this is dependent on the activity of the insulin receptor (IR) homologue in

C. elegans, *daf-2* (abnormal *da*uer *f*ormation-2) and the IIS pathway (Hsin and Kenyon, 1999).

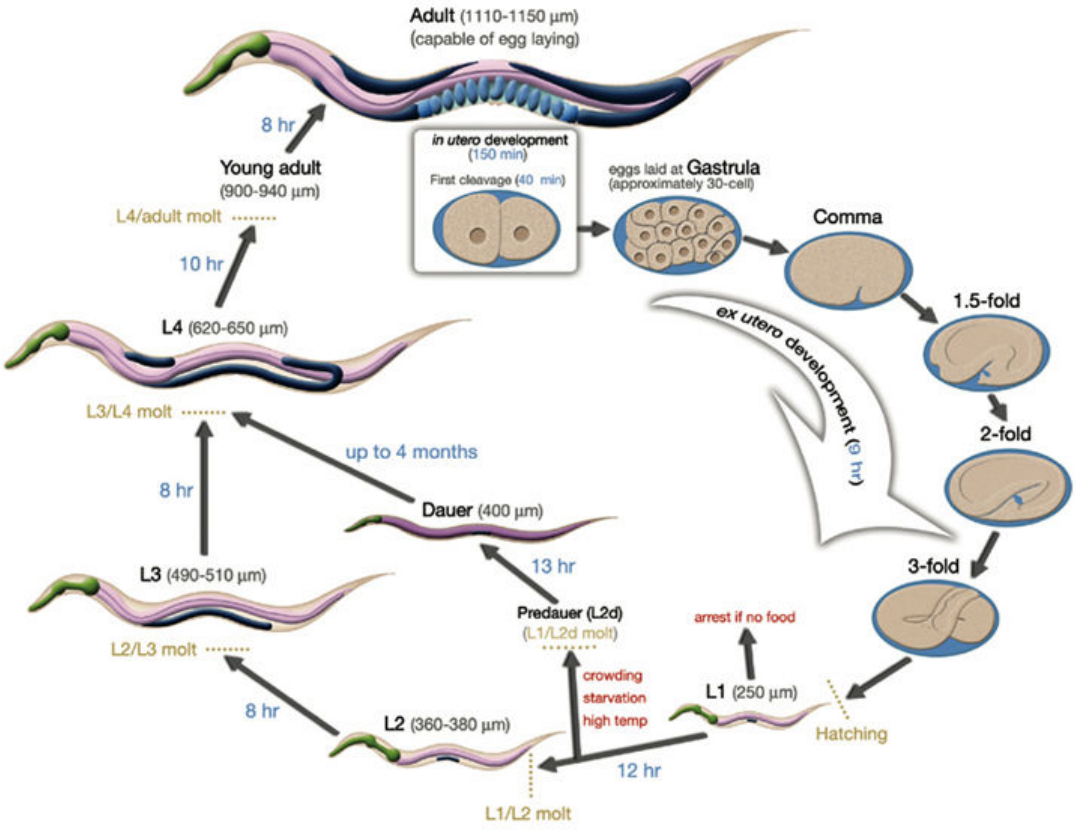


Figure 1 Normal Life Cycle and Dauer Diapause of C. elegans

C. elegans normal lifecycle is usually 2-3 weeks and consists of four larval stages. Each hermaphrodite has ~200 progeny. Under crowded, starved or high temperature conditions, the animals can enter into a developmentally arrested dauer stage where they can live for many months. Upon re-introduction of food, decreased temperature or lessened crowding, the worm can re-enter the lifecycle at the L4 stage and produce progeny. Figure from wormatlas.org

1.3. The Insulin/Insulin-like Growth Factor-1 (IGF-1) Signaling (IIS) Pathway

The IIS pathway has diverse functions which include: regulation of metabolism (Saltiel and Kahn, 2001), dauer diapause (Vowels and Thomas, 1992; Dorman et al., 1995; Larsen et al., 1995), growth (Butler and Le Roith, 2001; Brogiolo et al., 2001), ageing (Clancy et al., 2001; Kenyon, 2011), development (Kimura et al., 1997), reproduction (Tatar et al., 2003), immunity (Singh and Aballay, 2006) and stress response (Morley and Morimoto, 2004). As a result of the many roles of the IIS pathway, gene alterations result in many diseases such as diabetes mellitus (Nandi et al., 2004) and certain forms of cancer (Massoner et al., 2010). While a disruption in genes of the IIS pathway results in disease in humans, single gene mutations that lower IIS result in extension of lifespan in the yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) (Barbieri et al., 2003), the nematode *C. elegans* (Kimura et al., 1997), the fruit fly *Drosophila melanogaster* (*Drosophila*) (Clancy et al., 2001) and the mouse *mus musculus* (Selman et al., 2008; Selman et al., 2011), suggesting an evolutionary conservation between species (Figure 2; Piper et al., 2008).

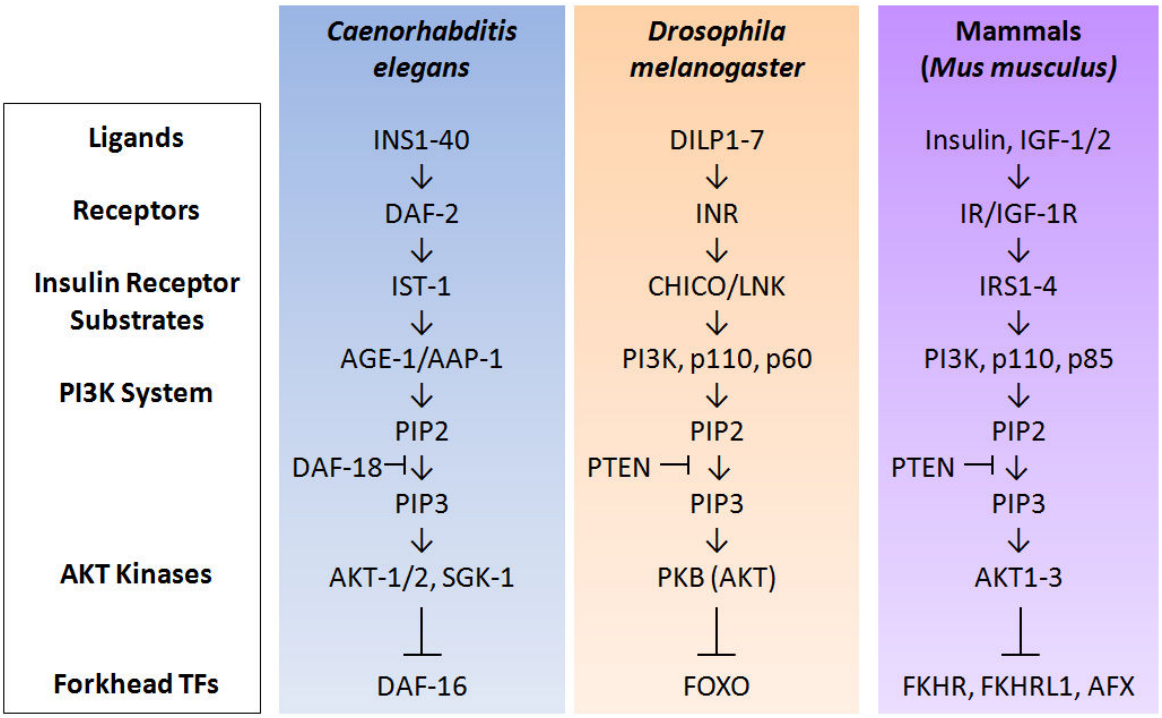


Figure 2 The Core Components of the IIS Pathway of C. elegans, Drosophila and Mammals are Conserved

Conservation of many of the core components of the IIS pathway is shown at the various levels of the signaling cascade from the extracellular ligands to the forkhead transcription factors (FTFs) which can translocate to the nucleus. Positive interactions are depicted with arrows while negative interactions are indicated with lines. Figure adapted from Garofalo, 2002.

The IIS pathway signaling cascade components in worms, flies and mice include: ligands, receptors, insulin receptor substrates, the PI3-kinase system, AKT kinases and FTFs (Figure 3). *C. elegans* and *Drosophila* both contain a single insulin-like receptor (DAF-2 in *C. elegans* and INR in *Drosophila*) and a single forkhead transcription factor (DAF-16 in *C. elegans* and FOXO in *Drosophila*), while mammals exhibit multiple genes of many components of the IIS pathway (Figure 3; Broughton and Partridge, 2009).

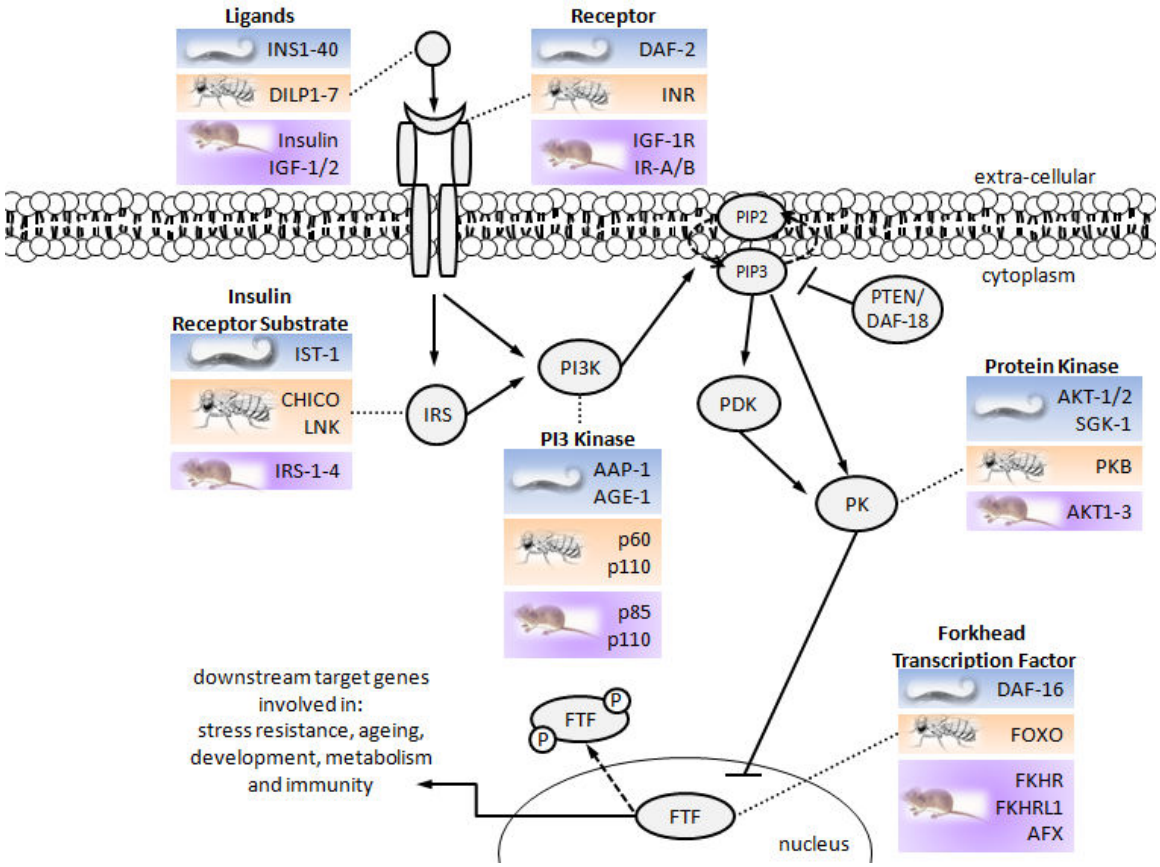


Figure 3 The Insulin/Insulin-like Signaling Pathway in Worms, Flies and Mice

The conserved IIS pathway components in *C. elegans*, *Drosophila* and mammals. Proteins of the IIS pathway are shaded in grey with arrows indicating positive interactions and lines conveying negative regulation. Homologues for each component of the IIS pathway can be found attached to the protein by dashed lines and are colour-coded for each organism: blue, pink and purple for worms, flies and mice, respectively. Figure adapted from Broughton and Partridge, 2009.

1.3.1. The Insulin/Insulin-like Ligands

Mammals have three ligands that activate the IIS pathway: one insulin and two insulin-like growth factor (IGF) ligands, IGF-1 and IGF-2 (Broughton and Partridge, 2009). Insulin is produced in the β-cells of the pancreas and is secreted into the circulatory system in response to elevated blood glucose levels (Myers and White, 1996). Insulin binds to and activates the insulin receptor (IR) on the cell surface in various tissues, where it promotes glucose influx and metabolism in muscle cells and adipocytes while suppressing gluconeogenesis in the liver (Myers and White, 1996; White and Kahn, 1994). The mature form of insulin results from processing and begins as a single polypeptide, preproinsulin, which contains a signal peptide at the N-terminus that targets it to the endoplasmic reticulum, where it is cleaved into three units (Chan et al., 1976). Proinsulin is the resulting protein and contains two peptide chains (A and B) joined by a C-peptide (Bell et al., 1980). Mature insulin results from cleavage of the C-chain and disulphide linkage of the A and B chains (Bell et al., 1980). IGF-1/2 are mainly produced by the liver with a small amount produced in the brain and function to regulate organismal growth in response to growth hormones (de la Monte and Wands, 2005). IGF-1/2 contain approximately 50% amino acid similarity to insulin, however, IGF-1/2 retain the C-chain that is cleaved from insulin during processing (de la Monte and Wands, 2005). Insulin and IGF-1/2 can bind and activate the IR and the insulin-like growth factor-1 receptor (IGF-1R) or hybrids of the receptors (Taguchi and White, 2008).

The *Drosophila* genome was searched for genes encoding insulin-like peptides and seven insulin-like peptides were identified (Brogiolo et al., 2001). *Dilp1-7* for *Drosophila i*nsulin-*l*ike *p*eptides, are structurally similar to preproinsulin and contain a signal peptide, an A chain, a B chain and a C peptide (Brogiolo et al., 2001). It is suggested that the peptides are more similar to insulin rather than IGF-1 and IGF-2 due to consensus cleavage sites between the A and B chains (Brogiolo et al., 2001). DILP2 is 35% identical to mature insulin and has been genetically shown to interact with *Inr*, the *Drosophila* insulin receptor (Brogiolo et al., 2001). DILPs1-6 are cleaved like mammalian insulin and all of the 7 DILPs can act redundantly and are evolutionarily conserved (Grönke et al., 2010). Partridge's lab constructed null mutants for all seven DILPs and found that loss of DILP2 increased lifespan and loss of DILP6 reduced growth and DILP3 is required for normal expression of DILPs 2 and 5 in brain neurosecretory cells

(Grönke et al., 2010). In addition, DILP6 expression in fat body compensated for the loss of median neurosecretory cell (MNC) DILP2, 3 and 5, suggesting a complex feedback system between peripheral fat tissues and the central nervous system (Grönke et al., 2010).

To date, approximately 40 insulin-like (INS) molecules have been identified in *C. elegans*, the functions of which are widely unknown. However, it is thought that the *ins* genes act as either agonists or antagonists of *daf-2* in a number of tissues throughout the worm to regulate ageing (Panowski and Dillin, 2009; Kaletsky and Murphy, 2010). Fourteen of the *ins* genes are expressed in amphid sensory neurons, as revealed by GFP fusion experiments, with *ins-1* also expressed in intestine and other *ins* genes expressed in the pharynx, vulval muscle, vulva and hypodermis (Pierce et al., 2001). Insulin-like gene 1 (*ins-1*) is the most similar to human insulin in terms of primary sequence similarity and structural homology and is one of two INS proteins that, like human insulin, contains a cleavable C peptide (Panowski and Dillin, 2009). Both *ins-1* and *ins-18* act as antagonists of *daf-2*, as overexpression of either *ins-1* or ins-18 decrease *daf-2* signaling and cause arrest at the dauer stage and increased lifespan in wild-type worms (Pierce et al., 2001).

Some agonists of *daf-2* include *daf-28* and *ins*-7. *Daf-28* dominant-negative mutant allele worms show increased DAF-16 nuclear localization, extended lifespan and tendency for dauer arrest (Malone et al., 1996). Knockdown of *ins-7* by RNAi resulted in extended lifespan and increased DAF-16 nuclear localization (Murphy et al., 2003; McElwee et al., 2003; Murphy et al., 2007). *Ins-7* likely acts in a positive feedback loop that increases DAF-16 activity in the intestine to inhibit *ins-7* expression and thus lower *daf-2*/insulin signaling throughout the entire organism (Panowski and Dillin, 2009; Murphy et al., 2003). Reduced DAF-16 activity results in increased INS-7 levels whereas overexpression of DAF-16 in the intestine increases DAF-16 nuclear localization in other tissues by inhibiting *ins-7* expression (Murphy et al., 2007).

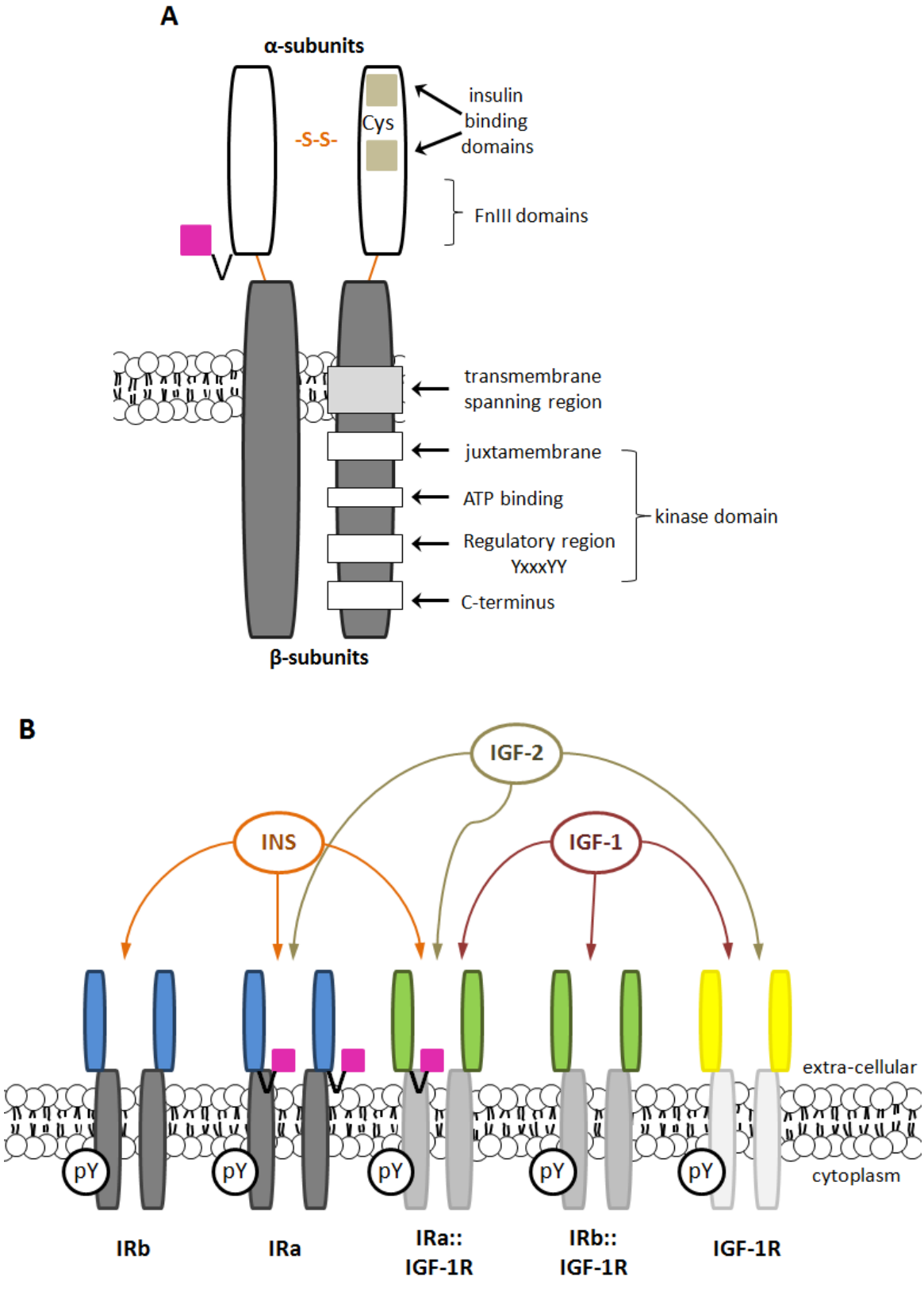

A
α-subunits
-S-S-
Cys
insulin
binding
domains
FnIII domains
transmembrane
spanning region
juxtamembrane
ATP binding
Regulatory region
YxxxYY
kinase domain
C-terminus
β-subunits
B
IGF-2
INS
IGF-1
pY
extra-cellular
cytoplasm
IRb
IRa
IRa::
IGF-1R
IRb::
IGF-1R
IGF-1R

Figure 4 Insulin Receptor Structural Features and Isoforms

Structural features of the mammalian insulin receptor the *C. elegans* IR homologue DAF-2 and the five mammalian insulin receptor isoforms: IRb, IRa, IRa::IGF-1R, IRb::IGF-1R and IGF-1R. A) The mammalian insulin receptor is composed of two α-subunits and two transmembrane β-subunits linked together by disulfide bonds (orange lines). FnIII are fibronectin type III domains. B) The five mammalian insulin receptor isoforms and the ligands that preferentially bind to them. IRa retains exon 11 (pink box) while IRb omits it. pY represents phosphotyrosine. A is adapted from Myers and White, 1996. B is adapted from Taguchi and White, 2008.

1.3.2. The Insulin Receptor

Insulin principally binds to and activates a heterotetrameric, cell-surface receptor named the insulin receptor (Figure 4A; White and Kahn, 1994) while IGF-1 and IGF-2 mainly activate the IGF-1R (Ullrich et al., 1986). There are two different isoforms of the mammalian insulin receptor: IRa and IRb and two hybrid receptors of IR and IGF-1R: IRa::IGF-1R and IRb::IGF-1R (Figure 4C; White and Kahn, 1994). The insulin receptor is composed of two α-subunits that are linked to each other and to a β-subunit by disulfide bonds (Figure 4A; White and Kahn, 1994). The IR results from a proreceptor that is cleaved during translation to be composed of an α and a β-subunit that are linked together by disulfide bonds and assemble as heterotetramers with another cleaved α and a β-subunit (Figure 4A; White, 2003). The IR is present in virtually all vertebrate tissues with varying concentrations from around 40 receptors on circulating erythrocytes to more than 200,000 receptors on adipocytes and hepatocytes (White and Kahn, 1994). Insulin binds to the cysteine rich extracellular α-subunits to activate the tyrosine kinase domains on the intracellular portion of the transmembrane β-subunits (Figure 4A; White, 2003; Taguchi and White, 2008). The IR gene contains 22 exons and the two isoforms of the IR are generated by alternative splicing of exon-11: IRa retains exon-11 while IRb omits exon-11 (Figure 4B; Taguchi and White, 2008). Between the IR and IGF-1R, there exist 84% amino acid sequence identity in the β-subunits and 48% amino acid sequence similarity in the α-subunits (Ullrich et al., 1986). Once activated, the receptors activate a cascade of intracellular proteins to regulate gene expression, protein synthesis, cell proliferation or death and glucose and lipid metabolism (Boucher et al., 2010).

In humans, patients with the syndrome leprechaunism contain mutations in one or both alleles of the IR gene and are characterized by severe growth retardation, insulin resistance, subcutaneous lipoatrophy, acanthosis nigricans, hirsutism and paradoxical fasting hypoglycaemia (Takahashi et al., 1997). A patient born of parents who are cousins who are heterozygous for the IR mutation and have type A insulin resistance, showed co-existence of both leprechaunism and type A insulin resistance syndrome and a marked decrease in tyrosine kinase activity of the insulin receptor (Takahashi et al., 1997). This patient is reported to have a homozygous missense mutation at position 1092 (glutamine→arginine) in the tyrosine kinase domain of the insulin receptor gene. Interestingly, another single amino acid substitution in the tyrosine kinase domain of the

Drosophila IR has resulted in severe growth retardation (Brogiolo et al., 2001). It is hypothesized that the patients are viable due to some compensatory effects of IGF-1 signaling which is known to have some insulin action in intact cells and through the IGF-1R, can phosphorylate downstream insulin receptor substrate IRS-1 and subsequently transduce insulin signaling (Takahashi et al., 1997). Another hypothesis is that perhaps residual tyrosine kinase activity was present during fetal development, as IR function was not completely abolished (Takahashi et al., 1997).

While mammalian systems have IRa, IRb, IGF-1R and their hybrids (Figure 4B), *Drosophila* and *C. elegans* only contain one IR (Figure 3). The *Drosophila* homologue of the mammalian insulin receptor gene is *inr* and loss of function mutations in this gene lead to embryonic lethality (Fernandez et al., 1995) while varying *inr* activity during *Drosophila* development regulates organ size by altering cell number and size in a cell-autonomous manner (Brogiolo et al., 2001). The structure of the *Drosophila inr* is similar to both the mammalian IR and the IGF-1R (Fernandez et al., 1995), having two α- and two β-subunits and a cytoplasmic tyrosine kinase in the β-subunit that was activated upon insulin binding (Fernandez-Almonacid and Rosen, 1987). A difference between *Drosophila* and mammalian systems is that the proreceptor of *Drosophila* INR is processed differently than the mammalian insulin or IGF-1 receptor precursors (Fernandez et al., 1995). The *Drosophila* INR proreceptor is processed proteolytically to generate an insulin-binding α-subunit and a β-subunit that contains a novel 60kDa (~400 amino acids) extension in the C-terminal side of the tyrosine kinase domain that contains multiple potential tyrosine autophosphorylation sites (Fernandez et al., 1995). Binding sites for downstream components similar to IRS are found in the C-terminal extension (Yenush et al., 1996) and when phosphorylated, the C-terminal domain can bind IRS-1 (Marin-Hincapie and Garofalo, 1999; Poltilove et al., 2000) and PI3K (Yenush et al., 1996).

Daf-2 encodes the *C. elegans* insulin receptor and regulates both longevity and dauer diapause (Kimura et al., 1997). When *daf-2* function is reduced, the lifespan of the animal more than doubles compared to wild-type and remarkably, is not only long-lived, but remains healthy and viable for longer periods of time and has increased stress resistance (Kenyon et al., 1993; Kimura et al., 1997). *Daf-2* is required for growth into adulthood, as animals with a complete loss of *daf-2* do not survive to adulthood (Kenyon,

2011). The *daf-2* gene has been known to influence dauer formation since the early 1980's and the ability of *daf-2* mutants to become dauer was shown to require the *daf-16* gene (Riddle et al., 1981).The partial loss of function mutation, *daf-2(e1370)*, is in a constant state of dauer which is termed dauer constitutive (Kenyon et al., 1993). *Daf-2* is the only insulin-like/IGF-1 transmembrane receptor in worms and it signals a highly conserved phosphatidylinositol 3-kinase (PI 3-kinase)/AGE-1 pathway (Figure 3; Lin et al., 2001).

1.3.3. Insulin Receptor Substrates

In mammals, activation of the insulin receptor causes tyrosine phosphorylation of the IRS proteins which propagates the insulin signal. There are four IRS family members, IRS1-4 (Lavan et al., 1997). IRS proteins contain a pleckstrin homology (PH) domain adjacent to a phosphotyrosine-binding (PTB) domain in the N-terminus and numerous tyrosine phosphorylation sites in the C-terminus (Lavan et al., 1997; White, 2003). The PH domain couples IRS proteins to the activated receptors, the PTB domain binds directly to the phosphorylated NPXY-motif in activated receptors and the C-terminus tyrosine phosphorylation sites bind to PI3K (White, 2003). IRS proteins function as docking proteins for Src-Homology 2 (SH2) domain containing proteins such as PI3K (Lavan et al., 1997; White, 2003). Global deletion of the insulin receptor substrate, *Irs1*$^{-/-}$, in mice, significantly extends lifespan in both female and male mice (Selman et al., 2008; Selman et al., 2011).

Drosophila has two IRS homologues, *chico* and *lnk* (Clancy et al., 2001; Werz et al., 2009). Mutation of *chico* in *Drosophila* extends lifespan by 48% in homozygotes and 36% in heterozygotes (Clancy et al., 2001). Chico, Spanish for "small boy", was identified in a screen for mutations that cause a reduction in body size (Böhni et al., 1999). Genetic evidence suggested that the *Drosophila* IR can signal in the absence of c*hico* (Böhni et al., 1999) and *lnk* was predicted to exist during *chico* analysis (Poltilove et al., 2000). Further work confirmed the presence of *lnk*, and like *chico*, contained a PH domain, an SH2 binding motif and unlike *chico*, a highly conserved C-terminal *Cbl* (*c*asitas *B*-lineage *l*ymphoma) recognition motif (Werz et al., 2009). Also unlike *chico*, *lnk* does not contain the YXXM consensus binding site to interact with PI3K (Werz et al., 2009). *Chico* and *lnk* double mutants were lethal (Werz et al., 2009).

There is one putative *C. elegans* IRS homologue, IST-1 (*i*nsulin receptor *s*ubs*t*rate-1), which contains a PH and PTB domain and potential docking site for SH2 domain-containing proteins, such as PI3K (Wolkow et al., 2002). Experiments using knockdown of *ist*-1 by RNAi reveal that IST-1 can potentiate a *daf-2* signal but it is not essential for signaling under normal conditions and may be functioning in a parallel pathway downstream of *daf-2* (Wolkow et al., 2002).

1.3.4. PI3K System and AKT/PKB Kinases

The IIS is transduced either directly (in worms and flies) or indirectly (in worms, flies and mice) from the receptor via the IRS to phosphorylate phosphoinositol 3-kinase (PI3K/p110) and its adaptor subunit p85 (p60 in *Drosophila*) (Figure 3; Broughton and Partridge, 2009). Once activated, PI3K is recruited to the cell membrane where it converts PIP_2 (phosphatidylinositol (4,5)-biphosphate) into the second messenger PIP_3 (phosphatidylinositol (1,4,5)-triphosphate) (Figure 3; Broughton and Partridge, 2009). This activity is antagonized by the tumour suppressor PTEN (phosphatase and tensin homologue deleted on chromosome 10), which dephosphorylates the position 3 of the inositol ring of PIP_3 to be converted to PIP_2 (Maehama and Dixon, 1998).

The serine-threonine kinase Akt/PKB was first identified with properties resembling both PKA and PKC and as an oncogene (Staal et al., 1987). There exist three mammalian isoforms of PKB: Akt1-3 which are similar to one another in amino acid identity but show broad tissue expression and have both overlapping but also distinct functions (Garofalo et al., 2003).

In *C. elegans*, *daf-2* signals through PI3K that consists of a p55-like subunit, AAP-1 (Wolkow et al., 2002) and a p110 catalytic subunit, AGE-1 (Morris et al., 1996). The AAP-1/AGE-1 complex phosphorylates PIP_2 into PIP_3 and is antagonized by DAF-18, the *C. elegans* homologue of PTEN (Figure 3; Ogg and Ruvkun, 1998; Larsen et al., 1995; Suzuki and Han, 2006). PIP_3 then activates PDK-1 (phosphoinositide-dependent kinase), the homologue of mammalian AKT-2, which phosphorylates AKT-1, AKT-2 and SGK-1 (serum and glucocorticoid inducible kinase-1) (Hamilton et al., 2005). SGK-1 is involved with development, stress response and longevity (Hamilton et al., 2005). The two serine-threonine Akt/PKB (protein kinase B) homologues, AKT-1 and AKT-2,

primarily function to target and activate the DAF-16 transcription factor and are involved in dauer formation (Paradis and Ruvkun, 1998). Mutations of *daf-2* and *age-1* result in extended lifespan (Kenyon et al., 1993; Friedman and Johnson, 1988). Lifespan extension of *daf-2* mutants requires the transcription factors DAF-16 and HSF-1 (Hsu et al., 2003; Lin et al., 2001; Ogg et al., 1997).

1.3.5. Forkhead Transcription Factors

In mammals, the family of three forkhead transcription factors FKHR (*F*or*kh*ead in *r*habdomyosarcoma), AFX (acute-lymphocytic-leukaemia-1 fused gene from chromosome X) and FKHRL1 (FKHR-like 1) which are termed FKHR isoforms and are also called FOXO1, FOXO3 and FOXO4, are all directly phosphorylated by AKT/ PKB in cells, thus inhibiting the FKHR isoforms from the nucleus and subsequently preventing them from regulating genes involved in growth control, apoptosis, DNA repair and oxidative stress (Woods and Rena, 2002; Birkenkamp et al., 2003).

In *Drosophila*, Akt only has one target homologue of the FOXO transcription factors, dFOXO (Puig et al., 2003; Kramer et al., 2003). Similar to mammals, upon insulin signaling, Akt phosphorylates FOXO, which inhibits FOXO nuclear translocation and also transcriptional activity (Puig et al., 2003). Under low levels of insulin signaling, FOXO translocates to the nucleus and is predicted to regulate close to 2000 genes, half of which are regulated in a tissue-specific manner (Gershman et al., 2007). Components of the translational apparatus as well as mitochondrial components are among the genes regulated by FOXO (Teleman, 2010). A specific target of dFOXO is the translational repressor, 4E-BP (4E-*b*inding *p*rotein) which binds eIF4e (*e*ukaryotic *i*nitiation *f*actor 4E) and blocks the ribosome from being recruited to the 5' end of mRNAs, thereby reducing cellular translation and elevating metabolic rates (Teleman et al., 2005).

In *C. elegans*, the *daf-2* signaling cascade through AKT-1/2 negatively regulates the main target DAF-16/FOXO, which encodes a forkhead transcription factor, that translocates to the nucleus depending on its phosphorylation level (Figure 3; Lin et al., 2001). DAF-2 signaling prevents nuclear accumulation of DAF-16::GFP (Lin et al., 2001). When all four consensus phosphorylation sites for *Akt* were mutated from serine

or threonine to alanine in DAF-16::GFP animals, strong nuclear accumulation of DAF-16 similar to nuclear accumulation in *daf-2(1370)* mutants, was seen (Lin et al., 2001).

DAF-16 activity is required to enter dauer diapause and is also required for the constitutive dauer formation and extended adult longevity of *daf-2* mutants, as mutations in *daf-16* can suppress the mutational effects of *daf-2* and *age-1* by bypassing the need for the IIS pathway and resulting in worms that are unable to enter into the dauer stage as larvae (Ogg et al., 1997; Larsen et al., 1995; Dorman et al., 1995).

DAF-16 responds to environmental cues as well as genetic. Animals carrying an integrated DAF-16::GFP transgene are longer lived, and more stress resistant than animals carrying the integration marker alone (Henderson and Johnson, 2001). In conditions rich in food, *daf-2* and *akt-1/2* prevent DAF-16::GFP from entering the nucleus while starvation, heat and oxidative stress cause rapid nuclear localization of DAF-16::GFP (Henderson and Johnson, 2001).

Just as mammalian FOXO genes have overlapping and different functions, the *C. elegans* DAF-16 has 3 isoforms: a, b, and d/f, that display overlapping, specific and cooperative functions (Partridge, 2011; Kwon et al., 2010). mRNA abundance from a downstream target gene of DAF-16, *hsp-12.6*, was measured by qRT-PCR (reverse transcription polymerase chain reaction) and it was found that the three isoforms showed overlapping regulation of this target gene (Kwon et al., 2010). Recently, it was determined that two of these DAF-16 isoforms, a and d/f are necessary for full extension of lifespan (Kwon et al., 2010).

In addition to the IIS pathway, there is a parallel c-*J*un *N* terminal *k*inase (JNK-1) signaling pathway that results in the phosphorylation of different sites of DAF-16 to usher it into the nucleus in *C. elegans* (Figure 5; Oh et al., 2005). In *C. elegans jnk-1* mutants, lifespan is decreased in a manner dependent on DAF-16, as JNK-1 promotes the translocation of DAF-16 into the nucleus in response to heat stress (Oh et al., 2005). Conversely, overexpression of *jnk-1* extends lifespan of *C. elegans* (Oh et al., 2005).

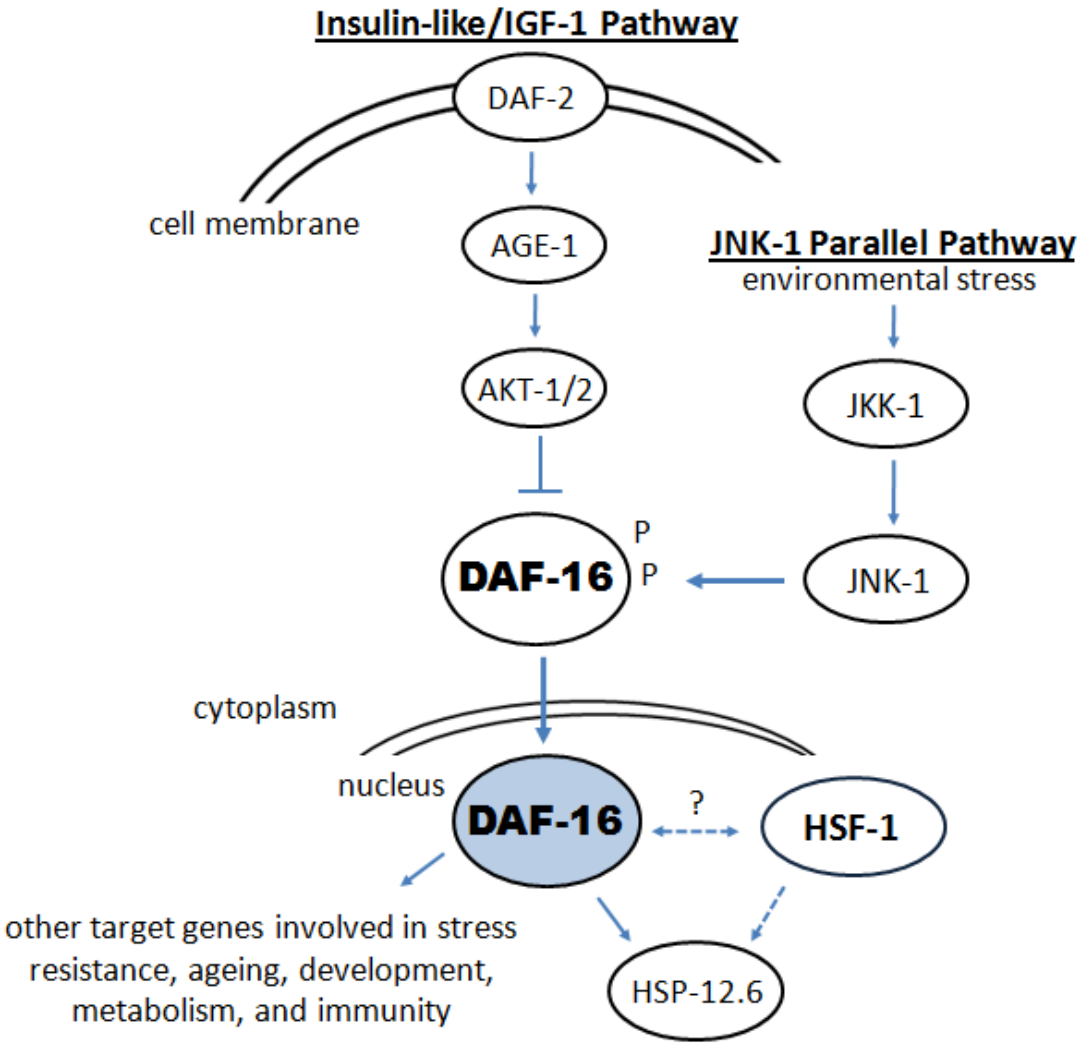


Figure 5 The JNK-1 Parallel Pathway Converges onto DAF-16

The conserved *daf-2*/IIS signaling pathway and JNK-1 parallel pathway both converge onto the transcription factor DAF-16 in *C. elegans*. At low *daf-2* levels or upon environmental stress, DAF-16 is sequestered to the nucleus, where it acts upon downstream genes that are involved in ageing, development, metabolism and stress resistance. Figure adapted from Oh et al., 2005.

1.3.5.1. Downstream Targets of DAF-16

Upon environmental stresses such as heat shock, starvation or under low insulin signaling conditions, DAF-16 is translocated to the nucleus (Figure 5; Henderson and Johnson, 2001; Lin et al., 2001). Once inside the nucleus, DAF-16 upregulates many target genes that are involved in metabolism, ageing, immunity, stress resistance and development (Lee et al., 2003; Murphy et al., 2003; McElwee et al., 2003).

Expression profiling such as serial analysis of gene expression (SAGE) and genome-wide searches such as DNA microarrays for DAF-16 binding sites in gene regulatory regions have uncovered many downstream targets of DAF-16 (Jensen et al., 2006).

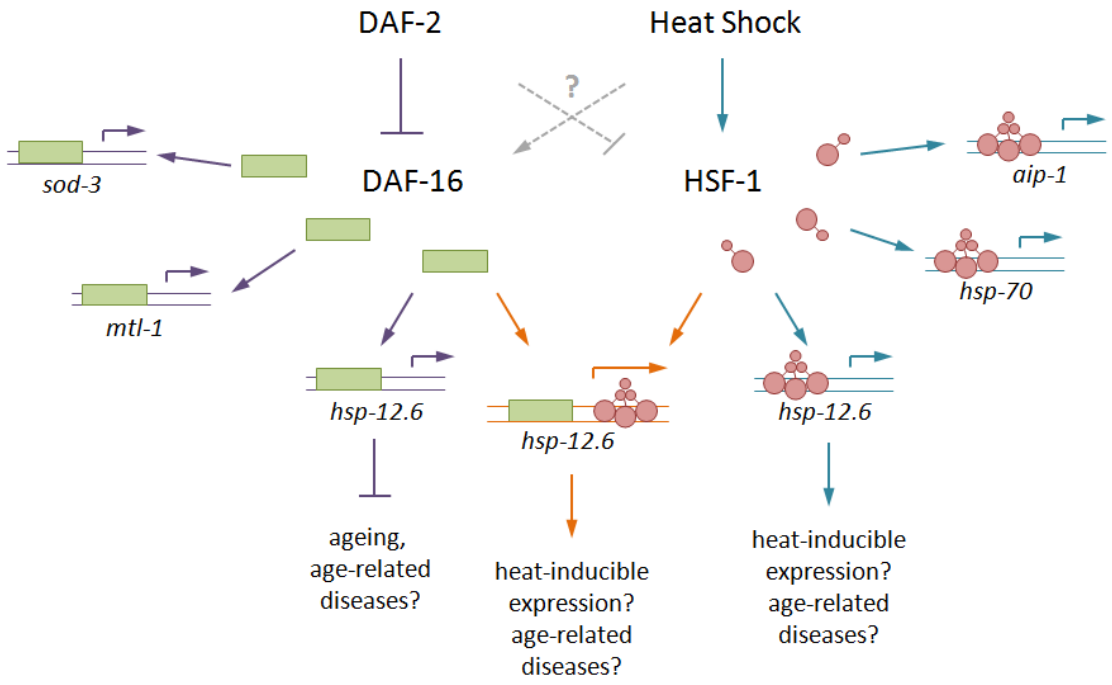


Figure 6 Downstream Targets of the Transcription Factors DAF-16 and HSF-1

DAF-16 targets *sod-3* and *mtl-1* while HSF-1 targets *aip-1* and *hsp-70* (Hsu et al., 2003). *Hsp-12.6* is a downstream target of both transcription factors DAF-16 and HSF-1. Together, DAF-16 and HSF-1 direct heat-inducible expression of *hsp-12.6* (Hsu et al., 2003). Only DAF-16 is necessary for *hsp-12.6*-directed regulation of longevity. It is possible that HSF-1, either alone or together with DAF-16, directs *hsp-12.6* to influence age-related diseases. Figure adapted from Hsu et al., 2003.

DAF-16 affects expression of a set of genes during early adulthood and many of these genes influence ageing (Murphy et al., 2003). Downstream targets of DAF-16 involved in longevity were found by microarray analysis (Murphy et al., 2003). Murphy et al. were looking for genes whose products directly influence ageing and discovered several genes that are expressed in response to cellular stress. The genes encode small heat shock proteins (smHSPs) including members of the 16kDa family, *hsp-16.1* and *hsp-16.49,* and two members of the 12kDa family, *hsp-12.3* and *hsp-12.6*, that were found to be upregulated in *daf-2(mu150)* animals with reduced *daf-2* activity and downregulated in animals with reduced *daf-16(RNAi);daf-2(RNAi)* activity (Murphy et al., 2003). In addition, stress response genes such as *sod-3*, (a mitochondrial iron/manganese *s*uper*o*xide *d*ismutase) that may function against oxidative stress and promote normal lifespan, were also upregulated by *daf-2(RNAi)* (Figure 6; Murphy et al., 2003).

In addition to stress proteins, normal *daf-16* levels upregulated pathogen resistance factors but downregulated gene classes involved in neuronal signaling, RNA binding and DNA replication, which antagonize lifespan and are downregulated in *daf-2* mutants (Murphy et al., 2003). It is important to note that these downstream genes of DAF-16 seemed to have a cumulative effect on ageing as there was no other RNAi treatment other than *daf-16(RNAi)* that totally suppressed the lifespan extension of *daf-2* mutants (Murphy et al., 2003).

Specific targets of DAF-16 are *sod-3*, and the metallothionein gene, *mtl-1*, while HSF-1 specifically targets *hsp-70* and the arsenite inducible protein, *aip-1* (Figure 6; Hsu et al., 2003).

1.3.6. The HSF-1 Transcription Factor

Heat shock factor-1 (HSF-1) transcriptional regulators exist in many organisms from yeast to humans (Clos et al., 1990; Guertin et al., 2010). There are four HSF family members in mammals (HSF1-4) while *Drosophila* and *C. elegans* contain only one known HSF each (Akerfelt et al., 2010). HSF-1 is the principal regulator of the heat shock response, and HSF-2 can also bind to HSP gene promoters in the presence of HSF-1 that contain intact DNA-binding domains (DBD) (Akerfelt et al., 2010; Östling et

al., 2007). HSF-4 has not been implicated in the heat shock response, but has been found to compete with HSF-1 common targets in mouse lens epithelial cells (Akerfelt et al., 2010; Fujimoto et al., 2004). HSF-3 is found in avian species where it is activated at higher temperatures and in murine species where it binds to genes other than HSPs (Akerfelt et al., 2010; Fujimoto et al., 2010). Yeast requires HSF and in *Drosophila* HSF is necessary for the heat shock response and stress tolerance, with a loss in HSF resulting in an early developmental phenotype (Jedlickha et al., 1997). Mice lacking HSF-1 can develop normally and attain adulthood; however, they exhibit multiple defects such as increased prenatal lethality, growth retardation and female infertility (Xiao et al., 1999). HSF s were first identified to upregulate HSP transcription under heat shock conditions, but recent studies have uncovered roles for HSFs in development, metabolism, lifespan and as a therapeutic target for neurodegenerative diseases and pancreatic cancer (Akerfelt et al., 2010).

Hsf contains conserved domains in *S. cerevisiae*, *Drosophila*, *C. elegans*, mus musculus and humans: a DBD which is responsible for HSF to bind to the heat shock element (HSE) of target proteins, an 80 residue hydrophobic heptad repeat (HR-A/B) essential for trimer formation and a transcriptional activation domain that is responsible for transcription at the HS promoter (Hadju-Cronin et al., 2004; Morimoto, 1998). Except in yeast and human HSF-4, a HR-C domain is present and may have a role in suppressing HSF trimer formation (Nakai and Morimoto, 1993; Rabindran et al., 1993). The HSE is conserved from yeast to humans (Fernandes et al., 1994; Trinklein et al., 2004; Guertin and Lis, 2010).

Except in *S. cerevisiae*, HSF exists either in the cytosol or nucleus as an inactive monomer under unstressed conditions (Clos et al., 1990; Westwood and Wu, 1993; Morimoto, 1998). Stresses such as heat stress, oxidative stress or the presence of misfolded proteins activates HSF by causing it to trimerize and bind to the HSE of HSPs with high affinity (Figure 6; Morimoto, 1998). Activated HSF is relocated to the nucleus and functions to induce HSP transcription (Morimoto, 1998).

In *C. elegans*, HSF-1 regulates the heat shock response and promotes longevity, as mutants of *hsf-1* have markedly reduced lifespans (Hsu et al., 2003). While reduced *hsf-1* activity decreases lifespan, overexpression of *hsf-1* extends lifespan (Hsu et al.,

2003). The transcription factors HSF-1 and DAF-16 are both required for *daf-2* mutants to extend lifespan. For example, reduction of function mutations of *daf-2*, *daf-2(e1370)* and *daf-2(mu150)* were not able to extend the short lifespan phenotype of *hsf-1(RNAi)*-treated animals (Hsu et al., 2003). Additionally, in short-lived *daf-16* mutants, *daf-2* mutations were unable to increase lifespan (Gems and Partridge, 2001; Guarente and Kenyon, 2000; Lin et al., 2001). HSF-1 and DAF-16 can function independently of one another. *Hsf-1(RNAi)* did not prevent nuclear accumulation of DAF-16 in *daf-2* mutants, nor did *hsf-1(RNAi)* diminish expression of known downstream genes of *daf-16*, *mtl-1* and *sod-3*, indicating the DAF-16 can function independently of HSF-1 (Figure 6; Hsu et al., 2003). Moreover, *daf-16* mutations did not diminish heat-inducibility of downstream targets of *hsf-1*, *aip-1*, *unc-33* and the homologue of HSP-70, suggesting that HSF-1 can function independently of DAF-16 (Figure 6; Hsu et al., 2003).

While they can function independently of one another, there is much evidence that suggest that HSF-1 and DAF-16 together activate expression of specific genes that in turn promote longevity, such as *hsp-16.1*, *hsp-16.49*, *hsp-12.6* and *sip-1* (*s*tress *i*nduced *p*rotein) (Hsu et al., 2003). These four genes were identified to be upregulated in the DAF-2 pathway in a DNA microarray analysis, as their expression sharply increased in animals with reduced *daf-2* activity and decreased in animals with reduced *daf-16* activity (Murphy et al., 2003). Hsu et al. confirmed the microarray data that these genes are upregulated in the DAF-2 pathway using RT-PCR. In addition, they identified that HSF-1 functioned in the IIS pathway as *hsf-1(RNAi)* on both *daf-2(e1370)* and N2 animals showed a decrease in the mRNA of the stress proteins (Hsu et al., 2003). Moreover, the investigators found that DAF-16 functioned in the heat shock response, as mRNA of the stress proteins decreased under heat shocked *daf-16* conditions versus N2 or *daf-16* animals without HS (Hsu et al., 2003). Furthermore, all four genes contained upstream regulatory sequences identical to consensus DAF-16 and HSF-1 DNA binding sites. It is thought that in *daf-2* animals and wild-type animals under heat shock, DAF-16 and HSF-1 bind directly to regulatory sequences in these stress proteins (Hsu et al., 2003). Finally, the investigators found a decrease in longevity when the animals were treated with RNAi towards these stress proteins in *daf-2* animals. Thus, a role for DAF-16 and HSF-1 is uncovered in lifespan regulation that may be a result of turning on downstream target genes involved in the heat stress response.

1.4. Small Heat Shock Proteins (smHSPs)

HSPs were first discovered in *Drosophila*, after a unique set of puffs could be induced in larvae following a HS treatment (Ritossa, 1962). Now, expression of heat shock proteins is known to be tightly regulated during development in multiple organisms from amoebae, brine shrimps, nematodes, flies and plants to mammals (Morrow and Tanguay, 2012). SmHSPs can be roughly divided according to their molecular masses and sequence similarity into four members of HSP-12s, six members of HSP-16s, HSP-25, HSP-43, HSP-17.5 and SIP-1 (Ding and Candido, 2000a). Some smHSPs are expressed constitutively, such as the HSP-12kDa family while others such as the HSP-16kDa family respond to environmental factors such as heat stress (Ding and Candido, 2000a; Leroux et al., 1997a). Stress-induced expression of smHSPs is regulated by HSFs while constitutive expression of smHSPs is regulated by other transcription factors, which suggests that some smHSPs have specialized functions (Morrow and Tanguay, 2012). *Hsp-16.1*, *hsp-16.2*, *hsp-16.41* and *hsp-16.48* are activated only in response to heat shock and heavy metals, paraquat and the fungicide captan and the chemical agents captan, cadmium, methanol and ethanol (Stringham et al., 1992; Jones et al., 1996). HSP-12.6 levels in first-stage larvae treated to the same stressors were not altered when analysed by Western blot with the anti-HSP-12.6 polyclonal antibody (Leroux et al., 1997a). Other stressors, such as hyper- and hypo-osmotic shock and cold shock did not alter HSP-12.6 levels as well (Leroux et al., 1997a). Overexpression of smHSPs in cultured cells confers thermotolerance (Landry et al., 1989) and this mechanism is thought to depend on, at least in part, the chaperone-like activity of the smHSPs (Horwitz, 1992). α-crystallins in the eye lens and smHSPs such as *hsp-16.1*, *hsp-16.2*, *hsp-16.41* and *hsp-16.48* in *C. elegans* function as molecular chaperones, preventing the aggregation of denatured proteins and guiding misfolded proteins to refold to their native state (Horwitz, 1992; Leroux et al., 1997a). The 16-kDa family of smHSPs, specifically *hsp-16.1*, *hsp-16.2*, *hsp-16.11* and *hsp-16.49* also have a role in the regulation of longevity (Hsu et al., 2003; Murphy et al., 2003). A greater amount of research has been done on the larger smHSPs compared to smaller 12-kDa family of smHSPs in *C. elegans,* and as a result, there is not as much knowledge surrounding the 12-kDa family.

The 16-kDa and 12-kDa HSP families have been studied biochemically *in vitro* and message levels have been measured throughout *C. elegans* development (Leroux et al., 1997a; Jones et al., 1989). Control of HSP-16 synthesis has been studied using *Hsp-16-lacZ* fusions in transgenic animals (Stringham et al., 1992; Jones et al., 1996). HSP-16 is over-accumulated in worms with longevity mutations following thermal stress (Walker et al., 2001). Monitoring of *hsp-16.2::GFP* transgenic animals in response to heat shock and response to juglone showed a measure of *hsp-16* induction following these stresses (Link et al., 1999). *Hsp-16* is also implicated in longevity (Walker and Lithgow, 2003).

Cross-linking and sedimentation velocity analyses indicated that recombinant HSP-12.6 is monomeric and HSP-12.6 does not function as a molecular chaperone *in vitro*, at least it does not prevent the thermally unfolded citrate synthase (Leroux et al., 1997a). In addition, immunohistochemical staining has been performed using the polyclonal anti-HSP-12.6 antibody (Ding and Candido, 2000a). However, no other *in vivo* expression studies on *C. elegans* have been performed with regards to HSP-12.6 until now.

1.4.1. The 12kDa Family of Heat Shock Proteins

1.4.1.1. Structure of the 12kDa Family of Heat Shock Proteins

The smallest known HSPs in *C. elegans* are the four members of the 12kDa family of heat shock proteins (Kokke et al., 1998). HSP-12.1, HSP-12.2, HSP-12.3 and HSP-12.6 basically consist of the conserved smHSP family 80-100 amino acid α-crystallin domain but also have very short hydrophobic N-terminal regions (25-26 residues) and almost lack the polar C-terminal tail altogether (Figure 7; Kokke et al., 1998; Leroux et al., 1997a).

HSP-12.2 and HSP-12.3 are the most divergent of the 12kDa heat shock proteins with only 43% sequence identity (Figure 7; Kokke et al., 1998). Rather than forming large multimeric complexes as is typical of other smHSPs, HSP-12.2 and HSP-12.3 form tetramers while HSP-12.6 was found to occur as a monomer based on sedimentation velocity data (Kokke et al., 1998; Leroux et al., 1997a). HSP-12.6 is 48%

and 67% identical to HSP-12.2 and HSP-12.3, respectively (Figure 7; Kokke et al., 1998).

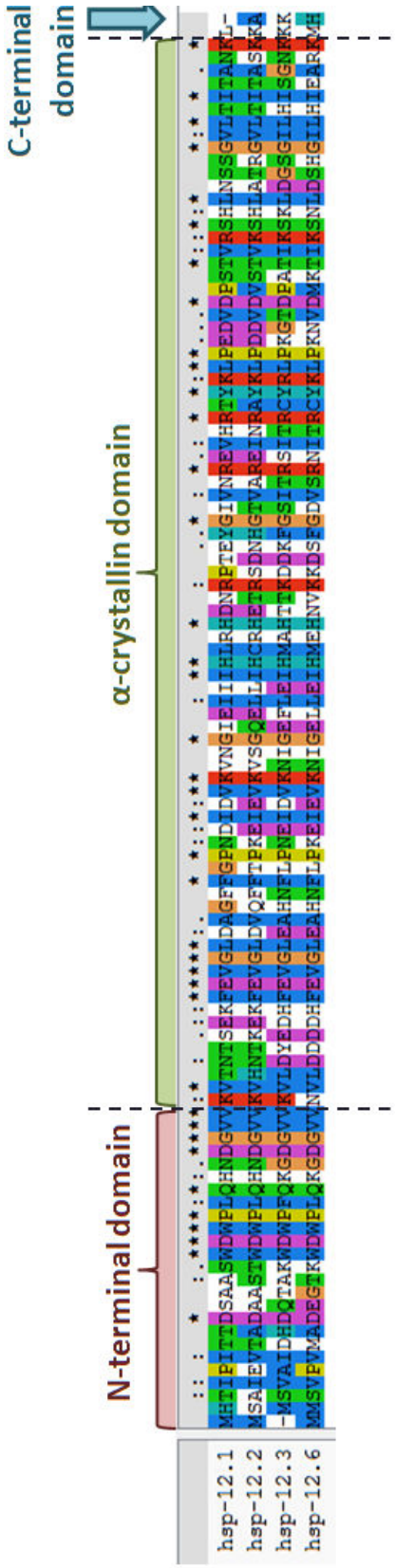

C-terminal domain
α-crystallin domain
N-terminal domain
hsp-12.1
hsp-12.2
hsp-12.3
hsp-12.6

Figure 7 Amino Acid Sequence Alignment of the HSP-12kDa Family

Multiple alignments (CLUSTAL) of the four members of the hsp-12kDa family of proteins: HSP-12.1, HSP-12.2, HSP-12.3 and HSP-12.6. All four members of the HSP-12kDa family of heat shock proteins are represented and aligned according to their domains as outlined in Leroux et al., 1997a. *Hsp-12.6* contained a conserved α-crystallin domain, a truncated N-terminal domain and basically lacked a C-terminal domain when compared to α-crystallin (Leroux et al., 1997a). (*) Indicated identical amino acids, (:) indicated strongly similar amino acids (those in the same group) and (.) indicated weakly similar amino acids (both polar, but have different charges). Amino acids were colour coded based on polarity and charge (Gnanasekar et al., 2008).

1.4.1.2. Function of the 12kDa Family of Heat Shock Proteins

Unlike typical smHSPs, HSP-12.2, HSP-12.3 and HSP-12.6 reportedly do not function as molecular chaperones *in vitro* (Kokke et al., 1998; Leroux et al., 1997a). An excess of recombinant *hsp-12.2* or recombinant *hsp-12.3* did not prevent the aggregation of thermally unfolding citrate synthase (CS) when assayed at 43°C (Kokke et al., 1998). Moreover, HSP-12.6 did not prevent the thermally induced aggregation of CS at 45°C (Leroux et al., 1997a).

It is speculated that the lack of *in vitro* chaperone-like activity and the inability to form larger multimeric complexes may be due to the truncated N-terminal and lack of C-terminal tail when compared to α-crystallin (Leroux et al., 1997a; Kokke et al., 1998). Tetrameric and dimeric arrangements are building blocks for larger smHSP complexes and higher complex formation beyond the tetramer is indicated to be required for chaperone-like activity (Kokke et al., 1998; Kokke et al., 2001). This higher multimer formation is thought to be mediated through N-terminal domains (Kokke et al., 1998), therefore, a truncated N-terminal domain may be a reason that HSP-12.6 is monomeric and lacks *in vitro* chaperone-like activity (Kokke et al., 1998). The N-terminal domain has been implicated in substrate binding, as it has available hydrophobic binding sites while the polar and flexible C-terminal extensions improve chaperone activity by enhancing the solubility of the smHSP-substrate complex (Smulders et al., 1998; Leroux et al., 1997b).

1.4.2. IIS components and smHSPs in the Prevention of Poly-Q Aggregates

While some experiments suggest that *hsp-12.2*, *hsp-12.3* and *hsp-12.6* do not act as molecular chaperones *in vitro* (Leroux et al., 1997a; Kokke et al., 1998), these smHSPs may have a specialized function *in vivo* (Morrow and Tanguay, 2012). With respect to ageing, smHSPs could be acting as molecular chaperones *in vivo* to prevent misfolded or unfolded proteins from aggregating. Protein aggregation is useful as a biomarker for ageing and in the study of diseases such as Alzheimer`s and Huntington`s disease, which both involve specific proteins that form insoluble aggregates as organisms age, such as Huntington`s-like polyglutamine-repeat (poly-Q) protein aggregates (David et al., 2010; Morley et al., 2002). IIS pathway components seem to have a protective effect against the formation of these aggregates (David et al., 2010).

David et al., tested *daf-2* mutants for the presence of poly-Q aggregates at chosen days in the lifespan of the animals and found that *daf-2* mutants display fewer poly-Q protein aggregates than wild-type animals at days 12, 21 and 31 of their lifespan (David et al., 2010). Thus, as the worms are ageing, reduced IIS is thought to have a protective effect against age-dependent aggregates. Conversely, *daf-16* and *hsf*-1 RNAi accelerated aggregation formation (Hsu et al., 2003). Hsu et al. further examined whether or not smHSP RNAi might have the same effect and found that like *daf*-16 and *hsf*-1, RNAi of *hsp-16.1*, *hsp-16.49*, *hsp-12.6* and *sip-1* accelerated poly-Q aggregation, although not to as great of an extent (Hsu et al., 2003). From this evidence, it is suggested that the transcription factors DAF-16 and HSF-1 may be acting to extend lifespan by preventing aggregate formation through smHSP effectors.

1.4.3. HSP-12.6 and α-crystallin

Hsp-12.6 is a member of the hsp-20/alpha crystallin family and is similar to alpha-β-crystallin (Murphy et al., 2003). The α-crystallin family consists of two genes: α-A-crystallin and α-B-crystallin, which have ~58% sequence similarity (Horwitz, 1992). α-A-crystallin and α-β-crystallins are major protein components of the mammalian eye lens, comprising ~50% of total lens structural protein mass (de Jong, 1981; Horwitz, 2003). α-A and α-B-crystallins are important proteins as they must maintain clarity of the eye lens for the duration of the organism's lifetime and inherited crystallin mutations are a common cause of childhood cataract while age-related cataract (the most common cause of blindness worldwide) and myopathy are due to mutations in the long-lived α-A and α-B-crystallins (Clark et al., 2012; Horwitz, 2003). A member of the smHSP family, α-crystallins possess chaperone-like function, suppressing thermally induced aggregation of β-crystallins and γ-crystallins, two other major mammalian structural lens proteins, in order to maintain eye lens clarity (Horwitz, 1992). The functions of α-A-crystallin and α-B-crystallin are not lens-specific, as they can also function outside the mammalian eye lens and are expressed under stress conditions (Horwitz, 1992). In addition to maintaining the proper refractive index in the eye lens, α-B-crystallins exhibit chaperone function, cytoskeletal stabilization and anti-apoptotic function in the muscles (smooth, skeletal and cardiac), brain, colon, kidney, liver, lung, stomach and testis and are overexpressed in many neurological diseases (Boncoraglio et al., 2012).

Apart from *C. elegans*, HSP-12.6 homologues have been identified in other nematode species including the parasitic nematodes *Brugia malayi* (*B. malayi*), *Nippostrongylus brasiliensis* (*N. brasiliensis*) and two hookworm *Ancylostoma* species. The parasite, *B. malayi,* was screened for identifying targets that bound to the human interleukin-10 receptor (huIL10R) and an insert was found that showed 53% homology to *C. elegans* HSP-12.6 (Gnanasekar et al., 2008). The gene was named *B. malayi* HSP-12.6 (BmHSP-12.6) and displayed the conserved α-crystallin domain, a short N-terminus and a small C-terminus (Gnanasekar et al., 2008). BmHSP-12.6 was differentially transcribed in the vertebrate stages of the parasite's life cycle (Gnanasekar et al., 2008). Interestingly, BmHSP-12.6 was found to bind huIL10R in a dose dependent fashion and inhibited human IL-10 (huIL10) from binding to huIL10R *in vitro* (Gnanasekar et al., 2008). RT-PCR revealed an up-regulation of *hsp-12.*6 expression in the parasitic nematode *N. brasiliensis* (*Nb-Hsp12.6*). *Nb-Hsp12.6* was temporally expressed in infective-L3 larvae and adults during the worm expulsion stage where the parasite is surviving the immunological response of the host (Arizono et al., 2011). cDNA from the infective L3 larvae stage of two *Ancylostoma* hookworm species, *A. caninum* (*Ancylostoma caninum*) and *A. ceylanicum* (*Ancylostoma ceylanicum*), abundantly represented *C. elegans hsp-12.6* transcripts (Mitreva et al., 2005). It has been suggested that *C. elegans* dauer larvae show striking morphological similarity to the infective-L3 stage of parasitic nematodes; both are developmentally arrested, thin and resistant to harsh conditions (Ogawa et al., 2009; Jensen et al., 2006). As *hsp-12.6* is up-regulated during the critical L3 infective stage of parasitic nematodes (Li et al., 2009; Arizono et al., 2011; Gnansekar et al., 2008), an understanding of the mechanism by which nematode survival is regulated would unveil potential parasite controls and greatly impact human health (Jensen et al., 2006).

SAGE data revealed that *hsp-12.6* was the most highly expressed transcript in a dauer population of N2 *C. elegans* (Jones et al., 2001). In another study, SAGE showed that *hsp-12.6* gene expression was higher in the reduction of function mutant, *daf-2(m41)* versus control animals (Halaschek-Wiener et al., 2005). The combination of SAGE and microarray data (Jones et al., 2001; Halaschek-Wiener et al., 2005; Murphy et al., 2003; McElwee et al., 2003) highlights *hsp-12.6* as reproducibly involved in *C. elegans* longevity.

1.4.3.1. HSP-12.6 in the IIS Pathway

To isolate downstream targets of DAF-16, Murphy et al. (2003) performed a microarray analysis to identify genes that were either upregulated in a *daf-2* mutant background or downregulated in a *daf-16* mutant background and then carried out functional analysis of these candidates using RNAi. HSP-12.6 was investigated as a result of this microarray that suggested *hsp-12.6* expression was increased in animals with reduced *daf-2* activity and decreased in animals with reduced *daf-16* activity (Murphy et al., 2003). RT-PCR analysis confirmed the microarray results as *hsp-12.6* mRNA levels increase in *daf-2(e1370)* animals and decrease in *daf-16(mu86)* (Hsu et al., 2003).

It is of interest to investigate where *hsp-12.6* fits into the IIS pathway (Figure 3; Figure 6). While *daf-16* and *hsf-1* are thought to possess independent functions, it is also thought that they may act together to promote longevity with regards to smHSPs and possibly other genes (Figure 6; Hsu et al., 2003). For example, at 20°C, *hsp-12.6(RNAi)* decreased longevity of HSF-1 overexpressing animals (Hsu et al., 2003), suggesting that HSP12.6 is a downstream target and effector of HSF-1 in ageing.

The role of *hsp-12.6* in the IIS pathway was studied with respect to ageing as *hsp-12.6* was pulled out in the microarray data (Murphy et al., 2003), SAGE (Halaschek-Weiner et al., 2005) and is implicated in ageing (Hsu et al., 2003). Lifespan of *hsp-12.6(RNAi)* treated *daf-2(mu150)* animals was reduced when animals were at 25°C and also in *daf-2(mu150)* animals shifted from 20°C to 25°C at L2 (Murphy et al., 2003). Moreover, *hsp-12.6(RNAi)* reduced the extended lifespan phenotype of *daf-2(e1370)* animals at 20°C by approximately 25% (Hsu et al., 2003). Although this reduction of lifespan by *hsp-12.6(RNAi)* was relatively small, it was found to be statistically significant (Hsu et al., 2003).

1.5. Thesis Overview

While the literature indicates that a reduction of function of *hsp-12.6* reduces lifespan, given that *hsp-12.6* does not appear to act as a molecular chaperone *in vitro* (Leroux et al., 1997a), the mechanism is ill defined. For this reason, the role of *hsp-12.6*

in ageing is attractive to investigate to see how it is affecting lifespan in the IIS pathway. This study aims to answer a few key questions regarding *hsp-12.6* in the role of lifespan regulation under non stressed conditions, such as; where does *hsp-12.6* fit into the IIS pathway? Is there functional redundancy among the hsp-12kDa family members? Also, where is HSP-12.6 expressed? These questions were addressed by building the *phsp-12.6::HSP-12.6::DSRED2* translational fusion and also characterizing the putative deletion *hsp-12.6(gk156)* allele. Longevity assays were performed to determine if *hsp-12.1*, *hsp-12.3* and *hsp-12.6* had roles in ageing and also where they fit in the IIS pathway.

The roles of the transcription factors DAF-16 and HSF-1 in *hsp-12.6* directed lifespan regulation was addressed. By characterizing HSP-12.6, which is regulated by DAF-16, more knowledge is gained concerning how the IIS pathway influences ageing. Although a lot of study is devoted to the IIS pathway in many organisms, there is still much to be discovered concerning the IIS pathway and its influence on ageing and age-related diseases. By taking advantage of the many features of *C. elegans* such as the short lifecycle and availability of tools such as RNAi clones, it is possible to continue to elucidate the genetic regulation of ageing.

2. Results

2.1. Tissue Expression Pattern of HSP-12.6

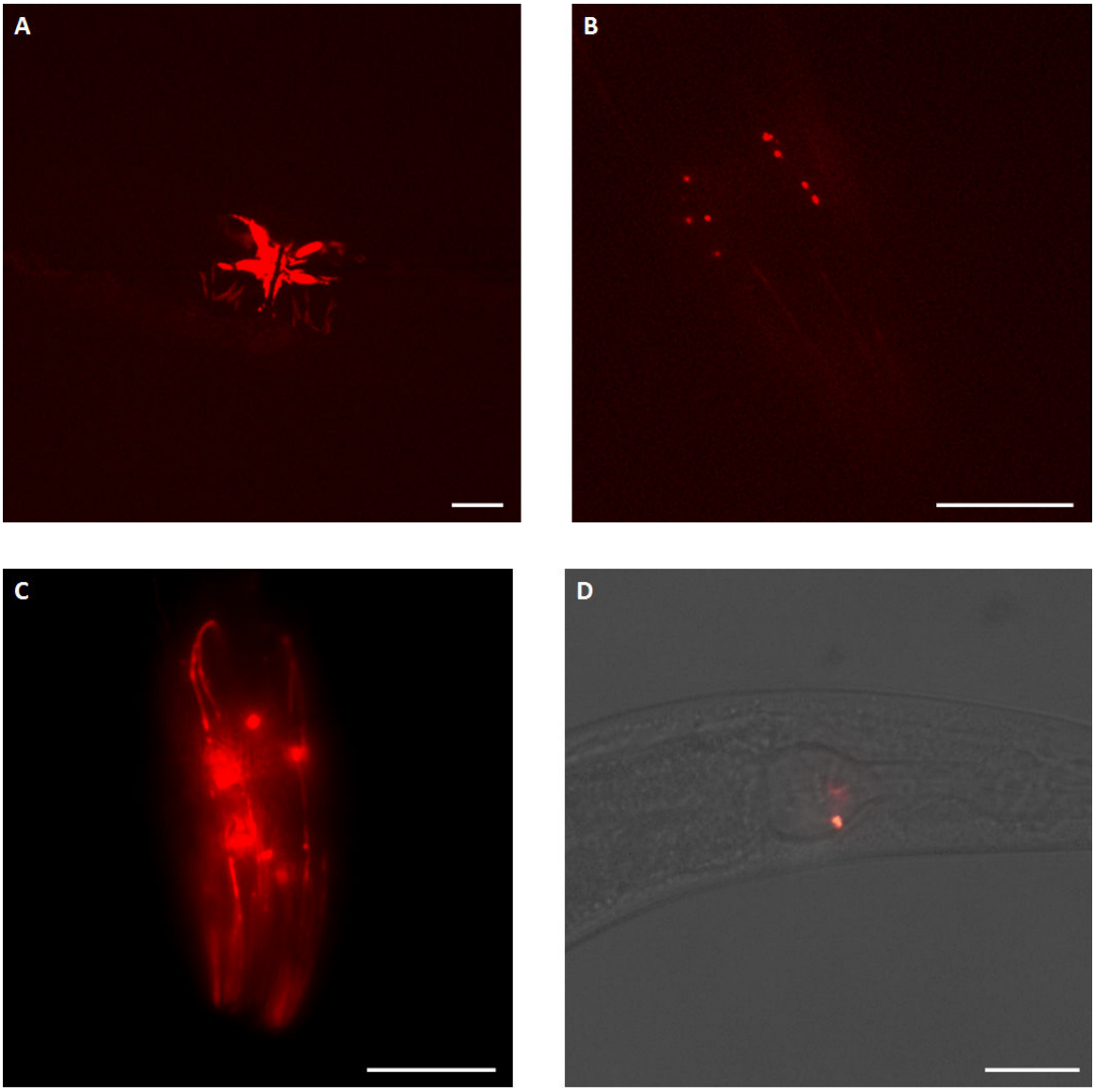
The wild-type expression pattern of HSP-12.6 was determined by constructing a transgenic strain carrying a translational fusion reporter fusion of *phsp-12.6::HSP-12.6::DSRED2*. As exogenous DNA typically forms extrachromosomal arrays which are not passed onto daughter cells with complete fidelity, the transgenic array was subsequently integrated into the nematode genome by X-ray mutagenesis to produce strain VA314. This strain was then outcrossed 6 times prior to subsequent analysis.

Expression of the DSRED2 fluorescent protein was observed in body muscle cells, the vulval and uterine muscles, and both anteriorly and posteriorly directed axons in the head and tail (Figure 8). This pattern partially overlaps the immunostaining observed by Ding and Candido (2000a) where a polyclonal anti-rabbit antibody against HSP-12.6 revealed expression in the vulval muscles, spermatids and spermatocytes under normal growth conditions.

Transgene expression was observed in both animals subjected to heat shock at 33°C (Figure 8A, B, E and G) as well as animals maintained at non-heat shock conditions (Figure 8C, D, F and H). This constitutive expression is consistent with that reported by Ding and Candido (2000a) where HSP-12.6 protein was detected by immunostaining in the vulval tissues of unstressed animals. In contrast to the study of Ding and Candido (2000a), expression of the translation HSP-12.6::DSRED2 reporter was detected in intestinal cells (Figure 8G) while no expression was observed in the spermatids or spermatheca.

A punctate pattern of expression was observed in the body muscle of heat shocked *phsp-12.6::HSP-12.6::DSRED2* animals (Figure 8E and F), a pattern reminiscent of the subcellular localization of mitochondria in body muscle reported by

Haynes et al. (2010).


A
B
C
D

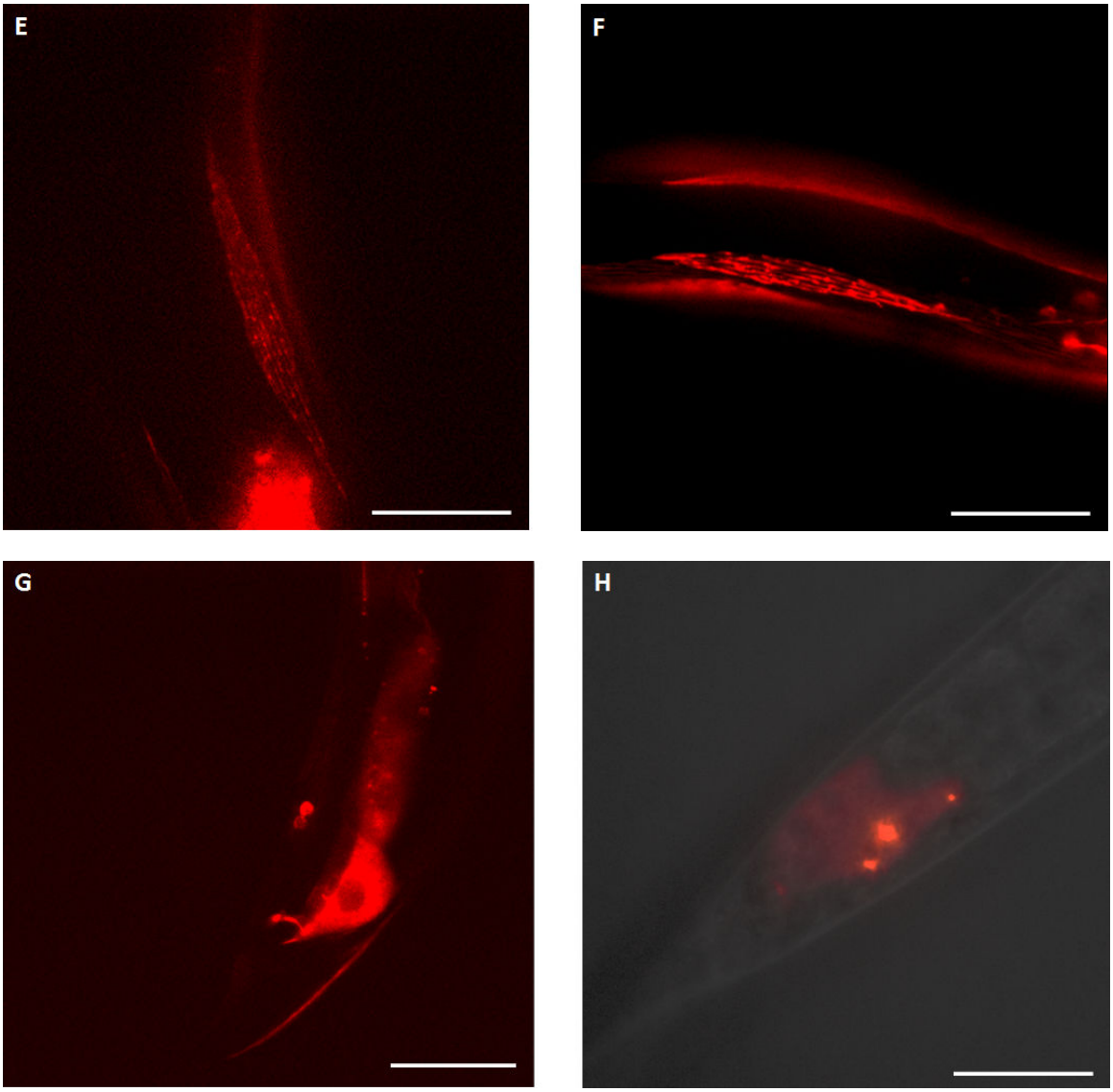


Figure 8 Expression of HSP-12.6 Translational Fusion Line

Animals in A, B, E and G were exposed to 2 two hour heat shock exposures of 33°C separated by a 30 minute recovery period at 20°C (adapted from Stringham et al., 1992). Animals in C, D, F and H were grown and assessed under non-heat shocked conditions at 20°C. Scale bars in C, D and E-H=50µm. A) Vulva muscles, scale bar=25µm. B) Anterior axons, scale bar=30µm. C) Anterior neurons. D) Anterior neurons cell body with DIC image of the head overlapped. E) Skeletal body muscle displaying punctate pattern. F) Skeletal body muscle displaying punctate pattern. G) Intestinal cells and neurons. H) Intestinal cells.

2.1.1. HSP-12.6 Does Not Co-Localize to Mitochondria

As the distribution of mitochondria in muscle cells results in a punctate pattern (Haynes et al., 2010) similar to the punctate pattern of HSP-12.6 expression observed in skeletal muscle (Figure 8E and F), it was hypothesized that HSP-12.6 could be localized to the mitochondria of these cells. To test this hypothesis, the *phsp12.6::HSP-12.6::DSRED2* translational fusion was crossed into the background of the cytoplasmic muscle specific GFP reporter *myo-3$_{pr}$::gfp^{cyt}(zcIs21)* to generate the control strain VA402 (Figure 9), or into the background of the mitochondrial muscle specific GFP reporter *myo-3$_{pr}$::gfp^{mt}(zcIs14)* to generate the test strain VA403 (Figure 10). The resulting double mutants were subsequently viewed under high magnification on a spinning disc confocal microscope to visualize the two fluorescent reporter proteins, GFP and DSRED2.

While expression of both the cytoplasmic GFP marker, *myo-3$_{pr}$::gfp^{cyt}* and *phsp-12.6::HSP-12.6::DSRED2* could be observed in the same muscle cell, no co-localization of the fluorescent proteins was seen at this resolution (Figure 9). This suggested that HSP-12.6 is not uniformly distributed to the cytoplasm of muscle cells and that it is possible that HSP-12.6 may still be cytoplasmic (Figure 9). Moreover, no co-localization was seen between the mitochondrial marker *myo-3$_{pr}$::gfp^{mt}* and *phsp-12.6::HSP-12.6::DSRED2* which suggested that HSP-12.6 is not localized to mitochondria in muscle cells (Figure 10). We conclude that the punctate expression pattern observed does not correspond to mitochondrial organelles but may be a result of overexpression of proteins or tagging with DSRED2 protein.

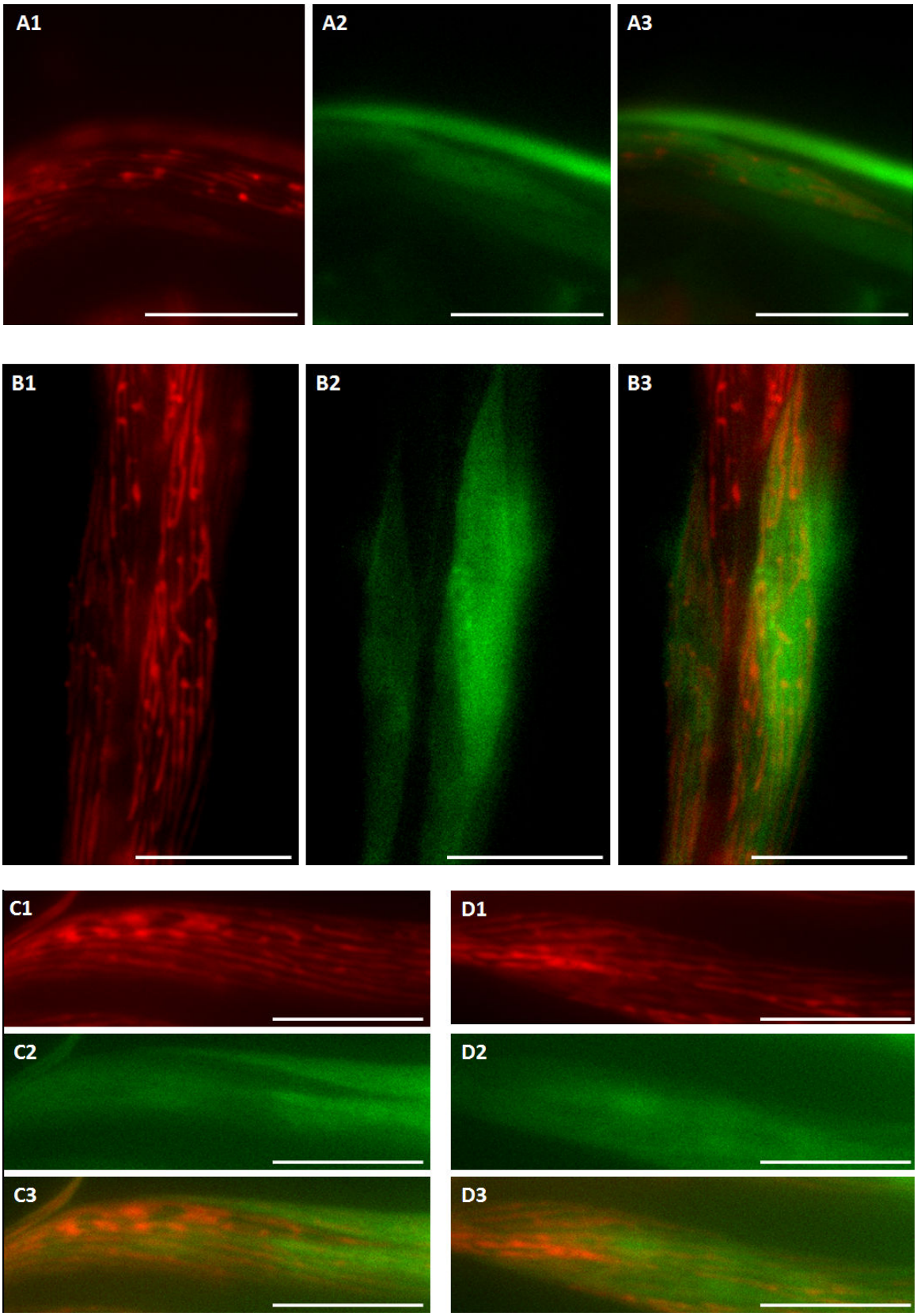
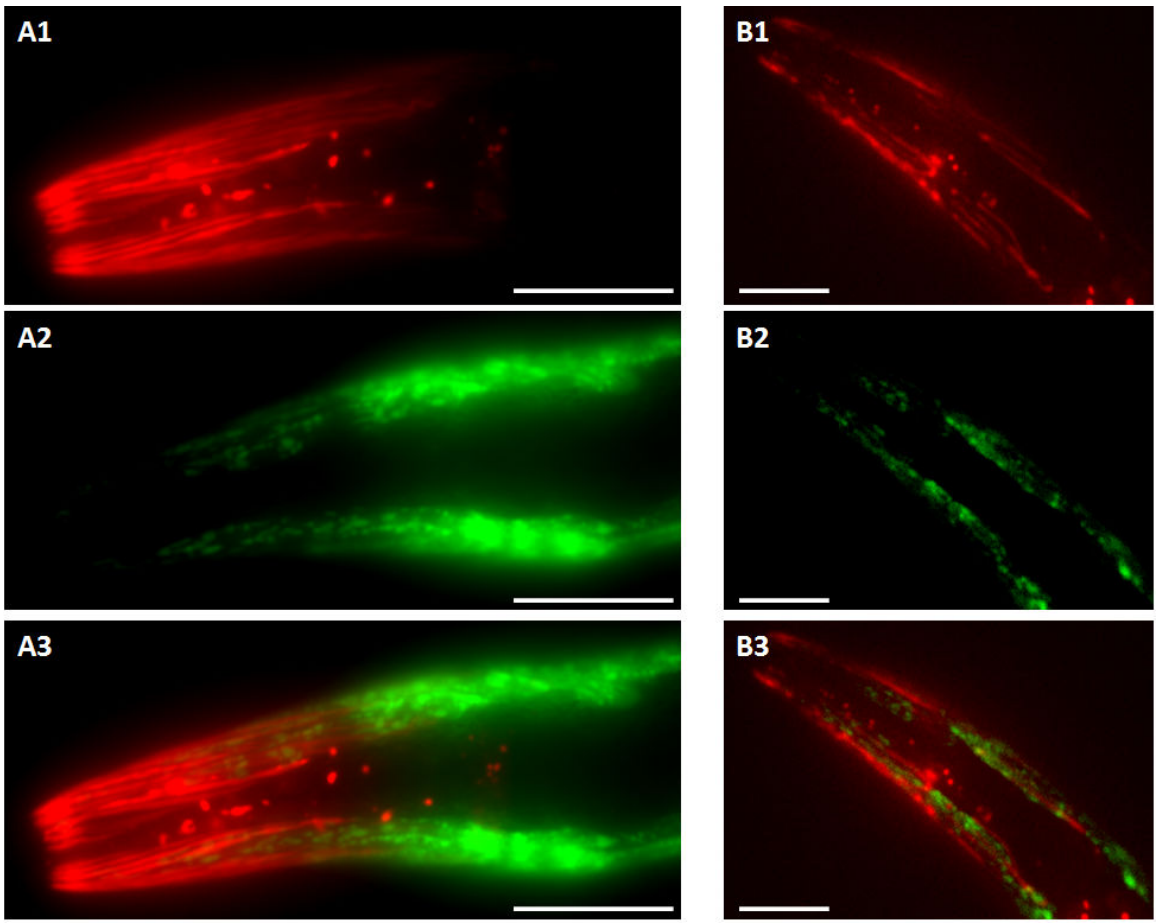

A1
A2
A3
B1
B2
B3
C1
C2
C3
D1
D2
D3

Figure 9 Cytoplasmic GFP myo-3$_{pr}$::gfp^{cyt}(zcIs21) Crossed with pshp-12.6::HSP-12.6::DSRED2 Showed No Co-localization of the Two Fluorescent Proteins

Head and midbody muscle of *myo-3$_{pr}$::gfp^{cyt};phsp-12.6::HSP-12.6::DSRED2* animals. *myo-3$_{pr}$::gfp^{cyt}* (panels 2) was expressed in the cytoplasm of body muscles while *phsp-12.6::HSP-12.6::DSRED2* (panels 1) was expressed in the muscle fibers. No co-localization of the two fluorescent proteins was seen (merge panels 3). All scale bars represent 25µm. A and B) Midbody wall muscle. C and D) Anterior body wall muscle.


A1
B1
A2
B2
A3
B3

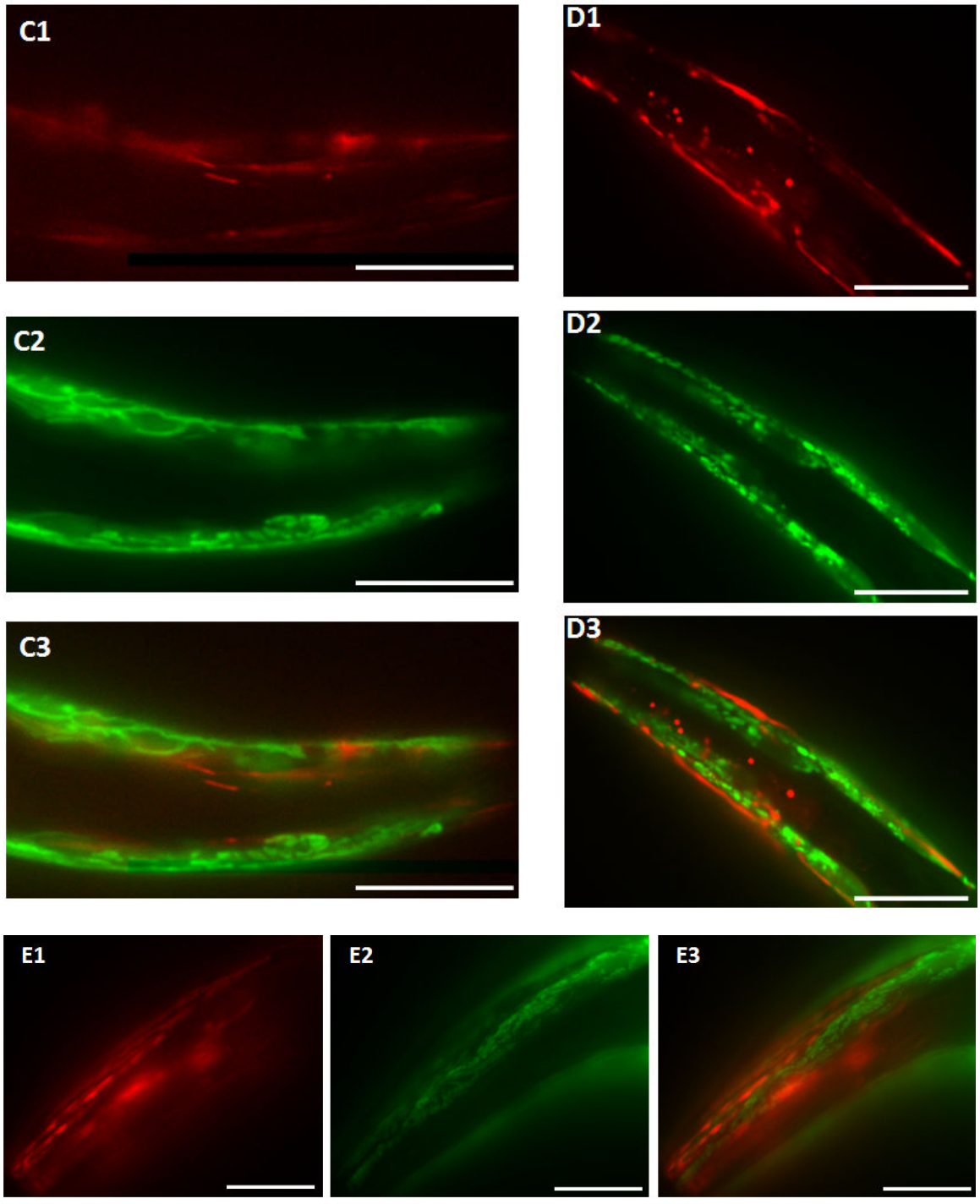

C1
D1
C2
D2
C3
D3
E1
E2
E3

Figure 10 Mitochondrial GFP myo-3$_{pr}$::gfp^{mt}(zcIs14) Crossed with pshp-12.6::HSP-12.6::DSRED2 Showed No Co-localization of the Two Fluorescent Proteins

Head regions of *myo-3$_{pr}$::gfp^{mt};phsp-12.6::HSP-12.6::DSRED2* animals under non heat stressed conditions at 20°C. All scale bars represent 20µm. Part 1 of each panel was *phsp-12.6::HSP-12.6::DSRED2* expression. Part 2 of each panel was *myo-3$_{pr}$::gfp^{mt}*. Part 3 of each panel was a merge of the two fluorescent proteins. Each example showed no co-localization of the two fluorescent proteins.

2.2. HSP-12.6 Can Be Specifically Reduced by RNAi

In order to confirm that the translational fusion *phsp-12.6::HSP-12.6::DSRED2* was indeed over-expressing HSP-12.6 *in vivo*, *hsp-12.6(RNAi)* was fed to the transgenic animals in order to reduce HSP-12.6 and HSP-12.6::DSRED2 expression. Animals were scored for the presence or absence of DSRED2 expression under a fluorescent stereomicroscope at approximately 250x magnification (Figure 11). It was found that *hsp-12.6(RNAi)* significantly reduced expression of *phsp-12.6::HSP-12.6::DSRED2* when compared to *phsp-12.6::HSP-12.6::DSRED2* treated with an empty vector control pPD129.36 (Figure 12). *phsp-12.6::HSP-12.6::DSRED2* animals were counted for low, medium/high signal when treated with control, *hsp-12.6(RNAi)*, *hsp-12.3(RNAi)* and *hsp-12.1(RNAi)* (Figure 11; Table 1). *p* values were calculated by comparing n values between low signal or between med/high signal between various groups as outlined in Table 1. Both qualitative and quantitative data were specifically measured in the tail neurons of animals, as this gave the most consistent DSRED2 expression compared to other cell types (Figures 21 and 22 in Appendix B).

Due to the amino acid similarity of the HSP-12kDa family (Figure 7), it is possible that the members may be functionally redundant. Therefore, *hsp-12.1* or *hsp-12.3* RNAi were fed to *phsp-12.6::HSP-12.6::DSRED2* animals to determine if expression was affected. It was found that neither *hsp-12.1(RNAi)* nor *hsp-12.3(RNAi)* significantly altered *phsp-12.6::HSP-12.6::DSRED2* expression, suggesting that despite their amino acid sequence similarity, individual members of the HSP-12 family can be targeted using RNA interference (Figure 12).

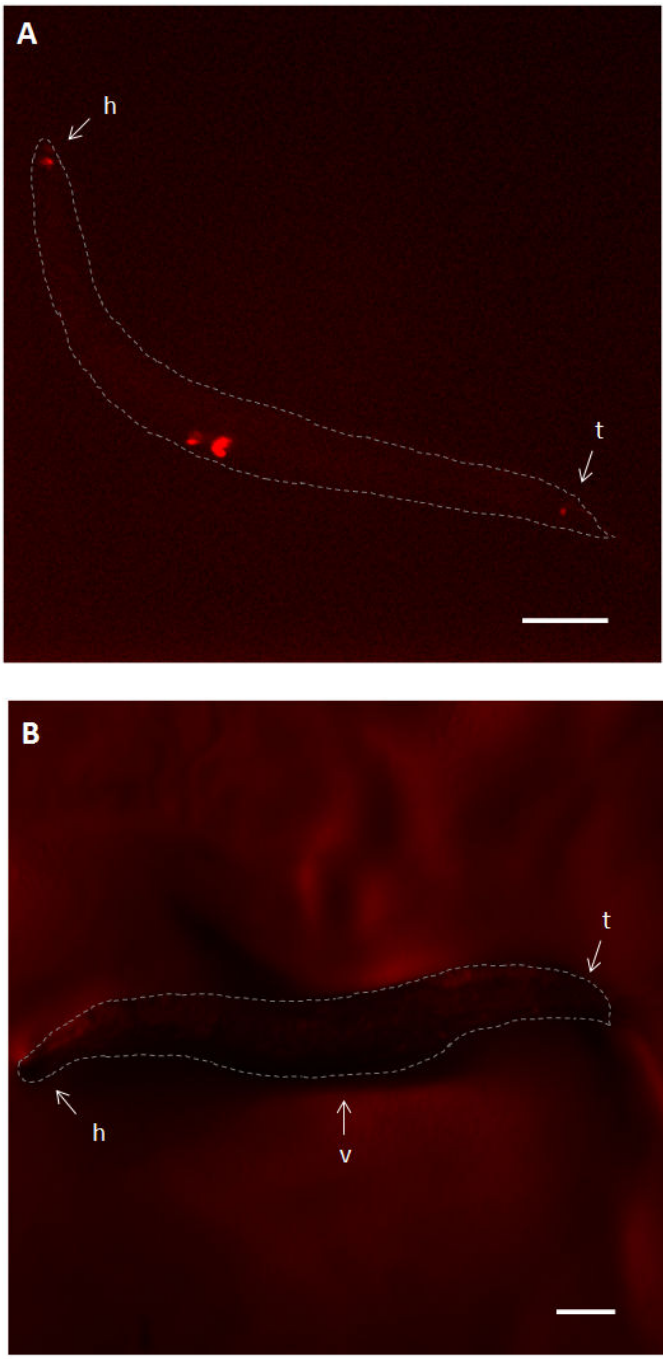


Figure 11 Qualitative Expression of hsp-12.6 after RNAi

Qualitative difference between observed expression or no expression of *phsp-12.6::HSP-12.6::DSRED2* for quantitative assessment of specificity of *hsp-12* family RNAi clones. In both panels, anterior is on the left and dorsal towards the top. Arrows point to head (h), vulva (v) and tail (t). Scale bars represent 100µm. Animals were grown and assessed at 20°C and observed under a fluorescent stereomicroscope at approximately 250x magnification. A) Example of positive expression in the head, vulva and tail at arrows. B) Example of no expression in the head, vulva and tail at arrows.

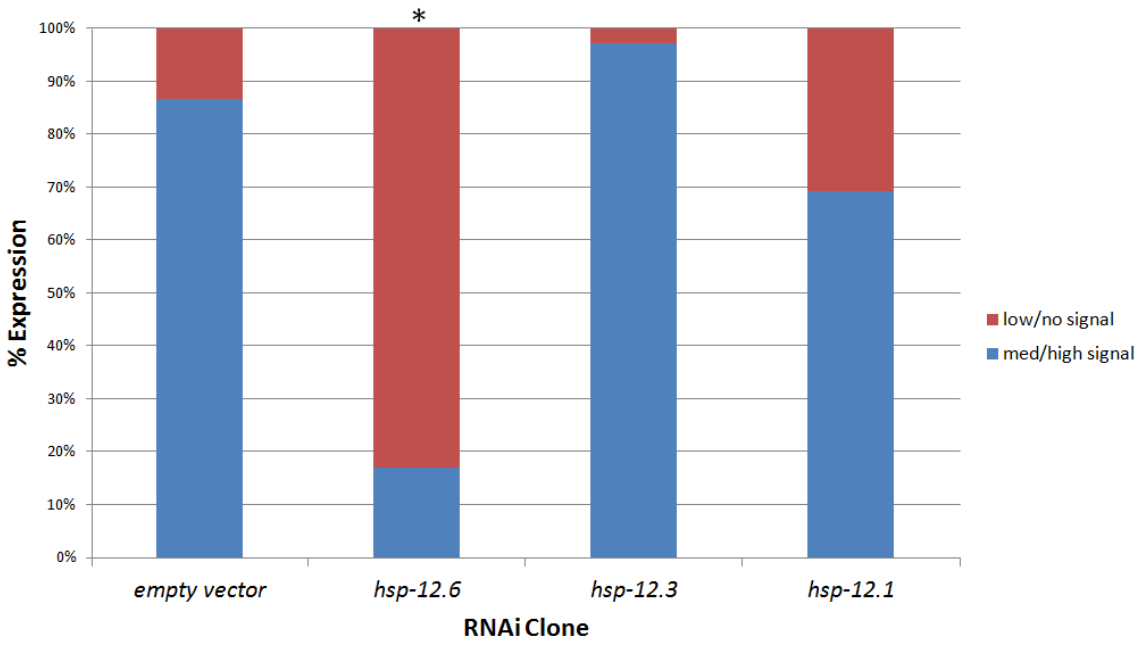


Figure 12 Hsp-12.6(RNAi) Reduces HSP-12.6 Expression

Quantitative assessment of *hsp-12.1(RNAi)*, *Hsp-12.3(RNAi)*, *Hsp-12.6(RNAi)* and pPD129.36 (empty vector control) on *phsp-12.6::HSP-12.6::DSRED2* animals. Expression of *phsp-12.6::HSP-12.6::DSRED2* in the tail was quantified. *Hsp-12.6(RNAi)* knocks down expression of *pshp-12.6::HSP-12.6::DSRED2* while *hsp-12.3(RNAi)* and *hsp-12.1(RNAi)* do not significantly lower reporter gene expression when compared to animals fed empty vector. n=45 for empty vector control, n=41 for *hsp-12.6(RNAi)*, n=37 for *hsp-12.3(RNAi)* and n=52 for *hsp-12.1(RNAi)*. Asterix indicates significance $p<0.001$.

Table 1 p values for pshp-12.6::HSP-12.6::DSRED2 Animals Treated with hsp-12.1, hsp-12.3 and hsp-12.6 RNAi

RNAi clone	*p* value	versus
pPD129.36 empty vector	*0.0001	*hsp-12.6(RNAi)*
	1.21E-01	*hsp-12.3(RNAi)*
	5.26E-02	T22A3.2 *hsp-12.1(RNAi)*
F38E11.2 *hsp-12.6(RNAi)*	*0.0001	*hsp-12.3(RNAi)*
	*0.0001	*hsp-12.1(RNAi)*
F38E11.1 *hsp-12.3(RNAi)*	*0.0007	*hsp-12.1(RNAi)*

*$p<0.05$

2.3. Longevity Assays

2.3.1. Optimizing Conditions for Longevity Assay Experiments: 20°C Utilizing S. Basal Medium and FUDR

A series of experiments were performed to optimize conditions in order to obtain reliable results for longevity assays. Consistent with previous reports, utilizing S. Basal medium and 5-fluoro-2'deoxyuridine (FUDR) resulted in the most reproducible data with regards to lifespan and does not directly influence lifespan (Mitchell et al., 1979; Alper et al., 2010; Arantes-Oliveira et al., 2002). When the animals were not staged to L1 by using S. Basal, lifespans were inconsistent between triplicate experiments. In addition, the use of FUDR to cause sterility ensured that only the F1 animals were scored and eliminated the risk of accidental counting of F2 progeny on plates.

Due to the nature of HSP-12.6 being a heat shock protein, it was important to determine an appropriate temperature at which to conduct longevity assays. While it was was found that assays performed at either 20°C and 30°C were consistent (Figure 13), to eliminate the possibility of any confounding heat shock temperature effects, it was decided to focus on the role of HSP-12.6 in ageing under normal growth conditions at 20°C.

2.3.1.1. Optimizing Temperature for Longevity Assays: 20°C vs. 30°C

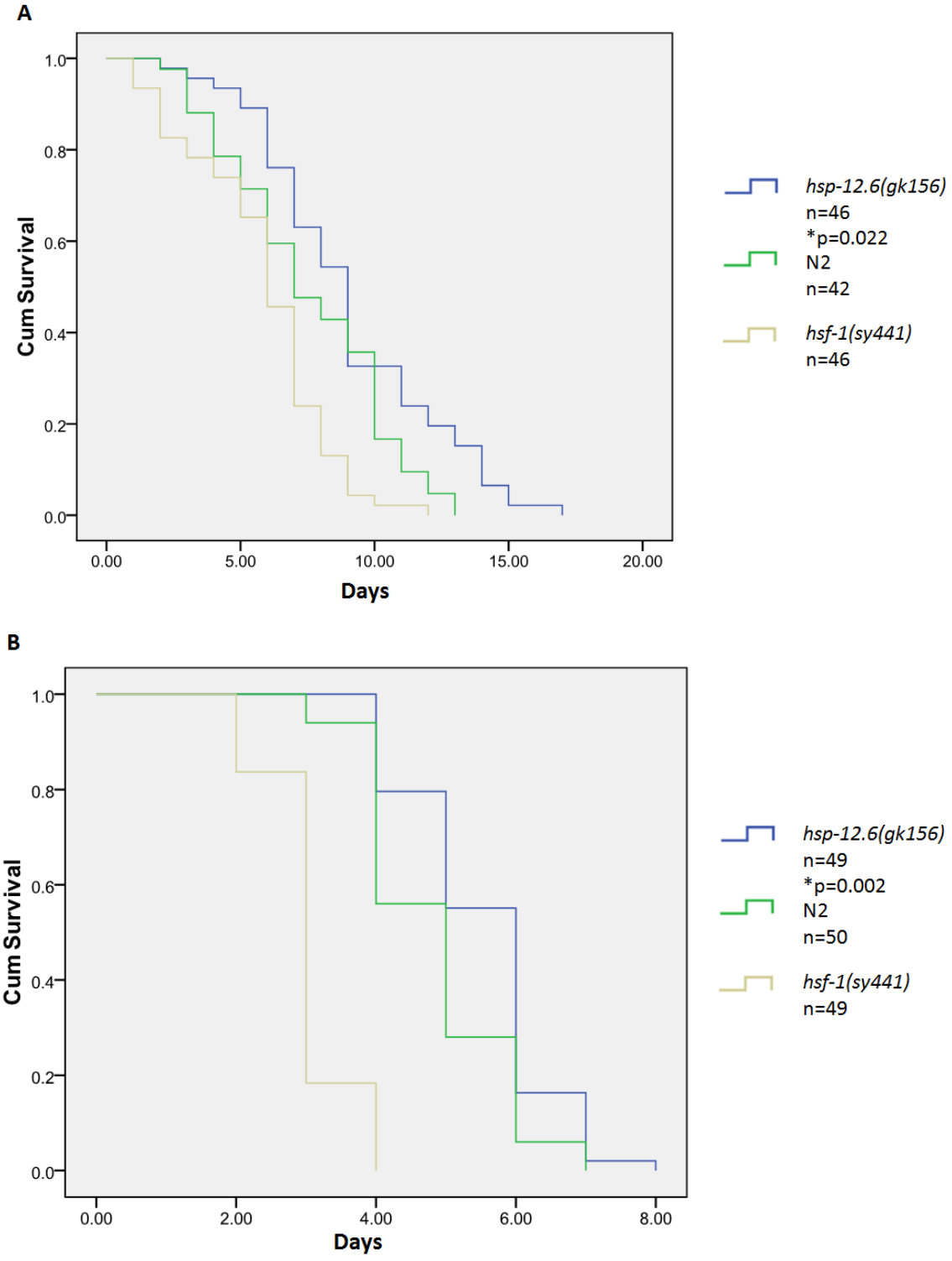


Figure 13 Longevity Assay of hsp-12.6(gk156) at 20°C and 30°C

Longevity assays using N2, *hsp-12.6(gk156)* and *hsf-1(sy441)* at both 20°C and 30°C. Longevity assays were performed in triplicate, however, each data line shown is one representative assay. An asterix (*) indicates statistical significance. A) Longevity assay performed at 20°C. N2 and *hsf-1(sy441)* controls behaved as expected: *hsf-1(sy441)* had a median survival of 6 days, which is lower than the median survival of N2 which was 7 days. Median survival for *hsp-12.6(gk156)* was 9 days, an extension of lifespan by 2 days when compared to N2 (p=0.022). B) One of three longevity assays performed at 30°C. Each of the three assays showed statistical significance between *hsp-12.6(gk156)* and both N2 and *hsf-1(sy441)* (p<0.05). Median survival of N2 was 5 days, while *hsf-1(sy441)* was 3 days and *hsp-12.6(gk156)* was 6 days.

2.4. HSP-12.6 Promotes Longevity

2.4.1. Overexpression of HSP-12.6 Extends Lifespan

Hsp-12.6 has been implicated to have a role in longevity as *hsp-12.6(RNAi)* reduces lifespan of N2, *daf-2(e1370)*, *daf-2(mu150)* and HSF-1 overexpressing animals (Hsu et al., 2003; Murphy et al., 2003). *hsp-12.6(RNAi)* shortened the extended lifespan phenotype of *daf-2(e1370)* by approximately 25% and *daf-2(mu150)* animals significantly (Hsu et al., 2003; Murphy et al., 2003).

Since *hsp-12.6(RNAi)* reduced lifespan, it was expected that an overexpression of *hsp-12.6* would extend lifespan. To test this hypothesis, longevity assays of animals carrying the *phsp-12.6::HSP-12.6::DSRED2* translational fusion were tested alongside a loss of function allele *hsf-1(sy441)* to act as a negative control. As predicted, animals carrying the overexpressing *phsp-12.6::HSP-12.6::DSRED2* translational fusion lived two days longer compared to wild-type animals (Figure 14). In contrast, loss of HSF-1 in the allele *hsf-1(sy441)* shortened lifespan markedly (Figure 14). This result is consistent with the role of HSF-1 in the transcription of ageing effectors. Thus when there is a reduction of cellular HSP-12.6, the lifespan of *C. elegans* is decreased and conversely, when HSP-12.6 is overexpressed, lifespan of *C. elegans* is extended. These results suggest that HSP-12.6 has a small, but statistically significant positive role in longevity.

Due to the fact that *hsp-12.6(RNAi)* reduces lifespan, it was expected that a deletion allele of *hsp-12.6* would similarly reduce lifespan. However, this was found not to be the case, as *hsp-12.6(gk156)* extended lifespan in a similar manner to the overexpressing transgenic *phsp-12.6::HSP-12.6::DSRED2* line (Figure 14). *Hsp-12.6(gk156)* significantly extended lifespan when compared to both N2 ($p< 0.0001$) and the negative control *hsf-1(sy441)* ($p< 0.0001$). There was no significant difference in lifespan between the *hsp-12.6(gk156)* allele and *phsp-12.6::HSP-12.6::DSRED2* ($p=0.2509$), suggesting that they both extended lifespan to a similar degree.

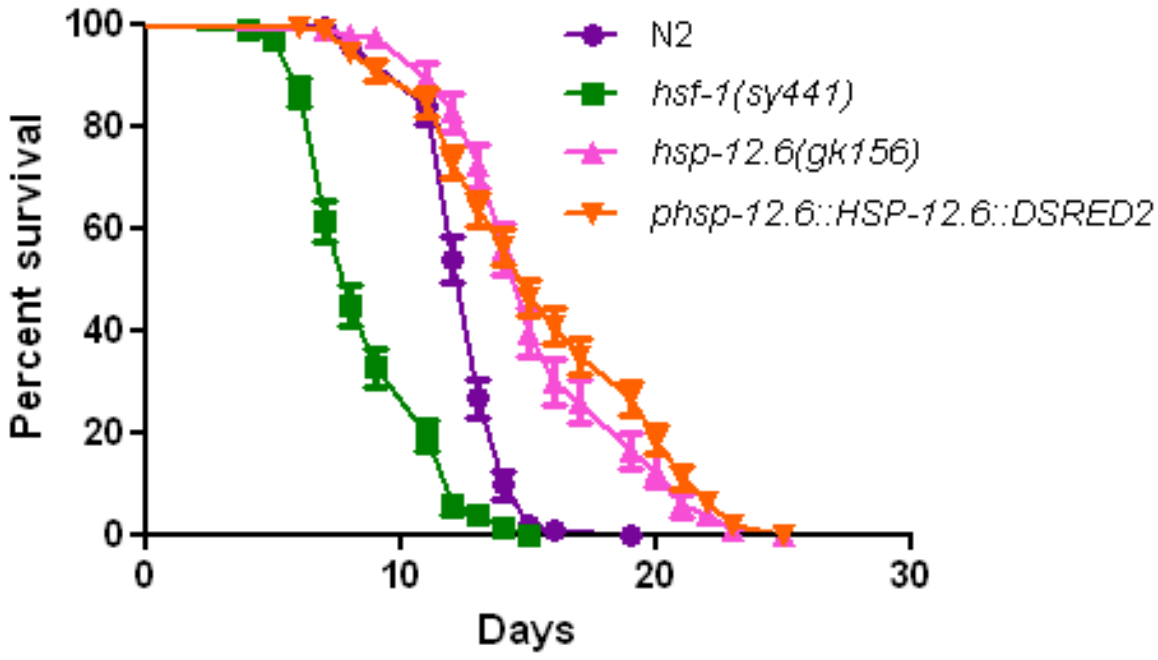


Figure 14 Longevity Assay of hsp-12.6(gk156) and phsp-12.6::HSP-12.6::DSRED2 at 20°C in S. Basal Medium and FUDR

Hsp-12.6(gk156) causes a statistically significant extension of lifespan compared to wild-type animals. *p* value is <0.0001 for *hsp-12.6(gk156)* versus both N2 and *hsf-1(sy441)*. *pshp-12.6::HSP-12.6::DSRED2* causes a statistically significant extension of lifespan. *p* value is < 0.0001 between *phsp-12.6::HSP-12.6::DSRED2* and both N2 and *hsf-1(sy441)*. *hsp-12.6(gk156)* and *pshp-12.6::HSP-12.6::DSRED2* are not statistically significant from one another (*p*=0.2509). *p* value is < 0.0001 between *phsp-12.6::HSP-12.6::DSRED2* and both N2 and *hsf-1(sy441)* and also between *hsp-12.6(gk156)* and both N2 and *hsf-1(sy441)*. Median survival days are as follows: 8 days for *hsf-1(sy441)*, 13 days for N2 and 15 days for both *hsp-12.6(gk156)* and *pshp-12.6::HSP-12.6::DSRED2*. Experiments were performed three times and data is representative of an average of these three assays.

Additional *p* and n values and median survival for strains N2, *phsp-12.6::HSP-12.6::DSRED2*, and *hsf-1(sy441)* longevity assays at 20°C from Figure 14.

Table 2 p and n values and Median Lifespan for Figure 14 Longevity Assay of N2, hsp-12.6(gk156), phsp-12.6::HSP-12.6::DSRED2 and hsf-1(sy441) at 20°C

Strain	Genotype	Median survival (days)	n	P value	versus
BC49	N2	13	124		
VC281	*hsp-12.6(gk156)*	15	104	*< 0.0001	BC49
				*< 0.0001	hsf-1
				0.2509	VA314
VA314	*phsp12.6::HSP-12.6::DSRED2*	15	195	*< 0.0001	BC49
				*< 0.0001	hsf-1
HSF-1	*hsf-1(sy441)*	8	150	*< 0.0001	BC49

Data is representative of triplecate experiment. $p<0.005$ is significant and indicated by an asterix.

2.4.2. Hsp-12.6(gk156) Carries A Possible Duplication of hsp-12.6

Strain VC281 with genotype *hsp-12.6(gk156)* was obtained from the *Caenorhabditis* Genetics Center (CGC), who received it from the *C. elegans* Reverse Genetics Core Facility at UBC, which is part of the International *C. elegans* Gene Knockout Consortium. *Hsp-12.6(gk156)* was out-crossed to N2 Bristol three times and made homozygous before it was used in experiments. According to WormBase (WS230), the *gk156* allele is a 743 bp deletion that removes 300 bp upstream of and the entire conserved *hsp-12.6* α-β-crystallin domain and 100 bp of intronic sequence downstream of this exon, leaving only the second exon (Figure 15A).

Because of the surprising result that VC281 *hsp-12.6(gk156)* deletion seemed to extend lifespan (Figure 14), the allele was further investigated to ensure it was a simple deletion and did not contain a complex chromosomal rearrangement. Single worm PCR (swPCR) was performed on wild-type N2 and VC281 *hsp-12.6(gk156)* individual animals to determine the presence of a deletion in *hsp-12.6(gk156)*. Primers were designed to flank 430 bp upstream of the 743 bp *gk156* deletion (external left primer) and 485 bp

downstream of the deletion (external right primer) (Figure 15A; Table 3 in Materials and Methods). In N2 animals, when the external left and right primers are combined in swPCR, a band of 1,659 bp was expected and in *hsp-12.6(gk156)* animals, it was expected to see a band of 916 bp (external left and right primer product minus the deletion 1,659-743=916 bp). As expected in N2 animals, a 1,659 bp band was seen, and in *hsp-12.6(gk156)* animals, a 916 bp band was seen which indicated the deletion was present. However, in *hsp-12.6(gk156)* animals, an additional band at 1,659 bp was seen. This additional band of 1,659 bp suggested the presence of a wild-type copy of *hsp-12.6* somewhere in the genome (Figure 15B).

The internal right primer was designed to begin 74 bp downstream of the deletion sequence (Figure 15A), therefore in a homozygous deletion mutant; there would be no sequence present for the internal primer to anneal to. When the internal right primer was combined with the external left and right primer, product sizes of both 1,659 bp and 650 bp were expected in N2 animals and 916 bp only in *hsp-12.6(gk156)* animals. It seemed that there was preferential selection for the internal right primer rather than the external right when combined with the external left primer as individual N2 animal lanes resulted in 650 bp bands only and not the expected 1,659 bp band also (Figure 15C). The presence of 650 bp bands indicates the presence of a wild-type sequence in N2 worms. Intriguingly, *hsp-12.6(gk156)* animal lanes also resulted in 650 bp bands only and not the expected 916 bp band only as would be expected in a deletion mutant (Figure 15C). The presence of a 650 bp band in *hsp-12.6(gk156)* animals indicates that the internal right primer is able to anneal to a wild-type copy somewhere in the genome.

Together, these swPCR results indicated that although the 743 bp deleted copy of the gene is likely inserted in the genome, there is a strong possibility that a wild-type copy is also present somewhere in the genome (Figure 15B and C).

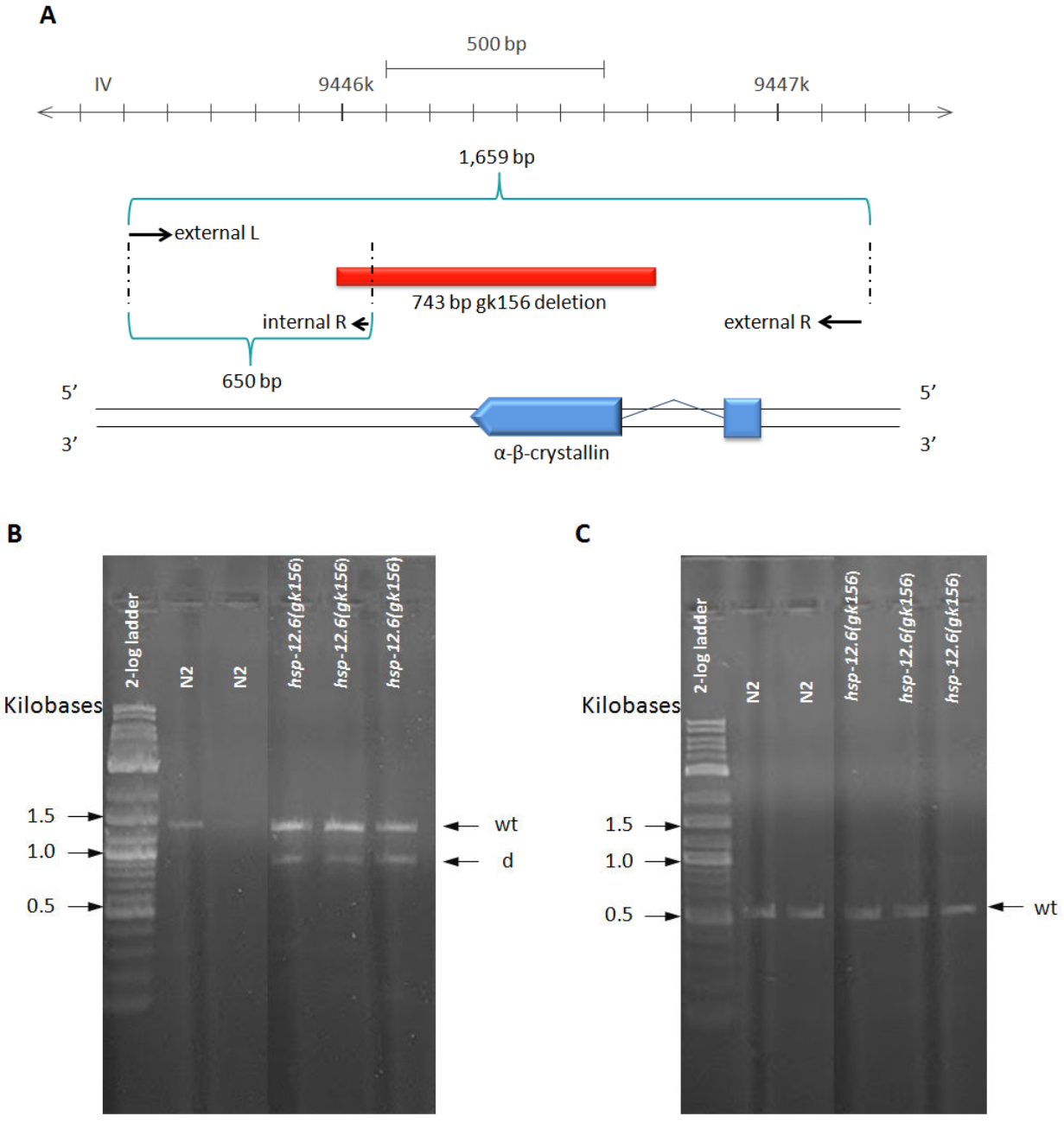

A
500 bp
IV
9446k
9447k
1,659 bp
external L
743 bp gk156 deletion
internal R
external R
650 bp
5'
3'
5'
3'
α-β-crystallin
B
Kilobases
2-log ladder
N2
N2
hsp-12.6(gk156)
hsp-12.6(gk156)
hsp-12.6(gk156)
1.5
1.0
0.5
wt
d
C
Kilobases
2-log ladder
N2
N2
hsp-12.6(gk156)
hsp-12.6(gk156)
hsp-12.6(gk156)
1.5
1.0
0.5
wt

Figure 15 Genomic Characterization of VC281 hsp-12.6(gk156)

VC281 *hsp-12.6(gk156)* genomic characterization. *hsp-12.6(gk156)* carries a possible duplication of *hsp-12.6*. Single worm PCR electrophoresed on an 0.8% agarose gel ran at 110 Volts for ~30 minutes. See Materials and Methods for details. A) *hsp-12.6(gk156)* locus and position of external left and right primers and internal right primer. *hsp-12.6(gk156)* was provided by the *C. elegans* Reverse Genetics Core Facility at UBC, which is part of the International *C. elegans* Gene Knockout Consortium and was received from the CGC and out-crossed 3 times. The *gk156* allele contains a 743 bp deletion (red box), removing the conserved α-β-crystalllin domain (blue box) and 100 bp of downstream intronic sequence (WormBase WS230). The *gk156* allele is found on chromosome IV and spanned the following base pairs: 9,445,978-9,446,720 (WormBase WS230). The external left primer began 430 bp upstream of the deletion while the external right primer began 485 bp downstream of the deletion which resulted in a product size of 1,659 bp and eliminates both exons of *hsp-12.6* (blue boxes). The internal (poison) right primer began 74 bp downstream of the deletion sequence which, when combined with the external left primer, created a product size of 650 bp. B) External left and right primers only, showed 1,659 and 916 bp bands. Lanes 1 and 2 were individual N2 animals while lanes 3-5 were individual *hsp-12.6(gk156)* animals. C) External left and right primers and internal poison right primer, showed 650 bp bands. It seemed that there was a preferential selection for the internal right primer rather than the external right when combined with the external left primer. If *hsp-12.6(gk156)* contained only a deletion and not a wild-type copy of *hsp-12.6*, only a 916 bp and not a 650 bp band would be expected.

2.4.3. Hsp-12.6 Operates in a daf-16 Pathway

Given that the *hsp-12.6*(*gk156)* allele was suspected of containing a wild-type copy of the gene somewhere in the genome, and having confirmed that *hsp-12.6(RNAi)* specifically knocks down the expression of the *phsp12.6::HSP-12.6::DSRED2* expression in transgenic animals (Figures 11 and 12), *hsp-12.6(RNAi)* was utilized in subsequent longevity experiments to reduce *hsp-12.6* expression *in vivo*.

Hsu et al. (2003) reported that *hsp-12.6(RNAi)* shortened the lifespan of N2 animals. Consistent with this result, we found that *hsp-12.6(RNAi)* decreases lifespan of wild-type animals by 2 days (Figure 16A). While the median survival of wild-type animals was 19 days, median survival of *hsp-12.6(RNAi)* animals was 17 days. This result suggested that a reduced function of *hsp-12.6* decreased lifespan of *C. elegans*.

Further, it was of interest to investigate whether the extended lifespan phenotype of animals overexpressing HSP-12.6 could be reduced by *RNAi*. This was found to be the case, as *hsp-12.6(RNAi)* reduced the extended lifespan of *phsp-12.6::HSP-12.6::DSRED2* animals by 1 day (Figure 16B).

Hsp-12.6(RNAi) was also used in longevity assays to place HSP-12.6 into the IIS pathway with respect to the transcription factors DAF-16 and HSF-1. It was found that *hsp-12.6(RNAi)* further reduced the already shortened lifespan of *hsf-1(sy441)* animals by 1 day (Figure 16C). While the median survival of *hsf-1(sy441)* was 13 days, lifespan was only 12 days when *hsf-1 (sy441)* animals were fed *hsp-12.6(RNAi)*. This result suggests that *hsp-12.6* regulation of lifespan is at least not solely dependent on the HSF-1 transcription factor, even though sequences identical to consensus HSF-1 binding sites were found upstream of *hsp-12.6* (Hsu et al., 2003).

In sharp contrast, *hsp-12.6(RNAi)* on *daf-16(mu86)* animals did not alter lifespan (Figure 16D), suggesting that the DAF-16 transcription factor is necessary and sufficient for HSP-12.6 function with regards to lifespan. The median lifespan was 12 days for *daf-16(mu86)* on both empty vector control and on *hsp-12.6(*RNAi). This result was consistent with the *hsp-12.6* promoter containing upstream DNA sequences identical to consensus binding sites for DAF-16 (Hsu et al., 2003).

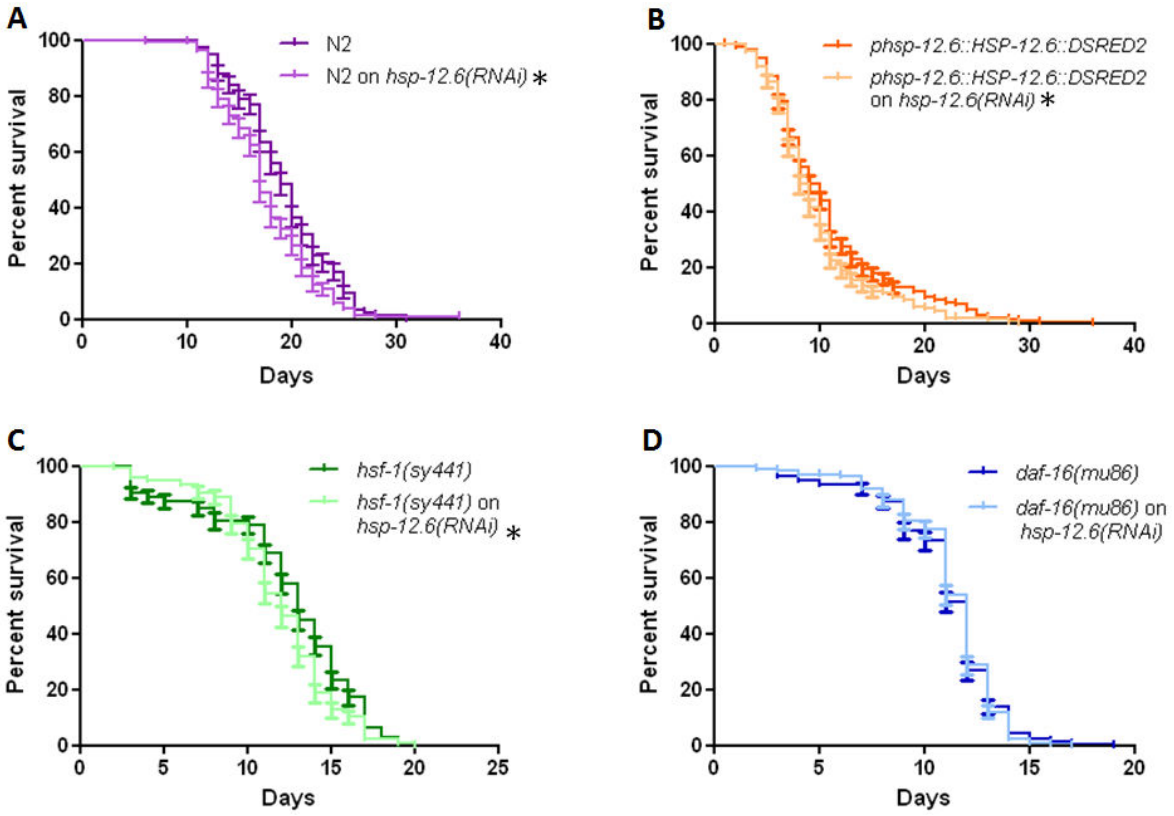


Figure 16 Hsp-12.6(RNAi) Decreases Lifespan in a daf-16 Mediated Pathway and May Function Independently of hsf-1

N2, *phsp-12.6::HSP-12.6::DSRED2*, *hsf-1(sy441)* and *daf-16(mu86)* on *hsp-12.6(RNAi)* Data is representative of the average of three trials. A) *Hsp-12.6(RNAi)* decreased lifespan of wild-type animals by 2 days p=0.0007. Median survival of N2 on empty vector pPD129.36 was 19 days and on *hsp-12.6(RNAi)* was 17 days. B) *Hsp-12.6(RNAi)* decreased lifespan of *pshp-12.6::HSP-12.6::DSRED2* by 1 day p=0.0090. C) *Hsp-12.6(RNAi)* decreased lifespan of *hsf-1(sy441)* by 1 day p=0.0064. Median survival of *hsf-1(sy441)* on empty vector control was 13 days while on *hsp-12.6(RNAi)* was 12 days. D) *Hsp-12.6(RNAi)* did not significantly alter lifespan of *daf-16(mu86)* p=0.9775. Median lifespan was 12 days for *daf-16(mu86)* on both empty vector control and on *hsp-12.6(RNAi)*. * indicates statistical significance p<0.05

2.5. Other Members of the HSP-12 Family May Not Have a Role in Ageing

HSP-12.6 is a member of a family of small 12-kDa heat shock proteins that share considerable amino acid sequence similarity (Figure 7; Kokke et al., 1998). Therefore, it was of interest to investigate whether there was any functional overlap between the members of the HSP-12 family. To address this issue, *hsp-12.1(RNAi)* was utilized in longevity assays on wild-type, *phsp-12.6::HSP-12.6::DSRED2*, *hsf-1(sy441)* and *daf-16(mu86)* animals (Figure 17).

Surprisingly, there was no alteration of lifespan in N2 animals treated with *hsp-12.1(RNAi)* (Figure 17A). The median survival for N2 animals on both empty vector and *hsp-12.1(RNAi)* was 23 days. Moreover, the extended lifespan phenotype of *phsp-12.6::HSP-12.6::DSRED2* was unaltered at 26 days when treated with *hsp-12.1(RNAi)* (Figure 17B). This result confirmed that the *hsp-12.1(RNAi)* treatment did not target the overexpressed HSP-12.6 (as previous results in Figure 12 indicated) and suggested also that *hsp-12.1* is not a determinant of lifespan.

Loss of function mutations in the transcription factors HSF-1 and DAF-16 were also treated with *hsp-12.1(RNAi)* and it was found that neither strain showed a significant change of lifespan. The median lifespan for *hsf-1(sy441)* animals was 10 days on empty vector and 11 days on *hsp-12.1(RNAi)*, however, this was not statistically significant (p=0.9946). The median lifespan was 12 days for both empty vector and *hsp-12.1(RNAi)* on *daf-16(mu86)* animals (p=0.3594).

Taken together, these results suggest that, in contrast to *hsp-12.6*, *hsp-12.1* may not have a role in ageing. However, this hypothesis would need to be confirmed by demonstrating that the *hsp-12.1(RNAi)* clone utilized was actually reducing the levels of *hsp-12.1* RNA and/or protein *in vivo*.

Another HSP12 family member*, hsp-12.3* was previously identified in a microarray analysis as being upregulated when *daf-2* activity is reduced and downregulated when *daf-16* activity is reduced (Murphy et al., 2003). However, no functional analysis with regards to lifespan was performed with *hsp-12.3*. For these

reasons, *hsp-12.3* was of special interest to investigate if it had a role in longevity. This was observed by using *hsp-12.3(RNAi)*.

However, N2 animals treated with *hsp-12.3(RNAi)* did not display a statistically significant alteration of lifespan (Figure 18A). Median lifespan was 23 days on empty vector and 24 days on *hsp-12.3(RNAi)* (p=0.3468). *Hsp-12.3(RNAi)* did not seem to have an effect on *phsp-12.6::HSP-12.6::DSRED2-12.6* lifespan either (Figure 18B). Similarly, neither *hsf-1(sy441)* nor *daf-16(mu86)* animals treated with *hsp-12.3(RNAi)* displayed a significant change in lifespan (Figure 18C and D). As was the case for *hsp-12.1*, these results suggest that *hsp-12.3* may not have a role in longevity; however, it is crucial that the *hsp-12.3(RNAi)* clone be tested to ensure that it is reducing endogenous *hsp-12.3* activity prior to making conclusions.

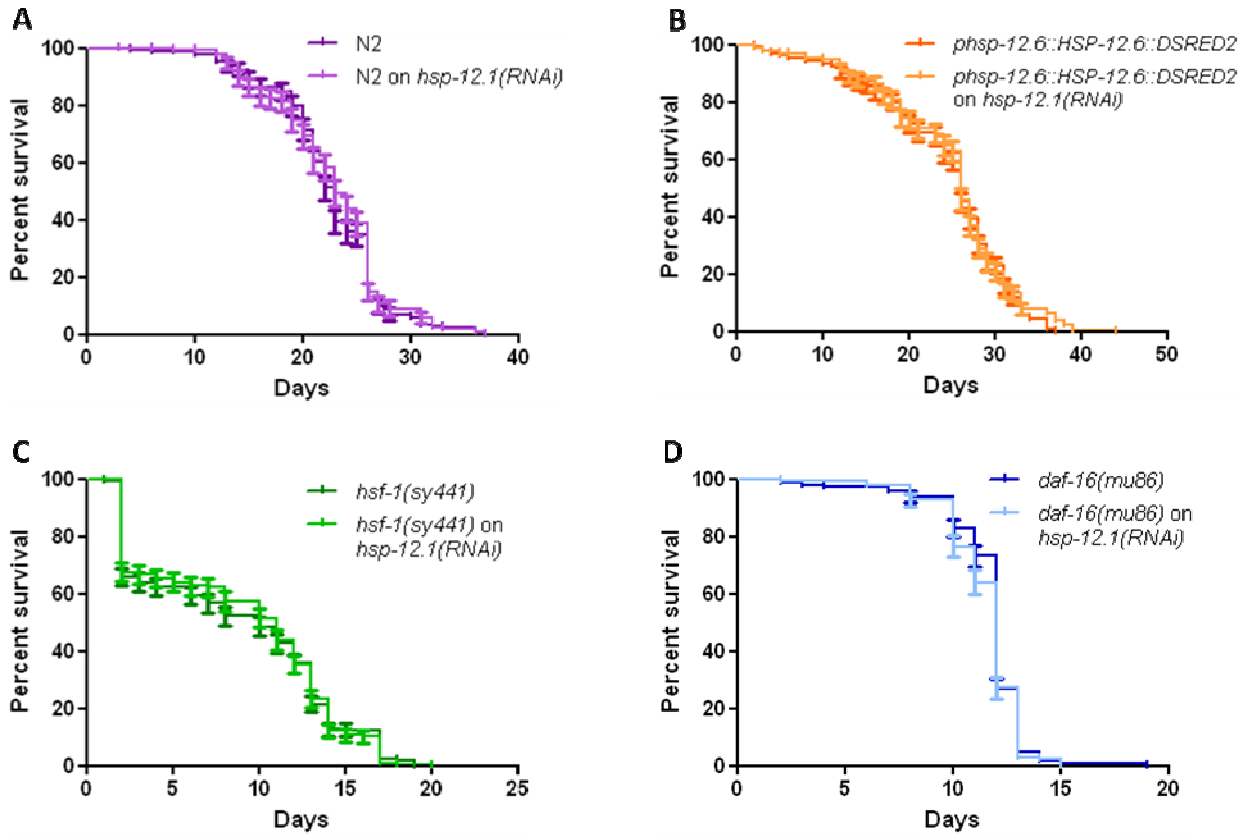


Figure 17 Hsp-12.1(RNAi) Does not Reduce Lifespan

N2, *phsp-12.6::HSP-12.6::DSRED2*, *hsf-1(sy441)* and *daf-16(mu86)* subjected to *hsp-12.1(RNAi)* A) *hsp-12.1(RNAi)* did not significantly affect wild-type lifespan p=0.5460. Median survival was 23 days for both empty vector and *hsp-12.1(RNAi)* treated animals B) *hsp-12.1(RNAi)* did not significantly alter lifespan of *phsp-12.6::HSP-12.6::DSRED2* p=0.3283. Median lifespan was 26 days for both empty vector and *hsp-12.3(RNAi)* treated animals. C) *hsp-12.1(RNAi)* did not significantly affect lifespan of *hsf-1(sy441)* p=0.9946. Median lifespan was 10 days for empty vector and 11 days for *hsp-12.1(RNAi)* treated animals. D) *hsp-12.1(RNAi)* did not significantly alter lifespan of *daf-16(mu86)* p=0.3594. Median lifespan was 12 days for both empty vector and *hsp-12.1(RNAi)* treated animals. Data is representative of the average of three trials.

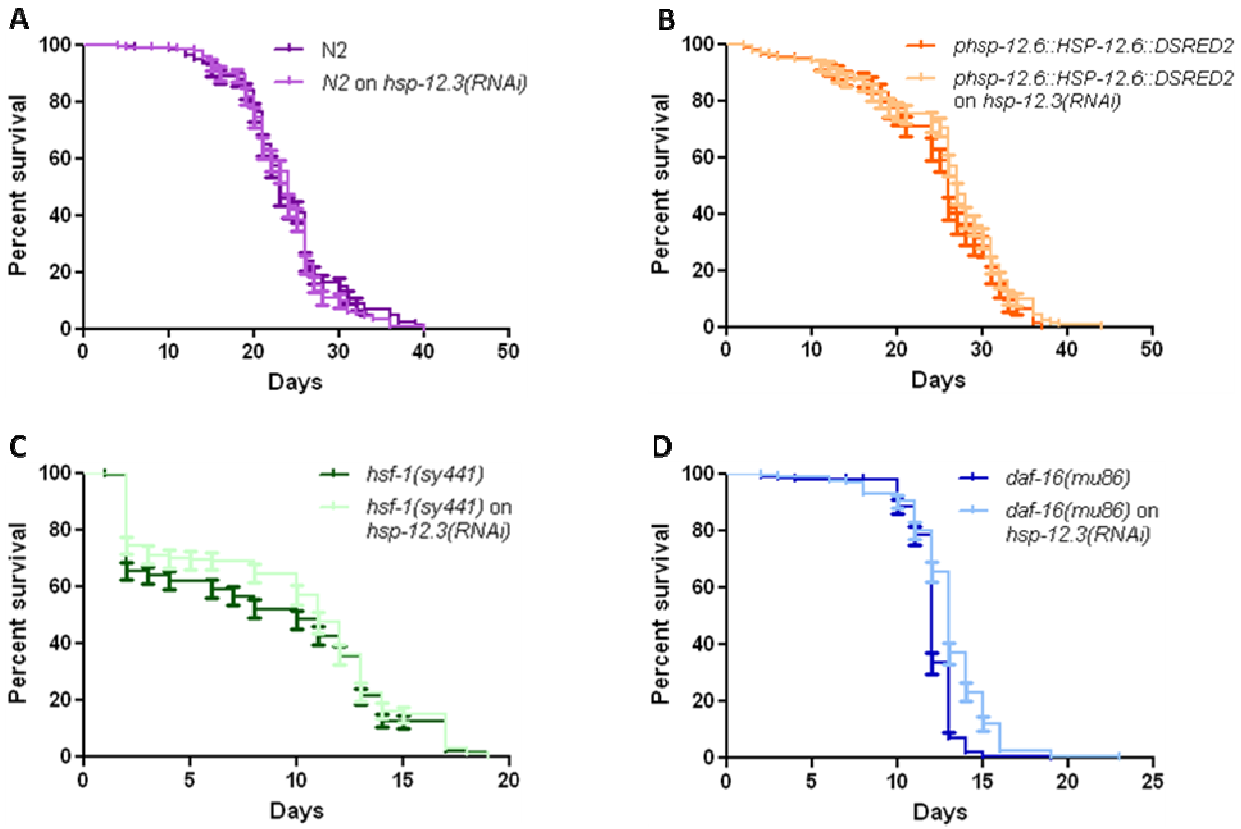


Figure 18 Hsp-12.3(RNAi) Does not Reduce Lifespan

N2, *phsp-12.6::HSP-12.6::DSRED2*, *hsf-1(sy441)* and *daf-16(mu86)* on *hsp-12.3(RNAi)* A) *hsp-12.3(RNAi)* did not significantly alter lifespan of wild-type animals p=0.3468. Median survival was 23 days for empty vector and 24 days for *hsp-12.3(RNAi)* treated animals. B) *hsp-12.3(RNAi)* did not significantly alter lifespan of *phsp-12.6::HSP-12.6::DSRED2* p=0.0677. Median survival was 26 days for empty vector and 27 days for *hsp-12.3(RNAi)* treated animals. C) *hsp-12.3(RNAi)* did not significantly affect lifespan of *hsf-1(sy441)* p=0.2351. Median survival was 10 days for empty vector and 11 days for *hsp-12.3(RNAi)* treated animals. D) *hsp-12.3(RNAi)* increased *daf-16(mu86)* lifespan by 1 day p<0.0001. Median lifespan was 12 days on empty vector and 13 days for *hsp-12.3(RNAi)* treated animals. Data is representative of the average of three trials.

3. Discussion

3.1. Tissue Expression of HSP-12.6

Until now, only immunohistochemical staining using the polyclonal anti-HSP-12.6 antibody had been performed in order for expression of HSP-12.6 to be observed (Ding and Candido, 2000a). In order to view expression of HSP-12.6 *in vivo* in real time, a transgenic line containing a translational DSRED2 fusion was constructed and integrated. This strain, VA314 with genotype *phsp-12.6::HSP-12.6::DSRED2*, can be utilized in many future experiments to further investigate HSP-12.6 function and specific tissue expression and subcellular localization.

It is helpful to have both antibody staining and a translational fusion line to observe HSP-12.6 expression as both techniques exhibit advantages and disadvantages. Immunohistochemical staining was employed to examine normal tissue distributions of endogenous HSP-12.6 (Ding and Candido, 2000a). Although tissue specificity can be determined by antibody staining, the polyclonal anti-HSP-12.6 antibody cross-reacts with other HSP-12s (Leroux et al., 1997a; Kokke et al., 1998), therefore expression represented is not exclusively specific to HSP-12.6 but shows HSP-12kDa expression. An advantage of immunohistochemical staining is that germ cells can be examined: at higher magnification, the distribution of HSP-12 cytoplasmic staining in sperm cells was seen (Ding and Candido, 2000a). In contrast, transgenes tend to poorly express in germline tissues as was seen by generational silencing of *let-858* transgenes (Kelly et al., 1997). This could be a reason why sperm cells were observed by Ding and Candido, 2000 using the HSP-12.6 antibody but not in *phsp-12.6::HSP-12.6::DSRED2* animals in this study. Transgene expression is not necessarily an accurate picture of expression because some control elements such as repressor elements could be eliminated and therefore, tissues that may not normally express the gene of interest may be expressing. However, an advantage of using translational fusion

strains to observe expression of a gene of interest is to view expression levels *in vivo* as the animal develops under different conditions.

Consistent with the results of Ding and Candido (2000a), this study found that HSP-12.6 is constitutively expressed in vulva muscles, suggesting that HSP-12.6 has a normal role in unstressed cells.

Several unknown head neurons were also observed to express HSP-12.6. To determine the identity of these neurons, a cross between *phsp-12.6::HSP-12.6::DSRED2* with neuronal markers expressing GFP or another fluorescent protein could be performed and co-localization could be observed. Sensory cilia are implicated to regulate normal ageing in *C. elegans*, as mutations that affect sensory cilia or their support cells or in sensory signal transduction extend lifespan (Apfeld and Kenyon, 1999). Defects in the IIS pathway in sensory neurons, such as *ifta-2*, result in extended lifespan and defective dauer formation (Schafer et al., 2006). *Ifta-2* is expressed solely in the ciliated sensory neurons in the head and tail of *C. elegans*, as shown in *pifta-2::gfp* animals (Schafer et al., 2006). A cross of *phsp-12.6::HSP-12.6::DSRED2* with *pifta-2::gfp* animals could show whether *hsp-12.6* is expressing in ciliated sensory neurons.

3.2. HSP-12.6 Does Not Localize to Mitochondria

The tissue specificity and unique pattern of sub-cellular distribution in muscle cells gave possible clues as to HSP-12.6 function. More specifically, the punctate pattern of expression seen in body muscle was reminiscent of mitochondrial expression seen in *myo-3*$_{pr}$*::GFP*^{mt} animals (Haynes et al., 2010).

The mitochondria is directly involved in ageing as it regulates oxidative phosphorylation and is involved with heat shock proteins in the unfolded protein response in mitochondria (UPR^{mt}) (Haynes et al., 2010). Therefore, it was hypothesized that the role of HSP-12.6 in longevity might be mediated through mitochondria.

Co-localization between HSP-12.6 and the cytoplasmic fluorescent marker was not seen, suggesting that HSP-12.6 was not uniformly distributed to the cytoplasm of muscle cells. However, it is possible that HSP-12.6 is expressed in the cytoplasm of

muscle cells which may be seen in *myo-3$_{pr}$::GFP^{cyt};phsp-12.6::HSP-12.6::DSRED2* strain at a higher resolution than observed in this study.

DSRED2 is the second generation of the DSRED fluorescent proteins which were derived from the sea anemone *Discosoma striata* (Matz et al., 1999). DSRED is an obligate tetramer and has a tendency to form oligomers which can lead to protein aggregation (Baird et al., 2000). Although DSRED2 has been mutated to increase solubility and reduce aggregation when compared to DSRED (Living Colors^{TM} DsRed2, 2001), it is possible that the puncta seen in muscle cells is due, at least in part, to some aggregation of DSRED2. Use of a monomeric red fluorescent protein such as mCherry would help elucidate whether the puncta are aggregates of the fluorescent protein due to forming oligomers (Shaner et al., 2005).

It is possible that HSP-12.6 could be localized to another part of the muscle such as the muscle spindle fibers. *phsp-12.6::HSP-12.6::DSRED2* appeared to be expressed as striations (parts 1 in each panel of Figure 10). To confirm that HSP-12.6 is expressed in muscle fibers, a cross between a known muscle spindle marker fused to GFP with *phsp-12.6::HSP-12.6::DSRED2* could be performed and observed at high magnification.

While the cause of the punctate staining pattern remains elusive, one possibility is that the puncta correspond to structures within the myofibrils of the body muscle. For example, HSP-25, which also shares homology to α-crystallins and is constitutively expressed, was found to be localized to the dense bodies and M-lines of myofibrils in *C. elegans* (Ding and Candido, 2000b). This punctate pattern of expression was observed in both heat stressed and starved animals. Perhaps the punctate pattern of expression seen in these states is aggregation of proteins that accumulate in aged or stressed animals or perhaps simply an accumulation of DSRED2. Body muscle and neurons are two tissues greatly affected by ageing: muscle cells of young worms are largely resistant to heat stress (due to the stress response largely mediated by HSF-1) and young neurons are highly susceptible to chronic heat stress but can refold or disaggregate proteins during recovery from heat shock (Kern et al., 2010).

3.3. Specificity of *hsp-12.6(RNAi)* Clone

To quantify the specificity of *hsp-12.6(RNAi)*, *phsp-12.6::HSP-12.6::DSRED2* animals were treated with empty vector, *hsp-12.1(RNAi)*, *hsp-12.3(RNAi)* and *hsp-12.6(RNAi)*. Tail expression was the most easily assayed tissue due to the fact that it is brighter than head and vulva expression under the fluorescent stereomicroscope and therefore gave the most consistent data when compared to vulva and head expression data.

HSP-12.6 expression in the tail of animals treated with *hsp-12.6(RNAi)* was reduced while HSP-12.6 expression was not reduced with *hsp-12.1(RNAi)* or *hsp-12.3(RNAi)*, illustrating specificity of the *hsp-12.6(RNAi)* clone. This indicates little to no overlap between the hsp-12kDa family members, and validates both the use of *hsp-12.6(RNAi)* in subsequent experiments as an accurate measure of reducing *hsp-12.6* activity and also that *hsp-12.6* tissue expression in this study was accurately expressing HSP-12.6.

3.4. Optimizing and Non Heat Shock Conditions for Longevity Assays

Longevity assay conditions were optimized with the strains used in this study to obtain the most consistent data with respect to temperature of the assay, the use of S. Basal to synchronize populations and the sterilizing agent FUDR (Mitchell et al., 1979; Alper et al., 2010; Hamilton et al., 2005). *Hsp-12.6* is not significantly up-regulated by heat stress and expression is constitutive (Ding and Candido, 2000a; Leroux et al., 1997a). Using 20°C and 30°C as temperatures for longevity assays produced results where triplicate experiments were consistent with one another. However, to eliminate any possible heat shock temperature effects and in order to compare values to the literature because both *hsp-12.6(RNAi)* experiments in longevity were performed at either 20°C or 25°C (Hsu et al., 2003; Murphy et al., 2003), this study focused on the role of *hsp-12.6* in longevity under unstressed conditions. Future experiments could include longevity assays at the elevated heat shock temperature of 30°C.

3.5. HSP-12.6 in the Regulation of Lifespan

Daf-2 mutant animals live more than twice as long as wild-type (Kenyon et al., 1993). From microarray data, it was seen that *hsp-12.6* was up-regulated when *daf-2* activity was reduced and down-regulated when *daf-16* activity was reduced (Murphy et al., 2003). Therefore, it was hypothesized that, as a downstream target of the IIS pathway, *hsp-12.6* may be contributing to the regulation of longevity. Murphy et al. (2003) tested *hsp-12.6(RNAi)* in *daf-2(mu150)* animals and found that the extended lifespan phenotype was reduced. Furthermore, *hsp-12.6(RNAi)* also decreased the extended lifespan phenotype of *daf-2(e1370)* animals (Hsu et al., 2003).

Consistent with the literature, this study found that the lifespan of N2 animals was decreased in animals treated with *hsp-12.6(RNAi)*. Hsu et al. (2003) found that the lifespan of N2 animals was decreased when treated with *hsp-12.6(RNAi)*. Murphy et al. (2003) also found a decrease in N2 lifespan when treated with *hsp-12.6(RNAi)*. This result further suggests that *hsp-12.6* has a small, but statistically significant role in the regulation of lifespan.

Based on these experiments, it was hypothesized that an overexpression of HSP-12.6, *phsp-12.6::HSP-12.6::DSRED2*, would extend lifespan. While this study did not confirm that *phsp-12.6::HSP-12.6::DSRED2* is indeed overexpressing HSP-12.6, translational fusion strains have been known to act as overexpression lines (Frokjaer-Jensen et al., 2008; Kuwahara et al., 2005). Injection of DNA into the gonad of *C. elegans* forms an extrachromosomal array that is eventually incorporated into the nucleus (Frokjaer-Jensen et al., 2008). These arrays may contain hundreds of copies of the injected DNA, and the genes are overexpressed (Frokjaer-Jensen et al., 2008).

Using *phsp-12.6::HSP-12.6::DSRED2* as a likely overexpression strain, it was found that overexpression of HSP-12.6 extended lifespan of *C. elegans* by two days. This result was consistent with the hypothesis that an overexpression of HSP-12.6 would increase lifespan just as *hsp-12.6(RNAi)* was found to decrease lifespan.

Overexpression of HSP-12.6 extended lifespan of *C. elegans* and *hsp-12.6(RNAi)* selectively reduced *phsp-12.6::HSP-12.6::DSRED2* expression. *Hsp-12.6(RNAi)* shortened the extended phenotype of HSP-12.6 overexpression line *phsp-*

12.6::HSP-12.6::DSRED2. Although this is a small reduction, it is statistically significant and further suggested *hsp-12.6(RNAi)* is specific to HSP-12.6.

Just as overexpression of HSP-12.6 increases lifespan, it was expected that the deletion mutant, *hsp-12.6(gk156)*, would decrease lifespan. However, it was found that *hsp-12.6(gk156)* actually extended lifespan by 2 days when compared to wild-type. Both N2 and *hsf-1(sy441)* control strains acted in a consistent manner with regards to lifespan, as *hsf-1(sy441)* animals showed a much farther reduction in lifespan than N2 animals as was consistent with literature. Subsequent PCR analysis showed that *hsp-12.6(gk156)* carries a wild-type copy of *hsp-12.6* somewhere in the genome. Thus, a significant conclusion is that although *hsp-12.6(gk156)* may contain the deletion, there is a strong possibility that a wild-type duplication exists elsewhere in the genome and this allele cannot be used as a loss of function mutant.

The extended longevity phenotype of *hsp-12.6(gk156)* may be due to multiple wild-type copies inserted in the genome or positional effects of where the extra copy was inserted. Depending on where the wild-type copy inserted, it may be affected by the promoter of another gene, thus changing transcription. To determine if the extended longevity phenotype of *hsp-12.6(gk156)* could reflect multiple copies of HSP-12.6, it would be beneficial to create a mutant that contains an additional copy of HSP-12.6. A single copy insertion of HSP-12.6 in *C. elegans* could be generated by Mos 1-mediated single-copy insertion (MosSCI) described in Frokjaer-Jensen et al. 2008. The single-copy transgenic strain could be assayed to observe a change in lifespan and compared to the *hsp-12.6(gk156)* longevity phenotype. A slightly higher than wild-type longevity phenotype would be expected in the single copy transgenic while an extended phenotype in *hsp-12.6(gk156)* animals would suggest that more than one copy of HSP-12.6 is present.

Due to this finding, *hsp-12.6(gk156)* was not utilized in subsequent longevity assays. It is difficult to know where the wild-type duplication is in the genome, if it is close to the deletion it is unlikely *hsp-12.6(gk156)* could be out-crossed to remove the duplication. If the duplication contains a SNP not contained in the deletion, the duplication could be mapped against SNPS in the region.

3.6. HSP-12.6 Downstream of DAF-16 in the IIS Pathway

Another question posed in this thesis is where *hsp-12.6* fits into the IIS pathway. This was done by treating IIS pathway mutants with *hsp-12.6(RNAi)* in longevity assays. Specifically, the regulation of *hsp-12.6* by the transcription factors HSF-1 and DAF-16 were of interest to investigate as identical sequences to consensus HSF-1 and DAF-16 binding sites were found upstream of *hsp-12.6* (Hsu et al., 2003). Moreover, these two transcription factors are major players in the regulation of lifespan by the IIS pathway as *daf-16* and *hsf-1* are required for the extended lifespan phenotype of *daf-2* mutants (Garigan et al., 2002; Hsu et al., 2003).

A reduction in the extended longevity of HSF-1 overexpressing animals has been seen when they are treated with *hsp-12.6(RNAi)* (Hsu et al., 2003). This result suggests that *hsp-12.6* is acting downstream of *hsf-1* and it was thought that *hsp-12.6(RNAi)* would not further decrease a reduced lifespan in *hsf-1(sy441)* animals. However, when *hsf-1(sy441)* animals were treated with *hsp-12.6(RNAi)*, the already shortened lifespan was further reduced by 1 day. This result suggested that while *hsp-12.6* could still be operating downstream of the *hsf-1* transcription factor, it is possible that under normal growth conditions, *hsp-12.6* does not necessarily require *hsf-1* to alter lifespan of *C. elegans*.

DAF-16 has many target genes that affect lifespan (McElwee et al., 2003; Murphy et al., 2003). While some of these target genes act as molecular chaperones or in the prevention of radical oxygen species to regulate ageing, the function of many other target genes in the regulation of lifespan is not known (Murphy et al., 2003). smHSPs have been implicated to be involved in ageing (Hsu et al., 2003; Murphy et al., 2003). *Hsp-12.6(RNAi)* did not significantly alter lifespan of *daf-16(mu86)*. This result suggested that *daf-16* is required for *hsp-12.6* regulation of lifespan. It can be inferred that as a downstream target of DAF-16, *hsp-12.6* contributes to the regulation of lifespan, although we do not know the mechanism of HSP-12.6 action.

In addition to longevity assays, *hsp-12.6* can be placed in the IIS pathway by crossing *phsp-12.6::HSP-12.6::DSRED2* with *daf-2* mutants. For example, *phsp-12.6::HSP-12.6::DSRED2* can be crossed with *pdaf-16::GFP*. Upon heat shock, DAF-16

enters the nucleus (Henderson and Johnson, 2001), the animals could be heat shocked and then changes in *hsp-12.6* expression levels could be observed.

Discrepancy between N2 longevity in various lifespan assays was seen in this study. Literature values for N2 lifespan is 20.9 ± 0.6 days (Hsu et al., 2003). All of the longevity assays utilizing RNAi in this work are consistent with this value. However, lifespan was decreased in the longevity assay of *phsp-12.6::HSP-12.6::DSRED2* and *hsp-12.6(gk156)*. Although care was taken to ensure that parameters of the assay were controlled such as temperature, it is likely that some variable altered lifespan of the strains. However, all of the strains exhibited a consistent decrease in lifespan, lifespans of the *hsf-1(sy441)* negative control and overexpression line were also consistent relative to N2 and the assays were done in triplicate, therefore, lifespan relative to the controls were analysed.

3.7. *Hsp-12.1* and *hsp-12.3* in Ageing

Due to the amino acid sequence similarity of the HSP-12kDa family and that the 12-kDa family members have been shown to interact with one another (e.g. *hsp-12.2* and *hsp-12.3* form heterotetramers) (Kokke et al., 1998), it was hypothesized that since *hsp-12.6* had a role in the regulation of lifespan, the other members may share a similar function. In addition, like *hsp-12.6*, *hsp-12.3* was identified in the microarray analysis and found to be upregulated when *daf-2* activity is reduced and decreased when *daf-16* activity is reduced (Murphy et al., 2003). No functional lifespan analysis was performed on *hsp-12.3* by that research group. For these reasons, it was hypothesized that *hsp-12.1* and *hsp-12.3* are likely to have a role in longevity or at least be acting downstream of *daf-16*. However, the results obtained in this study for *hsp-12.1(RNAi)* and *hsp-12.3(RNAi)* did not support these possible roles.

There was no alteration of lifespan in N2, *phsp-12.6::HSP-12.6::DSRED2*, *hsf-1(sy441)* or *daf-16(mu86)* animals treated with either *hsp-12.1(RNAi)* or *hsp-12.3(RNAi)*. However, it is not certain that these RNAi clones are functioning to specifically target *hsp-12.1* and *hsp-12.3*. It is necessary to confirm that *hsp-12.1(RNAi)* and *hsp-*

12.3(RNAi) are specifically reducing *hsp-12.1* and *hsp-12.3*, respectively. This could be done by RT-PCR.

It is possible that the four members of the 12-kDa family of smHSPs could be functionally redundant or that they would present a stronger longevity phenotype in combination with one another. If the *hsp-12.1* and *hsp-12.3* RNAi clones were confirmed to be specific against their target genes, future longevity assays using combinations of all of the hsp-12kDa RNAi clones should be performed.

It was hypothesized that due to sequence similarity of *hsp-12.3* and *hsp-12.6*, and the fact that *hsp-12.3* was identified in a microarray analysis as a possible downstream target of *daf-16* in the IIS pathway, that *hsp-12.3* would have a role in longevity. That was not seen here, but, if it was confirmed that *hsp-12.3* alone did not have a role in longevity, it is possible that *hsp-12.3* requires *hsp-12.2* in order to be active in longevity or some other function, as they are known to form heterotetramers (Kokke et al., 1998). Future experiments using *hsp-12.3* in various combinations with the other hsp-12kDa family members would help to elucidate the hypothesis that *hsp-12.3* does not function alone and requires other family members to function.

If specificity of the hsp-12kDa RNAi clones was confirmed, then the result that *hsp-12.1(RNAi)* and *hsp-12.3(RNAi)* do not alter lifespan would indicate that *hsp-12.6* is functioning independently of the other 12-kDa family members.

3.8. Conclusion

As most of the previous information concerning *hsp-12.6* came from biochemical *in vitro* studies (Leroux et al., 1997a), antibody staining (Ding and Candido, 2000a) and *in vivo* functional studies of *hsp-12.6(RNAi)* on a few IIS pathway mutants (Murphy et al., 2003; Hsu et al., 2003), this study has added knowledge concerning characterization and function of *hsp-12.6*.

12-kDa smHSPs are not significantly up-regulated by various stressors including heat shock (Ding and Candido, 2000a; Leroux et al., 1997a). This is consistent with the finding that *phsp-12.6::HSP-12.6::DSRED2* expression is constitutive at 20°C. However,

in comparison to previous expression analysis that used antibody staining, this study illustrated that expression of HSP-12.6 can be seen *in vivo* in real time using the translational fusion *phsp-12.6::HSP-12.6::DSRED2* created. Expression in vulva, anterior neurons and muscle cells was observed. The *phsp-12.6::HSP-12.6::DSRED2* is a new tool that can be used in many future experiments, for example, to determine which tissues are specifically expressing HSP-12.6 at a given stage in the *C. elegans* lifecycle under varying environmental conditions. The IIS pathway functions in *C. elegans* adult lifespan, but HSP-12.6 *in vitro* expression was found to be highest in L1 animals (Leroux et al., 1997a), so is *hsp-12.6* functioning in larval development to set the stage for lifespan extension? As an eye lens protein that protects from cataract formation, α-crystallin is necessary for the duration of the organism's lifetime (Clark et al., 2012; Horwitz, 2003). It is possible that *hsp-12.6* has *in vivo* roles in early developmental stages of *C. elegans* to prevent ageing or age-related disease. *phsp-12.6::HSP-12.6::DSRED2* can be used to view expression changes over the lifespan of the animal to observe real time *in vivo* translation of HSP-12.6.

This study elaborated on *in vivo* functional studies using *hsp-12.6(RNAi)*. It was previously shown that *hsp-12.6(RNAi)* reduced the lifespan of N2, *daf-2(mu150)*, *daf-2(e1370)* and HSF-1 overexpression strains (Hsu et al., 2003; Murphy et al., 2003). Now, it has been shown that the extended phenotype of *phsp-12.6::HSP-12.6::DSRED2* is reduced when *hsp-12.6* activity is reduced. It is remarkable that a small heat shock protein with no *in vitro* chaperone activity (Leroux et al., 1997a) showed small but statistically significant regulation of lifespan. While HSP-12.6 has no *in vitro* chaperone activity, it is possible that it may still have a chaperone-like function *in vivo*. Perhaps the *hsp-12.6* associated prevention of poly-Q aggregates is coupled with longevity regulation (Hsu et al., 2003). A role for hsp-12.6 in the prevention of age-related disease is a topic that is worth further study.

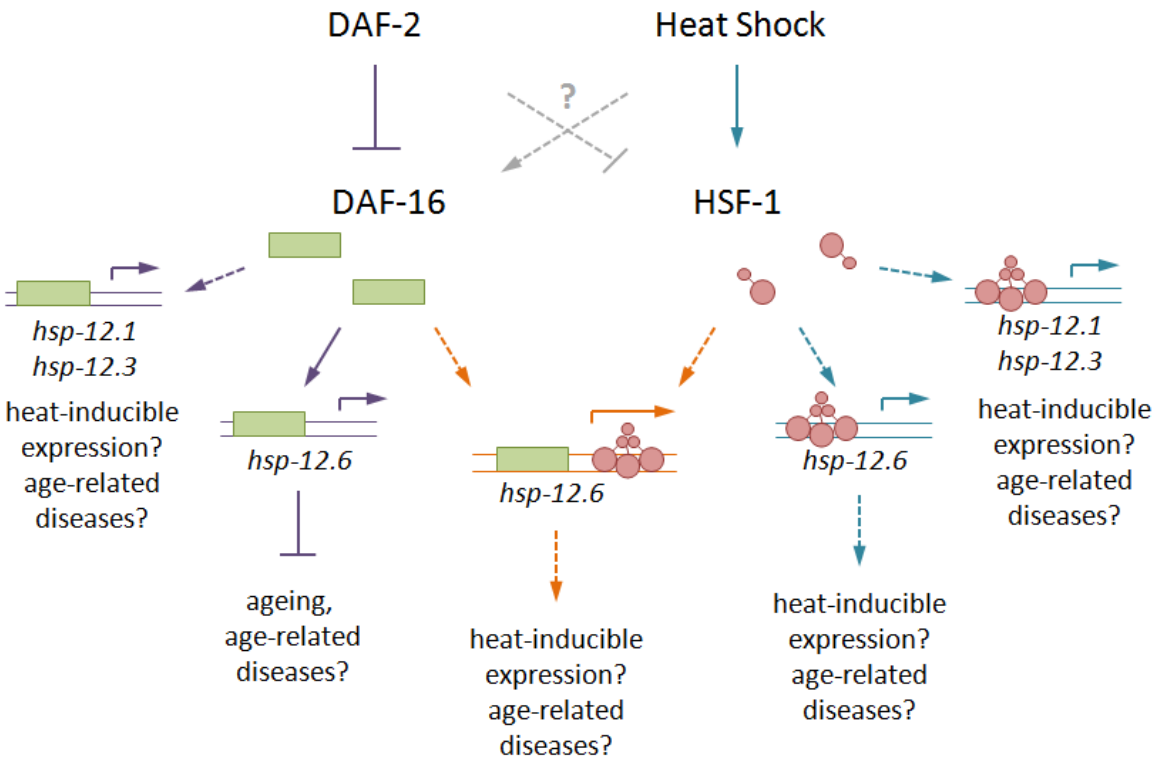


Figure 19 Model for the Role and Placement of hsp-12.6 in the IIS Pathway

As a downstream target of DAF-16, *hsp-12.6* regulates longevity. HSF-1 is not required for *hsp-12.6* directed regulation of lifespan but it may be necessary for *hsp-12.6* heat-inducible or stress-induced expression or *hsp-12.6* age-related disease prevention. *Hsp-12.1* and *hsp-12.3* do not seem to be involved in the regulation of lifespan in conjunction with either DAF-16 or HSF-1. Figure adapted from Hsu et al., 2003.

Hsp-12.6(RNAi) did not alter the lifespan of *daf-16(mu86)* animals which placed *hsp-12.6* as a downstream target of *daf-16* in the IIS pathway (Figure 19). As a downstream target of DAF-16, it is of interest to find out whether DAF-16 is necessary for HSP-12.6 heat-inducible expression by observing *pdaf-16::GFP;phsp-12.6::HSP-12.6::DSRED2* animals under heat shock conditions. It is hypothesized that as *daf-16* enters the nucleus after heat shock, HSP-12.6 expression would increase.

These longevity assays led to fitting *hsp-12.6* into the IIS pathway; it is strongly suggested that the transcription factor DAF-16 is required for *hsp-12.6* directed regulation of lifespan. However, it seemed that the transcription factor HSF-1 is not necessary for HSP-12.6 directed regulation of lifespan (Figure 19). HSF-1 may still play a role in *hsp-12.6* function as sequences identical to consensus HSF-1 binding sites upstream of *hsp-12.6* exist (Hsu et al., 2003). Longevity assays under heat shock conditions could be performed to see if the role of *hsf-1* with *hsp-12.6* is increased under heat shock.

This study raises many questions concerning smHSPs and the regulation of ageing. While this work suggests that *hsp-12.1* and *hsp-12.3* do not have a role in the regulation of lifespan, further work is necessary to confirm these results. It is still possible that the other 12-kDa family members contribute to ageing but members of this family show an overlap or redundancy of function (Figure 19).

This study also added that *hsp-12.6(gk156)* does not function as a deletion mutation due to the suspected presence of a wild-type copy of *hsp-12.6* somewhere in the genome. This allele, if characterized further by mapping and sequencing, might be useful as an overexpression allele.

There is now more knowledge concerning *hsp-12.6* and where it fits into the IIS pathway. This new knowledge is important with regards to elucidating not only the role of *hsp-12.6* in longevity, but also that of DAF-16 within the IIS pathway. The IIS pathway is involved in the regulation of longevity through the transcription factor DAF-16 and it seemed that this is influenced at least in part, by smHSPs like *hsp-12.6*. By characterizing *hsp-12.6* as a downstream target gene of DAF-16, a better understanding of how the IIS pathway influences ageing is gained. The mechanism of action by which

hsp-12.6 influences ageing is yet to be determined and future experiments should be performed to elucidate this function.

4. Materials and Methods

4.1. Strains and Maintenance of *C. elegans*

C. elegans strains were maintained and cultured on nutrient growth media plates seeded with *Escherichia coli* strain OP50 according to Brenner (1974). Unless otherwise stipulated, all genetic crosses and experiments were conducted at 20°C.

The wild-type *C. elegans* strain used in this study was N2 Bristol (Baillie Laboratory strain, BC49) and was a gift from Dave Baillie (Simon Fraser University).

C. elegans strains provided by the *Caenorhabditis* Genetics Center used in this work were: CF1038 *daf-16(mu86)*; PS3551 *hsf-1(sy441)*; and BW163 *him-8(e1489)*.

C. elegans strain VC281 *hsp-12.6(gk156)* was provided by the *Caenorhabditis* Genetics Center who obtained it from the C. *elegans* Reverse Genetics Core Facility at UBC, which is part of the International *C. elegans* Gene Knockout Consortium.

C. elegans strains SJ4157 *myo-3$_{pr}$::gfp^{mt}(zcIs14)* V and SJ4103 *myo-3$_{pr}$::gfp^{cyt}(zcIs21)* were gifts from Cole Haynes (Sloan-Kettering Institute).

The following double mutants were constructed for this work: VA403 *myo-3$_{pr}$::GFP^{mt}*; *phsp-12.6::HSP-12.6::DSRED2* and VA402 myo-*3$_{pr}$::GFP^{cyt}*; *phsp-12.6::HSP-12.6::DSRED2*. The presence of each transgene in the cross was determined by visual inspection of fluorescent reporters.

4.2. Generation and Integration of Transgenic Line VA314 *phsp-12.6::HSP-12.6::DSRED2*

The translational fusion *phsp-12.6::HSP-12.6::DSRED2* was constructed using a PCR fusion approach (Figure 20; Hobert 2002). A and B primers were designed to flank

the *hsp-12.6* gene of interest, with primer A beginning 2kb upstream (Figure 20; Tables 3 and 4). This PCR amplified the *hsp-12.6* genomic region, generating amplicon #1 (Figure 20). Reagent concentrations for PCR #1 were: 1.5µl DNA, 10µl of 5x HF buffer for Phu Long, 1.5µl of 10mM dNTPs, 2.5µl of 20mM of both primers A and B (to a final concentration of 0.5µM each), 0.5µl Phu Long enzyme and dH_2O to 40µl.

A second PCR product using primers C and D amplified the reporter construct, DSRED2 and unc-54 3' UTR, resulting in amplicon #2 (Figure 20; Tables 3 and 5). Primer B adds a 24 bp overlap in frame to the DSRED2 coding region (Figure 20). Reagent concentrations for PCR #2 were: 1µl DNA, 10µl of 5x HF buffer for Phu Long, 1.5µl of 10mM dNTPs, 2.5µl of 20mM of primers C and D (to a final concentration of 0.5µM each), 0.5µl Phu Long enzyme and dH_2O to 40µl.

Nested primers A* and D* were used to fuse PCR amplicons 1 and 2 (Figure 20; Tables 3 and 6). Reagent concentrations for fusion PCR were: 1µl DNA from amplicon #1, 1µl DNA from amplicon #2, 10µl of 5x HF buffer for Phu Long, 1.5µl of 10mM dNTPs, 2.5µl of 20mM of both primers A* and D* (to a final concentration of 0.5µM each), 0.5µl Phu Long enzyme and dH_2O to 40µl. Concentration of amplicon #3 was estimated by gel electrophoresis and was purified using the PCR purification kit by Qiagen and subsequently used to inject into *C. elegans*.

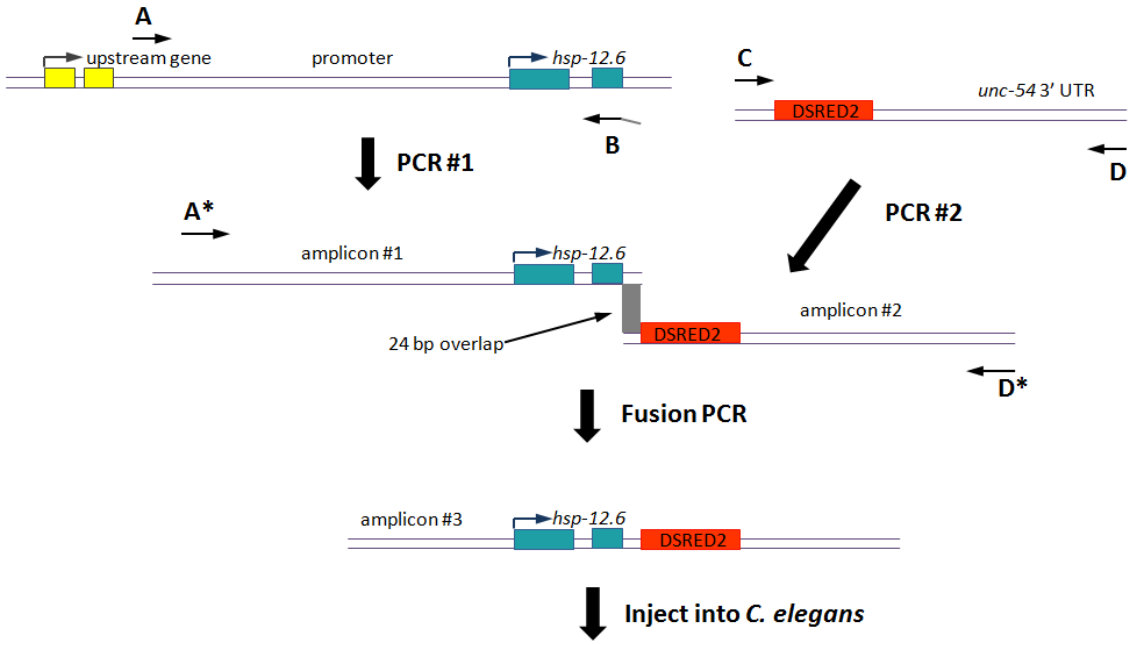


Figure 20 Outline of Fusion PCR Protocol for Generating the phsp-12.6::HSP-12.6::DSRED2 Translational Fusion

Amplicon #1 was generated by using primers A and B using genomic DNA from wild-type worms. Primer B adds a 24 bp overlap (grey box) in frame to the DSRED2 coding region. Primers C and D amplify the DSRED2 reporter gene and 3' UTR of *unc-54*, resulting in amplicon #2. Primers A* and D* are used to fuse amplicon #1 and amplicon #2. This results in amplicon #3 which can be directly injected into *C. elegans*. Figure adapted from Hobert, 2002.

Table 3 PCR Primer Sequences for the Construction of phsp-12.6::HSP-12.6::DSRED2 Translational Fusion

Name	Sequence	Tm
Primer A	5`-TTCTGAACTTCGCCCTCTTC-3`	63.8°C
Primer B	5`-TGACGTTCTCGGAGGAGGCCATATGCATTTTTCTTGCTTCAA-3`	84.1°C
Primer C	5`-ATGGCCTCCTCCGAGAACGTCA-3`	73.3°C
Primer A*	5`-CAAAGAATGTTCTCGATGCAAA-3`	64.3°C

Table 4 Fusion PCR Protocol

Temperature	Duration (minutes)	Repeat
98°C	40 seconds	1x
98°C	10 seconds	30x
55°C	15 seconds	
72°C	1:30 for PCR #1 30 seconds for PCR #2 2 for fusion PCR	
72°C	10	1x
4°C	∞	

The transgene was microinjected at a concentration of 15ng/ml along with a wild-type selectable marker at 65ng/ml into strain *dpy-5(e907)* according to the method of Mello et al. (1991). A single transformed line that transmitted the transgene at the highest frequency was selected for integration. To integrate the transgenic extrachromosomal array, L4 animals were subjected to 1,500 rads of X-rays. Worms were left to recover for one hour at 20°C. 75 irradiated animals were picked onto 5 large plates and the F2 progeny screened to identify homozygous integrants. From this screen, a constitutively expressing line was identified and out-crossed a total of 6 times to generate strain VA314.

4.3. PCR Primers

Primers utilised to characterize the genotype of *hsp-12.6(gk156)* were designed using the program Primer3 (version 0.4.0) (http://frodo.wi.mit.edu/primer3/) (Table 5). The external left primer annealed to the template 430 bp upstream of the putative 743 bp *gk156* deletion (Figure 15A). The external right primer annealed to the template 485 bp downstream of the 743 bp *gk156* deletion. Amplification of the *gk156* deletion allele using this set of primers therefore should theoretically produce a PCR product size of 1,659 bp (Figure 15B). The internal right primer annealed to the template 74 bp upstream of the start of the *gk156* deletion which when combined with the external left primer resulted in a product size of 650 bp (Figure 15C). Tm calculations were

performed using the nearest neighbour method (Breslauer et al., 1986; http://www.finnzymes. fi/tm_determination.html).

Table 5 PCR Primers for the VC281 hsp-12.6(gk156) Duplication Detection

Name	Sequence	Tm
Hsp-12.6(gk156) external left	5`-gtgacgattcgagagcaaca-3`	64.6°C
Hsp-12.6(gk156) poison right	5`-tatcacgctaaccagtattatttacgg-3`	63.9°C
Hsp-12.6(gk156) external right	5`-cgatcccattgaaaacatca-3`	63.7°C

The primers were obtained from Invitrogen™ and were suspended in dH_2O to make a 50mM stock solution. Primers were then further diluted to 20mM for use in the single worm PCR protocol. Primer stock solutions were stored at -20°C.

4.4. Single Worm PCR

Prior to PCR amplification, 5-7 individual gravid worms were picked per strain into individual tubes that contained 7µl lysis buffer. 100µl lysis buffer stock made fresh contained: 10µl Thermopol buffer, 90µl dH_2O and 2.5µl proteinase K. Tubes were subjected to the single worm lysis protocol outlined in Table 3. Each 7µl tube of lysis DNA was then added to individual PCR tubes that contained 40µl of MasterMix. Reagent concentrations for the PCR MasterMix were: 5µl of 10x buffer, 1µl of 10mM dNTPs, 1µl of 20mM of appropriate primers (to a final concentration of 0.5µM), 0.5µl Taq polymerase enzyme and dH_2O to 40µl).

PCR reactions were performed in a BioRad MyCycler according to the protocol outlined in Table 6. The PCR product was then purified using the QIAquick PCR purification kit (Quiagen) and stored at -20°C.

Table 6 Single Worm Lysis Protocol

Temperature	Duration (minutes)
liquid N_2	20
65°C	75
95°C	20
4°C	∞

Table 7 Single Worm PCR Protocol

Temperature	Duration (minutes)	Repeat
95°C	5	1x
95°C	30 seconds	30x
50°C	30 seconds	
72°C	1	
72°C	7	1x
4°C	∞	

4.5. Longevity Assays

Longevity assays were conducted at either 20°C or 30°C, as indicated. Strains were grown at 20°C and were not starved or crowded before use in longevity assays.

All strains were assayed on 2% agarose plates (NGM plates substituted with 20g agarose). Where indicated, longevity assays were performed in the presence of 5µg/ml of the sterilizing agent 5-fluoro-2'deoxyuridine (Mitchell et al., 1979). For each animal assayed, time "0" was defined as L4 + 48 hours while time of death was defined as the point when the animal stopped responding to a prod with a platinum pick. Worms were censored if they crawled to the sides of the plate. Longevity assays were completed in triplecate. Data is representative of the three trials.

4.6. RNA Interference

RNA interference experiments were performed using the feeding method of Kamath et al. (2001). Briefly, *E. coli* HT115 bacteria transformed with RNAi vectors expressing dsRNA of the genes of interest were streaked onto LB plates containing 10ug/ml each of ampicillin and tetracycline and grown overnight at 37°C. 5ml overnight cultures of RNAi bacteria were inoculated in LB containing ampicillin and grown at 37° overnight with shaking at 200rpm.

Overnight cultures of RNAi clones (*hsp-12.1*, *hsp-12.3*, *hsp-12.6* or pPD129.36 empty vector control) were seeded onto IPTG plates and incubated for 24 hours at room

temperature prior to the addition of the animals. Gravid worms from each strain to be tested were picked onto the IPTG plates containing the appropriate RNAi clone, allowed to lay eggs for 24hrs and subsequently removed. The resulting F1 progeny were allowed to grow to adults and were then picked to fresh RNAi plates. F1 worms grew gravid and were screened after 2 days. Representative pictures were taken three days after reaching the adult stage.

T22A3.2 *hsp-12.1*, F38E11.2 *hsp-12.3* and F38E11.1 *hsp-12.6* RNAi clones were gifts from Dave Baillie (Simon Fraser University).

Experiments were blinded and animals were scored for presence or absence of DSRED2 signal. The Fisher exact probability test was used to determine statistical significance $p<0.001$.

4.7. RNAi Longevity Assays

For RNA interference survival assays, plates seeded with bacteria harbouring the appropriate RNAi clone were prepared as previously described, with the exception that FUDR at a final concentration 5mg/ml was included to prevent fertilization of eggs (Alper et al., 2010; Hamilton et al., 2005).

To synchronise animals, eggs were initially hatched in S. Basal medium (to arrest growth at L1) and then allowed to grow for 48hrs on freshly seeded NGM + OP50 plates before being transferred to fresh RNAi + IPTG + FUDR plates. For survival assays this was considered time “0”. Worms were scored for survival every day and transferred to fresh RNAi plates every 5 days.

4.8. Statistical Analysis and Software

The amino acid sequences of the HSP-12kDa family members were aligned using ClustalW2 software (Figure 7).

Nematode survival was analyzed using GraphPad Prism 5 and the Mantel-Cox log-rank test was used to determine statistical significance of differences in survival. *p*-<0.05 was considered statistically significant. SPSS software was used to determine the statistical significance of the longevity assays in Figure 13.

The fisher exact probability test 2x2 was used to determine the exact p value for Table 1 (vassarstats.net).

Confocal images were collected with an Olympus IX80 spinning disk confocal microscope and MetaMorph for Olympus basic software. The low resolution images in Figure 11 were taken with a QImaging Retiga 2000R Fast 1394 camera on Qcapture Pro version 6.0.0.412 software.

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Appendices

Appendix A.

Genome Sequences for the hsp-12kDa Family

The genome sequences for the hsp-12kDa family are below (WormBase WS230). Exons are highlighted in grey.

Genome Sequence for *hsp-12.1* on Chromosome I

ATGCACACAA TTCCAATTAC AACTGACTCG GCGGCTTCTT GGGATTGGCC CCTTCAGCAT
AACGATGGTG TCGTGAAGGT GACCAACACT TCGGAGAAAT TCGAAGTTGG ACTTGATGCT
GGATTCTTTG GACCAAATGA TATTGATGTG AAGGTGAACG GAATCGAAAT TATCATTCAT
TTGCGACATG ATgtgagttt tggaactgcc ggagccgtat attttagtcg gttagggggc accagggaaa agccatttcc
atgcatttat gctaattcta cttttattat tccactttta aagtattgct tacagttttc agaaggtctt aaagatttct aacatcctgc
aattttgaat aacatctgat ttttgctgaa tttccggatt ttagtttatt gtgaaacaaa aataaaatcg gattttatca cactaactcc
agtggaccaa tttatttctt ttcctgaaac atttttcttg aattaaataa aatttttga acttagatta gtggagtatt accagcagag
aaaataacaa aaaccaactc tgtggtgcta aaataaaaca aaaaatacat tttttcggaa tgtttttatt tacagccaaa
agacgtccaa gtttgaaaaa aaatcagcaa tttaccaaaa catagaatga aaactttgaa aagccttttt tacaaattag
catcacaatt tcttatacct cattcattgt catttctata tagagatcag agtctttttt caatcaatta ttaataaaat agttattgca
gAATCGTCCA ACCGAGTACG GAATCGTCAA TCGTGAGGTT CATCGTACTT ATAAACTTCC
AGAAGATGTA GATCCATCAA CTGTTAGATC TCATCTCAAC TCATCCGGAG TTCTCACAAT
CACTGCCAAT AAACTTTAAa aatgttttaa catattcgaa tttttttttc ttctaaattt ttaatttctg ctgaattctg
cttgccataa atgaatgtac aaataaaagt caatattttg g

Genome Sequence for *hsp-12.2* on Chromosome III

atattttcac acttttaatt ttctagagaa aATGTCCGCT ATCGAGGTGA CCGCTGACGC CGCTTCCACC
TGGGATTGGC CACTTCAACA CAACGACGGA GTTGTCAAGg ttcgggatat tttttctctt taatagaagg
ctctgcaatt tttatgagcc aaaatttaat aaaaacgctc atttttctt tttggttaaa tttggaatat aaaccaacca cgacacttta
gtaacttaga ctacctactc ttgaaaaaat aaaacaacaa ttttcgaatg acgctacagt acccctacct taacactact
tcataggaaa cgtaattttt gatattaata agttcacaaa tatatagtaa atcatcaaat ttatcaatac agtgaaaaga tgttgtaata
tgttcagaaa taccttagaa aacttactat gatcgcaata gaagctgtat caaaattaaa gaaaactata tttattttga tagcttctat
ctggattttc gataaattgc atttgtatac tggaatgaat aactagaata gattatgagt ttttcatata agcatagttt caaattacac
ttgaaaaatc agcatgttct gtaatcatat agacacactg taaaatacag tactcttcgc ctagagacga agagtacagt
agtttacagt gtggaaaagt actctgacag tcgtaatgtt aactcgtgta aattctgact gaatattctt aaatttcagG
TCCACAACAC CAAGGAAAAG TTCGAGGTCG GACTTGACGT TCAATTCTTC ACTCCAAAGG
AAATCGAGGT CAAGGTTTCC GGACAAGAGC TTCTCATCCA CTGCCGTCAC GAGgtaagag
ttataattgg tatttataaa attaggaggc tccttttttcg acttcaactc actgaaactt acccattttc tgaattatat aaaatctaac
aaaagaatga tacctatggt agcattgaat atttgttcca aaatatcaaa ttataaaact aaatacagta atatcgtcct
tcaaaaattt ccactgaaca tatgacgtag tagttaaaag acaacaatat gaaccatatt ttgggtctcg ccacgcggac
aatgatttac agaatgattt acagtagctc gtgatgccgt atcttctttt tcaacttaag cagcacagat ttgtttaaag aaaaatatgc
tgaactagat agataattct tataaataga agattaattg tataactctc aactttccag ACCCGCAGTG ACAACCATGG
AACCGTTGCC CGTGAAATCA ACCGTGCCTA CAAACTTCCA GATGACGTTG ATGTTTCCAC
CGTCAAATCC CACCTCGCCA CCCGCGGAGT TCTCACCATC ACTGCTTCCA AGAAGGCTTA
Aatgtctaga acatgattac aaccaaaaaa aacaatattt ttctcaattg aataatatca aattattctt cacttaaata ctttacattt
ctttttgact aatcaaatat tgcaatactt tcaataaatg tacgacttta tg

Genome Sequence for *hsp-12.3* on Chromosome IV

agattcaaca agacttggga ctttttgaaa ctattttctg ataaaATGTC TGTTGCTATT GATCACGATC AAACTGCTAA GTGGGACTGG CCATTCCAGA AAGGAGATGG AGTTGTTAAG gttggttttt taattccaag agagttgaaa tgcttattca aacaattatt agGTTCTGGA CTACGAAGAC CATTTCGAGG TTGGACTTGA GGCTCACAAC TTCTTGCCAA ACGAGATTGA TGTGAAGAAC ATTGGAGAAT TCCTTGAGAT CCACATGGCT CACACAACCA AAGACGATAA GTTTGGATCT ATCACCCGCA GTATTACCAG ATGCTATAGA CTTCCAAAGG GAACCGATCC AGCTACCATC AAGAGCAAGT TGGATGGCAG CGGAATCCTT CACATCTCCG GAAACAAGAA AAAGTAAata caacaatgaa tctgtagtca ttttgttaga aattacataa ctggatgtat cactttctgt tattcatttg acaactgccg tttacaataa atgaatttgc attcata

Genome Sequence for *hsp-12.6* on Chromosome IV

aaaagggtga gcattctgtg aatggacaac aatttcctct ctttctaata ctaacttctg aaaatttcag aaaaatccc aaaaactttt gccaagATGA TGAGCGTTCC AGTGATGGCT GACGAAGGAA CCAAGTGGGA TTGGCCACTT CAAAAGGGAG ATGGAGTTGT CAATgtgagt ttaatattaa tgatttaaaa attattatac ttgaattcca tatcaatcct ttatgaatca caaatataac cgcattaaaa attaaattag aatttcaatt tttttgagca aaaaagaata tcgacagaaa tcaacttcta tttttcatta cttgattcca gGTCCTCGACG ACGATGATCA CTTCGAAGTA GGCCTAGAAG CCCATAACTT CCTCCCAAAA GAGATTGAAG TCAAGAACAT TGGAGAACTT CTTGAAATCC ATATGGAGCA CAATGTAAAG AAGGATTCAT TTGGAGATGT CTCTCGTAAC ATTACTCGTT GTTACAAACT TCCAAAGAAT GTTGATATGA AGACAATCAA GAGCAACTTG GATTCACATG GAATTCTTCA CATTGAAGCA AGAAAAATGC ATTAA

Appendix B.

Reduction of Expression in the Head and Vulva of *phsp-12.6::HSP-12.6::DSRED2* Animals by hsp-12kDa RNAi

RNAi reduction of function was observed for presence or absence of signal in the head and vulva of *phsp-12.6::HSP-12.6::DSRED2* animals treated with *hsp-12.1(RNAi)*, *hsp-12.3(RNAi)*, *hsp-12.6(RNAi)* and empty vector control pPD129.36. However, expression in the head and vulva are not consistent. For example, when *hsp-12.6* is compared to the empty vector of the vulva (Figure 21). Expression is expected to be reduced with *hsp-12.6(RNAi)*, however, the expression of the empty vector was low to begin with, therefore, it was difficult to determine further reduction in expression. Therefore, expression seen in the tail of *phsp-12.6::HSP-12.6::DSRED2* animals was used to determine specificity of *hsp-12.6(RNAi)* as tail expression was higher in empty vector animals.

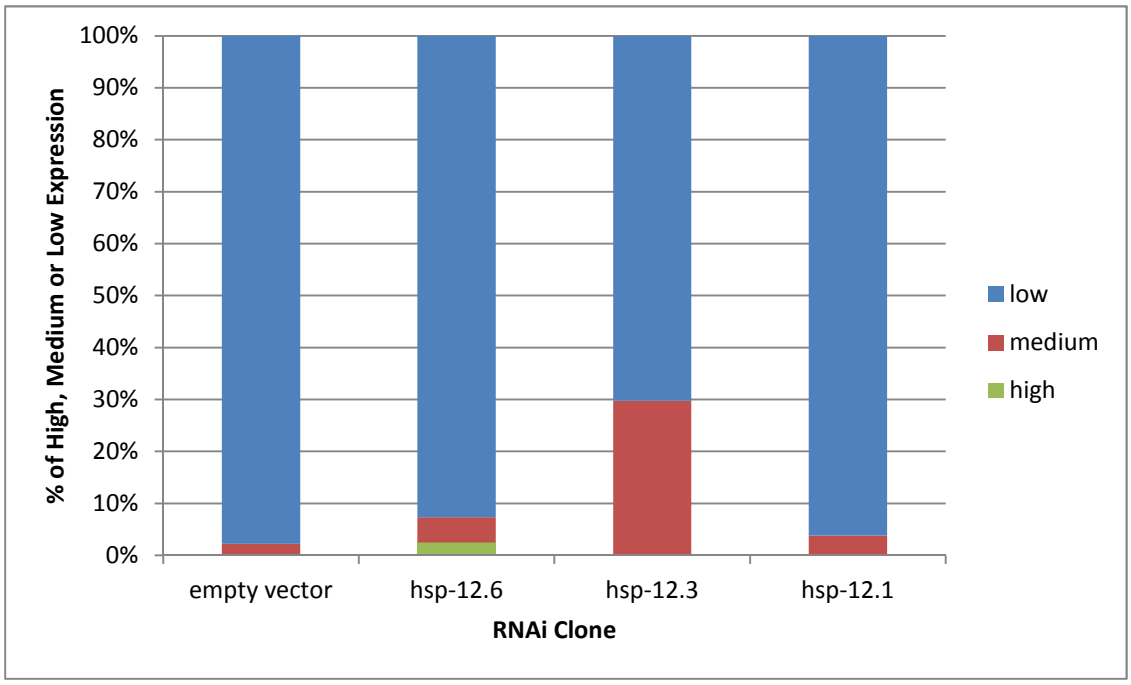

Figure 21 Observed RNAi Reduction of hsp-12.1, hsp-12.3, hsp-12.6 and pPD129.36 (empty vector control) in the Head of phsp-12.6::HSP-12.6::DSRED2 animals

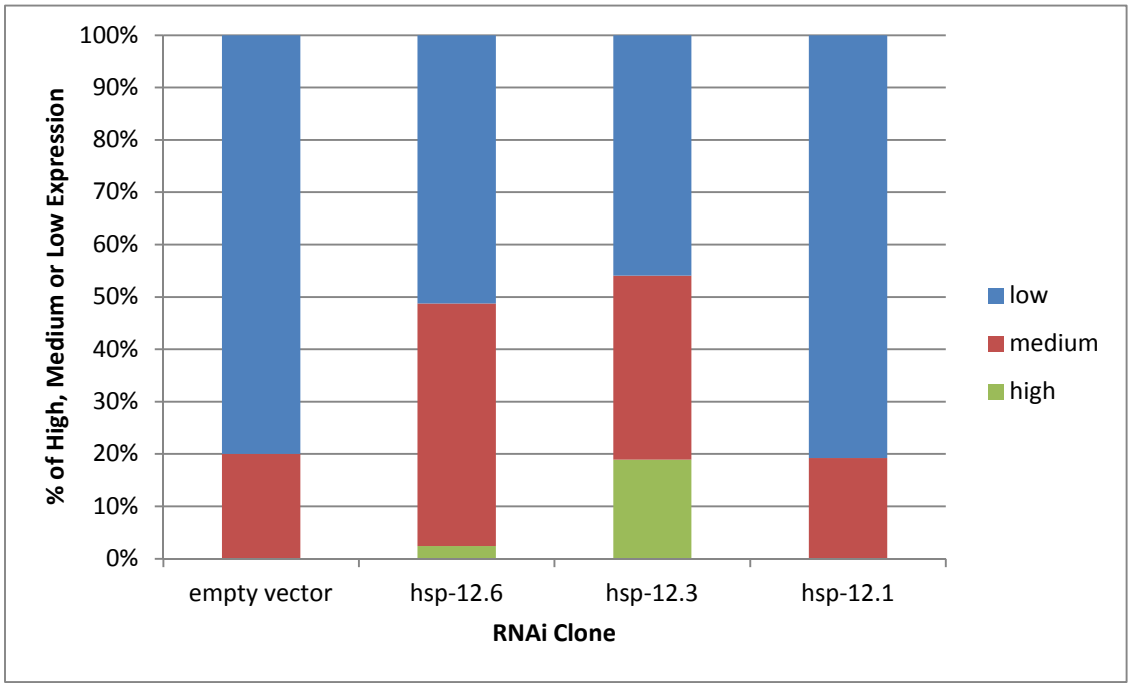

Figure 22 Observed RNAi Reduction of hsp-12.1, hsp-12.3, hsp-12.6 and pPD129.36 (empty vector control) in the Vulva of phsp-12.6::HSP-12.6::DSRED2