GENETIC ANALYSIS OF NOVEL CULLIN RING E3 UBIQUITIN LIGASE COMPLEXES ESSENTIAL IN REPRODUCTIVE DEVELOPMENT AND AGING IN *C. ELEGANS*

by

MOHAMMAD MOSTAFIZUR RAHMAN

(Under the Direction of Edward T. Kipreos)

ABSTRACT

The Cullin RING finger ligases (CRL) are the most abundant class of E3 ubiquitin ligases that facilitates ubiquitin-mediated proteolysis through the 26S proteosome. Cullin4 RING finger ubiquitin ligase (CRL4) is a key regulator of DNA repair and replication. DDB1 is the adapter component of the CRL4 complex, and binds diverse substrate recognition subunits (SRS). Inactivation of the CRL4 components CUL-4 or DDB-1 produces a fully penetrant germline nucleolus defect in *C. elegans*. The DNA content of the germ cells suggests an elongation of G1 phase, and there is no evidence of the DNA re-replication that is observed in somatic cells of *cul-4* or *ddb-1* mutants. We have identified DCAF-1 (DDB1 and CUL4-associated *f*actor) as the SRS specific for CRL4/DDB1 mediated germline function. *dcaf-1* mutant animals develop into sterile adults, however the DCAF-1 protein does not appear to be critical for the soma. We discovered that a reduction of FOG-1 activity rescues the *dcaf-1* germ cell nucleolar morphology defect. FOG-1 is a cytoplasmic polyadenylation element binding (CPEB) protein that is known to act in sperm fate specification in the *C. elegans* gonad.

Post-translational protein modifications (PTM) occurring alone or in different combinations greatly affect a protein's life by modulating its localization, activity, and interaction with other proteins. O-linked-β-N-acetylglucosamine (O-GlcNAc) modification is a regulatory, nucleo-cytoplasmic post-translational glycosylation of proteins associated with age-related diseases such as Alzheimer's, and type II diabetes. Cellular O-GlcNAc levels also influence global protein degradation through the 26S proteosome. Elevation of O-GlcNAc levels can induce insulin resistance in mammals. In *C. elegans*, attenuation of the insulin-like signal transduction pathway increases adult lifespan. We demonstrate that the O-GlcNAc cycling enzymes OGT and OGA, which add and remove O-GlcNAc, respectively, modulate insulin-mediated adult lifespan regulation in *C. elegans*. The O-GlcNAc-mediated effect on lifespan is dependent on the FoxO transcription factor DAF-16. DAF-16 is a key factor in the insulin signal transduction that regulates reproductive development, lifespan, and stress tolerance in *C. elegans*. Our data indicates that O-GlcNAc cycling selectively influences only a subset of DAF-16 mediated phenotypes, lifespan and oxidative stress resistance. Finally, we found that the Cullin1 RING finger ubiquitin ligase (CRL1), which is a key regulator of mitotic cell cycle exit, is also critical for DAF-16 protein stability in aging adults.

Keywords: C. elegans, cell culture, cullin, lifespan, O-GlcNAc, DDB-1, DCAF-1, nucleolus

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MOHAMMAD MOSTAFIZUR RAHMAN

B.Sc., Dhaka University, Bangladesh, 1994

MS, Long Island University, New York, 2002

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

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MOHAMMAD MOSTAFIZUR RAHMAN

Major Professor: Edward T. Kipreos

Committee: Michael McEachern

Jacek Gaertig

Stephen Dalton

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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DEDICATION

To my mentor Shah M Faruque

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CHAPTER 1

GENERAL INTRODUCTION

Intracellular Protein degradation

In mammalian cells, the majority of cellular proteins are continuously being hydrolyzed into amino acids and replenished by new protein synthesis. In a 70-kg adult about 280g of cellular proteins are being synthesized and degraded each day (Mitch and Goldberg 1996). Intracellular and extracellular proteins are being degraded at different half-lives starting from a few minutes for cell cycle regulatory cyclins, to a few days for muscle actin or myosin, to a few months for red blood cell hemoglobin protein turnover (Ciechanover 2005). Deletion of rate-limiting proteins is important for cellular growth regulation, e.g., periodic degradation of cyclins help accurate progression through the cell cycle, and in the absence of food enzymes for the glucose storage pathway are degraded, while during inflammation I-kB is quickly degraded to protect tissues from further injury by allowing activation of cellular tissue repair pathways (Mitch and Goldberg 1996). All these process are vital for mammalian cells to adjust to continuous changes in the extracellular environment. The process of immune response is another key example where protein degradation pathways play a decisive role (Rock and Goldberg 1999). Degradation of cellular proteins is also important for numerous cellular processes in addition to the gene expression regulation at the level of transcription and translation (Ciechanover 2005). Intracellular protein degradation is a very complex, and tightly regulated process as protein homeostasis is crucial to regulate key cellular processes, *e.g.,* signal transduction, transcription and translation, protein quality control, receptor mediated endocytosis, antigen presentation, and modulation of metabolic pathways in response to nutrient availability (Rock and Goldberg

1999; Ciechanover 2005; Grillari, Katinger et al. 2006; Lecker, Goldberg et al. 2006; Nakayama and Nakayama 2006). The balance between proteolysis and translation is key to normal cellular growth, development, and maintenance of healthy lifespan. Deregulation of the protein degradation pathways that could affect cell cycle regulation would result in uncontrolled cell proliferation and tissue growth, genomic instability, and finally cause cancer (Hershko and Ciechanover 1992; Mitch and Goldberg 1996; Rolfe, Chiu et al. 1997). A number of disorders related to muscle wasting, immunity, and inflammatory response are linked to aberrant proteolysis (Ciechanover 2005). The rate of proteolysis decelerates in aging tissues as the ability of the protein degradation machinery decreases with age in almost all organisms analyzed to-date (Martinez-Vicente, Sovak et al. 2005). Severe alteration in cellular protein homeostasis results in enhanced susceptibility to many diseases in old age compared to young animals (Figure 1.1). Most of the aging related neurodegenerative disorders are a consequence of progressive decline in protein degradation ability leading to impaired turnover of damaged proteins, and protein aggregation in aged tissues (Grillari, Katinger et al. 2006).

Metazoan cells utilize multiple protein degradation routes to maintain protein homeostasis (Figure 1.2). Identification of lysosome-dependent proteolysis in the mid-1950s was the first recognition of the significance of intracellular protein degradation in regulating various cellular processes and relevant biological functions [reviewed in (Ciechanover 2005)]. Lysosomes mostly degrade extracellular and cell surface proteins, as well as numerous cytosolic proteins that are engulfed into various autophagic vacuoles. The Lysosome contains mostly cystein proteases, which are important for the hydrolysis of old organelles within a cell (Hideshima, Bradner et al. 2005). In the early 1990s, the hypothesis that proteolysis results from direct substrate and protease interaction lead to the identification of the 26S proteosome as the ‘multi-catalytic protease’ for proteolysis of the majority of intracellular proteins in animal tissues (Grainger and Winkler 1989; Hoffman, Pratt et al. 1992). There are other proteolytic

machines also functional in animal cells, a) calpains function in tissue injury, necrosis, and autolysis; and b) caspases function in hydrolysis of cellular components during apoptosis (Salvesen and Dixit 1997; Goll, Thompson et al. 2003). In metazoa, the autophagy-lysosome pathway also accounts for a large number of protein turnover. In a multi-cellular organism, cellular protein synthesis and degradation is balanced precisely as aberrant synthesis or degradation could lead to pathological consequences. In most mammalian cells, lack of insulin and/or essential amino acids can induce autophagic vacuole formation, and subsequent lysosome-mediated protein degradation. Insulin-mediated activation of protein degradation can be reversed upon re-introduction of insulin in the target tissues (Kadowaki and Kanazawa 2003; Kanazawa, Taneike et al. 2004; Capel, Prod'homme et al. 2009). Insulin promotes cellular growth by inducing protein synthesis through activation of the translation machinery; and also increases the cellular capacity of protein synthesis by increasing the cellular content of ribosomes (Proud 2006). The rate of ribosome synthesis is directly affected by insulin-mediated signaling but the molecular details are still not clear how insulin signaling regulates the number of ribosomes in the cells of a target tissue. On the other hand, insulin also modulates 26S proteosome-mediated protein degradation efficiency in different tissues (Bennett, Hamel et al. 2000; Fawcett, Hamel et al. 2001). Thus the insulin signal transduction pathway plays a vital role in cellular protein homeostasis by regulating both anabolism and catabolism to support growth, and maintenance of animal cells.

Ubiquitin-mediated pathways

Ubiquitin-mediated pathways are known to govern an array of cellular processes, including cell division, cellular growth and differentiation, transcription, apoptosis, signal transduction, and metabolism through both non-proteolytic, and proteolytic mechanisms (King, Deshaies et al. 1996; Pickart 2001; Nakayama and Nakayama 2006). The highly conserved 76-amino acids long 8.5 kilodalton (kDa) ubiquitin molecule is usually attached to a lysine of the

target protein (Thrower, Hoffman et al. 2000). Ubiquitin itself has seven lysine residues (K6, K11, K27, K29, K33, K48, and K63), all of which can be conjugated to another ubiquitin molecule to form polyubiquitin chains similar to its target protein substrates (Ikeda and Dikic 2008). Usually a poly-ubiquitin chain is assembled at K48 of a ubiquitin attached to its target substrate. Once the poly-ubiquitin chain grows enough it can direct the protein substrate to the 26S proteosome for degradation, and the attached ubiquitin molecules are recycled (Hershko and Ciechanover 1992). The 26S proteosome, key to all non-lysosomal protein degradation, is composed of a cylindrical 20S core, which is attached to two 19S subunits at end of the 20S core cylinder, one each. The 19S complexes create a lid and a base that recognizes the poly-ubiquitin tagged protein, unfolds the tagged protein, and facilitates its entry into the 20S cylinder for degradation (Conaway, Brower et al. 2002).

Ubiquitination is a dynamic reversible covalent modification that can be disassembled from target proteins by deubiquitination enzymes (DUBs), which are also known as isopeptidases (Wilkinson 2000). Depending on the structure and nature of ubiquitin conjugation to its target proteins, ubiquitination of cellular proteins also serves many functions independent of 26S proteosome-mediated proteolysis. For, example, ubiquitin conjugation to a different site (K63) rather than the canonical K48 site is associated with protein kinase activation (Mukhopadhyay and Riezman 2007; Chun and Jin 2010). Mono-ubiquitination of histone proteins in regulating chromatin remodeling and gene transcription regulation; and membrane proteins to regulate vesicle trafficking are other well established examples of proteosome independent function of ubiquitin (Sigismund, Polo et al. 2004; Chen and Sun 2009). Ubiquitin conjugation also serves as signaling molecules (Shenoy, McDonald et al. 2001; Geetha, Jiang et al. 2005); and as a sorting signal for substrates to the endosome (Komada and Kitamura 2005). Although ubiquitin serves as a major signaling molecule to eliminate abnormally folded or damaged proteins that arise by synthesis errors or damage by oxygen radicals or by

denaturation. Antigen presentation is also dependent on ubiquitin-mediated proteosome function (Rock and Goldberg 1999). Thus aberrations in the protein ubiquitination process lead to many disorders like chronic kidney disease, loss of muscle protein in uremia, muscle atrophy, and especially failure to present foreign antigens efficiently (Lecker, Goldberg et al. 2006). Precise regulation of proteolysis of critical cell-cycle regulators by ubiquitin-mediated proteosome pathway works as an important mechanism to ensure accurate cell cycle progression; and the first line of defense against uncontrolled cell proliferation.

E3 ubiquitin ligase as a critical regulator in protein degradation

Protein degradation through the 26S proteosome is irreversible unlike most other regulatory mechanisms. The process starts with the activation of ubiquitin molecules by ubiquitin activating enzyme (E1), which catalyzes formation of thiol-ester bond between ubiquitin's C-terminal and a cysteine residue in the E1 enzyme (Haas, Warms et al. 1982). Next the activated ubiquitin is transferred to ubiquitin conjugating enzyme (E2). At last, a ubiquitin ligase (E3) transfers the E2 bound activated ubiquitin molecule to lysine residues either on its recruited substrate or on to another ubiquitin molecule already attached to its recruited substrate to form poly-ubiquitin chains (Figure 1.3). In human and budding yeast there is one major E1, shared by E2 enzymes. There are multiple E2s, e.g., 11 in budding yeast, which function as ubiquitin donors for hundreds of E3 ligases to transfer the activated ubiquitin molecules to target substrate proteins upon recruitment (Lecker, Goldberg et al. 2006). The Anaphase promoting complex (APC) and Skp2-Cullin-F-box (SCF) complexes are two well-studied examples of distinct multi-protein complexes that function in ubiquitin-mediated protein degradation (Bai, Sen et al. 1996; Tyers and Jorgensen 2000). The E3 ubiquitin ligases that work as the scaffold of multi-protein complexes are responsible for the substrate specificity and recruitment of targets to the ubiquitin conjugating complex (Pickart 2001). In eukaryotes, hundreds of E3s are reported, the majority of which fall into two large families, 1) HECT (Homologous to E6-AP

Carboxyl-Terminus) domain, and 2) RING (Really Interesting New Gene) finger domain containing proteins. The HECT domain E3 ubiquitin ligases can transfer ubiquitin directly to its substrate, while the RING finger domain facilitates substrate ubiquitination by recruitment to the complex (Pickart 2001; Rotin and Kumar 2009). While the HECT E3 ligases are involved in recycling and trafficking of cell surface receptors, channels, transporters, and viral proteins; the RING family E3 ubiquitin ligase, APC and Cullin-RING E3 (CRL) mediated complexes are known to function directly in cell cycle progression (Yu, Peters et al. 1998; Tyers and Jorgensen 2000; Petroski and Deshaies 2005; Rotin and Kumar 2009).

CRL complexes

The Cullin-RING E3 ligases (CRL) are the most abundant class of E3 ligases known to-date, and are responsible for ~20% of all the proteins degraded by the 26S proteosome (Petroski and Deshaies 2005). The discovery of Cul1 (Cdc53) from the genetic studies on cell division in the *S. cerevisiae* and *C. elegans* systems were later extended to the discovery of a large family of Cullin RING E3 ubiquitin ligase (CRL) throughout eukaryotes that are involved in a wide array of cellular and organismal processes beyond cell division regulation (Kipreos, Lander et al. 1996; Mathias, Johnson et al. 1996; Willems, Lanker et al. 1996). There are three CRLs in budding yeast, six in *C. elegans* and *D. melanogaster*, nine in *A. thialina*, and seven in mammals (Soucy, Smith et al. 2009). The CRLs utilizes specific adapter proteins with the help of numerous substrate recognition subunit (SRS) components identify, bind and recruit target substrate proteins in the multi-protein CRL complexes for proteosome mediated degradation (Petroski and Deshaies 2005). Thus, Cullin proteins facilitate binding to different family of SRSs, which can assemble hundreds of distinct CRLs to degrade thousands of unique cellular proteins separately. Biochemical insights into CRL complexes came from the solved X-ray crystal structure of the CRL complexes, and numerous separate protein-protein interaction studies (Zheng, Schulman et al. 2002; Petroski and Deshaies 2005; Angers, Li et al. 2006).

CRL1 is a typical example of a Cullin-RING E3 ubiquitin ligase complex based on a Cullin protein scaffold where the adapter molecule (Skp1) binds at the N-terminus close to helical Cullin repeats. On the other hand RING-H2 domain proteins, for example, Rbx1 binds to the C-terminus of Cullin proteins (Zheng, Schulman et al. 2002). Now the adapter molecules in an assembled CRL complex can function actively to recruit various SRSs bound to its target substrate proteins. Even though a variety of protein components are involved in distinct CRL complexes, current evidence implies that all CRL complexes share a more or less conserved modular structure across many different species (Pintard, Willems et al. 2004; Petroski and Deshaies 2005). In *C. elegans,* six distinct CRL complexes are reported but only the CRL1 and CRL4 complexes are shown to function in cell division and DNA replication. CUL1(Cdc53) was first identified in budding yeast as being essential for G1 to S transition by degradation of G1 cyclins, and separately identified in *C. elegans* where inactivation of a conserved CUL-1 function leads to hyperplasia in numerous tissues, while CUL4 has been reported to inhibit DNA replication licensing factors to ensure a single DNA replication event per cell cycle in metazoa (Kipreos, Lander et al. 1996; Kim and Kipreos 2007; Jackson and Xiong 2009).

Post-translation modification of proteins

A variety of chemical modifications observed in proteins are collectively known as post-translational modifications or PTM. The protein modifications are observed to occur alone or in different combinations at various time points during development in a protein's life or dependent on cellular signaling events. PTMs of proteins determine their secondary and tertiary structures, and regulate their activities and functions *in vivo*. Various PTMs of proteins make it possible to regulate metabolic enzymes and other proteins at different levels by modulating protein activity, protein-protein interaction, and/or subcellular localization (Huber and Hardin 2004). Different PTMs of proteins help 'fine tune' their cellular functions. Many of these PTMs are crucial in cellular signal transduction. For example, receptor tyrosine kinases are auto phosphorylated

upon ligand binding to its receptors. Catalytic domain phosphorylation leads to activation of the subsequent downstream signaling pathway by allowing SH2 and PTB domain proteins to recognize and bind the receptor to other effectors (Bevan 2001). PTMs of proteins thus allow various combinations of signaling networks and pathways to function in regulating diverse biological processes (Table 1.1). In addition to activating catalytic enzyme functions by phosphorylation, signaling proteins are also modified by myristoylation, farnesylation, cysteine oxidation, ubiquitination, acetylation, methylation, and nitrosylation (Mann and Jensen 2003). These series of transient and reversible PTMs allow signaling molecules to switch-on and -off network functions, and allow cross talk between convergent and divergent pathways to regulate biological functions efficiently.

Dynamic protein O-GlcNAcylation

O-GlcNAc is a ubiquitous PTM of cellular proteins occurring on numerous nucleo-cytoplasmic proteins in eukaryotic organisms (Wells, Vosseller et al. 2001). It's a single acetylglucosamine sugar molecule that can be covalently attached to either Ser or Thr residues of its target proteins by a conserved O-GlcNAc transferase (OGT) enzyme abundant in both the nucleus and the cytoplasm of most cells. Most other glycosylation of proteins occur in the luminal compartments and on the cell surfaces, unlike O-GlcNAc modified proteins. While other glycosylations are static in nature, a conserved O-GlcNAcase (OGA) can remove the PTM from modified target proteins in both nucleus and cytoplasm (Hart, Housley et al. 2007). Protein O-GlcNAc modification is similar to protein phosphorylation, as both occurs at either Ser or Thr residues, and both are dynamically added or removed from target proteins in response to extracellular signaling. In contrast to the static nature of other glycosylation events, O-GlcNAc cycles dynamically in response to environmental cues, such as availability of nutrients and other factors. For example, recently mammalian neutrophils were observed to rapidly become O-GlcNAcylated in response to chemotactic agents (Kneass and Marchase 2004). Protein O-

GlcNAcylation regulates cellular responses to hormones such as insulin, initiates protective response against stress, and modulates the cellular response to growth and cell cycle progression as supported by recent studies reporting that many transcription factors and RNA polymerase II are modified by O-GlcNAc (Slawson, Housley et al. 2006).

Protein degradation through the 26S proteosome is also globally regulated by dynamic protein O-GlcNAcylation. The reversible O-GlcNAc cycling integrates proteosome function to the general metabolic state of the cell. O-GlcNAc-mediated regulation of proteosome function facilitates an organism's response to metabolic status (Zhang, Su et al. 2003). O-GlcNAc modification acts as a protective signal for target substrates as well as inhibits the ATPase activity of the 19S cap of the proteosome to decelerate global protein degradation. The concentrations of numerous proteins are actively controlled by ubiquitin proteosome mediated degradation, including those oxidized and aggregated insoluble proteins as well as several pro-apoptotic factors key to cell survival (Chen, Chang et al. 2000). As proteosome efficiency can be actively modulated by dynamic O-GlcNAc cycling, many tissue proteolysis events could be directly affected by nutritional status, which would allow insulin-mediated signaling impinge on regulation of cellular protein homeostasis. For example, in muscle tissues general proteolysis can be inhibited by insulin, and amino acids as reported from *in vitro* studies but their exact role in protein degradation pathways *in vivo* remain to be elucidated (Capel, Prod'homme et al. 2009).

References

Angers, S., T. Li, et al. (2006). "Molecular architecture and assembly of the DDB1-CUL4A ubiquitin ligase machinery." Nature **443**(7111): 590-593.

Bai, C., P. Sen, et al. (1996). "SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box." Cell **86**(2): 263-274.

Bennett, R. G., F. G. Hamel, et al. (2000). "Insulin inhibits the ubiquitin-dependent degrading activity of the 26S proteasome." Endocrinology **141**(7): 2508-2517.

Bevan, P. (2001). "Insulin signalling." J Cell Sci **114**(Pt 8): 1429-1430.

Capel, F., M. Prod'homme, et al. (2009). "Lysosomal and proteasome-dependent proteolysis are differentially regulated by insulin and/or amino acids following feeding in young, mature and old rats." J Nutr Biochem **20**(8): 570-576.

Chen, F., D. Chang, et al. (2000). "Role of p53 in cell cycle regulation and apoptosis following exposure to proteasome inhibitors." Cell Growth Differ **11**(5): 239-246.

Chen, Z. J. and L. J. Sun (2009). "Nonproteolytic functions of ubiquitin in cell signaling." Mol Cell **33**(3): 275-286.

Chun, A. C. and D. Y. Jin (2010). "Ubiquitin-dependent regulation of translesion polymerases." Biochem Soc Trans **38**(Pt 1): 110-115.

Ciechanover, A. (2005). "Intracellular protein degradation: from a vague idea thru the lysosome and the ubiquitin-proteasome system and onto human diseases and drug targeting." Cell Death Differ **12**(9): 1178-1190.

Conaway, R. C., C. S. Brower, et al. (2002). "Emerging roles of ubiquitin in transcription regulation." Science **296**(5571): 1254-1258.

Fawcett, J., F. G. Hamel, et al. (2001). "Insulin and analogue effects on protein degradation in different cell types. Dissociation between binding and activity." J Biol Chem **276**(15): 11552-11558.

Geetha, T., J. Jiang, et al. (2005). "Lysine 63 polyubiquitination of the nerve growth factor receptor TrkA directs internalization and signaling." Mol Cell **20**(2): 301-312.

Goll, D. E., V. F. Thompson, et al. (2003). "The calpain system." Physiol Rev **83**(3): 731-801.

Grainger, J. L. and M. M. Winkler (1989). "The sea urchin multicatalytic protease: purification, biochemical analysis, subcellular distribution, and relationship to snRNPs." J Cell Biol **109**(2): 675-683.

Grillari, J., H. Katinger, et al. (2006). "Aging and the ubiquitinome: traditional and non-traditional functions of ubiquitin in aging cells and tissues." Exp Gerontol **41**(11): 1067-1079.

Haas, A. L., J. V. Warms, et al. (1982). "Ubiquitin-activating enzyme. Mechanism and role in protein-ubiquitin conjugation." J Biol Chem **257**(5): 2543-2548.

Hart, G. W., M. P. Housley, et al. (2007). "Cycling of O-linked beta-N-acetylglucosamine on nucleocytoplasmic proteins." Nature **446**(7139): 1017-1022.

Hershko, A. and A. Ciechanover (1992). "The ubiquitin system for protein degradation." Annu Rev Biochem **61**: 761-807.

Hideshima, T., J. E. Bradner, et al. (2005). "Intracellular protein degradation and its therapeutic implications." Clin Cancer Res **11**(24 Pt 1): 8530-8533.

Hoffman, L., G. Pratt, et al. (1992). "Multiple forms of the 20 S multicatalytic and the 26 S ubiquitin/ATP-dependent proteases from rabbit reticulocyte lysate." J Biol Chem **267**(31): 22362-22368.

Huber, S. C. and S. C. Hardin (2004). "Numerous posttranslational modifications provide opportunities for the intricate regulation of metabolic enzymes at multiple levels." Curr Opin Plant Biol **7**(3): 318-322.

Ikeda, F. and I. Dikic (2008). "Atypical ubiquitin chains: new molecular signals. 'Protein Modifications: Beyond the Usual Suspects' review series." EMBO Rep **9**(6): 536-542.

Jackson, S. and Y. Xiong (2009). "CRL4s: the CUL4-RING E3 ubiquitin ligases." Trends Biochem Sci **34**(11): 562-570.

Kadowaki, M. and T. Kanazawa (2003). "Amino acids as regulators of proteolysis." J Nutr **133**(6 Suppl 1): 2052S-2056S.

Kanazawa, T., I. Taneike, et al. (2004). "Amino acids and insulin control autophagic proteolysis through different signaling pathways in relation to mTOR in isolated rat hepatocytes." J Biol Chem **279**(9): 8452-8459.

Kim, Y. and E. T. Kipreos (2007). "The Caenorhabditis elegans replication licensing factor CDT-1 is targeted for degradation by the CUL-4/DDB-1 complex." Mol Cell Biol **27**(4): 1394-1406.

King, R. W., R. J. Deshaies, et al. (1996). "How proteolysis drives the cell cycle." Science **274**(5293): 1652-1659.

Kipreos, E. T., L. E. Lander, et al. (1996). "cul-1 is required for cell cycle exit in C. elegans and identifies a novel gene family." Cell **85**(6): 829-839.

Kneass, Z. T. and R. B. Marchase (2004). "Neutrophils exhibit rapid agonist-induced increases in protein-associated O-GlcNAc." J Biol Chem **279**(44): 45759-45765.

Komada, M. and N. Kitamura (2005). "The Hrs/STAM complex in the downregulation of receptor tyrosine kinases." J Biochem **137**(1): 1-8.

Lecker, S. H., A. L. Goldberg, et al. (2006). "Protein degradation by the ubiquitin-proteasome pathway in normal and disease states." J Am Soc Nephrol **17**(7): 1807-1819.

Mann, M. and O. N. Jensen (2003). "Proteomic analysis of post-translational modifications." Nat Biotechnol **21**(3): 255-261.

Martinez-Vicente, M., G. Sovak, et al. (2005). "Protein degradation and aging." Exp Gerontol **40**(8-9): 622-633.

Mathias, N., S. L. Johnson, et al. (1996). "Cdc53p acts in concert with Cdc4p and Cdc34p to control the G1-to-S-phase transition and identifies a conserved family of proteins." Mol Cell Biol **16**(12): 6634-6643.

Mitch, W. E. and A. L. Goldberg (1996). "Mechanisms of muscle wasting. The role of the ubiquitin-proteasome pathway." N Engl J Med **335**(25): 1897-1905.

Mukhopadhyay, D. and H. Riezman (2007). "Proteasome-independent functions of ubiquitin in endocytosis and signaling." Science **315**(5809): 201-205.

Nakayama, K. I. and K. Nakayama (2006). "Ubiquitin ligases: cell-cycle control and cancer." Nat Rev Cancer **6**(5): 369-381.

Petroski, M. D. and R. J. Deshaies (2005). "Function and regulation of cullin-RING ubiquitin ligases." Nat Rev Mol Cell Biol **6**(1): 9-20.

Pickart, C. M. (2001). "Mechanisms underlying ubiquitination." Annu Rev Biochem **70**: 503-533.

Pintard, L., A. Willems, et al. (2004). "Cullin-based ubiquitin ligases: Cul3-BTB complexes join the family." EMBO J **23**(8): 1681-1687.

Proud, C. G. (2006). "Regulation of protein synthesis by insulin." Biochem Soc Trans **34**(Pt 2): 213-216.

Rock, K. L. and A. L. Goldberg (1999). "Degradation of cell proteins and the generation of MHC class I-presented peptides." Annu Rev Immunol **17**: 739-779.

Rolfe, M., M. I. Chiu, et al. (1997). "The ubiquitin-mediated proteolytic pathway as a therapeutic area." J Mol Med **75**(1): 5-17.

Rotin, D. and S. Kumar (2009). "Physiological functions of the HECT family of ubiquitin ligases." Nat Rev Mol Cell Biol **10**(6): 398-409.

Salvesen, G. S. and V. M. Dixit (1997). "Caspases: intracellular signaling by proteolysis." Cell **91**(4): 443-446.

Shenoy, S. K., P. H. McDonald, et al. (2001). "Regulation of receptor fate by ubiquitination of activated beta 2-adrenergic receptor and beta-arrestin." Science **294**(5545): 1307-1313.

Sigismund, S., S. Polo, et al. (2004). "Signaling through monoubiquitination." Curr Top Microbiol Immunol **286**: 149-185.

Slawson, C., M. P. Housley, et al. (2006). "O-GlcNAc cycling: how a single sugar post-translational modification is changing the way we think about signaling networks." J Cell Biochem **97**(1): 71-83.

Soucy, T. A., P. G. Smith, et al. (2009). "An inhibitor of NEDD8-activating enzyme as a new approach to treat cancer." Nature **458**(7239): 732-736.

Thrower, J. S., L. Hoffman, et al. (2000). "Recognition of the polyubiquitin proteolytic signal." EMBO J **19**(1): 94-102.

Tyers, M. and P. Jorgensen (2000). "Proteolysis and the cell cycle: with this RING I do thee destroy." Curr Opin Genet Dev **10**(1): 54-64.

Wells, L., K. Vosseller, et al. (2001). "Glycosylation of nucleocytoplasmic proteins: signal transduction and O-GlcNAc." Science **291**(5512): 2376-2378.

Wilkinson, K. D. (2000). "Ubiquitination and deubiquitination: targeting of proteins for degradation by the proteasome." Semin Cell Dev Biol **11**(3): 141-148.

Willems, A. R., S. Lanker, et al. (1996). "Cdc53 targets phosphorylated G1 cyclins for degradation by the ubiquitin proteolytic pathway." Cell **86**(3): 453-463.

Yu, H., J. M. Peters, et al. (1998). "Identification of a cullin homology region in a subunit of the anaphase-promoting complex." Science **279**(5354): 1219-1222.

Zhang, F., K. Su, et al. (2003). "O-GlcNAc modification is an endogenous inhibitor of the proteasome." Cell **115**(6): 715-725.

Zheng, N., B. A. Schulman, et al. (2002). "Structure of the Cul1-Rbx1-Skp1-F boxSkp2 SCF ubiquitin ligase complex." Nature **416**(6882): 703-709.

Table 1.1: List of post-translational modifications and biological functions*

PTM (modification sites)	Biological function affected
Phosphorylation (Ser, Thr, Tyr)	Signaling, protein stability, activation and inactivation of enzyme activity, protein-protein interaction
Glycosylation N-linked (Asn) O-linked (Ser, Thr)	Protein stability, solubility, secretion signaling, protein-protein interaction, regulatory function
Acylation Palmitoylation Farnesylation Myristoylation	subcellular localization, membrane targeting, protein-protein interaction
Sulphation (Tyr)	Signal transduction, subcellular localization, protein-protein interaction, receptor-ligand interaction
Ubiquitylation (Lys)	degradation signal, signal transduction, subcellular localization, protein-protein interaction
Methylation (Lys, Arg)	Protein activity, protein-nucleic acid interaction, chromatin remodeling and gene transcription
Acetylation (N-terminal Lys)	Protein stability and activity, protein-protein interaction, protein-DNA interaction
Disulphide bond (Cys)	Stabilization of protein structure and activity
Oxidation (Met, Trp)	Protein activity, oxidative damage
Deamidation (Asn, Gln)	Protein activity and interaction, associated with aging

*compiled from Mann and Jensen, 2003 & Huber and Hardin, 2004.

Figure 1.1: Proteolysis is vital for clearing damaged or unfolded proteins in our cell. As we grow older, protein degradation machinery become weaker, and less efficient. Many age related disorders and/or pathlogies are linked to accumulation of aggregated proteins, in absence of efficient protein turnover.

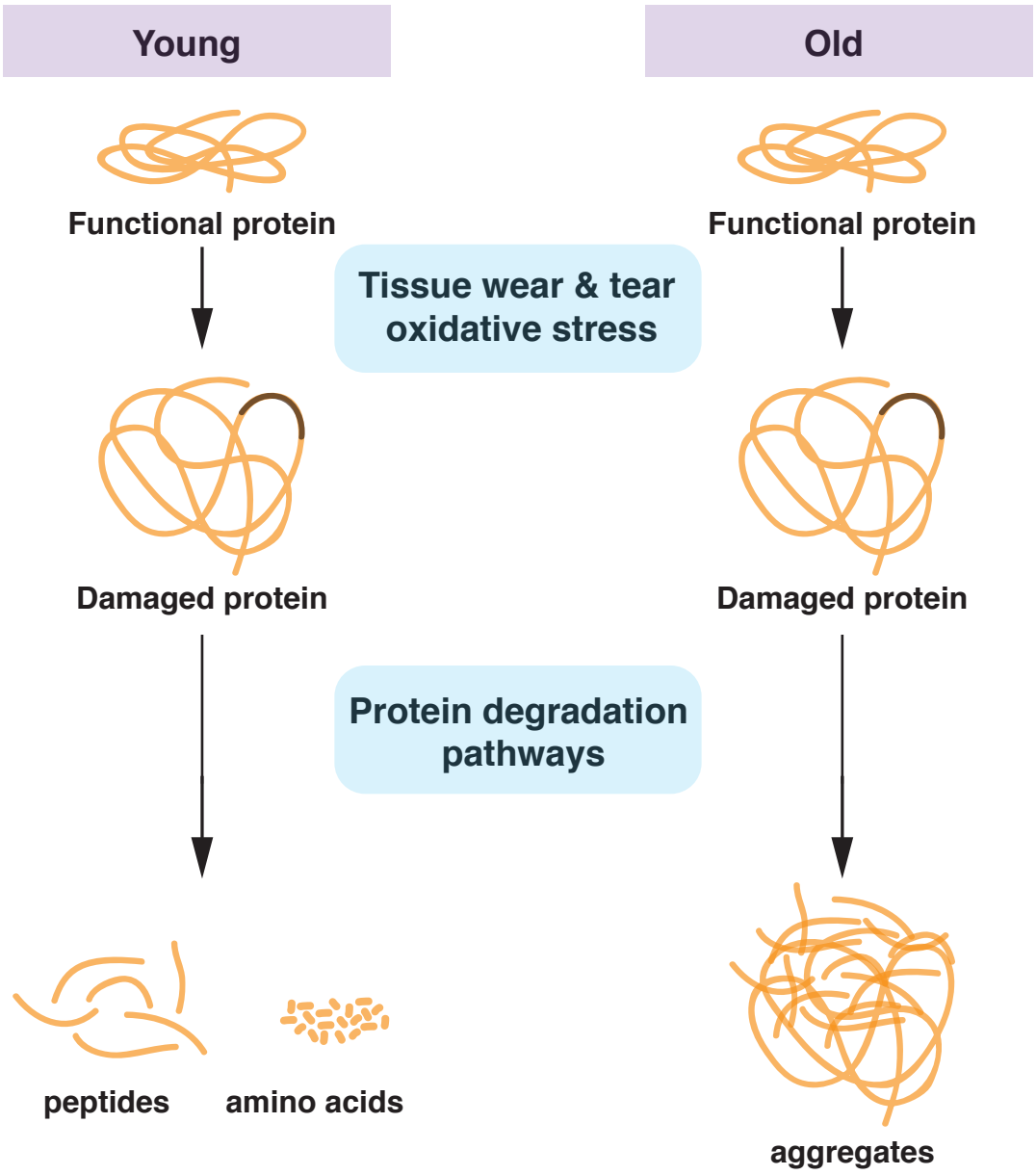

Young
Old
Functional protein
Functional protein
Tissue wear & tear
oxidative stress
Damaged protein
Damaged protein
Protein degradation
pathways
peptides
amino acids
aggregates

Figure 1.2: Metazoan cell utilizes different routes to degrade cellular proteins.

Proteins degradation by proteosome and other proteolytic systems

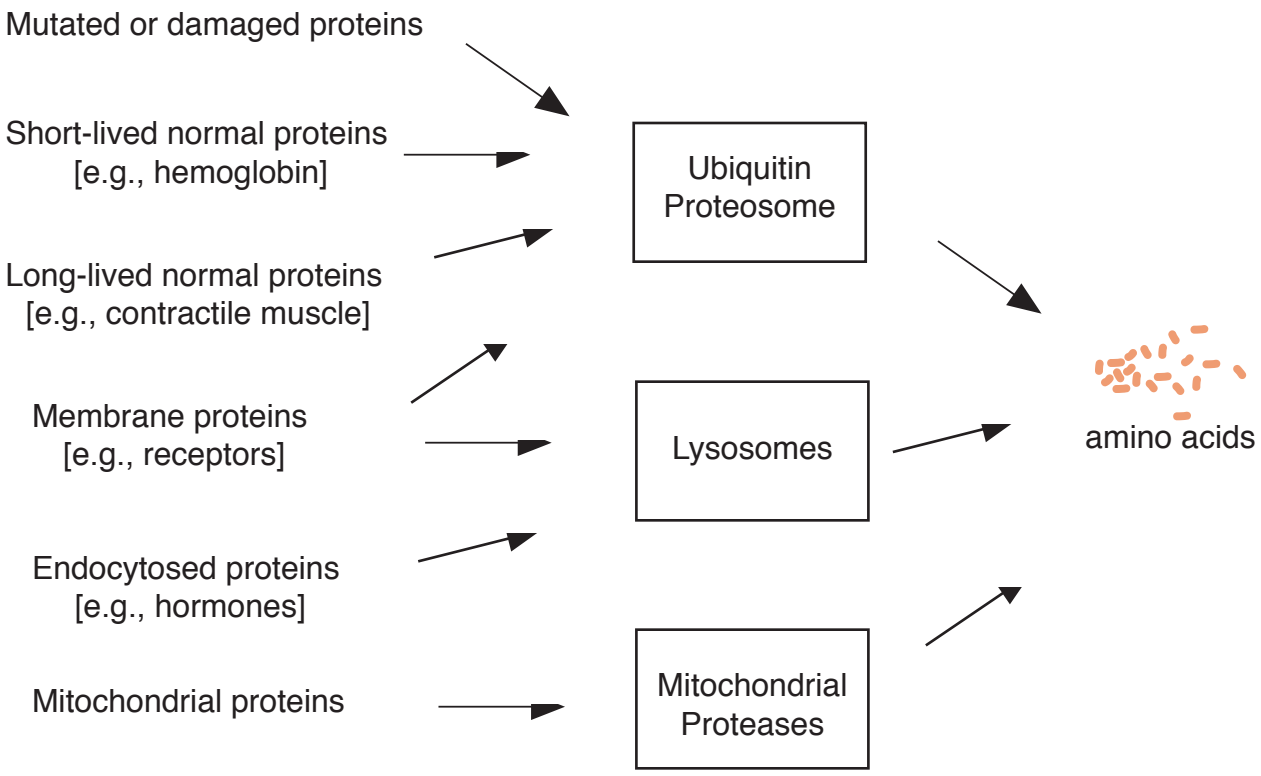
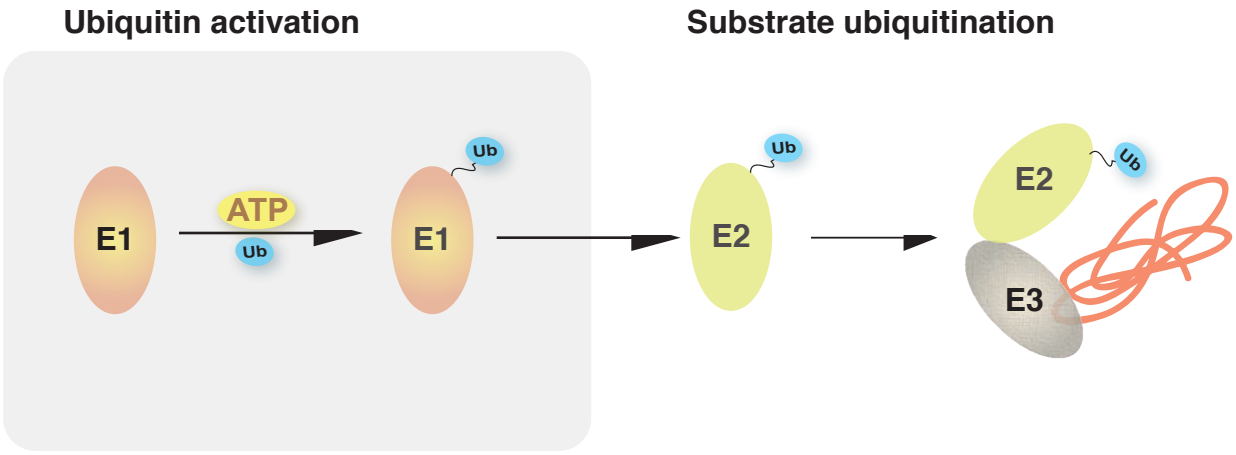
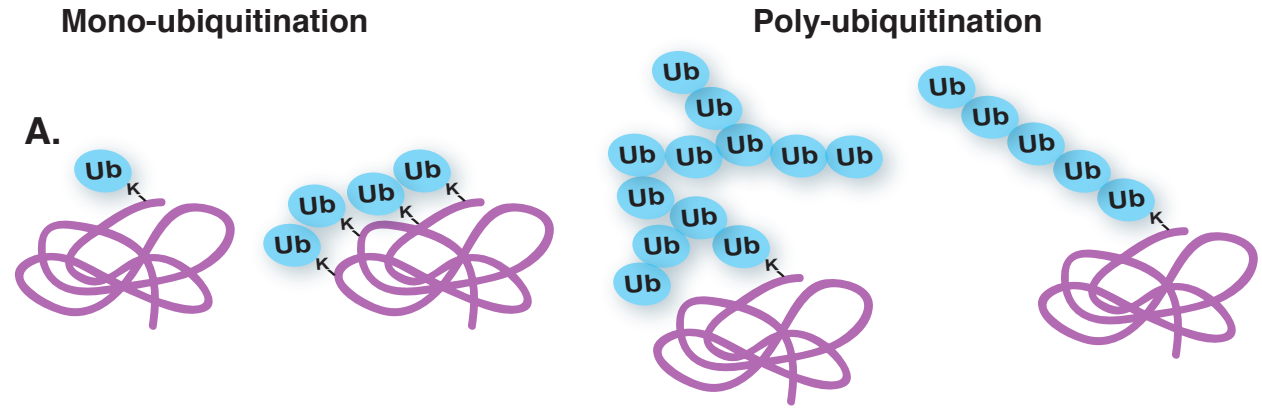
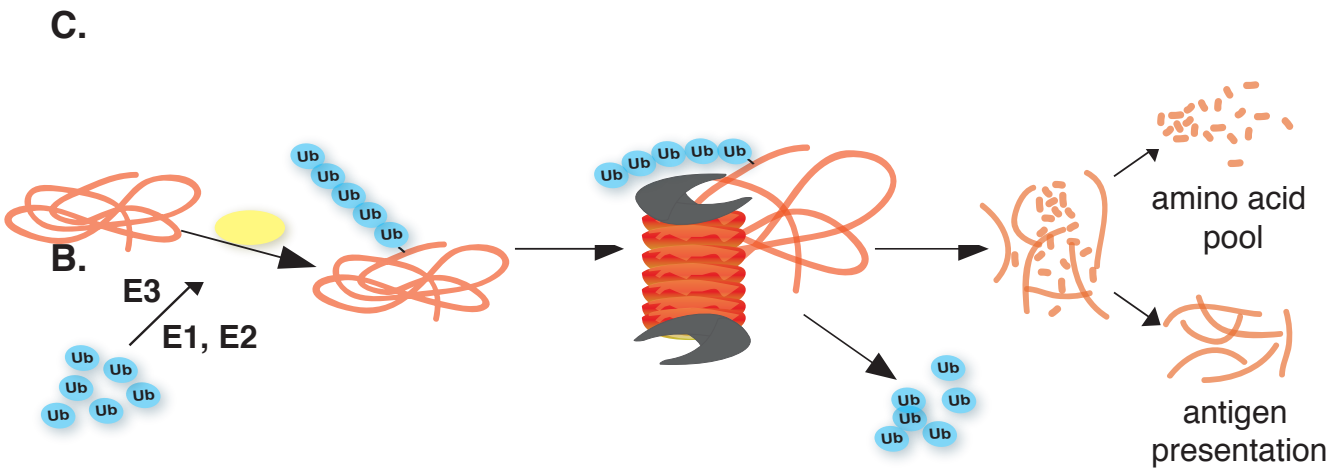


Figure 1.3: Ubiquitin conjugation to protein substrates. A. An activating enzyme (E1) first activates a ubiquitin molecule, which is transferred to a conjugating enzyme (E2). Later E2 can transfer the activated ubiquitin molecule to a protein recruited by ubiquitin ligase (E3) in a complex. B. Target proteins can be mono ubiquitinated at a single site or multiple sites. Similarly poly-ubiquitination of a protein can also be linear or branch chain. C. Poly-ubiquitin tagged target proteins can bind to the 26S proteosome where they are degraded into small peptides, and amino acids, while ubiquitin molecules are recycled back to its cellular pool.


Ubiquitin activation
Substrate ubiquitination
E1
ATP
Ub
E1
Ub
E2
Ub
E2
E3
Ub


Mono-ubiquitination
Poly-ubiquitination
A.
Ub
K


C.
B.
E3
E1, E2
Ub
amino acid
pool
antigen
presentation

CHAPTER 2

ESTABLISHMENT OF *IN VITRO* CELL CULTURE LINES FROM *C. ELEGANS*

Introduction

In 1965 Sydney Brenner first proposed *C. elegans* as a promising experimental model animal for genetic and developmental studies. In 2002, the Nobel Prize was awarded to Sydney Brenner, John Sulston, and H. Robert Horvitz who established *C. elegans* as an important model system; and later in 2006 the Nobel Prize was awarded to Andrew Fire and Craig Mello for establishing RNAi as a key tool for gene function analysis using *C. elegans* as a model organism. The invariant cell division and well-defined cell lineages have allowed critical insight into many developmental and cellular processes, e.g., polarity establishment, fate determination, apoptosis, morphogenesis, and cell cycle regulation. Approximately 40% of the genes associated with human diseases have homologs in *C. elegans*, a fact that makes it an excellent animal model system to study gene function *in vivo* (Culetto and Sattelle 2000; Segalat 2007).

Embryonic cells initially divide rapidly with a cell cycle going from mitosis to DNA synthesis, and then again into mitosis without any gap phases. Following the initial burst of rapid cell division, the rate gradually decreases as the embryonic cells start to differentiate into specific lineages (Edgar and O'Farrell 1990). A G2 phase is first observed at the 24-cell stage in embryonic development (Sulston, Schierenberg et al. 1983; Edgar and McGhee 1988; Park and Krause 1999). In *C. elegans,* unlike mammals, there is no multi-cell pluripotent stage (blastocyst in mammalian embryos). In *C. elegans,* the earliest cell division following fertilization is asymmetric, and is committed to generate dissimilar blastomere. Then a series of

asymmetric embryonic cell divisions generate six founder cells, and the embryonic cell divisions are largely completed within 350 minutes following fertilization (Molin, Schnabel et al. 1999). The L1 larvae hatches 8 hours post-fertilization with 556 somatic cells (113 cells underwent programmed cell death in the embryo). The invariant cell division in every embryo is defined by somatic cell lineages that limit proliferation to allow differentiation at selected time points during development, and generate exactly 959 somatic adult cells. The cell constancy is credited to an invariant pattern of cell cycle exit, timing of differentiation, and programmed cell death (Sulston, Schierenberg et al. 1983; Greenwood and Gautier 2005).

Early in mammalian embryonic development, the inner cell mass (ICM) differentiates from the trophoblast. The embryonic cells in the ICM are destined to develop into the embryo where the trophoblast develops as extra-embryonic tissue to support embryonic development. As embryogenesis proceeds, the ICM progressively differentiates into germinal layers, endoderm, mesoderm and ectoderm, which give rise to tissues and organs in an adult animal (Audesirk, Audesirk et al. 2004). Embryonic stem (ES) cells derived from the ICM can be maintained as pluripotent and can be differentiated into all three germ layers in culture similar to that occurs in embryos *in vivo* (Evans and Kaufman 1981). ES cells in suspension culture often turn into small aggregates of cells, termed as embryoid bodies (EB), that mimic early embryonic organization with an outer layer of primitive endoderm and an inner layer of primitive ectoderm (Keller 1995; Desbaillets, Ziegler et al. 2000; Chambers and Smith 2004). This expansive capacity for differentiation makes EBs a useful *in vitro* model to study differentiation and gene expression regulation in embryonic development (Leahy, Xiong et al. 1999). *C. elegans* embryonic development begins with a series of asymmetric divisions leading to six founder cells: AB, MS, E, C, D and P_4. These founder cells correspond to the germ layers, although not precisely, as embryonic endoderm is generated from E cells; mesoderm from MS, C and D cells, and ectoderm from AB and C cells. Most tissues in *C. elegans* adults are derived from

more than one founder cell except the intestine (only E lineage cells), and germ line (only P4 lineage cells) (Wood 1988).

Although there are no established *C. elegans* embryonic cell lines available, isolation techniques and embryo growth media (EGM) are well established. EGM supports normal embryonic cell division and growth from the 1-cell stage to approximately the 500-cell stage *in vitro* (Edgar 1995). In such transient culture, embryonic cells express proteins that are normally expressed *in vivo* (Edgar 1995; Maduro and Pilgrim 1995) but the embryonic cells divide at a notably slower pace (Goldstein 1993; Edgar 1995). *C. elegans* embryonic cells have been isolated for electrophysiological and other experiments in Leibovitz's (L-15) insect culture media (Christensen, Estevez et al. 2002; Zhang, Ma et al. 2002; O'Hagan, Chalfie et al. 2005). These non-proliferative cell populations are suitable for only limited usage, i.e., neuronal differentiation, gene expression arrays etc. All attempts to establish a *C. elegans* embryonic cell line encountered similar difficulties, limited cell proliferation and terminal differentiation into neurons within 24-hrs of isolation without any increase in total cell population. These studies also observed expression of markers analogous to L1 larval stage in isolated embryonic cells in culture media (Buechner, Hall et al. 1999; Leung, Hermann et al. 1999; Christensen, Estevez et al. 2002). *C. elegans* embryos without an egg-shell divide normally when placed in culture media but do not generate more cells than they do inside intact embryos *in vivo*. To surmount the limited proliferation potential of embryonic cells *in vitro,* we inactivated CUL-1 function, which is known to deregulate cell proliferation *in vivo,* and produce hyperplasia of all nematode tissues including the germline (Kipreos, Lander et al. 1996).

Currently there is no primary or immortalized tissue culture cell line available to the *C. elegans* research community. Establishment of a cell line would facilitate a variety of biochemical and molecular approaches in a homogenous cell population, such as, RNAi screens, gene expression analysis, and electrophysiological and pharmacological analyses of

channel function in neurons (Bianchi and Driscoll 2005). Establishment of an embryonic cell line would also facilitate exploration of early differentiation events. Such an experimental system would allow us to study the role of cell-cell signaling from embryogenesis to organogenesis to elucidate differentiation events *in vitro*. An embryonic cell line would also allow the investigation of gene functions otherwise lethal *in vivo* when inactivated that cannot currently be studied. Despite the considerable advantages offered for genetic studies in *C. elegans*, the model system is challenging for *in vitro* biochemical and molecular approaches due to the inability to isolate large quantities of any single tissue suitable for biochemical purification. A cell line would accelerate research all of these respects.

Experimental Methods and Materials

Nematode strains. Worms were handled according to standard procedures and maintained at 20°C with the exception of temperature sensitive (ts) mutants maintained at 16°C (Brenner 1974). Some nematode strains used in this work were provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR). The following strains were used in this study: Wild type N2 Bristol, *cul-1(e1756)/unc-69(e578), cul-1(e1756)/unc-69(e578);ced-3(n1286), cul-1(e1756) /unc-69(e578);lin-35(n745), cul-1(e1756)/unc-69(e578);cki-1(gk132)/mIn1[dpy10(e128)mIs14(myo-2::gfp)], fzr-1(ok380);lin-35(n745), glp-1(ar202); cul-1(e1756)/unc-69(e578);glp-1(ar202); gld-1(q485)gld-2(q497)/dpy-5(e61)unc-13(e51), gld-1(q485)gld-2(q497)/dpy-5(e61)unc-13(e51);glp-1(ar202), glp-1(ar202);teg-2(oz192), glp-1(ar202);puf-8(ok320); cul-1(e1756)/unc-69(e578);scm::*GFP.

Isolation of embryonic cells

To attain increased proliferation and disrupt the developmentally programmed differentiation pattern in embryos, we utilized *cul-1* mutant animals. Animals homozygous for *cul-1* mutation are not viable, however a genetically balanced heterozygote strain *cul-*

1(e1756)/*unc-69(e578)* is viable and fertile. Embryonic cells were isolated from *cul-1* feeding RNAi-treated *cul-1* heterozygote animals to inactivate maternal CUL-1 product in isolated embryos. In brief, embryos were collected from gravid adult animals by bleaching, embryonic cells were isolated by removing the eggshells using a combination of enzymatic (chitinase and chymotrypsin 5:1 mixture from Sigma) digestion, and manual dissociation by passing through 5μm sterile filters (Millipore) to separate single cells from large cell aggregates and animal debris prior to placing them into culture media (see in Table 1).

Isolation of germ cells

To attain increased proliferation and disrupt developmentally programmed differentiation pattern in the germline, we utilized temperature sensitive *glp-1(ar202)* mutant animals. Homozygous *glp-1(ar202)* mutant animals were raised at 25°C from a set of synchronized L1 stage larvae until they were two-day old adults. Animals were collected in M9 buffer with commercially available 1XPenicillin-Streptomycin solution (Sigma), and washed several times with 10 minutes incubation in between each wash. Clean animals were transferred into sterile axenic culture media (Rao, Carta et al. 2005), and incubated for several hours at 25°C prior to isolation of germ cells by crushing manually in a glass hand-homogenizer.

Isolation of hypodermal seam cells

To attain increased proliferation and disrupt developmentally programmed differentiation pattern in hypodermal seam cells we utilized *cul-1(e1756)*/*unc-69(e578); scm::GFP* mutant animals. Animals homozygous for the *cul-1* mutation are not viable, so heterozygote *cul-1(e1756)*/*unc-69(e578)* animals were grown on *cul-1* feeding RNAi. Embryonic cells were isolated similarly to what was described in the previous section with an additional 1-3 hours digestion in commercially available collagenase and dispase (1:1) mixture (Calbiochem). Cells were immediately resuspended into culture media without serum; and FACS sorted for GFP positive cells were separately collected in a sterile tube.

Optimization of cell culture media

C. elegans extract

C. elegans whole animal or embryo extract preparation was done in the following way. All animals were synchronized at L1 larval stage upon hatching into M9 buffer supplemented with 50ug/ml cholesterol solution. Animals were transferred to OP50-seeded plates and collected as gravid adults. Collected animals were washed 6 times in M9 buffer supplemented with a mixture of commercially available antibiotics and antifungal agents (Hyclone). Animals were incubated for 15 minutes between each wash. Prior to the final wash animals were transferred into sterile 10mM Tris.Cl (pH8.0) solution. After removing the excess liquid following the final wash, animals were flash frozen in liquid nitrogen.

Serum replacements

Mammalian ES cells can proliferate and maintain their pluripotent state similarly in knock-out serum replacement (KSR) plus bovine serum albumin (BSA) mixture instead of fetal bovine serum (FBS) in culture media (Mallon, Park et al. 2006). We also prepared *C. elegans* culture media with similar KSR (Invitrogen) and BSA (Sigma) mixture with and without 10% FBS (Hyclone). Each type of culture media was readjusted to ~340mOsm/kg by addition of trehalose.

Antioxidants

Mammalian ES cells and many somatic cells require β-mercaptoethanol (1mM) added to culture media for optimum growth (www.atcc.org). We examined various concentrations of available antioxidants reported in primary cell culture, i.e., β-mercaptoethanol, glutathione, ascorbic acid, N-acetyl-L-alanine and N-acetyl-L-cysteine to analyze the effect on *C. elegans* embryonic cell growth and proliferation *in vitro*.

Cell attachment

It is evident that mammalian ES cells grown in suspension culture spontaneously aggregate into EBs within 2-4 days (Desbaillets, Ziegler et al. 2000). ES cells can grow robustly in various matrices including matrigel, collagen I, collagen IV, fibronectin, laminin, gelatin and soft-agar, which support cell proliferation similar to a mouse feeder layer (Mallon, Park et al. 2006). We either prepared or collected similar culture plates from commercially available sources for *C. elegans* cell culture.

Feeder layer derived from invertebrate cell lines

Primarily mammalian ES cells were successfully derived and propagated on mouse feeder layers (Dani, Smith et al. 1997). We observed limited proliferation potential of *C. elegans* embryonic cells in suspension culture, and often these cells propagated in suspension culture as aggregates and differentiated into spheroid bodies spontaneously. There are commercially available insect cell lines, such as, Sf9/Sf21 from ovarian tissue of worm *Spodoptera frugiperda*, T.ni from ovarian cells of cabbage-worm *Trichoplusia ni*, embryonic cell line from flour moth *Ephestia kuehniella*, and numerous Drosophila embryonic cell lines (SL1 or Kc23 or D1) available from Drosophila Genomics Resource Center (Bloomington, IN). To utilize invertebrate cell lines as a feeder layer, we treated the cells in culture with mitomycin C and gamma irradiation to restrict proliferation in culture conditions using standard protocols. Later we plated isolated *C. elegans* embryonic cells on non-proliferative feeder layers, and analyzed the effect on *C. elegans* cell survival and proliferation *in vitro*.

Results

Following our improved protocol we have isolated several batches of embryonic cells from *cul-1 (e1756)* mutant animals. Isolated cells continue to proliferate in our current culture media for the first few days (Figure 2.1). Embryonic cell proliferation starts to slow down

following a week after isolation in our current culture media. A decrease in rate of cell division as well as cell death together reduces the total cell population to a low number (~$1X10^3$ to $5X10^3$ cells/ml) within 4 weeks (Figure 2.2) from a initial ~$2X10^6$ cells/ml plated following isolation (Figure 2.1, upper panel). Only in the first few days we did observe a rapid burst of cell division reaching ~$6X10^7$ cells (Figure 2.1 bottom panel). Within a few weeks after isolation we observed embryonic cells in culture to clump together into large aggregates (Figure 2.2, bottom panel) although we do not know whether these large clumps are aggregates of free, single cells or if the clumps were formed by proliferation of a single colony. Even after two months, we still observe the large clumps to survive as well as many small clumps, while most of the free, single cells started to disappear in the same culture (data not shown). We tested embryonic cells collected from such clumps with trypan blue, and found that the majority of the cells in these small and large clumps exclude trypan blue upon disruption by gentle pipetting. Disruption of large clumps into small aggregates or free, single cells did not improve cell survival or proliferation in the current culture conditions. Following four months in culture, a few cells were found to be dividing, and forming 2 or 4 cell clusters (Figure 2.3 & 2.4) that exclude trypan blue. After six months in culture, embryonic cell morphology starts getting crippled, and many cells at this point failed to exclude trypan blue suggesting that isolated embryonic cells in culture can not survival more than six months in our current culture condition (Figure 2.5). Later we incorporated *ced-3* and *lin-35*/Rb mutations in the *cul-1* mutant animals and isolated embryonic cells numerous times without any improvement in cell survival or proliferation potential. Upon inactivation of CED-3 mediated cell death pathways, we observed abnormally large embryonic cells in the *cul-1;ced-3* double mutant isolates following a month in culture (Figure 2.6).

Cyclin dependent kinase inhibitors (CKI) are critical in cell cycle exit, and inactivation of CKI-1 function leads to extra cell division in many nematode tissues (Buck, Chiu et al. 2009). We combined the *cul-1(e1756)* and *cki-1(gk132)* mutant alleles and isolated embryonic cells

from *cul-1(e1756);cki-1 (gk132)* double mutant animals fed with *cul-1* RNAi. We did not observe any improvement in cell survival or proliferation in embryonic cells in current culture conditions. In the following months, in embryonic cell cultures derived from *cul-1;cki-1* double mutants we occasionally observed comparatively dark, and enlarged group of cells in some embryonic cell isolations (Figure 2.7). These cell aggregates or large dark structure containing clusters were resistant to gentle pipetting. These embryonic-cell derived spheroid bodies vaguely resembles embryoid bodies (EBs) generated from mammalian embryonic stem cells under non-optimal culture conditions (Keller 1995).

Use of *C. elegans* extract and serum replacements (KSR and BSA with and without 10%FBS) at different concentrations and combinations plated on different attachment surfaces, such as, collagen, fibronectin, laminin, and gelatin, did not improve embryonic cell survival or proliferation potential in repeated experiments (data not shown). Previous studies to establish *C. elegans* embryonic cell culture tried peanut lectin and poly-L-Lysine to adhere isolated embryonic cells to the culture dish surface with limited success. Peanut lectin facilitated adherent cells to differentiate rapidly than non-adherent cells in L-15 based culture media (Christensen, Estevez et al. 2002). We also tested different combinations of modified culture media to plate isolated embryonic cells from different mutant backgrounds on feeder layers made from insect or vertebrate cell lines but this did not result in any improvement (data not shown).

Using a modified protocol established during following numerous trials, we were able to isolate germ cells from adult animals for the first time, and maintain these germ cells in culture without any bacterial contaminations for weeks (Figure 2.8). Isolated germ cells *from glp-1 (ar202)* mutant animals grown at 25°C never proliferated in the current culture media conditions; and completely disappeared from *in vitro* culture within two weeks after isolation (Figure 2.9).

Several attempts to isolate germ cells from many other tumorous germline mutants in the current culture media also resulted in similar failures (data not shown).

Finally, we attempted adult somatic hypodermal seam cells, which retain proliferative potential until the L4 larval stage in *C. elegans*. We utilized a scm::GFP (seam cell) marker to study deregulation of hypodermal (seam) cell upon inactivation of CUL-1 function. We were able to deregulate seam cell proliferation in adult animals upon inactivation of CUL-1 function (~2X more seam cells than in wild type) function (Figure 2.10). In *C. elegans* the POP-1 protein works in the Wnt signaling pathway to regulate binary cell fate decision (Owraghi, Broitman-Maduro et al. 2010). We observed a dramatic increase in seam cell numbers *in vivo* when we inactivated POP-1 function by RNAi in a *cul-1 (e1756)* mutant background (16 vs. 87 seam cells in wild type and *cul-1;pop-1* double mutant respectively). Isolated seam cells in current culture media never proliferated, and died within few days following isolation. Even following enrichment of seam cells in culture through FACS sorting to GFP-tagged seam cells from embryonic cells did not improve cell survival or proliferation potential (data not shown).

Discussion

Isolated embryonic cells from the *cul-1* cell cycle exit mutant divide slowly in cell culture media, and survived for six months with a progressive decline in cell division rate in later months. No noticeable proliferation in culture media was observed in embryonic cells isolated from wild-type strains. Wild-type embryonic cells were dead by a week post-isolation. Only the *cul-1* derived embryonic cells survived as long as six months in current culture conditions with occasional cell division observed in later months. Initial experiments with *cul-1* mutant derived embryonic cells in culture suggested that cells in clumps or aggregates survived longer in the current culture conditions compared to free, single, separated cells in the same culture wells. As examined by trypan blue exclusion, these small clusters of embryonic cells remain refractile

under phase contrast microscope for months without any significant decrease in viability. Individual cells dissociated from the large clumps died rapidly. The *cul-1* derived embryonic cells in culture rarely generated neuron-like long processes. Earlier studies to establish *C. elegans* embryonic cell culture also reported a decline in the number of mitotic cells to only 4% within 24 hours post-isolation from wild-type embryos, and total cell death after a week without any increase in total cell number (Christensen, Estevez et al. 2002; Zhang, Ma et al. 2002).

Leukemia Inhibitory Factor (LIF) and Bone Morphogenic Proteins (BMP) are critical morphogens required by mouse ES cells in culture to restrict cells from differentiation, and maintain their pluripotent state *in vitro* (Chambers 2004). Human ES cells require basic Fibroblast Growth Factor (bFGF) to restrict differentiation, and maintain their pluripotent state on a mouse feeder layer *in vitro* (Xu, Rosler et al. 2005). No homologs for mammalian LIF, BMP or bFGF are known in *C. elegans*. We tried to improve our current culture conditions to establish a *C. elegans* embryonic cell line using extract from wild type animals to supplement with unknown growth factors, if any. Our trials did not result in any improvement. In the future, whole animal or embryo extracts from hyper-proliferative *cul-1* mutant animals should be tested in a similar way using different extraction protocols as reported previously (Shatilla and Ramotar 2002). Also lipid soluble fractions from the *C. elegans* extracts need to be tested in future studies (Gill, Held et al. 2004). Antioxidant treatment could restrain senescence-like cell division arrest in cultured cells following acute oxidative stress when primary cells are grown at atmospheric oxygen (21%) levels (Levitt, Zhu et al. 2007). Acute oxidative stress could cause DNA damage anywhere, however telomeres are preferential targets; and telomeres are deficient in repairing such oxidative DNA breaks in primary cell cultures (von Zglinicki 2000; von Zglinicki 2002). Our preliminary experiments show that only glutathione [0.6mg/ml] improved the survival rate of embryonic cells following isolation but did not improve proliferation potential in our current study.

In addition to the *cul-1* mutation, several other gene mutations, *e.g.*, *lin-35*, *fzr-1*, *cki-1,* were reported to cause hyperplasia in nematode tissues (Hong, Roy et al. 1998; Nilsson and Hoffmann 2000; Fay, Keenan et al. 2002; Saito, Perreault et al. 2004). Retinoblastoma protein (Rb) in the mammalian system is a critical G1 to S phase regulator that modulates E2F mediated transcription in S phase (Stevaux and Dyson 2002). Fizzy-related proteins (Fzr) are components of the APC/C ubiquitin ligase that are active in late mitosis to regulate G1 cyclin degradation (Harper, Burton et al. 2002). Inactivation of either *lin-35*/Rb or *fzr-1*/FZR in *C. elegans* independently does not cause any phenotype but simultaneous inactivation leads to hyper proliferation in all nematode tissues (Fay, Keenan et al. 2002). We isolated embryonic cells from *lin-35;fzr-1 and cul-1;lin-35* double mutant animals without any success (data not shown). It would be interesting to test embryonic cell isolations from a *cul-1; lin-35; fzr-1* triple inactivation in future studies.

The limitation to embryonic cell division is imposed by differentiation events and apoptotic cell death at set time points in development. In *C. elegans,* CED-3 is the only caspase that initiates cell death in 131 cells generated during embryonic development. The *C. elegans* embryonic cell-autonomous apoptosis pathway shapes the differentiation pattern as a majority of cell death is seen preferentially in ectodermal cells, only a few in mesodermal cells, and none in endodermal cells (Yuan, Shaham et al. 1993). In *ced-3* mutants, the cells that would normally be programmed to die now continue to survive and differentiate instead. In a double mutant we expected hyper-proliferation in all nematode tissues, including those cells that were earlier programmed to die. There is no previous report showing how the developmental pattern might be affected by the proliferation of particular cells otherwise destined to die early in embryogenesis. We generated a *cul-1(e1756);ced-3(e1286)* double mutant, and isolated embryonic cells from *cul-1;ced-3* double mutant animals without much success (Figure 2.6).

C. elegans embryonic cells in prolonged culture often generate large cell aggregates and acquire a smooth exterior surrounding the cell aggregates with a dense darker interior morphology that in part vaguely resembles the embryoid bodies (EB) reported in mammalian ES cell culture (Desbaillets, Ziegler et al. 2000). The encapsulated cell aggregates derived from *C. elegans* embryonic cells are resistant to physical disruption by pipetting. Harsh manual treatment sometimes results in long processes to protrude from these structures indicating the presence of viable cells within these structures that are capable of changing morphology even after months in culture. Embryonic cells derived from *cul-1;cki-1* mutants were primarily isolated and maintained in EGM culture media containing 40% FBS which facilitated spheroid body-like structure formation. Further optimization of culture conditions is needed to generate a sufficient number of spheroid bodies for histochemical analysis. A detailed analysis would reveal any similarities to the *in vivo* embryonic development pattern. Mammalian EBs mimic early embryonic development in multiple aspects, *e.g.*, basement membrane assembly, endoderm differentiation and pro-amniotic cavity formation. It would be interesting to investigate whether *C. elegans* spheroid bodies formed in embryonic cell culture in sub-optimal conditions can similarly recapitulate embryonic development.

Germ cells isolated from *glp-1(ar202)* mutant animals grown at 25°C survived in culture for a few weeks but we never observed cells to proliferate in culture media. The majority of germ cells were gradually lost in the culture media within a week after isolation. Addition of different supplements that improved embryonic cell survival did not affect germ cell survival. A similar fate was also observed in hypodermal seam cell culture trials. We could easily separate GFP positive seam cells through FACS sorting, and enrich in culture but never observed GFP positive cells to increase in number in our current culture conditions.

References

Audesirk, G., T. Audesirk, et al. (2004). Biology: Life on Earth. San Francisco, CA, Benjamin Cummings.

Bianchi, L. and M. Driscoll (2005). Culture of embryonic C. elegans cells for eletrophysiological and pharmacological analyses, Wormbook.

Brenner, S. (1974). "The genetics of Caenorhabditis elegans." Genetics **77**(1): 71-94.

Buck, S. H., D. Chiu, et al. (2009). "The cyclin-dependent kinase inhibitors, cki-1 and cki-2, act in overlapping but distinct pathways to control cell cycle quiescence during C. elegans development." Cell Cycle **8**(16): 2613-2620.

Buechner, M., D. H. Hall, et al. (1999). "Cystic canal mutants in Caenorhabditis elegans are defective in the apical membrane domain of the renal (excretory) cell." Dev Biol **214**(1): 227-241.

Chambers, I. (2004). "The molecular basis of pluripotency in mouse embryonic stem cells." Cloning Stem Cells **6**(4): 386-391.

Chambers, I. and A. Smith (2004). "Self-renewal of teratocarcinoma and embryonic stem cells." Oncogene **23**(43): 7150-7160.

Christensen, M., A. Estevez, et al. (2002). "A primary culture system for functional analysis of C. elegans neurons and muscle cells." Neuron **33**(4): 503-514.

Culetto, E. and D. B. Sattelle (2000). "A role for Caenorhabditis elegans in understanding the function and interactions of human disease genes." Hum Mol Genet **9**(6): 869-877.

Dani, C., A. G. Smith, et al. (1997). "Differentiation of embryonic stem cells into adipocytes in vitro." J Cell Sci **110 (Pt 11)**: 1279-1285.

Desbaillets, I., U. Ziegler, et al. (2000). "Embryoid bodies: an in vitro model of mouse embryogenesis." Exp Physiol **85**(6): 645-651.

Edgar, B. A. and P. H. O'Farrell (1990). "The three postblastoderm cell cycles of Drosophila embryogenesis are regulated in G2 by string." Cell **62**(3): 469-480.

Edgar, L. G. (1995). "Blastomere culture and analysis." Methods Cell Biol **48**: 303-321.

Edgar, L. G. and J. D. McGhee (1988). "DNA synthesis and the control of embryonic gene expression in C. elegans." Cell **53**(4): 589-599.

Evans, M. J. and M. H. Kaufman (1981). "Establishment in culture of pluripotential cells from mouse embryos." Nature **292**(5819): 154-156.

Fay, D. S., S. Keenan, et al. (2002). "fzr-1 and lin-35/Rb function redundantly to control cell proliferation in C. elegans as revealed by a nonbiased synthetic screen." Genes Dev **16**(4): 503-517.

Gill, M. S., J. M. Held, et al. (2004). "Lipophilic regulator of a developmental switch in Caenorhabditis elegans." Aging Cell **3**(6): 413-421.

Goldstein, B. (1993). "Establishment of gut fate in the E lineage of C. elegans: the roles of lineage-dependent mechanisms and cell interactions." Development **118**(4): 1267-1277.

Greenwood, J. and J. Gautier (2005). "From oogenesis through gastrulation: developmental regulation of apoptosis." Semin Cell Dev Biol **16**(2): 215-224.

Harper, J. W., J. L. Burton, et al. (2002). "The anaphase-promoting complex: it's not just for mitosis any more." Genes Dev **16**(17): 2179-2206.

Hong, Y., R. Roy, et al. (1998). "Developmental regulation of a cyclin-dependent kinase inhibitor controls postembryonic cell cycle progression in Caenorhabditis elegans." Development **125**(18): 3585-3597.

Keller, G. M. (1995). "In vitro differentiation of embryonic stem cells." Curr Opin Cell Biol **7**(6): 862-869.

Kipreos, E. T., L. E. Lander, et al. (1996). "cul-1 is required for cell cycle exit in C. elegans and identifies a novel gene family." Cell **85**(6): 829-839.

Leahy, A., J. W. Xiong, et al. (1999). "Use of developmental marker genes to define temporal and spatial patterns of differentiation during embryoid body formation." J Exp Zool **284**(1): 67-81.

Leung, B., G. J. Hermann, et al. (1999). "Organogenesis of the Caenorhabditis elegans intestine." Dev Biol **216**(1): 114-134.

Levitt, P. S., M. Zhu, et al. (2007). "Genome maintenance defects in cultured cells and mice following partial inactivation of the essential cell cycle checkpoint gene Hus1." Mol Cell Biol **27**(6): 2189-2201.

Maduro, M. and D. Pilgrim (1995). "Identification and cloning of unc-119, a gene expressed in the Caenorhabditis elegans nervous system." Genetics **141**(3): 977-988.

Mallon, B. S., K. Y. Park, et al. (2006). "Toward xeno-free culture of human embryonic stem cells." Int J Biochem Cell Biol **38**(7): 1063-1075.

Molin, L., H. Schnabel, et al. (1999). "Complexity of developmental control: analysis of embryonic cell lineage specification in Caenorhabditis elegans using pes-1 as an early marker." Genetics **151**(1): 131-141.

Nilsson, I. and I. Hoffmann (2000). "Cell cycle regulation by the Cdc25 phosphatase family." Prog Cell Cycle Res **4**: 107-114.

O'Hagan, R., M. Chalfie, et al. (2005). "The MEC-4 DEG/ENaC channel of Caenorhabditis elegans touch receptor neurons transduces mechanical signals." Nat Neurosci **8**(1): 43-50.

Owraghi, M., G. Broitman-Maduro, et al. (2010). "Roles of the Wnt effector POP-1/TCF in the C. elegans endomesoderm specification gene network." Dev Biol **340**(2): 209-221.

Park, M. and M. W. Krause (1999). "Regulation of postembryonic G(1) cell cycle progression in Caenorhabditis elegans by a cyclin D/CDK-like complex." Development **126**(21): 4849-4860.

Rao, A. U., L. K. Carta, et al. (2005). "Lack of heme synthesis in a free-living eukaryote." Proc Natl Acad Sci U S A **102**(12): 4270-4275.

Saito, R. M., A. Perreault, et al. (2004). "The CDC-14 phosphatase controls developmental cell-cycle arrest in C. elegans." Nat Cell Biol **6**(8): 777-783.

Segalat, L. (2007). "Invertebrate animal models of diseases as screening tools in drug discovery." ACS Chem Biol **2**(4): 231-236.

Shatilla, A. and D. Ramotar (2002). "Embryonic extracts derived from the nematode Caenorhabditis elegans remove uracil from DNA by the sequential action of uracil-DNA glycosylase and AP (apurinic/apyrimidinic) endonuclease." Biochem J **365**(Pt 2): 547-553.

Stevaux, O. and N. J. Dyson (2002). "A revised picture of the E2F transcriptional network and RB function." Curr Opin Cell Biol **14**(6): 684-691.

Sulston, J. E., E. Schierenberg, et al. (1983). "The embryonic cell lineage of the nematode Caenorhabditis elegans." Dev Biol **100**(1): 64-119.

von Zglinicki, T. (2000). "Role of oxidative stress in telomere length regulation and replicative senescence." Ann N Y Acad Sci **908**: 99-110.

von Zglinicki, T. (2002). "Oxidative stress shortens telomeres." Trends Biochem Sci **27**(7): 339-344.

Wood, W. B. (1988). Embryology. New York, Cold Spring Harbor Laboratory.

Xu, C., E. Rosler, et al. (2005). "Basic fibroblast growth factor supports undifferentiated human embryonic stem cell growth without conditioned medium." Stem Cells **23**(3): 315-323.

Yuan, J., S. Shaham, et al. (1993). "The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme." Cell **75**(4): 641-652.

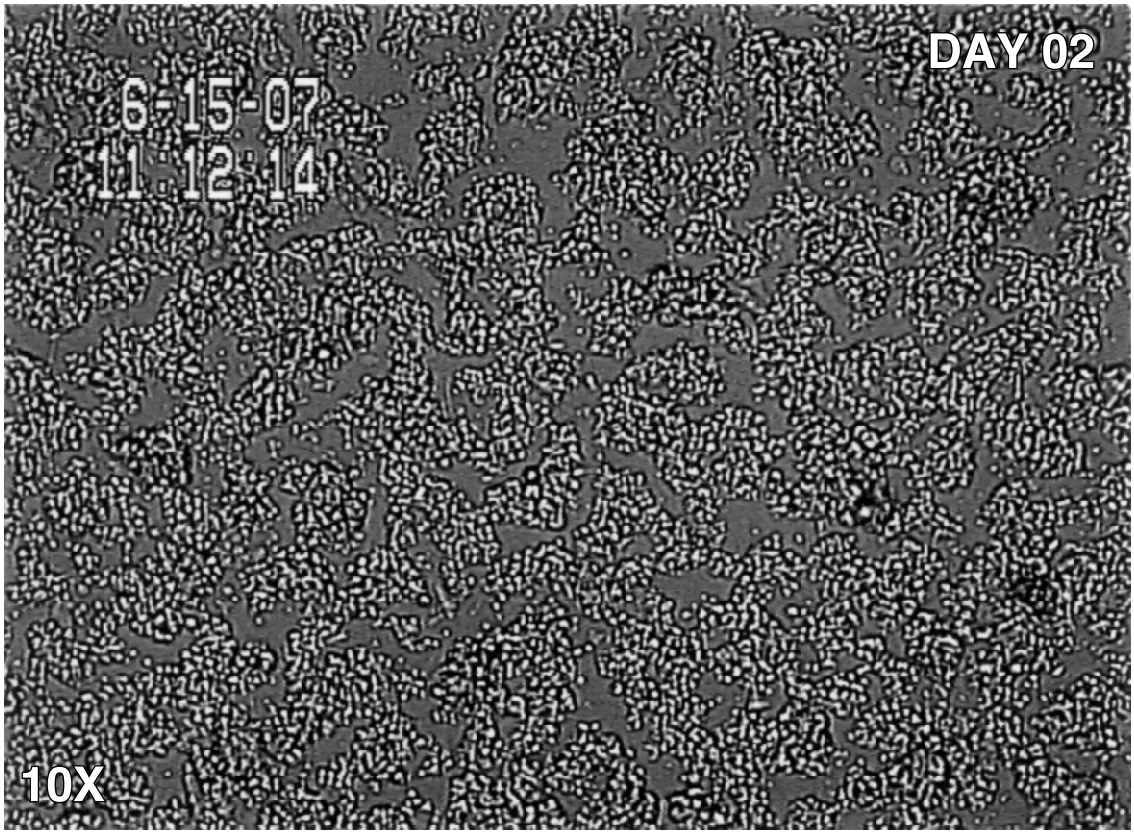
Zhang, Y., C. Ma, et al. (2002). "Identification of genes expressed in C. elegans touch receptor neurons." Nature **418**(6895): 331-335.

Table 2.1: Preparation of cell culture media

		100ml
PVP		500mg
L-Tyr		5mg
Stock salts		8.4ml
0.25M HEPES, pH7.4		10ml
Inulin (5mg/ml stock)		10ml
Amino acid (50X)		2ml
Pen-Strep (100X)		1ml
Base mix		1ml
Galactose		1ml
BME vitamins (100X)		500ul
Na2HPO4 (0.5M)		400ul
MgSO4 (1M)		200ul
CaCl2 (1M)		200ul
Lactate syrup		100ul
Trace minerals		100ul
Phenol red		1mg
Hemin-Cl		250ul
water, cell culture grade		24ml
Adjust osmolality to 345mOsm		
Defined FBS		40ml
L-glutamine (10(	-20C	1ml
Pyruvic acid (14ı	FRESH	200ul
Add Glutathione		60mg
Adjust pH back to 7.2-7.4		
Add cholesterol		500ul
[25mg/ml slurry in 50%EtOH]		

Add 500ul normocin to 100ml media, filter and aliquot into 15ml tubes

Figure 2.1: Isolated embryonic cells in culture. Embryonic cells isolated from *cul-1(e1756)* mutant animals initially plated ~1X10^6 cells/ml in a standard 6-well plate (in upper panel) while the bottom panel shows an increase in total cell population at day 5 following isolation.


DAY 02
6-15-07
11:12:14
10X

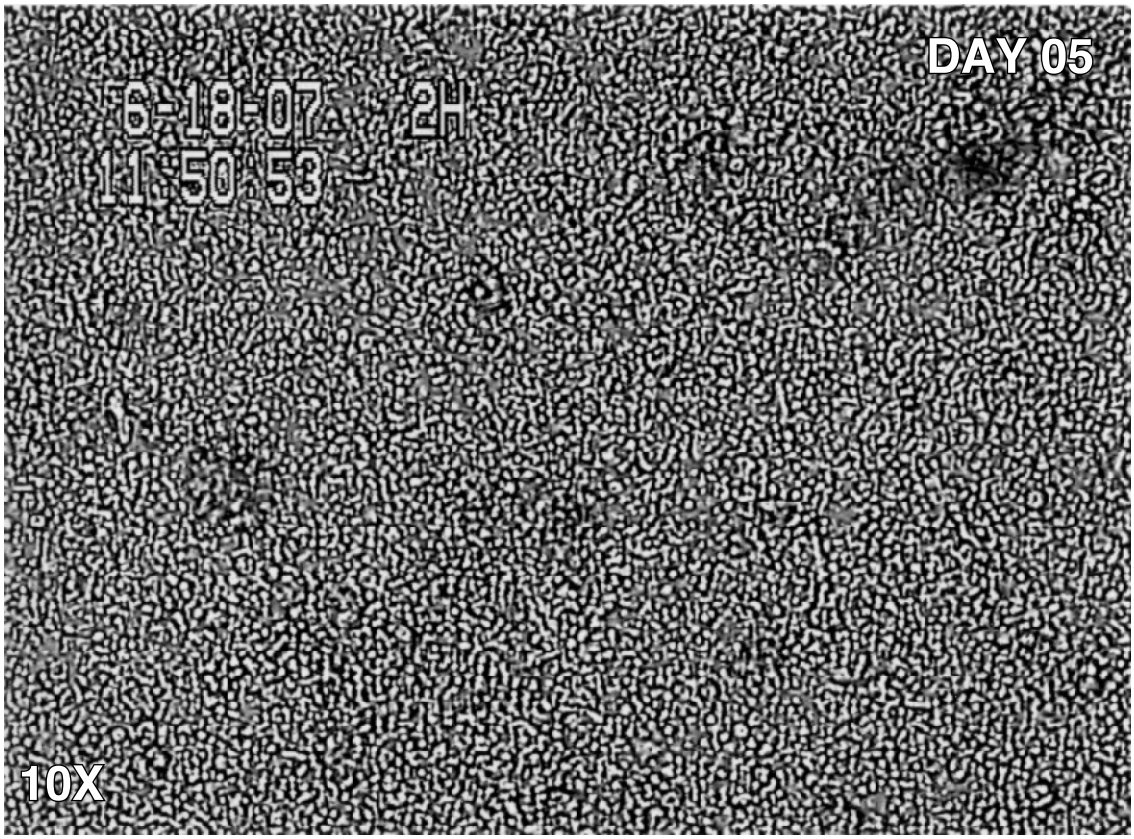
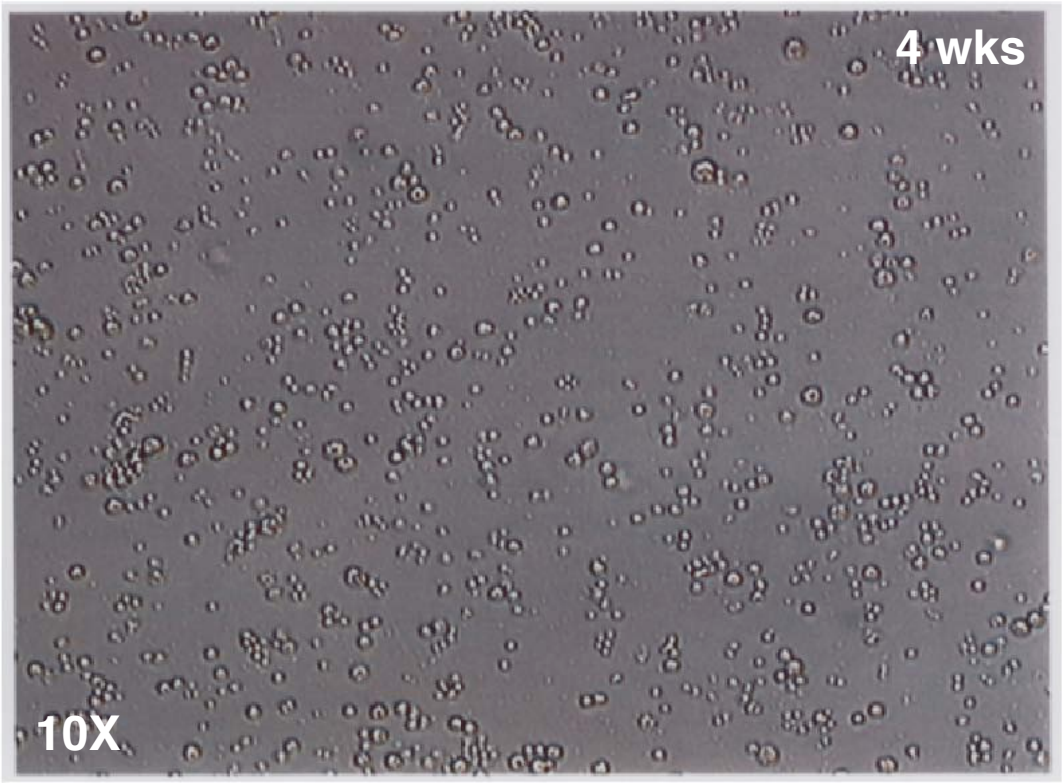

DAY 05
6-18-07 2H
11:50:53
10X

Figure 2.2: Isolated embryonic cells survive for months in culture without differentiation. Embryonic cells isolated from *cul-1* mutant animals proliferate briefly in current culture conditions (upper panel). Total embryonic cell population drops to ~4 to $6X10^4$ cells/ml from a initial plate of $2X10^6$ cells/ml. In later months, embryonic cell population continues to decrease but large cell clumps are observed to form (bottom panel).


4 wks
10X

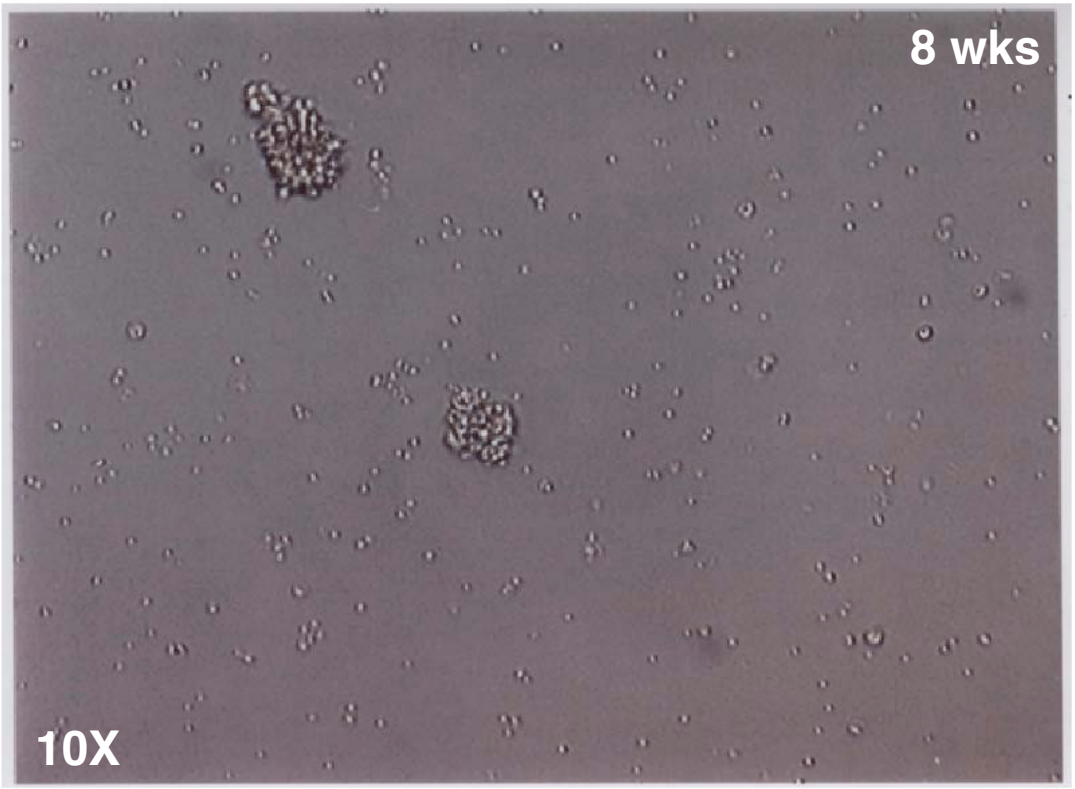

8 wks
10X

Figure 2.3: Isolated embryonic cells survive for months in culture. Embryonic cells isolated from *cul-1* mutant animals survive more than two months in current culture condition. Upon trypan blue exclusion test we found a majority of the cells in large clumps and small clusters are live cells. At this stage, most of the cells are not attached to the plastic surface of standard 6-well plate.

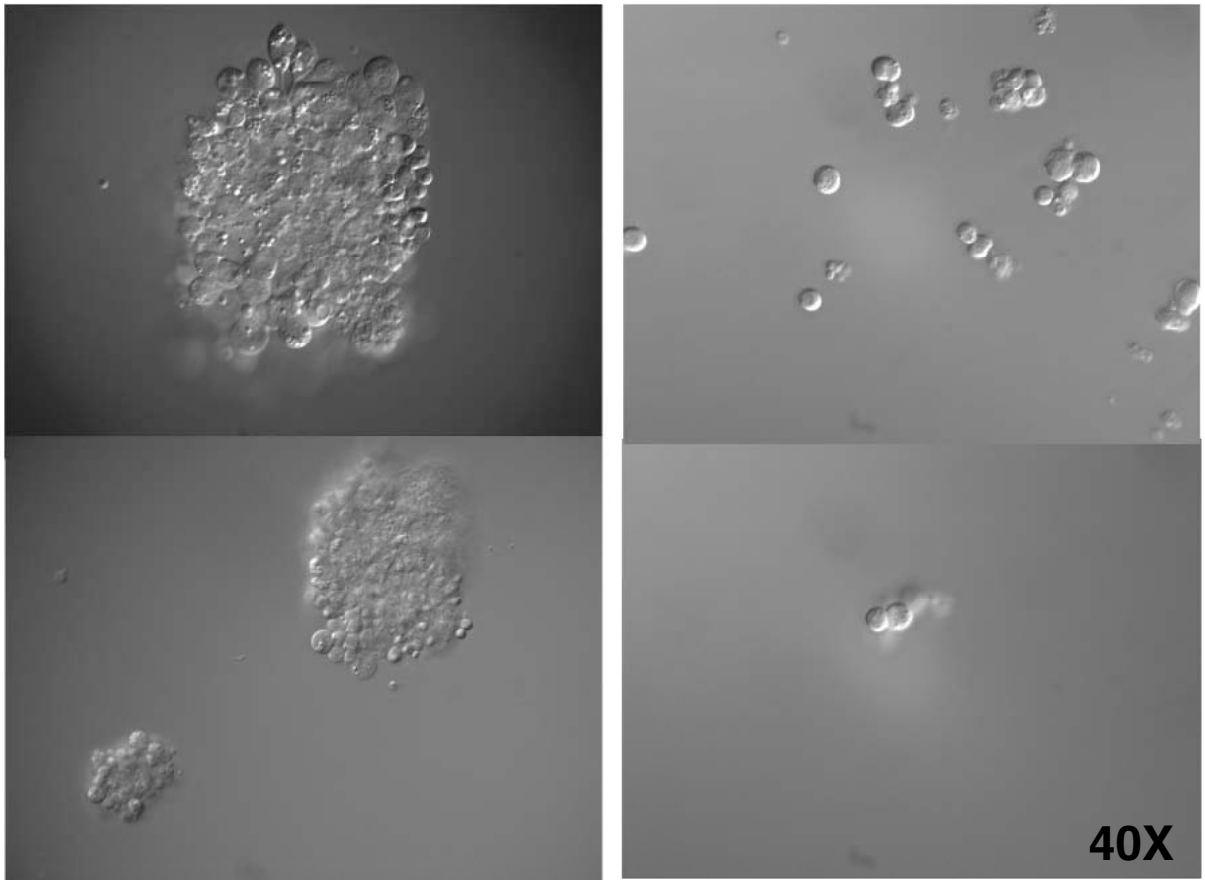

40X

Figure 2.4: Isolated embryonic cells survive for months in culture. Embryonic cells isolated from *cul-1* mutant animals survive more than four months in current culture condition. After four months in culture embryonic cell morphology starts to deform. At the stage, we observed many of these deformed cells to uptake trypan blue suggesting cells are dead although other healthy looking cells still exclude trypan blue. Following the 4-6 weeks in culture, the *cul-1* mutant embryonic cells in culture maintained a steady cell population between $2X10^4$ to $5X10^4$ cells/ml.

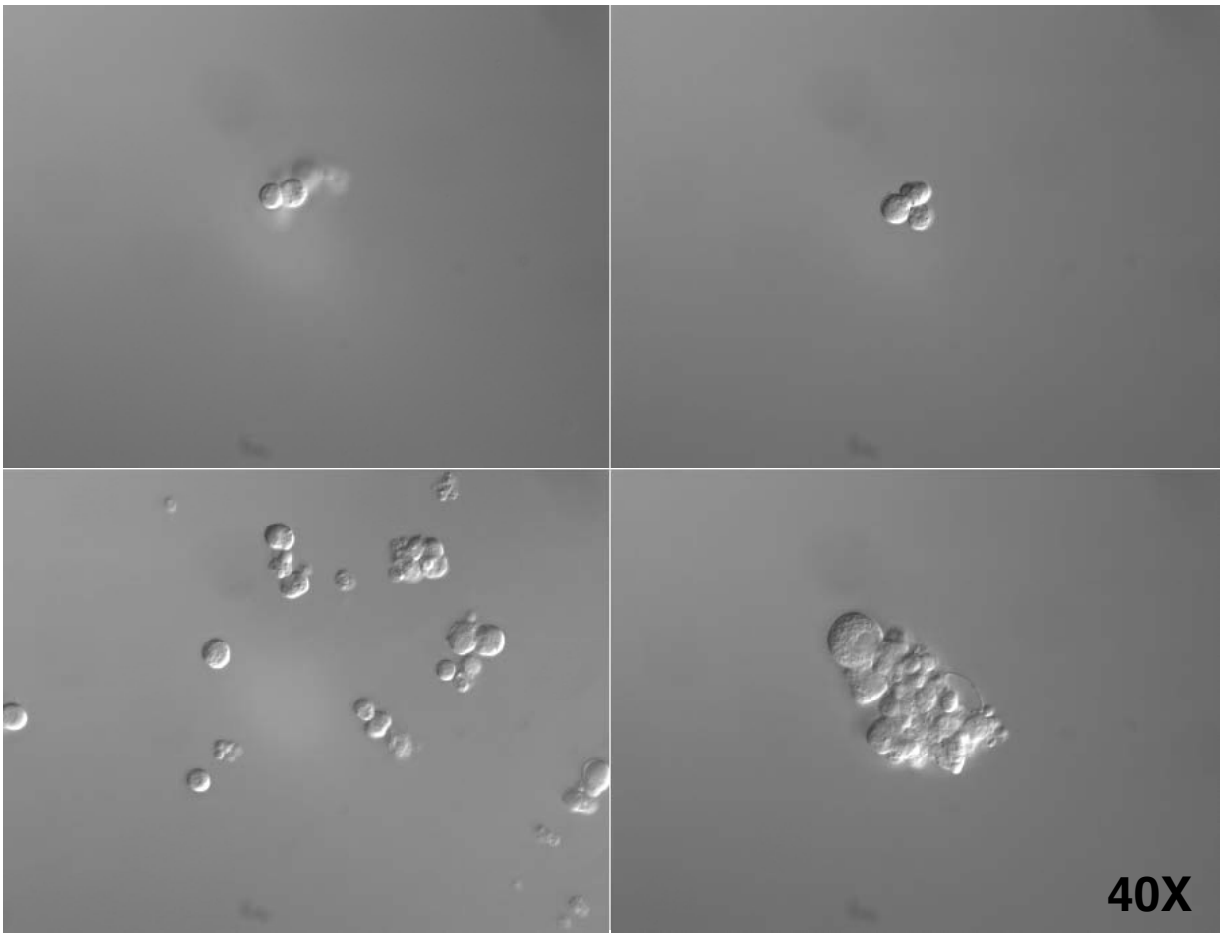
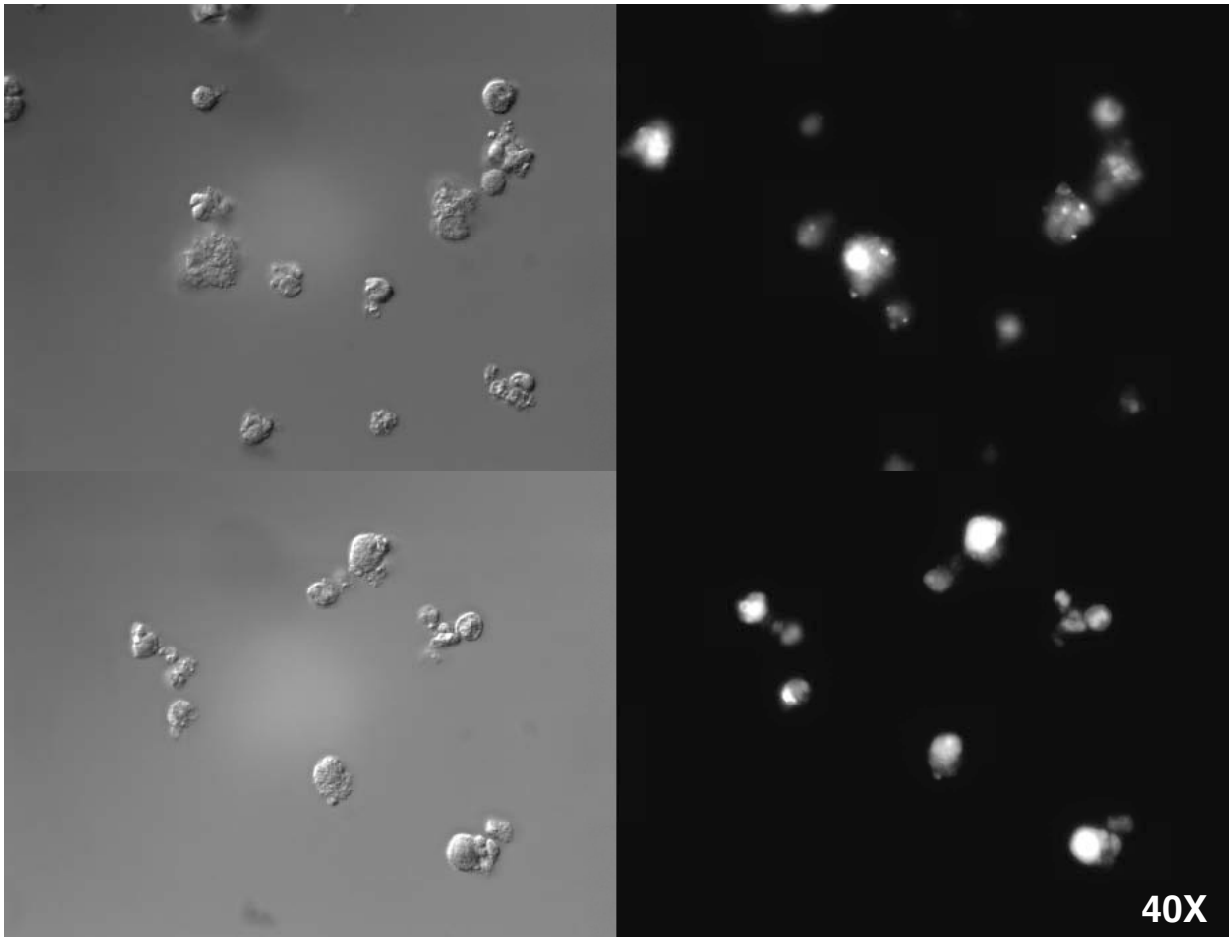
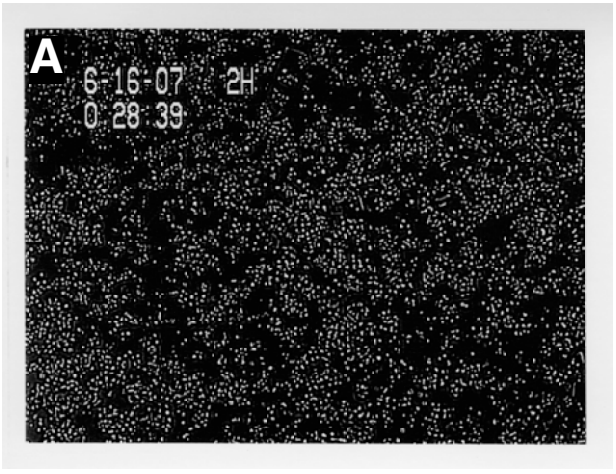

40X

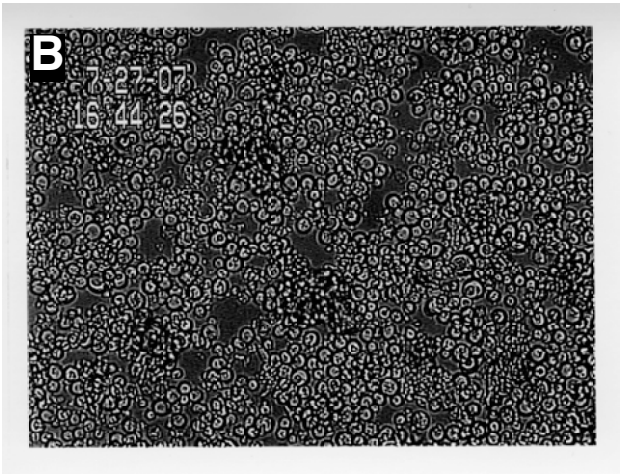
Figure 2.5: Embryonic cells in culture after 6 months. Embryonic cells isolated from a *cul-1* mutant animals survive more than six months in current culture condition but 100% of the cells in culture show auto-fluorescence characteristic of *C. elegans* intestine cells (derived from E lineage). Gut cells are the only cells in *C. elegans* known to auto-fluorescence in adult animals. After four months in culture embryonic cell morphology starts to deform possibly due to differentiation, and then cells start to die.

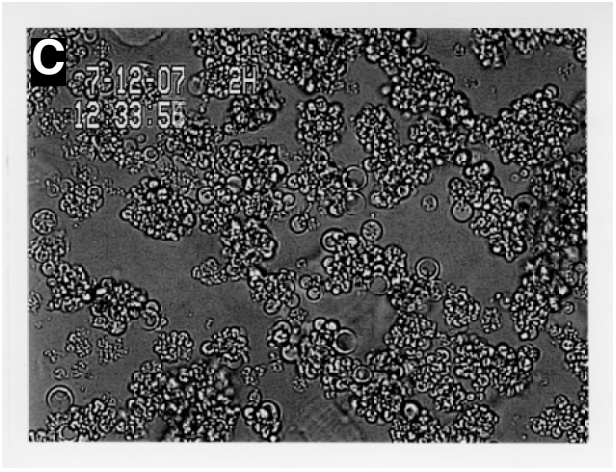


normal light **UV light**

Figure 2.6: Embryonic cell isolation from *cul-1;ced-3* double mutant animals. A. Embryonic cells in culture 2 days after isolation. B. Embryonic cells in culture 3 weeks after isolation. Many cells in current culture condition seem to enlarge abnormally in volume without any significant increase in the total cell population. C. Embryonic cells in culture (of a different *cul-1;ced-3* double mutant derived population than demonstrated in Figure 2.6A & 2.6B) following 6 weeks in culture media. Abnormally large cells were prominent in the culture as well as few smaller clumps of healthy cells. D. Eventually all the abnormal, enlarged embryonic cells derived from *cul-1;ced-3* double mutant died, and cleared off the culture leaving slowly dividing healthy cells in culture after 3 months.


A
6-16-07 2H
0 28 39


B
7-27-07
16 44 26


C
7-12-07 2H
12 33 55

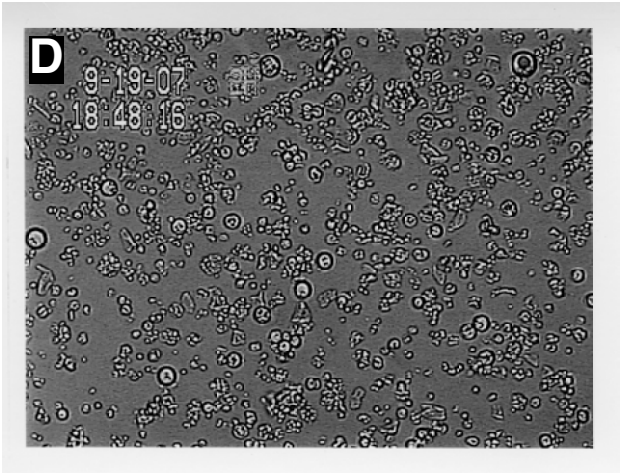

D
9-19-07
18 48 16

Figure 2.7: Phase contrast images of spheroid body-like structures. A. Image of individual cells and small clumps, representative of the culture for the first three months. B. Larger clumps of cells formed in the same culture well. Arrow points to a large clump of cells. C. Formation of spheroid bodies and large aggregation of cells in the process of making the smooth exterior (arrow). D. Spheroid bodies subjected to mechanical stress show long processes. E. Individual cell with neuron-like process following mechanical stress in the same culture well as in figure 2.7D. All images are the same magnification. The scale bar in C is 10 μm.

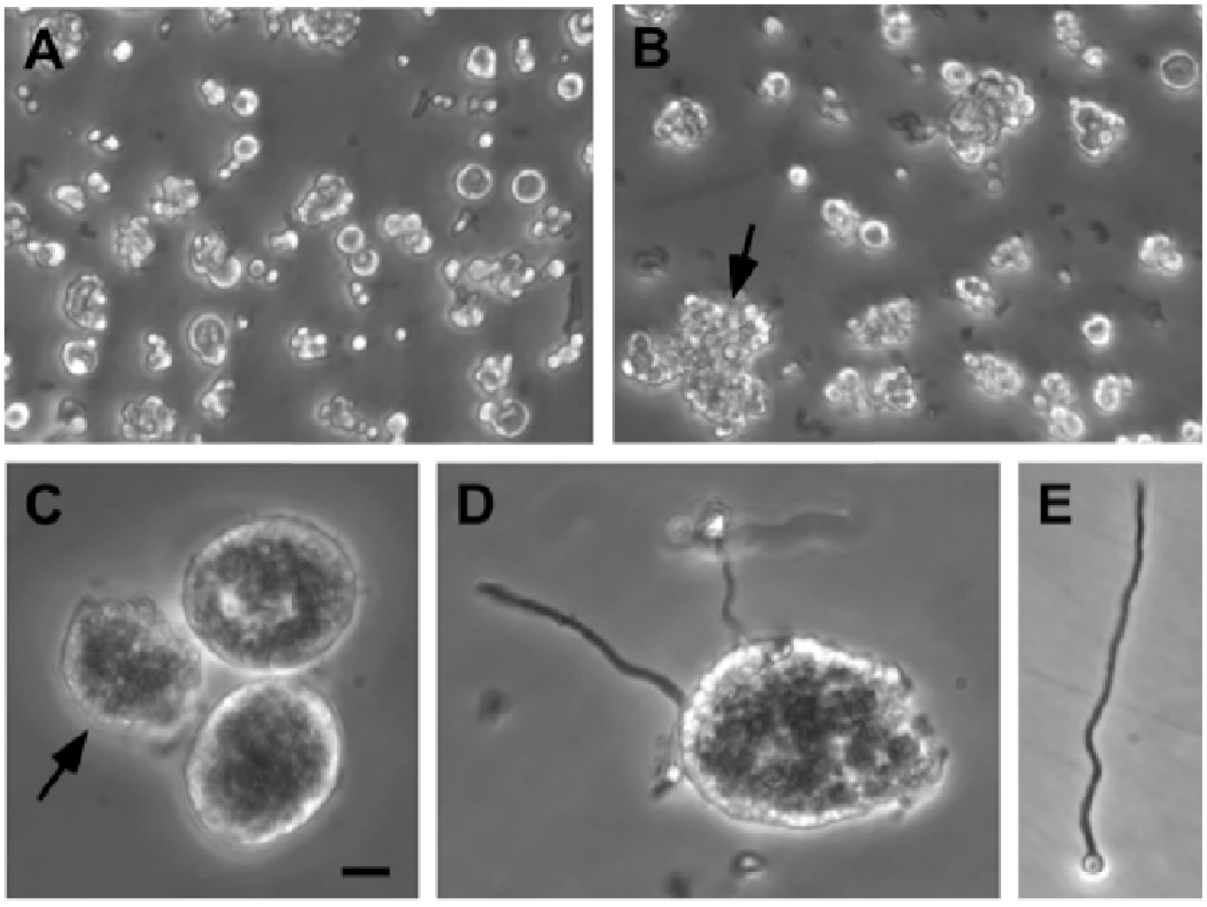

A
B
C
D
E

Figure 2.8: Germ cell isolation from *glp-1(ar202)* mutant animals. Cut gonad from germline 'tumor' mutant in culture media. Following days after isolation germ cells only dispersed into the culture media without any significant proliferation.

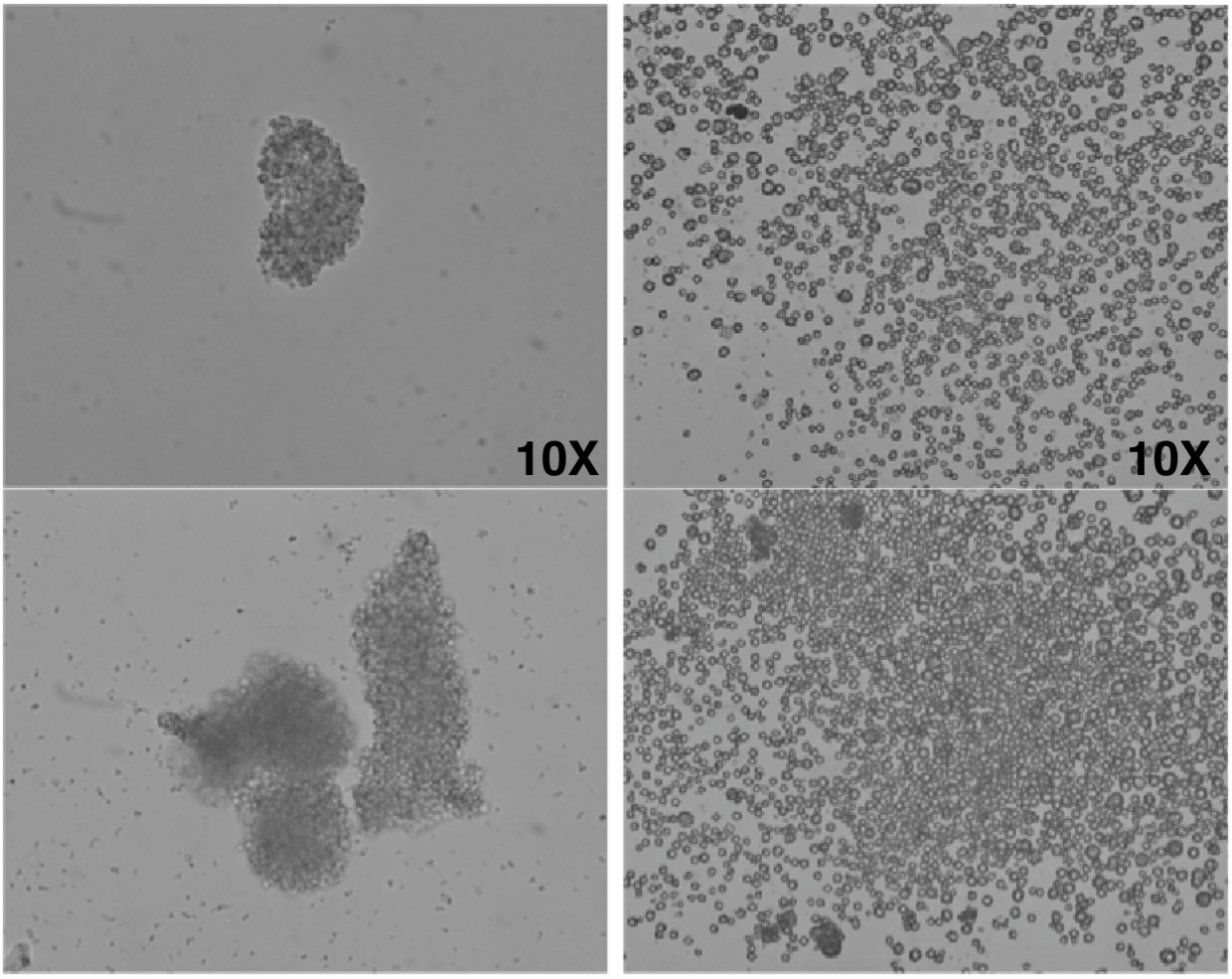
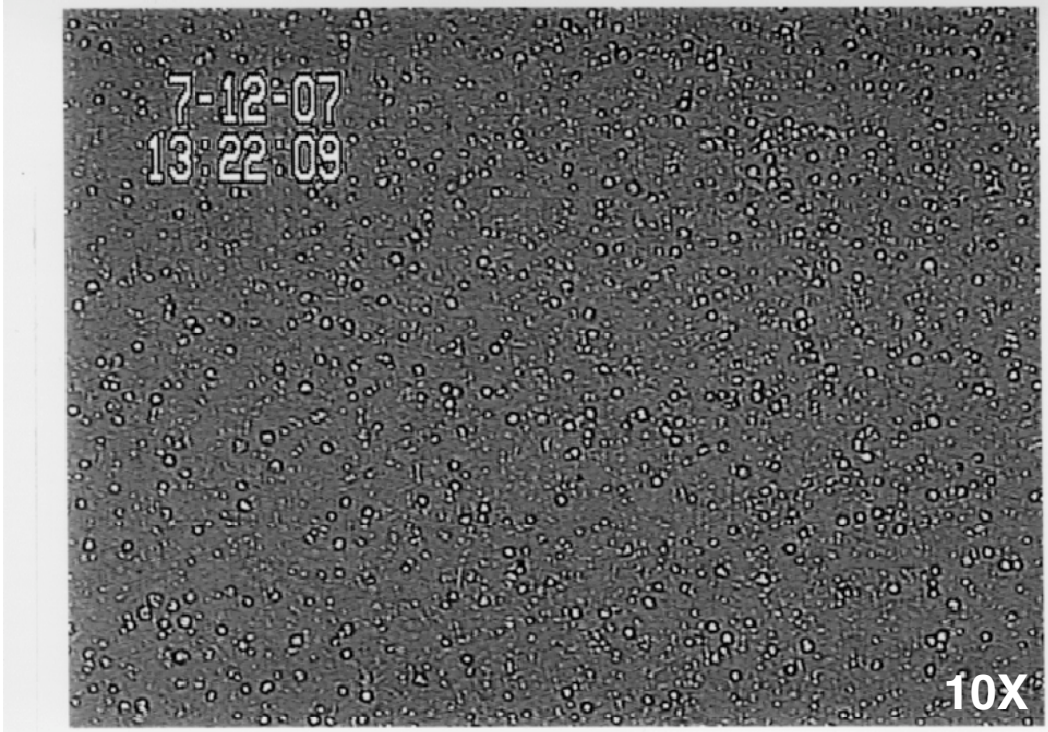

10X
10X
day 0
day 2

Figure 2.9: Large-scale germ cell isolation from *glp-1(ar202)* mutant animals. Germ cells were isolated from 10 large plates full of synchronized adult animals, and plated in a standard 6-well plate (~$2X10^5$ cells/ml). Upper panel shows isolated germ cells following 2 days in culture after isolation, and bottom panel shows all the isolated germ cells have been cleared off the culture media within two weeks after isolation.


7-12-07
13:22:09
10X

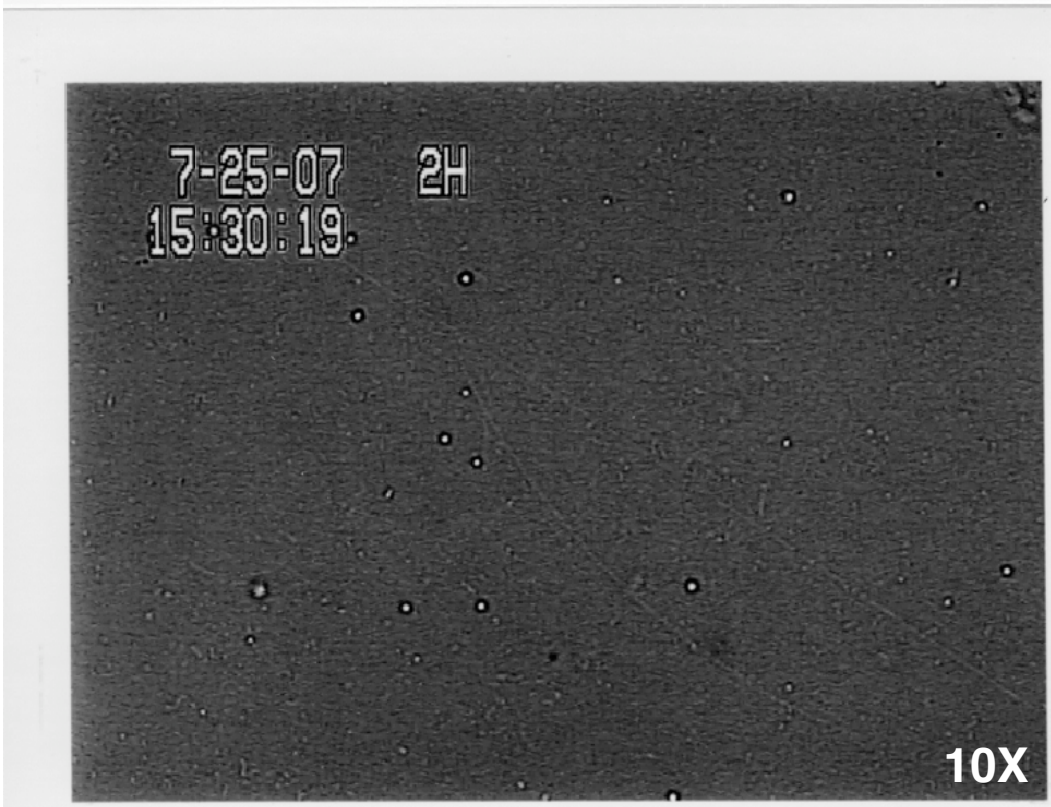
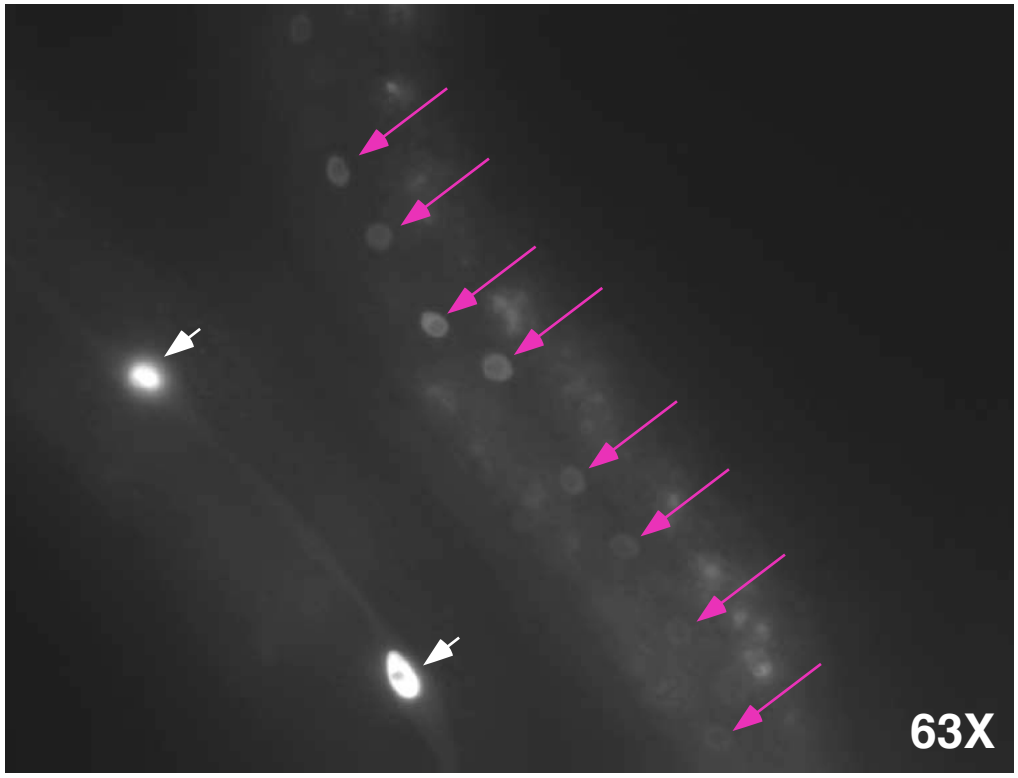

7-25-07 2H
15:30:19
10X

Figure 2.10: Deregulation in hypodermal cell proliferation upon CUL-1 inactivation. Two adult animals (side-by-side) carrying scm::GFP clearly marking hypodermal seam cell locations. We observed an increase in total seam cell numbers in *cul-1* RNAi fed animals compared to vector RNAi fed animals. In wild type short arrowheads mark seam cells while long arrows point to cells in *cul-1* RNAi fed animals.


63X

CHAPTER 3

MULTIPLE PATHWAY MODULATION OF DAF-16/FOXO MEDIATED LIFESPAN REGULATION IN *C. ELEGANS**

Introduction

Insulin resistance is a key event in both insulin dependent and non-insulin dependent diabetes mellitus. In addition to its role in diabetes, insulin resistance is linked to obesity, hypertension, and increased risk of atherosclerosis and other cardiovascular disorders in aging adults (Biddinger and Kahn 2006). Despite the fact that much of the insulin signaling pathway functions is known for decades, the molecular mechanisms underlying insulin resistance are complex and not fully understood. The links between insulin resistance and its aftereffects are critical in understanding diabetes, and associated age related disorders (Cohen and Dillin 2008).

The onset of insulin resistance is linked to nutrient excess, which leads to increased carbohydrate flux through the hexosamine biosynthesis pathway (HBP) and elevated UDP hexosamine levels (McClain, Lubas et al. 2002). Excess glucose in cells is normally converted into UDP-hexosamines, i.e., UDP-N-acetylglucosamine (GlcNAc) through HBP (Rossetti, Hawkins et al. 1995). Cellular UDP-GlcNAc levels are linked to dynamic post-translational modification of target proteins by a single GlcNAc sugar molecule at serine or threonine residues (Du, Edelstein et al. 2001; Vosseller, Wells et al. 2002). Metazoa have a highly conserved O-GlcNAc transferase (OGT) that catalyzes addition of O-GlcNAc molecules to target proteins (Kreppel, Blomberg et al. 1997), as well as a highly conserved O-GlcNAcase (OGA) that removes the sugar molecule from modified proteins (Wells, Gao et al. 2002; Hart,

*part of the experimental data is published in Aging 2010, 2(10):678-90.

Housley et al. 2007). Unlike other cellular glycosylation processes that occur mostly in the golgi and endoplasmic reticulum (ER), O-GlcNAc cycling occurs both in the nucleus and cytoplasm analogous to phosphorylation and dephosphorylation of cellular proteins (Hart, Housley et al. 2007). OGT and OGA enzymes are observed in both the nucleus and cytoplasm, occasionally in the same multi-protein complexes (Lubas, Frank et al. 1997). In the nucleus, OGT co-localizes with transcription initiation complexes and modulates gene transcription (Yang, Zhang et al. 2002). Numerous proteins have been reported to be O-GlcNAc modified, including transcription factors, nuclear pore complex proteins, kinases, and several metabolic enzymes (Whisenhunt, Yang et al. 2006). O-GlcNAc modification at critical sites alters enzyme activity, protein-protein interaction, protein stability, and gene transcription pattern (Toleman, Paterson et al. 2004; Zachara, O'Donnell et al. 2004; Love and Hanover 2005). Excess O-GlcNAc levels affect normal function of several proteins either by inhibiting general proteosome function (Zhang, Su et al. 2003; Liu, Paterson et al. 2004) or by masking critical phosphorylation sites that affect protein stability (Yang, Kim et al. 2006). Aberrant protein O-GlcNAcylation has been associated with insulin resistance in diabetes, and several neurodegenerative diseases (Dias and Hart 2007). In a mice model, inactivation of OGT function causes hypo-O-GlcNAcylation and subsequent hyper-phosphorylation of tau proteins (Liu, Iqbal et al. 2004). Conditional over-expression of OGT in mouse skeletal muscles and fat cells is also reported to induce an insulin resistance phenotype (McClain, Lubas et al. 2002).

In *C. elegans,* a highly conserved insulin-like signaling pathway regulates adult lifespan as well as post-embryonic and reproductive development, stress responses, and dauer formation (*daf*) in response to nutrient availability (Kenyon, Chang et al. 1993; Gems, Sutton et al. 1998). In general, upon insulin binding, the insulin receptor (IR) catalyzes receptor tyrosine phosphorylation events to activate phosphatidylinositol-3-OH kinase (PI3K) that in turn activates downstream effector kinases, namely PI3K-like dependent kinase (PDK), serum glutocorticoid

kinase (SGK), and protein kinase B (AKT) (Lizcano and Alessi 2002). Insulin pathway effector kinases functionally converge to antagonize a forkhead box (FoxO) transcription factor DAF-16, which integrates signals from multiple pathways to control a wide array of cellular processes essential for growth, and development including adult lifespan regulation in *C. elegans* (Mukhopadhyay, Oh et al. 2006). In *C. elegans,* DAF-2/IR signaling limits DAF-16/FoxO activity through inhibitory phosphorylation of DAF-16 by a highly conserved AKT-1 kinase at four Ser/Thr sites that restricts DAF-16 in the cytoplasm (Cahill, Tzivion et al. 2001; Lin, Hsin et al. 2001). A reduction in DAF-2-mediated signaling permits DAF-16 nuclear localization, and subsequent transcription of DAF-16 dependent genes involved in regulating post-embryonic growth, reproductive development, stress resistance, immunity, metabolism, and many novel processes that collectively affect lifespan in adult animals (Henderson and Johnson 2001; Kenyon 2005). Similar adult lifespan extension upon alteration of insulin or IGF signaling is also reported in *Drosophila,* mice and recently, in a human population with relatively high numbers of centurions in the population (Suh, Atzmon et al. 2008; Kenyon 2010).

Nuclear localization of DAF-16 is a key event in adult lifespan extension mediated by reduced insulin signaling in *C. elegans* (Lin, Hsin et al. 2001). Earlier studies have established that neither a constitutive nuclear localization of DAF-16 nor over-expression of DAF-16 was sufficient for significant lifespan extension (Hsin and Kenyon 1999; Henderson and Johnson 2001; Lin, Hsin et al. 2001; Libina, Berman et al. 2003). In addition, *daf-16* gene expression is not up regulated in long-lived insulin pathway mutants suggesting that changes in *daf-16* gene transcription is not used to regulate adult lifespan in *C. elegans* (Murphy, McCarroll et al. 2003). However, the mechanism remains ambiguous how insulin signaling, mediated through DAF-16, could regulate selectively biological processes in response to varying levels of insulin activity during development (Henderson and Johnson 2001; Cohen and Dillin 2008).

Our current work demonstrates that O-GlcNAc modification of cellular proteins help insulin-mediated signaling achieve its specificity to a subset of DAF-16 downstream targets implicated in the oxidative stress response, and adult lifespan regulation in *C. elegans*. In this study we also demonstrate that dynamic protein O-GlcNAcylation is essential for adult lifespan extension. Finally we have also identified a novel set of O-GlcNAc modified proteins in *C. elegans* (through affinity purification) of which the the majority are functionally regulated by the DAF-2 insulin-signaling pathway.

Genetic Methods and Materials

Nematode strains. Worms were handled according to standard procedures and maintained at 20°C with exception of temperature sensitive (ts) mutants maintained at 16°C (Brenner 1974). Some nematode strains used in this work were provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR). The following strains were used in this study: Wild type N2 Bristol, CF1041 *daf-2(e1370)*, CF1380 *daf-16(mu86)*, TJ1052 *age-1(hx546)*, VC345 *sgk-1 (ok538)*, GR1310 *akt-1(mg144),* GR1318 *pdk-1(mg142),* CL2070 *dvIs70(Phsp-16::gfp),* CF1553 *muIs84 (Psod-3::gfp),* ET422 *muIs109*[*Pdaf-16*::GFP::DAF-16 cDNA + *Podr-1*::RFP], JH2162 *axIs*[*Ppie-1::gfp:: cep-1* ORF]*,* ET434 *oga-1(ok1207)*, ET435 *ogt-1(ok1474)*, ET436 *oga-1(ok1207);ogt-1(ok1474)*, ET439 *oga-1(ok1207);daf-2(e1370)*, ET438 *ogt-1(ok1474);daf-2(e1370)*, ET437 *oga-1(ok1207);daf-16(mu86)*, ET468 *ogt-1(ok1474);daf-16(mu86)*, ET469 *oga-1(ok1207);age-1(hx546)*, ET393 *ogt-1(ok1474);age-1 (hx546)*, ET471 *oga-1(ok1207);sgk-1(ok538)*, ET470 *ogt-1(ok1474);sgk-1(ok538)*, ET472 *oga-1(ok1207); pdk-1(mg142)*, and ET445 *oga-1(ok1207);akt-1(mg144)*. The *oga-1(ok1207)* and *ogt-1(ok1474)* mutants in strains ET434 and ET435 were outcrossed 6 times against N2 in our laboratory. The mutations in *ogt-1(ok1474)* and *oga-1(ok1207)* alleles were verified using PCR primers provided by the C. elegans Gene-Knockout Project at OMRF, which is part of the International *C. elegans* Gene-Knockout Consortium. Different mutations in

double mutants generated in our laboratory were verified by standard PCR followed by restriction enzyme analysis or DNA sequencing spanning the region corresponding to the mutations, where appropriate.

Lifespan analysis. All lifespan assays were performed at 20°C using a total of 85-125 animals for each genotype per assay in NGM plates with OP50 bacteria following established procedures (Kenyon, Chang et al. 1993). In brief, gravid animals were allowed to lay eggs for a few hours at 20°C. Adults were transferred to new plates every alternate day during their fertile period, and later when necessary. Adults were split into 30-50 animals per plate. Adults were counted as dead when they failed to respond to repeated head and tail prodding. Each assay was repeated multiple times, and duplicate assays were pooled for final analysis.

Statistical analyses. Survival curves were analyzed by the Log-rank (Mantel-Cox) and Wilcoxon tests using GraphPad Prism (version 5.0) to determine the significance between mutant and wild-type controls as indicated in Table 3.1. In other experiments, statistical significance was determined by the unpaired two-tailed Student's t-test.

Stress tolerance assay. Oxidative stress resistance assays were performed in multi-well plates by immersing adults in M9 media containing 100 mM paraquat (1,1-dimethyl-4,4-bipyridinium dichloride) at 20°C using 10-20 animals for each genotype per assay (Olsen, Vantipalli et al. 2006). The complete absence of swimming movement was scored as dead. Death was confirmed by transferring the animals to NGM plates and observing no movement for next few hours. Death was scored every 3, 6, 9 and 12 hrs. Thermal resistance assays were performed on NGM plates pre-heated at 35°C using at least 50 animals for each genotype per assay (Arantes-Oliveira, Apfeld et al. 2002). Adults were incubated at 35°C and were scored every hour by response to touch until all animals were found dead (Lithgow, White et al. 1995). Experimental data from several assays were pooled together for final analysis.

Developmental time assay. Postembryonic developmental timing was assayed by collecting comma-stage embryos from animals raised at 20°C. Exact time of hatching was recorded, and then animals were transferred (mouth pipetted) into separate OP50 bacteria seeded NGM plates. After the third molt animals were inspected more frequently (every 15-20 minutes) to record the exact time of the fourth molt. Each experiment was repeated multiple times with at least 10 animals per genotype per assay, and multiple experiment data were pooled together for final analysis. For fecundity analysis, individual animals were placed on plates at 20°C. All offspring were counted by removing them onto fresh NGM plates one-by-one. Each experiment was repeated multiple times with at least 10 animals per genotype per assay, and multiple experimental data were pooled together for analysis.

Western immunoblotting. Adult animals were flash frozen in liquid nitrogen in lysis buffer (50mM HEPES pH 7.8 and 300mM NaCl). Frozen worms were crushed using a mortar and pestle pre-chilled with liquid nitrogen. The frozen ground worms were resuspended in cold lysis buffer (with DNaseI and protease inhibitor cocktail from Roche) and subjected to sonication 6 times for 8 seconds each followed by centrifugation at 16,000 rpm for 1 hour at 4°C. The soluble proteins from the middle layer of each tube were collected for further analysis. An equivalent amount of whole animal lysate was separated in a 10% NuPAGE Bis-Tris gel (Invitrogen) followed by transfer onto a PVDF membrane (Millipore). The membrane was probed with anti-O-GlcNAc antibody (1:2000 in 3% BSA dissolved in 1XTBS-0.05%Tween20) from Affinity Bioreagents. The immunoblot was visualized using a chemiluminescent substrate (super signal west pico from Pierce). The same membrane was later re-probed with anti-α-tubulin antibody (Sigma) to compare protein loading in different lanes of the gel.

RNAi experiments. Experiments were performed with *E. coli* strain HT115 transformed with pPD129.36 variant clones from RNAi library provided by Ahringer for gene inactivation experiments as previously described (Timmons, Court et al. 2001; Kamath, Fraser et al. 2003).

DAF-16::GFP nuclear localization assay. Animals were grown on different feeding RNAi plates for more than one generation. All imaged were taken at single shutter setting using a Hamammatsu ORCA-ER digital camera connected to Zeiss Axioscope. Equivalent nuclear and cytoplasmic areas were chosen for light intensity measurements using OpenLab (version 5.5) software purchased from Improvision Inc. An average of three mean light intensity measurement values per cell (in arbitrary units) for both nuclear and cytoplasmic regions were recorded for multiple cells in each treated or untreated animal. The assay was repeated multiple times with at least 10 animals per genotype in each assay. Multiple experimental data were pooled together for final analysis.

Microarray analysis. Adult animals were collected from ~10-15 large 3xNGM plates into individual tubes as 1-2ml of packed worm aliquots per genotype. Standard techniques were used to obtain RNAi (Trizol), and ~1 microgram of RNA was converted into cDNA (Qiagen Omniscript Kit) (Portman 2006). The cDNA samples from each genotype were sent to the Wells laboratory (CCRC, UGA) for labeling and hybridization reactions following standard protocols. Our collaborator used commercially available Affymetrix Genechip Expression Arrays, and professional technicians at a microarray shared access resource facility analyzed the gene expression data. Transcripts with a 4x change in expression compared to the controls were considered for the final comparisons presented in Table 3.2 and 3.3.

Affinity purification of O-GlcNAc modified proteins. Adult animals were collected and flash frozen in liquid nitrogen in 50mM HEPES pH 7.8 and 50mM NaCl buffer. Frozen worms were crushed with a mortar and pestle pre-chilled with liquid nitrogen. The frozen ground worms were re-suspended in the above-mentioned buffer (with DNaseI and protease inhibitor cocktail from Roche), and disrupted by sonication followed by centrifugation at 16,000 rpm for 1 hour at 4°C. Clear supernatant was collected and soluble O-GlcNAc modified proteins were identified following a standard protocol (Teo, Ingale et al. 2010). Briefly, affinity enrichment with mAb14 of

O-GlcNAc modified proteins was conducted, then the enriched proteins were reduced, alkylated and trypsin digested before mass spectrometry. Protein assignments made following tandem mass spectrometry on a linear ion trap using the *C. elegans* protein database from NCBI, and a false-discovery rate of less than 1% was used for filtering, as previously described (Teo, Ingale et al. 2010).

Results

In *C. elegans,* O-GlcNAcase (*oga-1*) complete loss-of-function (null) mutants are viable and fertile (Forsythe, Love et al. 2006). We observed an abnormally high levels of O-GlcNAc modified proteins in the western blot of whole animal lysate from *oga-1(ok1207)* animals probed with anti-O-GlcNAc antibody (Figure 3.1A). While the *oga-1(ok1207)* mutant appears overtly wild type, we noticed that the *oga-1(ok1207)* mutant adults live ~33% longer than wild-type adults at 20°C (Figure 3.1B). In *C. elegans,* O-GlcNAc transferase (*ogt-1*) loss-of-function mutant animals are also viable and fertile (Hanover, Forsythe et al. 2005). We observed a dramatic reduction in O-GlcNAc modified proteins in the western blot of *ogt-1(ok1474)* whole animal lysate probed with anti-O-GlcNAc antibody (Figure 3.1A). The *ogt-1(ok1474)* null mutant is also superficially wild type in appearance except that the adult lifespan is ~20% shorter than wild-type animals at 20°C (Figure 3.1B). We noted that *ogt-1(ok1474)* mutant animals also appear to age prematurely, as exemplified by the significant slowing down of the animal movement at day 12 of the adulthood relative to very active wild-type and *oga-1(ok1207)* adults at similar age (data not shown). Similar slow down in wild-type animal movement is only prominent at day 18 of adulthood or later (data not shown). Earlier, at day 6 of the adulthood, *ogt-1(ok1474)* mutant movement is indistinguishable from that of wild type (data not shown). To confirm that the adult lifespan extension observed in *oga-1(ok1207)* mutant animals results from increased levels of O-GlcNAc modified proteins, we analyzed the lifespan of animals deficient for both OGT-1 and OGA-1 function. In our current experiment we observed a significant

decrease in *oga-1(ok1207);ogt-1(ok1474)* double mutant lifespan compared to the *oga-1 (ok1207)* single mutant (15.5±4.9 and 20.8±2.2 days respectively, n=85-125 per genotype per assay, p<0.0001). Our current observation argues that the lifespan extension in *oga-1(ok1207)* mutant linked to its elevated levels of O-GlcNAc modified cellular proteins (Figure 3.1). Our findings strongly argue that protein O-GlcNAcylation is critical for normal lifespan in *C. elegans.*

Previous studies established that the FoxO-like transcription factor DAF-16 is essential for lifespan extension associated with the DAF-2 insulin signaling pathway mutants in *C. elegans* (Lin, Dorman et al. 1997). We asked whether lifespan extension in *oga-1(ok1207)* mutant animals also requires DAF-16 function. In our experiments we observed that lifespan extension in *oga-1(ok1207)* mutant adults is DAF-16 dependent. Adult lifespan of *oga-1 (ok1207);daf-16(mu86)* double mutant animals was similar to that of the *daf-16(mu86)* single mutant (Figure 3.2A), which is significantly different from *oga-1(ok1207)* single mutant alone (14.8±1.8 vs 20.8±2.2 days respectively, n= 85-125 per genotype, p<0.0001). The reduction in lifespan observed in *oga-1(ok1207)* mutant animals upon inactivation of DAF-16 suggested that changes in O-GlcNAc cycling might be additive to DAF-2-mediated insulin signaling pathway outcomes in *C. elegans*. Surprisingly our current experimental data reveals that inactivation of OGT-1 function significantly reduces the adult lifespan extension in *daf-2(e1370)* mutant animals as observed in *daf-2(e1370);ogt-1(ok1474)* double mutant animals (27.6±1.4 vs. 13.2±1.9 days respectively, n=85-125 per genotype, p<0.0001). Taken together we conclude that protein O-GlcNAcylation is essential for the lifespan extension observed in the *daf-2(e1370)* mutant (Figure 3.2B). Our experimental findings are in line with recent literature as other mutant alleles of OGT-1 also reduce long lifespan of the *daf-2(e1370)* mutant (Love, Ghosh et al. 2010).

In a conventional genetic analysis, mutations for genes that act in parallel pathways with a similar functional outcome generally demonstrate additive or synergistic interactions when

combined. To determine whether loss of O-GlcNAcase OGA-1 activity has additive or synergistic interactions with the DAF-2 insulin signaling pathway, we combined the long-lived *daf-2(e1370)* mutant allele with the long-lived *oga-1(ok1207)* mutant allele. We did not observe any additive effect, *i.e.,* further increase in adult lifespan, of the *daf-2(e1370);oga-1(ok1207)* double mutant compared to the *daf-2(e1370)* single mutant animals (Table 3.1). Loss-of-function mutants of DAF-2 insulin signaling pathway effector kinases AGE-1 or SGK-1 also extend adult lifespan in *C. elegans* (Dorman, Albinder et al. 1995; Hertweck, Gobel et al. 2004). Similar to *daf-2(e1370)* mutant, the extended lifespan observed in both *age-1(hx546)* and *sgk-1 (ok538)* mutants were not increased further when combined with the *oga-1(ok1207)* mutant allele (Figure 3.3C and 3.3D respectively). On the other hand, the extended lifespan of *age-1 (hx546)* and *sgk-1 (ok538)* mutants were reduced to the wild-type level upon combination with the *ogt-1(ok1474)* loss-of-function mutant allele (Figure 3.3A & 3.3B respectively, statistical analysis summarized in Table 3.1). The epistasis of OGT-1 and OGT-1 phenotypes suggested that the critical O-GlcNAcylation target proteins work in the same pathway, not in a parallel pathway, and is located genetically downstream of the insulin pathway effector kinases AGE-1 and SGK-1.

To further investigate where in the insulin pathway O-GlcNAc cycling impinges, we performed a set of genetic analysis with gain-of-function (gf) mutants of insulin signaling pathway, which constitutively activate the pathway irrespective of upstream activation signals. In *C. elegans,* the *akt-1(mg144)* mutant provides constitutive AKT-1 mediated phosphorylation of DAF-16 that is independent of upstream signaling from DAF-2 (Paradis and Ruvkun 1998; Gami, Iser et al. 2006). In our experiments we observed that the lifespan of the *akt-1(mg144)gf* mutant is shorter than that of wild-type adults at 20°C (Figure 3.3E). When the *akt-1(mg144)gf* allele is combined with the long-lived *oga-1(ok1207)* mutant allele constitutively active AKT-1 function could not reduce lifespan extension in a *oga-1(ok1207); akt-1 (mg144)* double mutant.

Adult lifespan of *oga-1(ok1207); akt-1(mg144)* double mutant was significantly longer than wild-type lifespan (19.8±4.2 and 15.9±3.6 days for *oga-1;akt-1* and wild type respectively, n= 85-125 animals per genotype per assay, p =0.0002) assayed at 20°C (Table 3.1). DAF-2 insulin pathway effector kinase PDK-1 also phosphorylates and increases AKT-1 activity in *C. elegans* (Alessi, James et al. 1997). A gain-of-function mutant allele of PDK-1 kinase [*pdk-1(mg142)*] was reported to have short lifespan (Paradis, Ailion et al. 1999). The *oga-1(ok1207);pdk-1 (mg142)* double mutant animals have significantly longer lifespan than *pdk-1(mg142)* alone, and wild-type animals assayed at 20°C (Table 3.1). Together our genetic data argues that adult lifespan extension mediated by unrestrained O-GlcNAc modification probably targets critical proteins located downstream of both AKT-1 and PDK-1 kinases. However neither *mg144* or *mg142* gain-of-function mutant alleles could suppress the *age-1(hx546)* mutant lifespan extension phenotype (Paradis and Ruvkun 1998).

The DAF-2 insulin pathway controls numerous biological functions that are associated with dauer entry, stress resistance, pathogen resistance, fertility, and development in *C. elegans* (Gems, Sutton et al. 1998). The lifespan extension observed in insulin signaling pathway mutants is also correlated with oxidative and thermal stress resistance as well as obvious changes in reproductive development (Gems, Sutton et al. 1998; Honda and Honda 1999). We asked the question whether aberrant alterations in O-GlcNAc cycling could affect all downstream functions controlled by the insulin pathway through DAF-16 /FoxO transcription factor. There is a strong but poorly understood link between longevity and oxidative stress resistance (Honda and Honda 1999). Upon investigation we discovered that *oga-1(ok1207)* mutant animals are resistant to oxidative stress (9hrs in 100mM paraquat) in a DAF-16 dependent manner (Figure 3.4A). Resistance to oxidative stress in *C. elegans* is also correlated with the expression of superoxide dismutase (*sod*) genes (Honda and Honda 1999), and similarly we observed increased *sod-3* expression levels in non-stressed adult animals upon

inactivation of OGA-1 function (Figure 3.4B). Notably, inactivation of OGT-1 in a *daf-2 (e1370);ogt-1(ok1474)* mutant significantly reduced its oxidative stress tolerance (Figure 3.4A) suggesting that protein O-GlcNAcylation is indispensable for oxidative stress resistance in the long-lived *daf-2(e1370)* mutant. Long-lived insulin pathway mutants are also thermal stress resistant (Hsu, Murphy et al. 2003). In contrast to DAF-2 pathway mutants, the long-lived *oga-1 (ok1207)* mutant was sensitive to thermal stress at 35°C (Figure 3.5A), and subsequently inactivation of OGA-1 function failed to induce heat shock protein (*hsp-16*) expression in non-stressed animals (Figure 3.5B). In addition to thermotolerance, we also observed significant differences in fecundity levels and post-embryonic developmental timing between the long-lived *daf-2(e1370)* and *oga-1 (ok1207)* mutants. Post-embryonic development is severely delayed in the *daf-2(e1370)* mutant while *oga-1(ok1207)* mutant animals develop normally (64.8±1.3 hrs vs. 43.5±3.9 hrs for *daf-2* and *oga-1* mutants respectively, n=10, $p < 0.0001$) similar to wild-type animals (41± 0.7 hrs) (Figure 3.6A). Additionally, *daf-2(e1370)* mutant animals demonstrate a reduced level of fecundity while the *oga-1 (ok1207)* mutant generates normal numbers of offspring (186±18 vs. 263±26 for *daf-2* and *oga-1* respectively, n=12, $p < 0.003$) similar to that observed in the wild-type animals (289±32) (Figure 3.6B). To confirm that increased thermotolerance, delayed post-embryonic development, and the reduced fecundity that are associated with the *daf-2(e1370)* mutant are independent of changes in O-GlcNAc cycling, we inactivated OGT-1 function in the *daf-2(e1370)* mutant. Consistently, we observed that thermotolerance, delayed post-embryonic development, and reduced fecundity phenotypes were unaltered upon loss of protein O-GlcNAcylation in the *daf-2(e1370);ogt-1(ok1474)* double mutant when compared to the *daf-2(e1370)* single mutant (Figure 3.5A, 3.6A & 3.6B respectively). Although inactivation of OGT-1 function significantly reduced *daf-2(e1370)* adult lifespan (28.6±3.5 vs. 13.2±1.9 days for *daf-2* and *daf-2;ogt-1* mutants respectively, n=85-125

animals per genotype, p <0.0001) implying that many cellular functions under DAF-2 insulin signaling pathway are regulated independently of the protein O-GlcNAc cycling.

To investigate our hypothesis that O-GlcNAc cycling can modulate a subset of DAF-16-mediated downstream activity under control of insulin signaling we analyzed the changes in DAF-16 dependent transcription profile in long-lived *daf-2(e1370)* mutant with and without functional OGT-1. In Affymatrix Genechip microarray experiment, we observed that ~32% of the DAF-16 dependent genes up regulated in *daf-2(e1370)* mutant require OGT-1 function (Table 3.2). At the same time we found that OGT-1 function is essential for the down-regulation of ~27% of DAF-16 dependent genes in the *daf-2(e1370)* mutant (Table 3.3). The experimental findings argue in favor for our hypothesis that O-GlcNAc cycling affects only a subset of the DAF-16 mediated downstream functions that are under the active control of the insulin signaling pathway *in vivo.*

DAF-16 is a key effector molecule in insulin signaling pathway-mediated regulation of the cellular processes associated with adult lifespan extension. The DAF-16 protein is usually evenly distributed between the cytoplasm and nucleus of several cell types including muscle, hypodermis, intestine, and head neurons in *C. elegans* (Henderson and Johnson 2001). Inactivation of insulin signaling pathway components results in complete nuclear localization of DAF-16, which is critical for lifespan extension (Lin, Hsin et al. 2001; Hertweck, Gobel et al. 2004). To understand how O-GlcNAcylation of cellular proteins could affect only a subset of DAF-16 mediated functions, we assayed DAF-16 nuclear localization upon inactivation of O-GlcNAc cycling enzymes. Interestingly we observed only a modest DAF-16 nuclear localization upon OGA-1 inactivation by RNAi (Figure 3.7C) in contrast to the complete nuclear localization of DAF-16 upon inactivation of insulin signaling pathway effectors kinases, such as, AGE-1 by feeding RNAi (Figure 3.7B). Surprisingly, inactivation of OGT-1 also caused similar levels of DAF-16 nuclear localization (Figure 3.7D, quantification in 3.7E). Taken together our current

experimental findings suggest that altering cellular O-GlcNAc cycling does not regulate DAF-16 downstream functions under DAF-2 insulin signaling predominantly through its sub-cellular localization.

In several occasions O-GlcNAc cycling is reported to affect protein stability linked to ubiquitin-mediated degradation through 26S proteosome (Zhang, Su et al. 2003). While mammalian FoxO is known to be O-GlcNAc-modified nothing is known about the *C. elegans* DAF-16 protein other than that it is also degraded though the ubiquitin-mediated proteosome pathway (Li, Gao et al. 2007; Kuo, Zilberfarb et al. 2008). A persistent decline in cellular protein degradation machinery is associated with aging and aging-related neurodegenerative diseases (Martinez-Vicente, Sovak et al. 2005) but how insulin signaling is linked to the regulation of cellular protein-degradation pathways is not understood well. CRL1 E3 ubiquitin ligase function is essential for DAF-16 transcriptional activity, and DAF-2 insulin pathway-mediated adult lifespan extension in *C. elegans* (Ghazi, Henis-Korenblit et al. 2007). In our current study we observed that inactivation of CUL-1 function similarly reduces wild-type lifespan as well as shorten *oga-1(ok1207)* long lifespan (Figure 3.8). In contrast to the published report by Ghazi *et al.*, (2007), we observed a significant reduction in DAF-16 protein (DAF-16::GFP translational fusion) levels upon CUL-1 inactivation in 12 day-old adults assayed at 20°C (Figure 3.9a & 3.9b). We investigated further, and found abnormally high levels of CEP-1/p53 protein accumulation in neuro-endocrine and other neuronal cells in adult animals upon CUL-1 inactivation (Figure 3.10). *C. elegans* SCF (skp-1/cul-1/Fbox) complexes negatively regulate CEP-1/p53 function, and inactivation of CEP-1 is reported to extend adult lifespan (Arum and Johnson 2007; Gao, Liao et al. 2008). Our current experimental findings propose a novel role of CRL1 E3 ubiquitin ligase complexes in regulating DAF-16 mediated functional outcomes of DAF-2 insulin signaling critical in development processes in response to availability of nutrients in *C. elegans*.

Finally in an effort to discover target proteins that are modified by O-GlcNAc in *C. elegans*, we affinity purified cellular proteins from *oga-1(ok1207)* and *ogt-1(ok1474)* mutants; and then identified O-GlcNAc modified proteins by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). In our current affinity purification of O-GlcNAc modified proteins, we identified 13 distinct proteins, encompassing 21 proteins in total when including paralogs with identical or nearly identical peptide sequences that could not be separately distinguished by mass spectrometry. The number of peptides leading to the identification of each protein (see Table 3.4) are as follows: PHI-37 (8); MDH-1 (7); ATP-2 (3); GPD-1 (2); GPD-2 (4); ACT-1 (5); TBA-2 (3); EFT-3 (6); VIT-6 (2); VHA-13 (2); CDC-48.2 (2); F27D4.1 (2); and C16A3.10 (3). Non-specific proteins would be present in our current affinity purification of O-GlcNAc modified proteins from both *ogt-1(ok1474)* and *oga-1(ok1207)* lysates, while O-GlcNAc-modified proteins (or proteins tightly-associated with O-GlcNAc modified proteins) would be specific for only *oga-1* mutant lysate. We reconfirmed the putative O-GlcNAc targets (in Table 3.4) are absent in the *ogt-1* mutant lysate. Significantly, expression of 8 of the 13 putative O-GlcNAc target proteins is regulated by the DAF-2 insulin signaling pathway, and therefore are located downstream of DAF-16 (Table 3.4). A substantial number of putative O-GlcNAc target proteins, which are metabolic enzymes, contribute to dauer entry, lifespan regulation, and oxidative stress response (Murphy, McCarroll et al. 2003; Halaschek-Wiener, Khattra et al. 2005; McElwee, Schuster et al. 2006; Schuster, McElwee et al. 2010). Our current experimental findings further demonstrate that downstream targets of DAF-2 insulin pathway are modified by O-GlcNAc as expected in our primary genetic assays.

Discussion

In the current study, we demonstrated that a dynamic post-translational protein modification, O-GlcNAcylation, is critical for adult lifespan in nematode *C. elegans*. We revealed that excessive protein O-GlcNAcylation down-regulates DAF-2 insulin signaling, which

is key to adult lifespan regulation in *C. elegans* (Kenyon 2010). We also demonstrated that excessive protein O-GlcNAcylation induces DAF-16 dependent oxidative stress resistance, and extends adult lifespan.

C. elegans O-GlcNAc cycling enzyme mutants are viable unlike in other animals (Hanover, Forsythe et al. 2005; Forsythe, Love et al. 2006). We observed that lack of O-GlcNAc transferase (OGT) activity reduces animal lifespan in *C. elegans*. However *ogt-1* null mutant animals develop normally, have an overtly wild-type appearance, and produce normal numbers of offspring, indicating no major anomalies in animal physiology upon complete loss of O-GlcNAc modification of cellular proteins. It suggests protein O-GlcNAcylation is not critical in nematode embryonic development. In line with the mammalian *in vitro* experimental findings that elevated O-GlcNAc levels augments cellular stress resistance (Ngoh, Watson et al. 2010; Zachara, Molina et al. 2010), we demonstrated that O-GlcNAc modification is essential for oxidative stress resistance in the nematode *C. elegans* (Brys, Vanfleteren et al. 2007). Importantly lack of O-GlcNAcase (OGA-1) activity, which leads to increased O-GlcNAc modified protein levels in *oga-1* mutant tissues, induces oxidative stress resistance albeit to a lesser extent than the insulin-pathway mutants. High levels of O-GlcNAc-modified proteins in *oga-1* mutant animals do not appear to cause any major anomaly the mutants develop normally into fertile adults, and generate normal numbers of offspring.

In mammalian cells, increased UDP-glucosamine levels activate OGT-1, which catalyzes the terminal step in the hexosamine biosynthesis pathway (HBP). HBP is a minor branch in glycolysis that diverts excess fructose-6-phosphate from glycolysis into glucosamine-6-phosphate leading to the generation of UDP-N-acetylglucosamines (McClain, Lubas et al. 2002). Cellular UDP-GlcNAc levels are highly sensitive to changing levels of glucose influx through glycolysis (Rossetti, Hawkins et al. 1995). A brisk increase in UDP-GlcNAc levels with changing cellular glucose concentration simultaneously increases protein O-GlcNAcylation, as

OGT functional activity is dependent on the concentration of its substrate UDP-GlcNAc (Haltiwanger, Holt et al. 1990). Thus GlcNAc acts as a nutrient sensor to adjust metabolism, and subsequent cellular processes in response to extracellular signaling (Lefebvre, Dehennaut et al. 2010). Such robust biochemical analysis is not evident in the current nematode literature. In *C. elegans,* nutrients trigger the release of insulin-like ligands and activate the signaling cascade for embryonic and reproductive development (Fielenbach and Antebi 2008). When food is restricted, the signaling is deactivated to repress development, and activate protective mechanisms to facilitate survival in harsh environmental conditions (Hertweck, Gobel et al. 2004; Ayyadevara, Alla et al. 2008). The loss of protein O-GlcNAcylation upon inactivation of O-GlcNAc transferase in long-lived *daf-2* mutant did not revert all *daf-2* phenotypes (increased thermotolerance, reduced fecundity, and delayed post-embryonic development) but only reduced oxidative stress resistance and extended lifespan of the *daf-2* mutant. Consistently, unrestrained protein O-GlcNAcylation mimics only a subset of *daf-2* phenotypes, for example, oxidative stress resistance and lifespan extension albeit a lesser extent than insulin pathway mutants. Our findings argue that O-GlcNAc modification antagonistically modulates a narrow branch of functional outcomes controlled by DAF-2 insulin signaling, in other words, O-GlcNAc cycling 'fine-tunes' DAF-2 insulin signaling-mediated responses in *C. elegans*.

Finally we have identified 13 putative O-GlcNAc target proteins in *C. elegans* through affinity purification (Table 3.4). Mammalian counterparts of 6 of the 13 proteins are known to be O-GlcNAc modified, suggesting that the putative *C. elegans* O-GlcNAc protein modifications are conserved in metazoa (Table 3.4). Significantly, the DAF-2 insulin pathway regulates expression of 8 of the 13 putative O-GlcNAc modified proteins. Strikingly 4 of the 13 putative O-GlcNAc target proteins are known to regulate lifespan. Inactivation of either of the F0F1-type ATP synthases ATP-2 or H28O16.1 extends adult lifespan, and increases the expression of the *sod-3* gene in *C. elegans* (Curran and Ruvkun 2007). Inactivation of the glyceraldehyde-3-

phosphate dehydrogenase GPD-2 also extends lifespan of *daf-2* mutant animals (Dong, Venable et al. 2007), while inactivation of the malate dehydrogenase MDH-1 shortens *daf-2* mutant lifespan (Samuelson, Carr et al. 2007). Our current experimental data contrasts with the mammalian *in vitro* cell culture studies where both upstream (IRS1 and AKT) and downstream (FoxO) components of the insulin signaling pathway are O-GlcNAc modified; and such modifications attenuate insulin signaling pathway outcomes *in vitro* (Housley, Rodgers et al. 2008; Yang, Ongusaha et al. 2008). Considering the limitation of any biochemical assay, we do not rule out the possibility of missing other O-GlcNAc proteins in *C. elegans* under our current assay conditions.

In mammalian cells, a reduction in O-GlcNAcase (OGA) activity or over-expression of O-GlcNAc transferase (OGT) leads to a reduced ability of insulin to activate glucose transport into the cells, *i.e.*, insulin resistance, which is prominent in both type I and type II diabetes patients (Arias, Kim et al. 2004). Increased levels of protein O-GlcNAcylation inhibits insulin signaling pathway mediated activation of AKT, and subsequently disrupts insulin-stimulated glucose transport in adipocytes, demonstrating a direct involvement of O-GlcNAc cycling in the attenuation of insulin-mediated signaling. Insulin resistance is marked by high glucose and high insulin levels in circulating blood, and aberrant insulin-regulated gene functions (Lizcano and Alessi 2002). Abnormalities in carbohydrate and fat metabolism are prominent in insulin-resistant tissues, and presumably cause elevated plasma sugar and fatty acid levels in type II diabetes patients (Lewis, Carpentier et al. 2002). In *C. elegans,* similar disarray in carbohydrate and fat storage is also reported in insulin signaling pathway mutants (Kimura, Tissenbaum et al. 1997), and upon disruption in O-GlcNAc cycling (Forsythe, Love et al. 2006). The O-GlcNAcase loss-of-function mutant phenotype is strikingly similar to the insulin resistance phenotype described in mammals even in absence of a blood circulation system, and the consequent need for blood glucose homeostasis in the nematode. Therefore *C. elegans* would provide a useful

model animal system to elucidate many roles of protein O-GlcNAcylation implicated in the insulin-resistance phenotype.

Similar to mammalian cells, a conserved insulin signaling pathway in *C. elegans* also regulates DAF-16/FoxO target gene transcription by restricting DAF-16 sub-cellular localization (Kenyon 2010). DAF-16 nuclear activity is essential for lifespan extension in all insulin pathway loss-of-function mutants (Lin, Hsin et al. 2001). Disruption of O-GlcNAc cycling causes a modest change in DAF-16 subcellular localization in contrast to the essentially complete DAF-16 nuclear localization that is observed in insulin pathway mutants (Lin, Hsin et al. 2001). In *C. elegans*, an increased level of O-GlcNAc staining is observed in the nuclear membrane close to nuclear pores (Forsythe, Love et al. 2006). In mammalian cells, several nuclear pore complex proteins are O-GlcNAc modified. The O-GlcNAc modification levels increase upon exposure to oxidative stress (Wells, Whelan et al. 2003), although in *C. elegans* such details are not known. Under the circumstances we cannot rule out the possibility that the increase in nuclear DAF-16 levels observed in *oga-1(RNAi)* and *ogt-1(RNAi)* animals might caused by compromised nuclear pore function upon disruption of normal O-GlcNAc cycling in *C. elegans*.

Protein aggregation is a hallmark in many aging-related neurodegerative disorders. Excessive protein aggregation can rise from decreased solubility or decreased protein degradation. Several studies have implicated dysfunctional ubiquitin-mediated protein degradation pathways in aging [reviewed in (Cohen and Dillin 2008)]. SCF (skp-1/cul-1/Fbox) complexes affect insulin-mediated aging process in *C. elegans* but the molecular pathways are not explained well in limited experimental approaches previously (Fielenbach, Guardavaccaro et al. 2007; Ghazi, Henis-Korenblit et al. 2007). Recently perturbations in the insulin signaling is also reported to significantly delay protein aggregation in *daf-2* mutants without any clear insight into the aging mechanism (David, Ollikainen et al. 2010). In our current study we found a novel function of CRL1 E3 ubiquitin ligase at the level of DAF-16 protein stability in *C. elegans*. A

highly conserved CUL-1 protein function is important during cell cycle exit in mitotic cells (Kipreos, Lander et al. 1996) but its role in post-mitotic adults cells was not investigated previously. Our experimental data argues for a novel CUL-1-mediated regulation of cryptic negative regulators of DAF-16 in post-mitotic intestinal cells (Figure 3.9a). Importantly we also demonstrated in our current study that inactivation of CUL-1 leads to abnormal accumulation of CEP-1, which is orthologus to the mammalian p53 protein, in neuro-endocrine and other neuronal cells (Figure 3.10). In *C. elegans,* neuro-endocrine cells induce production of TGF-beta and insulin-like ligands essential in development and adult lifespan regulation (Fielenbach and Antebi 2008). The neuronal cells (ASI, ADF, ASG, ASJ, ASK, AWA, AWC, and others) in the pharynx and head region help integrate environmental cues, such as nutrients and temperature; and convert these into endocrine signals. *C. elegans* CEP-1 is key to activation of cell death pathways, and also reported to negatively affect adult lifespan (Derry, Putzke et al. 2001; Arum and Johnson 2007; Scrable, Medrano et al. 2009). Atypical accumulation of CEP-1 in the pharynx region is expected to affect endocrine signaling but this requires further investigation. Interestingly, mammalian p53 is O-GlcNAc modified, and aberrant O-GlcNAc cycling is reported to affect its stability and function (Yang, Kim et al. 2006) but in *C. elegans* CEP-1 is not tested yet. Future studies might reveal the regulatory steps linking CUL-1 activity in post-mitotic, and terminally differentiated adult intestinal cells in response to insulin-mediated signaling. It would be also interesting to know whether O-GlcNAc modification of protein play any role.

Our results indicate that O-GlcNAc cycling in *C. elegans* is critical to a subset of downstream functions under insulin signaling that controls lifespan extension, and oxidative stress resistance but is not required for other functions regulated by insulin signaling. With many similarities between the conserved insulin signaling pathways in nematode and vertebrates it will be interesting to explore how O-GlcNAc modulates a subset of downstream

functions under the general control of insulin signaling in nematodes, and whether these results can be extended to vertebrates. Such an amenable genetic model will allow us a fuller exploration of the complexity of FoxO-mediated gene expression regulation in reproductive development, obesity, diabetes, hypertension, and associated pathologies prominent in aging adults.

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References

Alessi, D. R., S. R. James, et al. (1997). "Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha." Curr Biol **7**(4): 261-269.

Arantes-Oliveira, N., J. Apfeld, et al. (2002). "Regulation of life-span by germ-line stem cells in Caenorhabditis elegans." Science **295**(5554): 502-505.

Arias, E. B., J. Kim, et al. (2004). "Prolonged incubation in PUGNAc results in increased protein O-Linked glycosylation and insulin resistance in rat skeletal muscle." Diabetes **53**(4): 921-930.

Arum, O. and T. E. Johnson (2007). "Reduced expression of the Caenorhabditis elegans p53 ortholog cep-1 results in increased longevity." J Gerontol A Biol Sci Med Sci **62**(9): 951-959.

Ayyadevara, S., R. Alla, et al. (2008). "Remarkable longevity and stress resistance of nematode PI3K-null mutants." Aging Cell **7**(1): 13-22.

Biddinger, S. B. and C. R. Kahn (2006). "From mice to men: insights into the insulin resistance syndromes." Annu Rev Physiol **68**: 123-158.

Brenner, S. (1974). "The genetics of Caenorhabditis elegans." Genetics **77**(1): 71-94.

Brys, K., J. R. Vanfleteren, et al. (2007). "Testing the rate-of-living/oxidative damage theory of aging in the nematode model Caenorhabditis elegans." Exp Gerontol **42**(9): 845-851.

Cahill, C. M., G. Tzivion, et al. (2001). "Phosphatidylinositol 3-kinase signaling inhibits DAF-16 DNA binding and function via 14-3-3-dependent and 14-3-3-independent pathways." J Biol Chem **276**(16): 13402-13410.

Cohen, E. and A. Dillin (2008). "The insulin paradox: aging, proteotoxicity and neurodegeneration." Nat Rev Neurosci **9**(10): 759-767.

Curran, S. P. and G. Ruvkun (2007). "Lifespan regulation by evolutionarily conserved genes essential for viability." PLoS Genet **3**(4): e56.

David, D. C., N. Ollikainen, et al. (2010). "Widespread protein aggregation as an inherent part of aging in C. elegans." PLoS Biol **8**(8): e1000450.

Derry, W. B., A. P. Putzke, et al. (2001). "Caenorhabditis elegans p53: role in apoptosis, meiosis, and stress resistance." Science **294**(5542): 591-595.

Dias, W. B. and G. W. Hart (2007). "O-GlcNAc modification in diabetes and Alzheimer's disease." Mol Biosyst **3**(11): 766-772.

Dong, M. Q., J. D. Venable, et al. (2007). "Quantitative mass spectrometry identifies insulin signaling targets in C. elegans." Science **317**(5838): 660-663.

Dorman, J. B., B. Albinder, et al. (1995). "The age-1 and daf-2 genes function in a common pathway to control the lifespan of Caenorhabditis elegans." Genetics **141**(4): 1399-1406.

Du, X. L., D. Edelstein, et al. (2001). "Hyperglycemia inhibits endothelial nitric oxide synthase activity by posttranslational modification at the Akt site." J Clin Invest **108**(9): 1341-1348.

Fielenbach, N. and A. Antebi (2008). "C. elegans dauer formation and the molecular basis of plasticity." Genes Dev **22**(16): 2149-2165.

Fielenbach, N., D. Guardavaccaro, et al. (2007). "DRE-1: an evolutionarily conserved F box protein that regulates C. elegans developmental age." Dev Cell **12**(3): 443-455.

Forsythe, M. E., D. C. Love, et al. (2006). "Caenorhabditis elegans ortholog of a diabetes susceptibility locus: oga-1 (O-GlcNAcase) knockout impacts O-GlcNAc cycling, metabolism, and dauer." Proc Natl Acad Sci U S A **103**(32): 11952-11957.

Gami, M. S., W. B. Iser, et al. (2006). "Activated AKT/PKB signaling in C. elegans uncouples temporally distinct outputs of DAF-2/insulin-like signaling." BMC Dev Biol **6**: 45.

Gao, M. X., E. H. Liao, et al. (2008). "The SCF FSN-1 ubiquitin ligase controls germline apoptosis through CEP-1/p53 in C. elegans." Cell Death Differ **15**(6): 1054-1062.

Gems, D., A. J. Sutton, et al. (1998). "Two pleiotropic classes of daf-2 mutation affect larval arrest, adult behavior, reproduction and longevity in Caenorhabditis elegans." Genetics **150**(1): 129-155.

Ghazi, A., S. Henis-Korenblit, et al. (2007). "Regulation of Caenorhabditis elegans lifespan by a proteasomal E3 ligase complex." Proc Natl Acad Sci U S A **104**(14): 5947-5952.

Halaschek-Wiener, J., J. S. Khattra, et al. (2005). "Analysis of long-lived C. elegans daf-2 mutants using serial analysis of gene expression." Genome Res **15**(5): 603-615.

Haltiwanger, R. S., G. D. Holt, et al. (1990). "Enzymatic addition of O-GlcNAc to nuclear and cytoplasmic proteins. Identification of a uridine diphospho-N-acetylglucosamine:peptide beta-N-acetylglucosaminyltransferase." J Biol Chem **265**(5): 2563-2568.

Hanover, J. A., M. E. Forsythe, et al. (2005). "A Caenorhabditis elegans model of insulin resistance: altered macronutrient storage and dauer formation in an OGT-1 knockout." Proc Natl Acad Sci U S A **102**(32): 11266-11271.

Hart, G. W., M. P. Housley, et al. (2007). "Cycling of O-linked beta-N-acetylglucosamine on nucleocytoplasmic proteins." Nature **446**(7139): 1017-1022.

Henderson, S. T. and T. E. Johnson (2001). "daf-16 integrates developmental and environmental inputs to mediate aging in the nematode Caenorhabditis elegans." Curr Biol **11**(24): 1975-1980.

Hertweck, M., C. Gobel, et al. (2004). "C. elegans SGK-1 is the critical component in the Akt/PKB kinase complex to control stress response and life span." Dev Cell **6**(4): 577-588.

Honda, Y. and S. Honda (1999). "The daf-2 gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in Caenorhabditis elegans." Faseb J **13**(11): 1385-1393.

Housley, M. P., J. T. Rodgers, et al. (2008). "O-GlcNAc regulates FoxO activation in response to glucose." J Biol Chem.

Hsin, H. and C. Kenyon (1999). "Signals from the reproductive system regulate the lifespan of C. elegans." Nature **399**(6734): 362-366.

Hsu, A. L., C. T. Murphy, et al. (2003). "Regulation of aging and age-related disease by DAF-16 and heat-shock factor." Science **300**(5622): 1142-1145.

Kamath, R. S., A. G. Fraser, et al. (2003). "Systematic functional analysis of the Caenorhabditis elegans genome using RNAi." Nature **421**(6920): 231-237.

Kenyon, C. (2005). "The plasticity of aging: insights from long-lived mutants." Cell **120**(4): 449-460.

Kenyon, C., J. Chang, et al. (1993). "A C. elegans mutant that lives twice as long as wild type." Nature **366**(6454): 461-464.

Kenyon, C. J. (2010). "The genetics of ageing." Nature **464**(7288): 504-512.

Kimura, K. D., H. A. Tissenbaum, et al. (1997). "daf-2, an insulin receptor-like gene that regulates longevity and diapause in Caenorhabditis elegans." Science **277**(5328): 942-946.

Kipreos, E. T., L. E. Lander, et al. (1996). "cul-1 is required for cell cycle exit in C. elegans and identifies a novel gene family." Cell **85**(6): 829-839.

Kreppel, L. K., M. A. Blomberg, et al. (1997). "Dynamic glycosylation of nuclear and cytosolic proteins. Cloning and characterization of a unique O-GlcNAc transferase with multiple tetratricopeptide repeats." J Biol Chem **272**(14): 9308-9315.

Kuo, M., V. Zilberfarb, et al. (2008). "O-glycosylation of FoxO1 increases its transcriptional activity towards the glucose 6-phosphatase gene." FEBS Lett **582**(5): 829-834.

Lefebvre, T., V. Dehennaut, et al. (2010). "Dysregulation of the nutrient/stress sensor O-GlcNAcylation is involved in the etiology of cardiovascular disorders, type-2 diabetes and Alzheimer's disease." Biochim Biophys Acta **1800**(2): 67-79.

Lewis, G. F., A. Carpentier, et al. (2002). "Disordered fat storage and mobilization in the pathogenesis of insulin resistance and type 2 diabetes." Endocr Rev **23**(2): 201-229.

Li, W., B. Gao, et al. (2007). "RLE-1, an E3 ubiquitin ligase, regulates C. elegans aging by catalyzing DAF-16 polyubiquitination." Dev Cell **12**(2): 235-246.

Libina, N., J. R. Berman, et al. (2003). "Tissue-specific activities of C. elegans DAF-16 in the regulation of lifespan." Cell **115**(4): 489-502.

Lin, K., J. B. Dorman, et al. (1997). "daf-16: An HNF-3/forkhead family member that can function to double the life-span of Caenorhabditis elegans." Science **278**(5341): 1319-1322.

Lin, K., H. Hsin, et al. (2001). "Regulation of the Caenorhabditis elegans longevity protein DAF-16 by insulin/IGF-1 and germline signaling." Nat Genet **28**(2): 139-145.

Lithgow, G. J., T. M. White, et al. (1995). "Thermotolerance and extended life-span conferred by single-gene mutations and induced by thermal stress." Proc Natl Acad Sci U S A **92**(16): 7540-7544.

Liu, F., K. Iqbal, et al. (2004). "O-GlcNAcylation regulates phosphorylation of tau: a mechanism involved in Alzheimer's disease." Proc Natl Acad Sci U S A **101**(29): 10804-10809.

Liu, K., A. J. Paterson, et al. (2004). "Accumulation of protein O-GlcNAc modification inhibits proteasomes in the brain and coincides with neuronal apoptosis in brain areas with high O-GlcNAc metabolism." J Neurochem **89**(4): 1044-1055.

Lizcano, J. M. and D. R. Alessi (2002). "The insulin signalling pathway." Curr Biol **12**(7): R236-238.

Love, D. C., S. Ghosh, et al. (2010). "Dynamic O-GlcNAc cycling at promoters of Caenorhabditis elegans genes regulating longevity, stress, and immunity." Proc Natl Acad Sci U S A **107**(16): 7413-7418.

Love, D. C. and J. A. Hanover (2005). "The hexosamine signaling pathway: deciphering the "O-GlcNAc code"." Sci STKE **2005**(312): re13.

Lubas, W. A., D. W. Frank, et al. (1997). "O-Linked GlcNAc transferase is a conserved nucleocytoplasmic protein containing tetratricopeptide repeats." J Biol Chem **272**(14): 9316-9324.

Martinez-Vicente, M., G. Sovak, et al. (2005). "Protein degradation and aging." Exp Gerontol **40**(8-9): 622-633.

McClain, D. A., W. A. Lubas, et al. (2002). "Altered glycan-dependent signaling induces insulin resistance and hyperleptinemia." Proc Natl Acad Sci U S A **99**(16): 10695-10699.

McElwee, J. J., E. Schuster, et al. (2006). "Diapause-associated metabolic traits reiterated in long-lived daf-2 mutants in the nematode Caenorhabditis elegans." Mech Ageing Dev **127**(5): 458-472.

Mukhopadhyay, A., S. W. Oh, et al. (2006). "Worming pathways to and from DAF-16/FOXO." Exp Gerontol **41**(10): 928-934.

Murphy, C. T., S. A. McCarroll, et al. (2003). "Genes that act downstream of DAF-16 to influence the lifespan of Caenorhabditis elegans." Nature **424**(6946): 277-283.

Ngoh, G. A., L. J. Watson, et al. (2010). "Augmented O-GlcNAc signaling attenuates oxidative stress and calcium overload in cardiomyocytes." Amino Acids.

Olsen, A., M. C. Vantipalli, et al. (2006). "Checkpoint proteins control survival of the postmitotic cells in Caenorhabditis elegans." Science **312**(5778): 1381-1385.

Paradis, S., M. Ailion, et al. (1999). "A PDK1 homolog is necessary and sufficient to transduce AGE-1 PI3 kinase signals that regulate diapause in Caenorhabditis elegans." Genes Dev **13**(11): 1438-1452.

Paradis, S. and G. Ruvkun (1998). "Caenorhabditis elegans Akt/PKB transduces insulin receptor-like signals from AGE-1 PI3 kinase to the DAF-16 transcription factor." Genes Dev **12**(16): 2488-2498.

Portman, D. S. (2006). "Profiling C. elegans gene expression with DNA microarrays." WormBook: 1-11.

Rahman, M. M., O. Stuchlick, et al. (2010). "Intracellular protein glycosylation modulates insulin mediated lifespan in C.elegans." Aging (Albany NY).

Rossetti, L., M. Hawkins, et al. (1995). "In vivo glucosamine infusion induces insulin resistance in normoglycemic but not in hyperglycemic conscious rats." J Clin Invest **96**(1): 132-140.

Samuelson, A. V., C. E. Carr, et al. (2007). "Gene activities that mediate increased life span of C. elegans insulin-like signaling mutants." Genes Dev **21**(22): 2976-2994.

Schuster, E., J. J. McElwee, et al. (2010). "DamID in C. elegans reveals longevity-associated targets of DAF-16/FoxO." Mol Syst Biol **6**: 399.

Scrable, H., S. Medrano, et al. (2009). "Running on empty: how p53 controls INS/IGF signaling and affects life span." Exp Gerontol **44**(1-2): 93-100.

Suh, Y., G. Atzmon, et al. (2008). "Functionally significant insulin-like growth factor I receptor mutations in centenarians." Proc Natl Acad Sci U S A **105**(9): 3438-3442.

Teo, C. F., S. Ingale, et al. (2010). "Glycopeptide-specific monoclonal antibodies suggest new roles for O-GlcNAc." Nat Chem Biol **6**(5): 338-343.

Timmons, L., D. L. Court, et al. (2001). "Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in Caenorhabditis elegans." Gene **263**(1-2): 103-112.

Toleman, C., A. J. Paterson, et al. (2004). "Characterization of the histone acetyltransferase (HAT) domain of a bifunctional protein with activable O-GlcNAcase and HAT activities." J Biol Chem **279**(51): 53665-53673.

Vosseller, K., L. Wells, et al. (2002). "Elevated nucleocytoplasmic glycosylation by O-GlcNAc results in insulin resistance associated with defects in Akt activation in 3T3-L1 adipocytes." Proc Natl Acad Sci U S A **99**(8): 5313-5318.

Wells, L., Y. Gao, et al. (2002). "Dynamic O-glycosylation of nuclear and cytosolic proteins: further characterization of the nucleocytoplasmic beta-N-acetylglucosaminidase, O-GlcNAcase." J Biol Chem **277**(3): 1755-1761.

Wells, L., S. A. Whelan, et al. (2003). "O-GlcNAc: a regulatory post-translational modification." Biochem Biophys Res Commun **302**(3): 435-441.

Whisenhunt, T. R., X. Yang, et al. (2006). "Disrupting the enzyme complex regulating O-GlcNAcylation blocks signaling and development." Glycobiology **16**(6): 551-563.

Yang, W. H., J. E. Kim, et al. (2006). "Modification of p53 with O-linked N-acetylglucosamine regulates p53 activity and stability." Nat Cell Biol **8**(10): 1074-1083.

Yang, X., P. P. Ongusaha, et al. (2008). "Phosphoinositide signalling links O-GlcNAc transferase to insulin resistance." Nature **451**(7181): 964-969.

Yang, X., F. Zhang, et al. (2002). "Recruitment of O-GlcNAc transferase to promoters by corepressor mSin3A: coupling protein O-GlcNAcylation to transcriptional repression." Cell **110**(1): 69-80.

Zachara, N. E., H. Molina, et al. (2010). "The dynamic stress-induced "O-GlcNAc-ome" highlights functions for O-GlcNAc in regulating DNA damage/repair and other cellular pathways." Amino Acids.

Zachara, N. E., N. O'Donnell, et al. (2004). "Dynamic O-GlcNAc modification of nucleocytoplasmic proteins in response to stress. A survival response of mammalian cells." J Biol Chem **279**(29): 30133-30142.

Zhang, F., K. Su, et al. (2003). "O-GlcNAc modification is an endogenous inhibitor of the proteasome." Cell **115**(6): 715-725.

Table 3.1: Adult Lifespan Analysis.

Genotype	Median LS ±STDEV	*p*-value vs. wild type		Sample size n(censored)	Replicates N
		Log-rank	Wilcoxon		
wild type	15.9±3.6	-	-	404(59)	8
oga-1(ok1207)	20.9±4.1	<0.0001	<0.0001	317(43)	6
ogt-1(ok1474)	12.9±1.9	<0.0001	<0.0001	306(64)	6
oga-1(ok1207); ogt-1(ok1474)	15.5±4.9	0.030	0.040	174(35)	2
daf-2(e1370)	28.6±3.5	<0.0001	<0.0001	235(26)	3
oga-1(ok1207); daf-2(e1370)	21.6±0.6	<0.0001	<0.0001	201(20)	3
ogt-1(ok1474); daf-2(e1370)	13.2±1.9	<0.0001	<0.0001	218(28)	3
daf-16(mu86)	14.3±2.5	0.0001	<0.0001	228(60)	3
oga-1(ok1207); daf-16(mu86)	14.8±1.8	0.019	0.009	173(25)	2
ogt-1(ok1474); daf-16(mu86)	12.8±0.4	<0.0001	<0.0001	150(24)	2
age-1(hx546)	25.6±4.1	<0.0001	<0.0001	216(18)	3
oga-1(ok1207); age-1(hx546)	24.7±6.8	<0.0001	<0.0001	241(17)	3
ogt-1(ok1474); age-1(hx546)	15.9±1.6	0.424	0.590	258(31)	3
sgk-1(ok538)	29.8±3.2	<0.0001	<0.0001	235(16)	3
oga-1(ok1207); sgk-1(ok538)	26.2±2.1	<0.0001	<0.0001	209(30)	3
ogt-1(ok1474); sgk-1(ok538)	16.6±2.3	0.343	0.375	214(39)	3
pdk-1(mg142)gf	14.8±1.7	0.013	0.007	334(84)	5
oga-1(ok1207); pdk-1(mg142)	18.7±8.1[a]	<0.0001	<0.0001	284(43)	5
akt-1(mg144)gf	11.5±2.1	<0.0001	<0.0001	205(32)	2
oga-1(ok1207); akt-1(mg144)	19.8±4.2[b]	0.0002	0.0002	229(22)	2

a. *pdk-1* vs. *oga-1;pdk-1* p-value <0.0001
b. *akt-1* vs. *oga-1;akt-1* p-value <0.0001
N = number of replicates
n = pooled animals from N replicate experiments (censored animals were included in the Log-rank/Wilcoxon analysis)

Table 3.2

O-GlcNAc modification is essential for the following DAF-16 dependent genes upregulated in *daf-2(1370)* mutant

F15E11.15	F15E11.15a
H25K10.1	H25K10.1 /// status:Partially_confirmed
K07E8.3	SKN-1 Dependent Zygotic transcript /// locus:sdz-24
T05E12.6	T05E12.6
F37B4.7	F37B4.7 /// locus:folt-2
K03H6.2	K03H6.2 /// status:Partially_confirmed
T10H9.5	Peroxisomal Membrane Protein related pmp-5
PDB1.1	PDB1.1b
F28A12.4	peptidase
T16G1.5	T16G1.5 /// status:Partially_confirmed
F08A8.3	ACYL-COENZYME A OXIDASE, PEROXISOMAL (EC 1.3.3.6)
F46B6.8	lipase
F20G2.5	F20G2.5 /// status:Partially_confirmed
ZC412.3	ZC412.3 /// status:Confirmed
W05E7.1	GRounDhog (hedgehog-like family) /// locus:grd-3
B05I1.4	Temporarily Assigned Gene name /// locus:tag-344
LLC1.2	LLC1.2 /// status:Confirmed
F13H8.3	F13H8.3 /// status:Partially_confirmed
F41E7.1	F41E7.1 /// status:Partially_confirmed
B023.14	CYtochrome P450 family /// locus:cyp-34A8
Y40D12A.2	serine carboxypeptidase
C35A5.3	sodium/phosphate transport protein
F41H10.8	fatty acid ELOngation /// locus:elo-6
R07B1.3	membrane glycoprotein
C01G6.7	4-coumarate-coA ligase
T04D3.2	SKN-1 Dependent Zygotic transcript /// locus:sdz-30
B0024.4	status:Partially_confirmed
F44D12.9	status:Partially_confirmed
C03E10.6	locus:clec-222
F25B4.9	C-type LECtin /// locus:clec-1
T19D12.2	T19D12.2a
F27C8.1	Amino Acid Transporter /// locus:aat-1
W04E12.8	C-type LECtin /// locus:clec-50
B02I3.3	Neuropeptide-Like Protein /// locus:nlp-28
F39E9.11	locus:btb-17
C36B7.7	HEsitatioN behavior /// locus:hen-1
H12C20.3	Nuclear Hormone Receptor family /// locus:nhr-68
C55C3.3	status:Confirmed
Y53G8B.1	status:Confirmed
ZC412.4	status:Partially_confirmed
E04F6.7	DeHydrogenases, Short chain /// locus:dhs-7
T24H10.5	status:Predicted
F14E5.4	acid phosphatase
B02I3.4	Neuropeptide-Like Protein /// locus:nlp-29
Y38H6C.1	locus:dct-16
ZC47.7	F-box A protein /// ZC47.7 /// locus:fbxa-151 /// locus:fbxa-78
T07G12.5	permease
F25B4.8	F25B4.8b
ZK6.7b	lipase
F26G1.4	Toxin-regulated Target of p38MAPK /// locus:ttm-2
C32B5.5	status:Partially_confirmed
F28G4.1	CYtochrome P450 family /// locus:cyp-37B1
C53B4.7	BT (Bacillus thuringiensis) toxin Resistant bre-1
R10D12.9	status:Confirmed
F54D11.1	Phosphoethanolamine MethyTransferase /// locus:pmt-2
Y41C4A.11	WD domain, G-beta repeat
C09H5.2a	ATPase

F08H9.6	C-type LECtin /// locus:clec-57
C53B4.3	status:Partially_confirmed
Y50D4B.3	status:Partially_confirmed
F55E10.6	D-beta-hydroxybutyrate dehydrogenase (BDH)
ZK185.3	status:Partially_confirmed
F15B9.1	Fatty Acid/Retinol binding protein /// locus:far-3
F43H9.4	status:Confirmed
C08B6.1	UDP-GlucuronosylTransferase ugt-17
K11G9.6	MeTaLlothionein /// locus:mtl-1
C07G3.9	UDP-GlucuronosylTransferase /// locus:ugt-64
ZK550.6	status:Confirmed
R05F9.12	glycosyl hydralase, sucrase-isomaltase
C01B10.10	status:Partially_confirmed
F37H8.5	status:Confirmed
T02B5.3	carboxylesterase
F38E11.3	CyPIN (guanine aminohydrolase) homolog) /// locus:cpin-1
ZK1320.1	locus:gstk-1
T13F3.6	status:Confirmed
C05C10.4	acid phosphatase
C17H12.4	carboxylesterase
C07A9.8	Putative membrane protein
R12H7.2	ASpartyl Protease /// locus:asp-4
F27C8.4	SaPosin-like Protein family /// locus:spp-18
T03F7.7	T03F7.7a
R03G5.5	R03G5.5
F40F9.5	translocase
F44A2.3	status:Partially_confirmed
T07H3.6	BTB and MATH domain containing /// locus:bath-26
F08H9.7	C-type LECtin /// locus:clec-56
F32D8.12	F32D8.12b
C34H4.3	Temporarily Assigned Gene name /// locus:tag-244
F41H10.7	fatty acid ELOngation /// locus:elo-5
R57.1	R57.1a
Y39A1A.19	Flavin-containing MonoOxygenase family /// locus:fmo-3
F56F10.1	/// peptidase
C01H6.2	Ank repeat (2 domains)
C36A4.2	CYtochrome P450 family /// locus:cyp-25A2
M02D8.5	M02D8.5 /// status:Partially_confirmed
F35C5.8	C-type LECtin /// locus:clec-65
F54F7.4	MX region of TRA-2 Related /// locus:xtr-1
Y105E8A.4	Enoyl-CoA Hydratase /// locus:ech-7
C08F11.3	status:Confirmed
R07B7.5	Monooxygenase
C17G1.3a	UDP-glucosyltransferase
ZC376.3	carboxylesterase
ZK896.7	locus:clec-186
F37C4.6	phytoene desaturase
H37A05.2	status:Partially_confirmed
F01G10.2	Enoyl-CoA Hydratase /// locus:ech-8
C23H5.8	C23H5.8a
C01B10.4	status:Partially_confirmed
C25E10.5	status:Partially_confirmed
F57F4.3	GEI-4(Four) Interacting protein /// locus:gfi-1
C29F9.3	C29F9.3b
ZK688.2	status:Partially_confirmed
Y43F8C.1	Neuropeptide-Like Protein /// locus:nlp-25
W06D12.3	FATty acid desaturase /// locus:fat-5
W02B12.4	esterase
K06C4.8	G-protein coupled receptor
C29E4.7	locus:gsto-1
C04C3.5	abnormal DYe Filling dyf-3
F43D2.2	F43D2.2
C01H6.4	Flavin-binding monooxygenase-like
F19C7.1	status:Confirmed

C12D8.5	abnormal DAuer Formation /// locus:daf-36
ZK1320.3	status:Confirmed
VC5.3	Nematode Polyprotein Allergen related npa-1
F53H10.2	F53H10.2
C06B3.7	status:Partially_confirmed
T22F7.4	transposase
K04A8.1	status:Partially_confirmed
C01C10.2	C01C10.2b
C36A4.1	CYtochrome P450 family /// locus:cyp-25A1
T05E7.1	status:Partially_confirmed
F19C7.1	status:Confirmed

Table 3.3 O-GlcNAc modification is essential for the following DAF-16 dependent genes downregulated in *daf-2(1370)* mutant

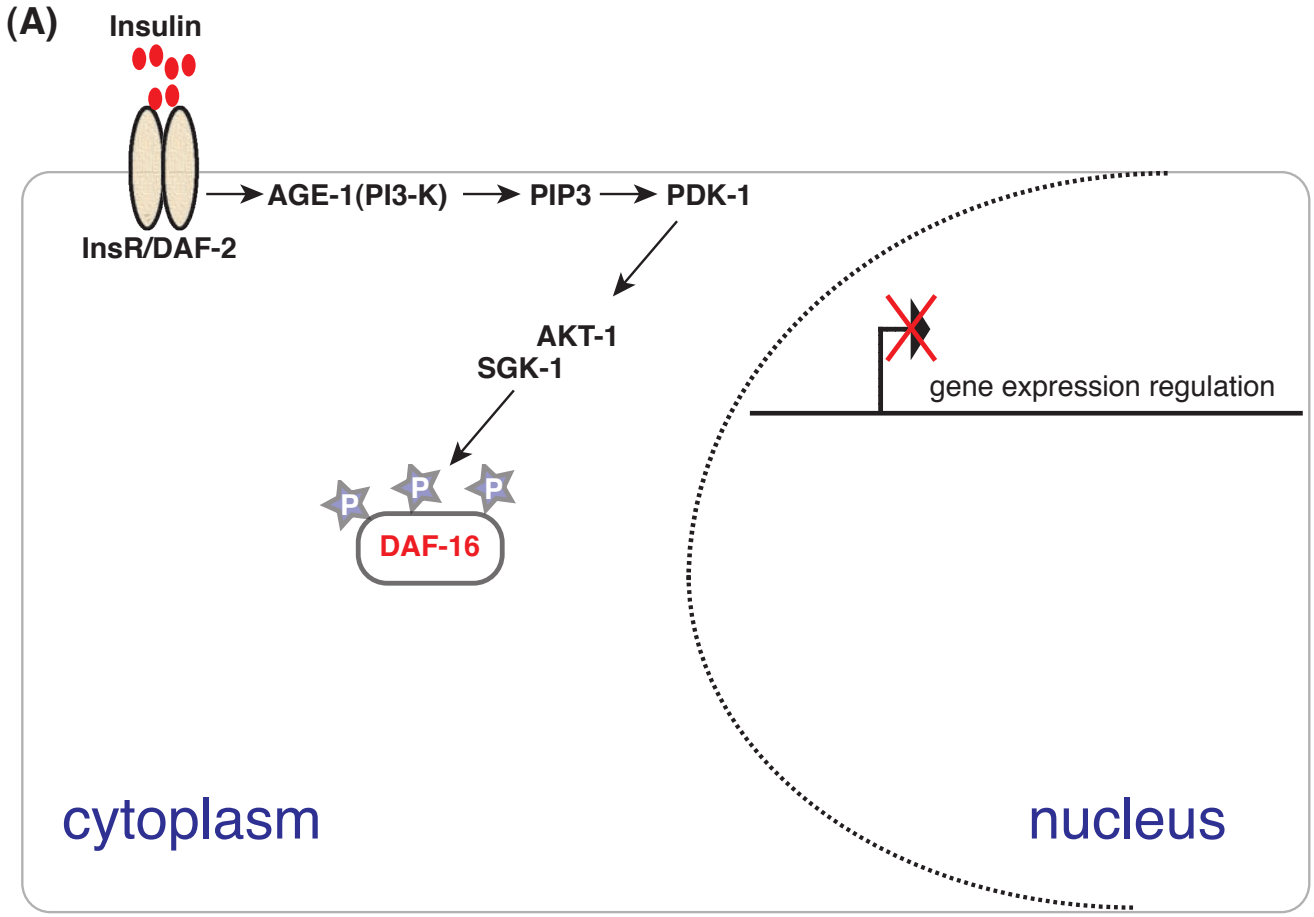
F46A8.7	F46A8.7 /// status:Partially_confirmed
F54D10.2	F-box A protein /// locus:fbxa-24
ZK131.4	HIStone /// locus:his-26 /// locus:his-14 /// locus:his-10 /// locus:his-64
Y41D4B.19	Nuclear Pore complex Protein npp-8
F59H6.7	CYclin A /// locus:cya-2
C37H5.8	Heat Shock Protein /// locus:hsp-6
F45F2.3	HIStone /// locus:his-5 /// locus:his-18
W06D11.2	status:Partially_confirmed
R04E5.8	R04E5.8a
R09H3.3	status:Predicted
W09C3.7	status:Confirmed
C48B6.3	status:Confirmed
Y75B7A1.4	Rho GTPase Activating protein rga-4
ZK1151.1	Variable ABnormal morphology vab-10
T24A6.1	status:Predicted
F55G1.3	locus:his-62
F11A5.10	Glutamate-gated ChLoride channel /// locus:glc-1
Y74C10AR.1	Eukaryotic Initiation Factor /// locus:eif-3.I
trehalase	C23H3.7 /// locus:tre-5
F11A5.9	sodium/phosphate transporter
Y67D8C.5	locus:eel-1
F35H10.1	HIStone /// locus:his-29
F17E9.9	HIStone /// locus:his-34
C04G2.8	status:Confirmed
K06C4.2	HIStone /// locus:his-28
F07B7.9	HIStone /// locus:his-50
R144.4	Wiskott-Aldrich syndrome protein (WASP)-Interacting Protein and gene assignment wip-1
F54E12.4	HIStone /// locus:his-58
H02I72.6	HIStone /// locus:his-66
C07E3.9	phospholipase A2
C15H9.11	status:Confirmed
ZK666.6	C-type LECtin /// locus:clec-60
F52H3.7	gaLECtin
Y43F8B.9	status:Partially_confirmed
F18G5.6	status:Confirmed
T12D8.1	Temporarily Assigned Gene name /// locus:set-16
Y47D3A.6	TRAnsformer : XX animals transformed into males tra-1
C12C8.3	abnormal cell LINeage lin-41
C34D4.14	status:Partially_confirmed
Y48E1A.1	Y48E1A.1b
B0035.9	HIStone /// locus:his-46 /// locus:his-48
F54E12.3	HIStone /// locus:his-56
K09H11.3	Rho GTPase Activating protein /// locus:rga-3
F53A3.4	Prion-like-(Q/N-rich)-domain-bearing protein pqn-41
T23D8.5	HIStone /// locus:his-67
T16G1.7	status:Partially_confirmed
F08F1.9	status:Predicted
F36A4.7	AMAnitin resistant /// locus:ama-1
F07C6.3	status:Partially_confirmed
F31D4.4	locus:clec-264
T01D3.6	T01D3.6a
F35E12.5	status:Predicted
W03D2.1	Prion-like-(Q/N-rich)-domain-bearing protein pqn-75
T06D8.1	T06D8.1a
C18A3.1	status:Partially_confirmed

Table 3.4. Summary of putative *C. elegans* O-GlcNAc targets

Proteins	Molecular Identity	Expression in *daf-2* mutant	O-GlcNAc modified in vertebrate homologs
PHI-37	F0F1-type ATP synthase	Downregulated	NR
ATP-2	F0F1-type ATP synthase	Downregulated	NR
MDH-1	Malate dehydrogenase	Downregulated	MDH1
GPD-2, GPD-3	Glyceraldehyde-3-phosphate dehydrogenase, major isozyme	Upregulated	GAPDH
GPD-1, GPD-4	Glyceraldehyde-3-phosphate dehydrogenase, minor isozyme	Downregulated	GAPDH
EFT-3, EFT-4	Translation elongation factor 1 alpha	Downregulated	EEF1AO
ACT-1, ACT-2, ACT-3, ACT-4	Actin	Downregulated	ACTG1
TBA-1, TBA-2, TBA-4	Alpha tubulin	Downregulated	TUBA1A
VIT-6	Vitellogenin	Downregulated	NR
VHA-13	Vacuolar protein translocating ATPase	NR	NR
CDC-48.2	AAA-type ATPase	NR	NR
F27D4.1	Electron transfer flavoprotein	NR	NR
C16A3.10	Ornithine aminotransferase	Downregulated	NR

EFT-3 and EFT-4 have identical protein sequences, as do GPD-2 and GPD-3. The peptides identified by LC-MS/MS did not distinguish between the almost identical ACT-1, ACT-2, ACT-3, and ACT-4; TBA-1, TBA-2, and TBA-4; or GPD-2 and GPD-3, respectively. NR, not reported.

Model 3.A: A conserved insulin signaling pathway in *C. elegans* regulates numerous functions including stress response, metabolism, growth, dauer formation, and reproductive development by restricting nuclear localization of DAF-16/FoxO transcription factor in response to nutrient availability. Upon ligand binding (top panel), the insulin-like receptor DAF-2 activates the AGE-1/PI3K that facilitates activation of downstream kinases PDK-1 and AKT-1. AKT-1 mediated phosphorylation sequesters DAF-16 in cytoplasm. In the absence of DAF-2 and PI3K/AKT mediated signaling (bottom panel) DAF-16 freely enters the nucleus, and regulates the expression of target genes to modulate numerous DAF-16 dependent processes.


(A)
Insulin
InsR/DAF-2
AGE-1(PI3-K)
PIP3
PDK-1
AKT-1
SGK-1
P
P
P
DAF-16
gene expression regulation
cytoplasm
nucleus

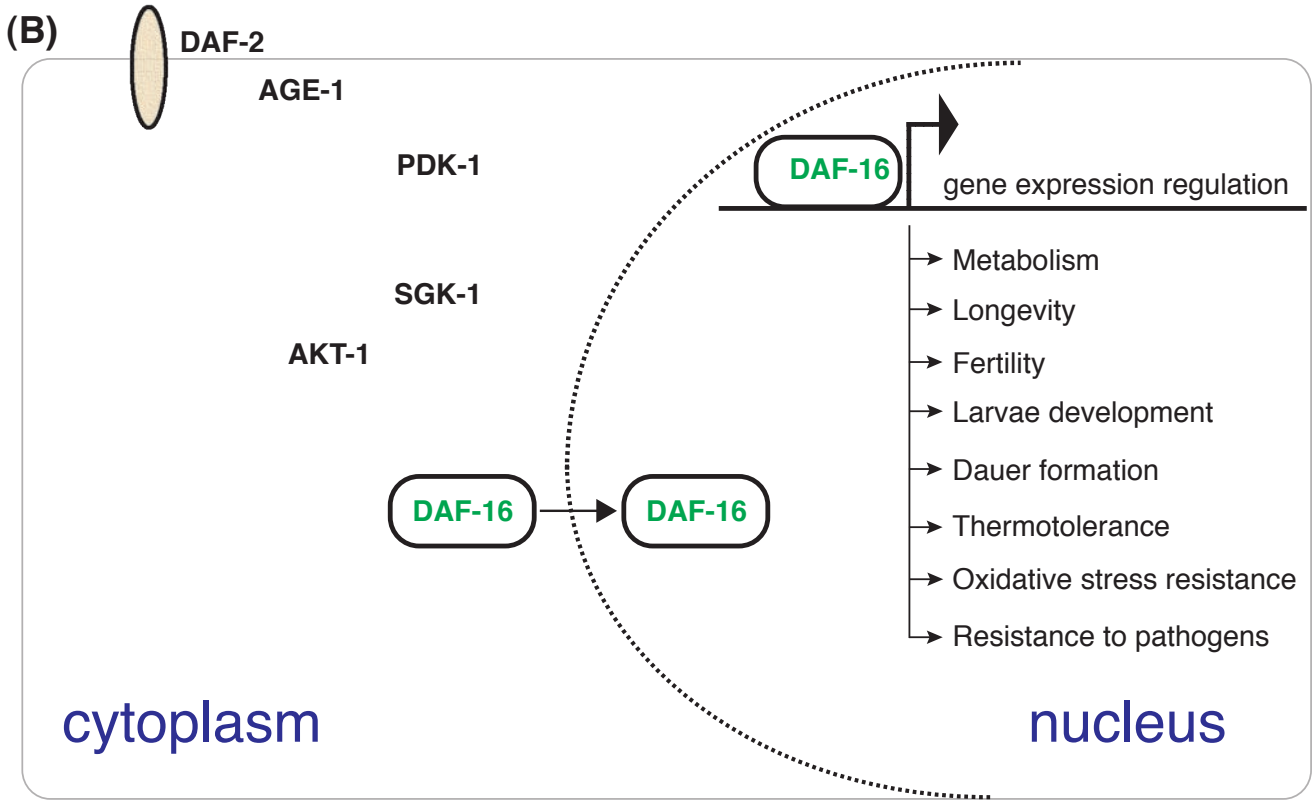

(B)
DAF-2
AGE-1
PDK-1
SGK-1
AKT-1
DAF-16
DAF-16
DAF-16
gene expression regulation
Metabolism
Longevity
Fertility
Larvae development
Dauer formation
Thermotolerance
Oxidative stress resistance
Resistance to pathogens
cytoplasm
nucleus

Figure 3.1: Increased levels of O-GlcNAc modified proteins linked to adult lifespan extension in nematode *C. elegans*. A) OGA-1 and OGT-1 regulate O-GlcNAc modified protein levels in *C. elegans*. Western blot of animal extracts from *ogt-1(ok1474)* and *oga-1 (ok1207)* mutants probed with specific antibodies against O-GlcNAc, and tubulin show the dramatic differences in O-GlcNAc modified protein levels. B) Representative adult lifespan curves of wild type, *ogt-1(ok1474),* and *oga-1(ok1207)* mutant animals assayed at 20°C. Note that *oga-1* mutant animals have extended lifespan while *ogt-1* mutant animals have reduced lifespan relative to the wild type control animals.

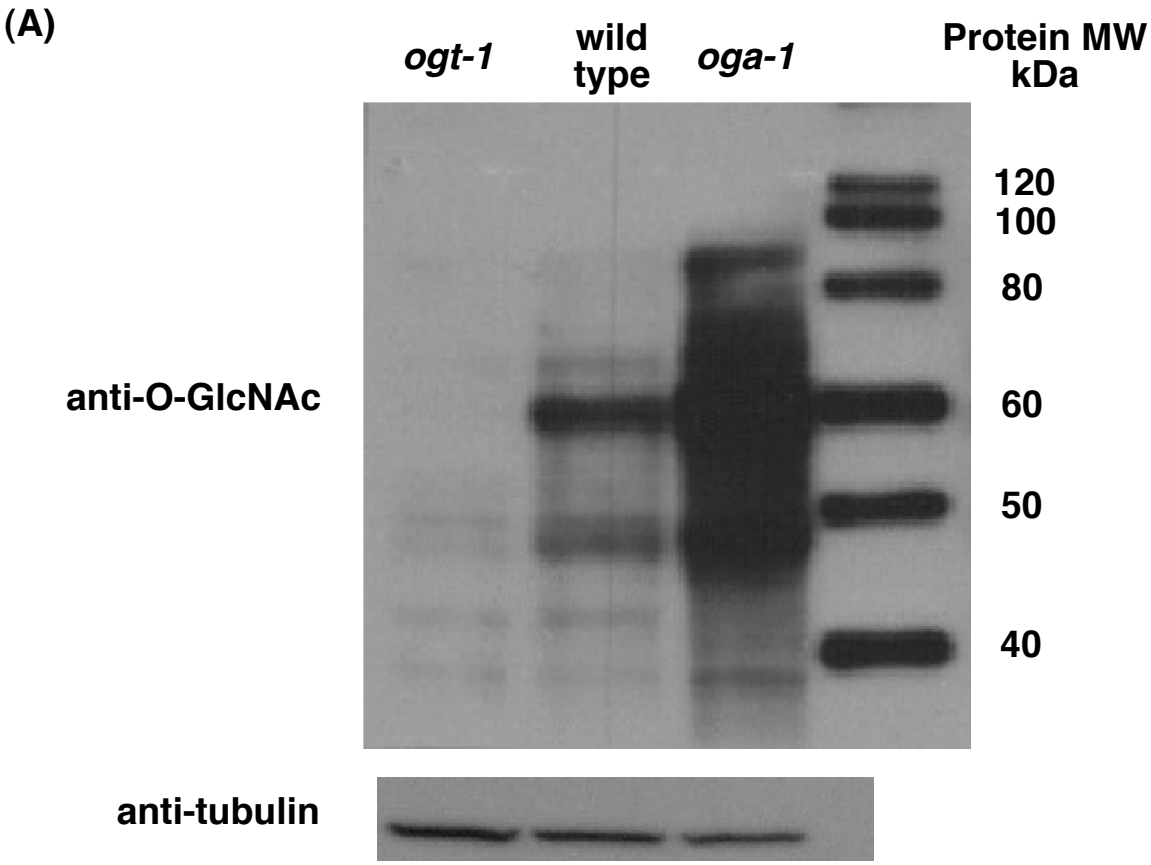
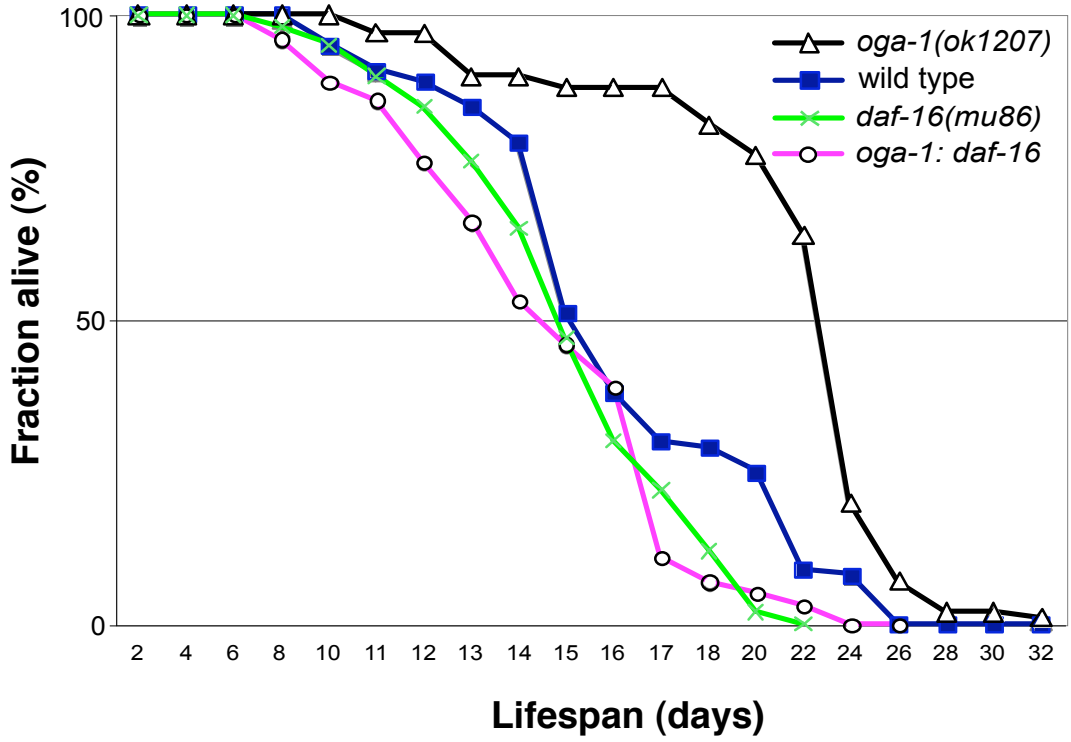

(A)
ogt-1
wild type
oga-1
Protein MW kDa
120
100
80
60
50
40
anti-O-GlcNAc
anti-tubulin
(B)
100
50
0
Fraction alive (%)
wildtype
ogt-1(ok1474)
oga-1(ok1207)
2 4 6 8 10 11 12 13 14 15 16 17 18 20 22 24 26 28 30 32
Lifespan (days)

Figure 3.2: Changes in protein O-GlcNAcylation levels negatively affect DAF-2 insulin-signaling mediated adult lifespan regulation. A) Lifespan extension in *oga-1(ok1207)* mutant animals is dependent on DAF-16 function as demonstrated in the short lifespan of the *oga-1 (ok1207);daf-16(mu86)* double mutant. B) Inactivation of protein O-GlcNAcylation in *daf-2 (e1370)* mutant animals reduced its typically long lifespan in the *daf-2(e1370);ogt-1(ok1474)* double mutant.

(A)


100
50
0
Fraction alive (%)
2 4 6 8 10 11 12 13 14 15 16 17 18 20 22 24 26 28 30 32
Lifespan (days)
oga-1(ok1207)
wild type
daf-16(mu86)
oga-1: daf-16

(B)

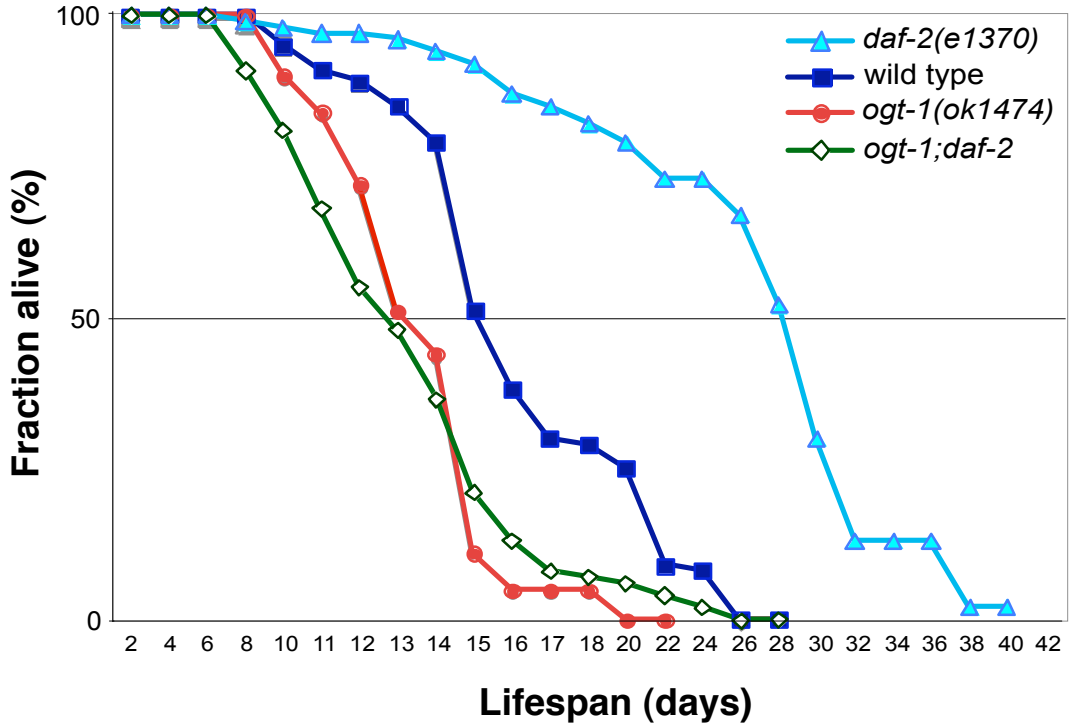
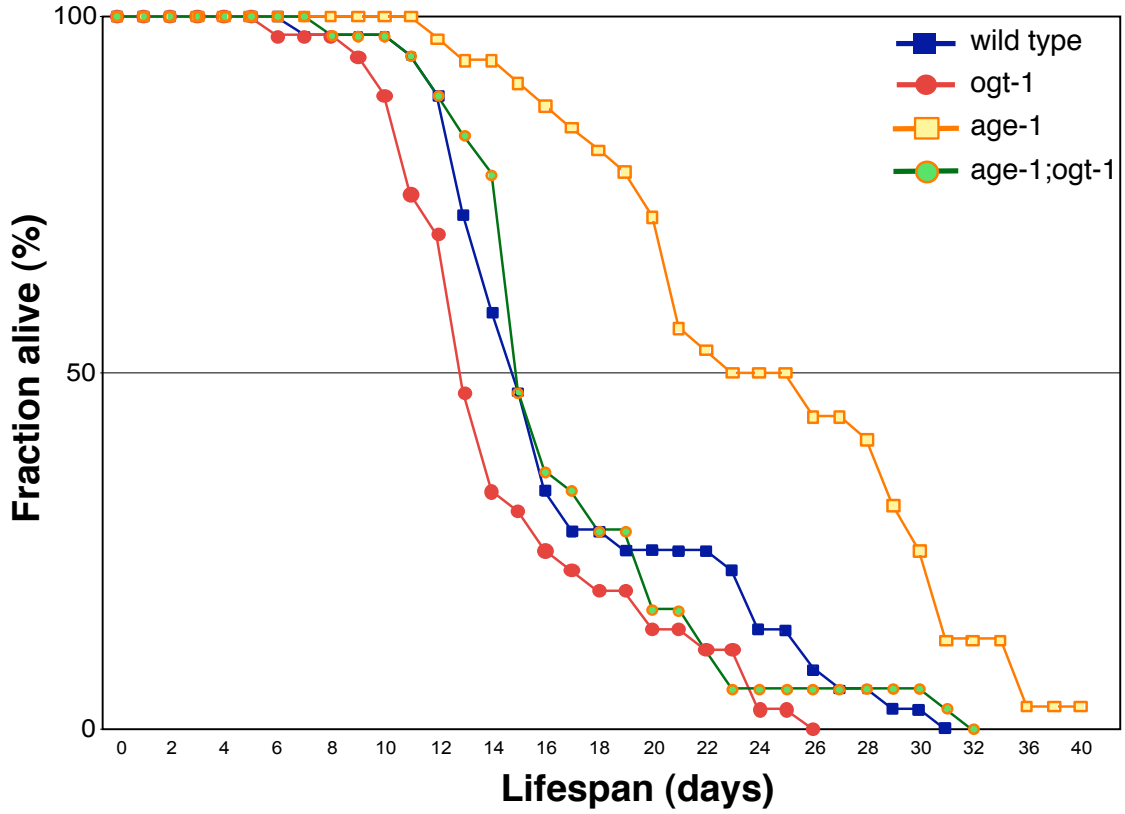
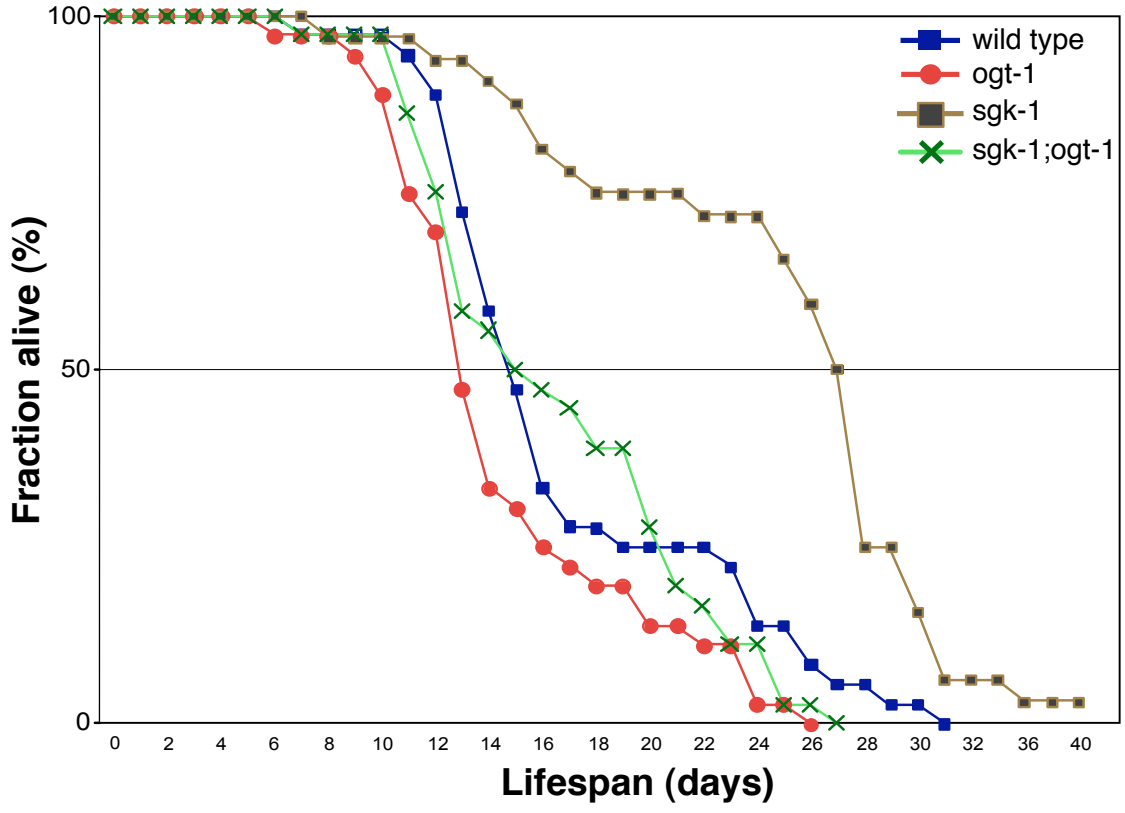

100
50
0
Fraction alive (%)
2 4 6 8 10 11 12 13 14 15 16 17 18 20 22 24 26 28 30 32 34 36 38 40 42
Lifespan (days)
daf-2(e1370)
wild type
ogt-1(ok1474)
ogt-1;daf-2

Figure 3.3: Elevated O-GlcNAc levels do not increase adult lifespan further in the long-lived insulin pathway mutants but OGT-1 function is essential for lifespan extension in the same mutants. The long lifespan of *age-1(hx546)* and *sgk-1(ok538)* mutants is dependent on protein O-GlcNAc modification as seen in *age-1(hx546); ogt-1(ok1474)* and *sgk-1(ok538); ogt-1(ok1474)* double mutants (3A & 3B respectively). The lifespan extension in *oga-1(ok1207)* mutant is not synergistic or additive with the long lifespan of *age-1(hx546)* and *sgk-1(ok538)* mutants (3C & 3D respectively). E) Elevated cellular protein O-GlcNAcylation level upon OGA-1 inactivation rescues the short lifespan of the *akt-1(mg144)* gain-of-function mutant as demonstrated in the *akt-1(mg144);oga-1(ok1207)* double mutant. Control curves were re-plotted to facilitate comparisons in panels A-E.

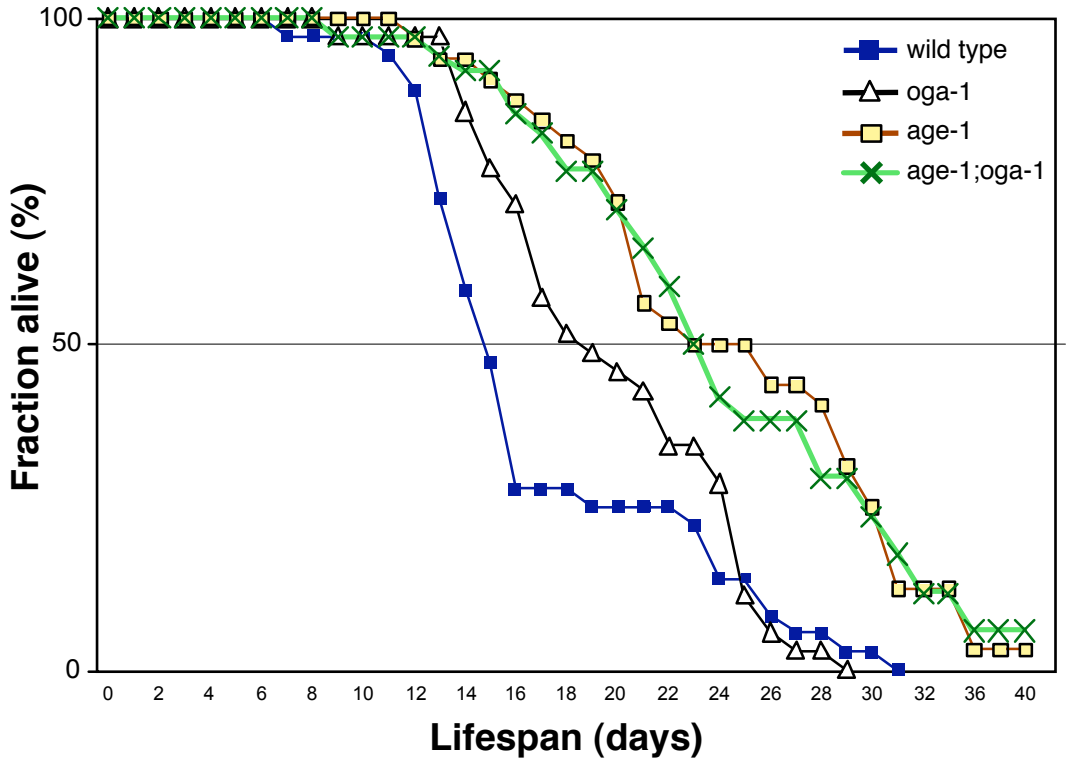
(A)


100
50
0
Fraction alive (%)
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 36 40
Lifespan (days)
wild type
ogt-1
age-1
age-1;ogt-1

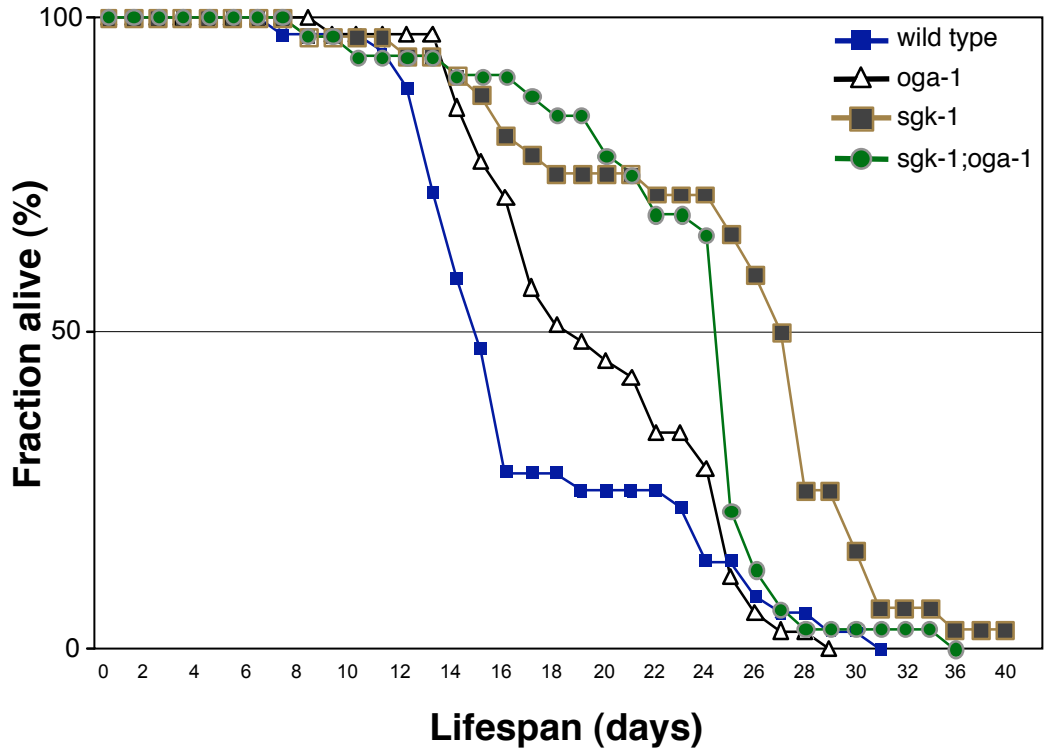
(B)


100
50
0
Fraction alive (%)
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 36 40
Lifespan (days)
wild type
ogt-1
sgk-1
sgk-1;ogt-1

(C)


100
50
0
Fraction alive (%)
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 36 40
Lifespan (days)
wild type
oga-1
age-1
age-1;oga-1

(D)


100
50
0
Fraction alive (%)
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 36 40
Lifespan (days)
wild type
oga-1
sgk-1
sgk-1;oga-1

(E)

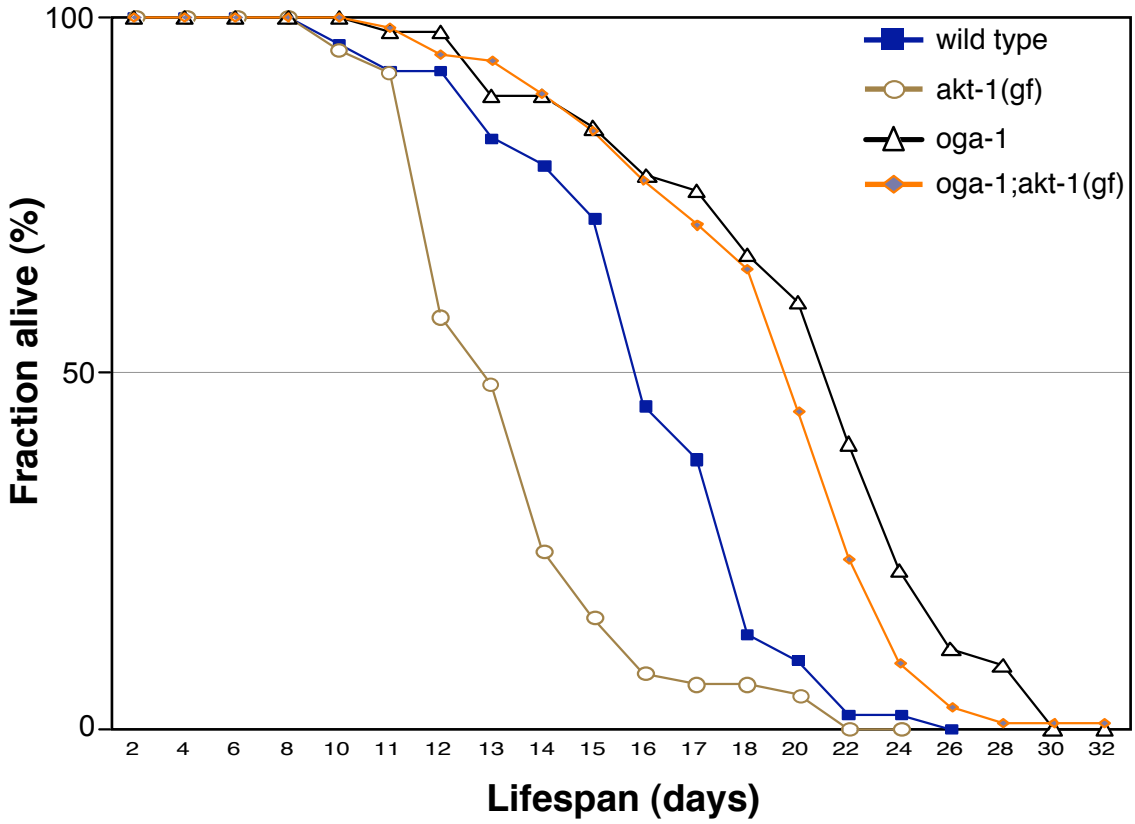
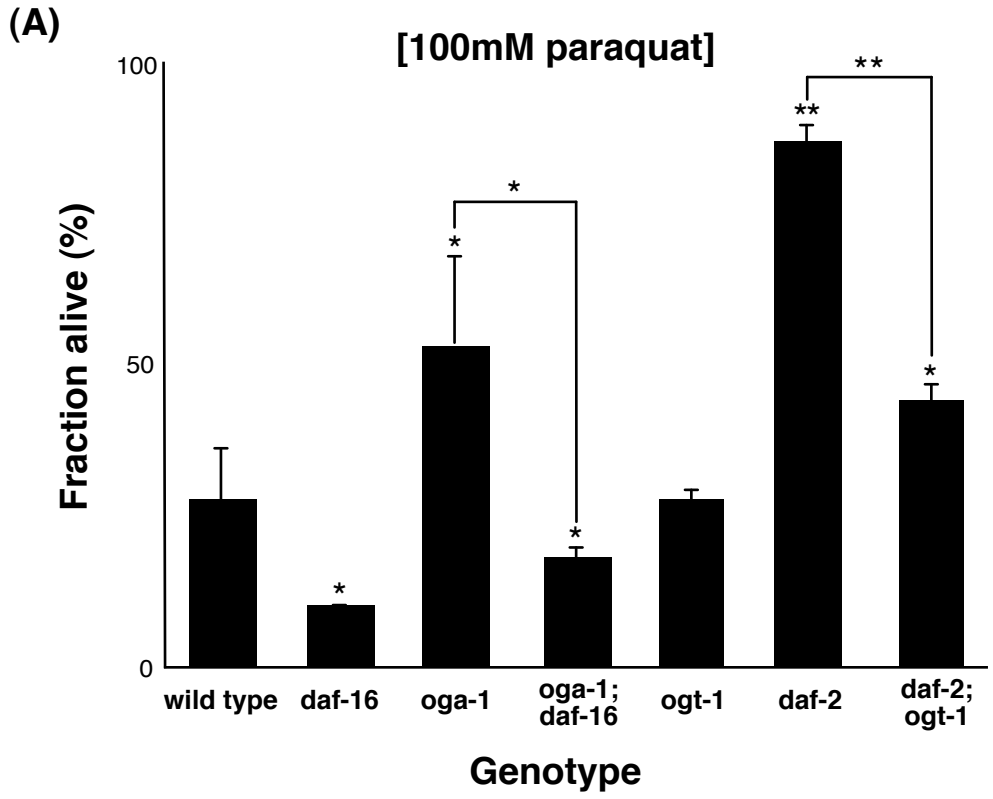

100
50
0
Fraction alive (%)
2
4
6
8
10
11
12
13
14
15
16
17
18
20
22
24
26
28
30
32
Lifespan (days)
wild type
akt-1(gf)
oga-1
oga-1;akt-1(gf)

Figure 3.4: Elevated levels of O-GlcNAc modified proteins in OGA-1 mutant affects oxidative stress resistance in adult animals is controlled by the insulin-signaling pathway. A) The *oga-1 (ok1207)* mutant is resistant to oxidative stress (100mM paraquat) while most of the wild type and *daf-16 (mu86)* mutant animals are dead by 9 hrs. Protein O-GlcNAcylation levels contribute to the oxidative stress resistance in *daf-2(e1370)* mutant animals as revealed by significant loss of resistance in the *daf-2 (e1370); ogt-1(ok1474)* double mutant animals assayed at 20°C. B) Quantification of SOD-3::GFP (driven by the *sod-3* promoter) upon inactivation of OGA-1 function by RNAi. Inactivation of the insulin pathway effector kinases, for example AGE-1, by feeding RNAi induces *sod-3* expression. Bar graphs show relative GFP signal intensity (deducted from the background) with arbitrary units in the Y-axis. Asterisks above bars denote statistically significant differences from wild type (* $p < 0.05$ and ** $p < 0.01$) while asterisks above the solid lines connecting two genotypes refer to statistically significant difference between the genotypes.


(A)
[100mM paraquat]
Fraction alive (%)
100
50
0
wild type
daf-16
oga-1
oga-1; daf-16
ogt-1
daf-2
daf-2; ogt-1
Genotype

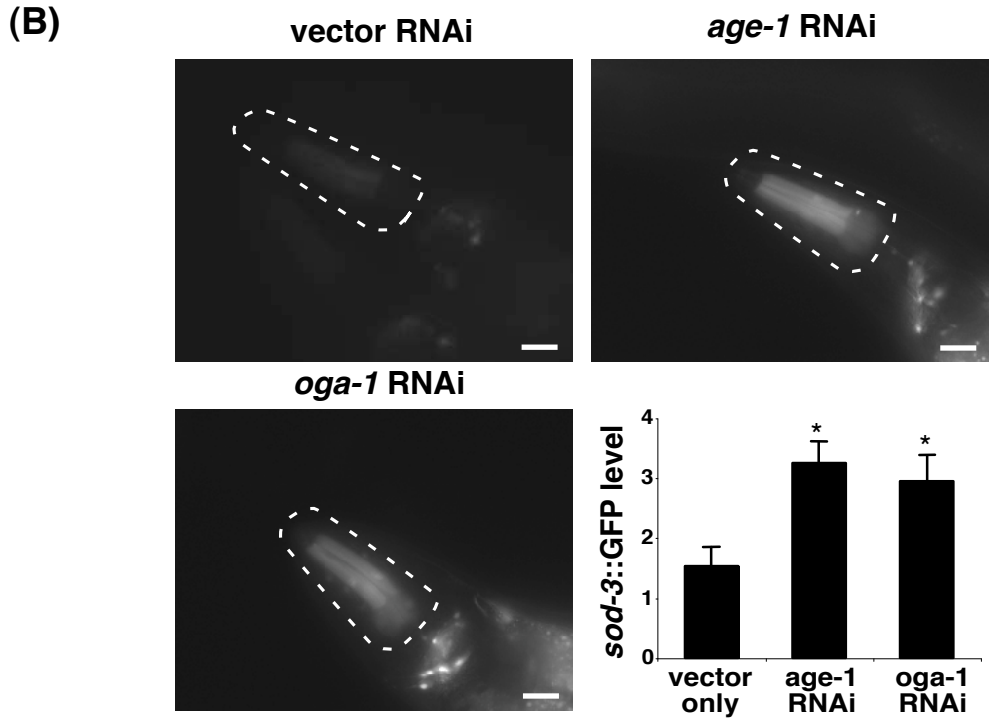
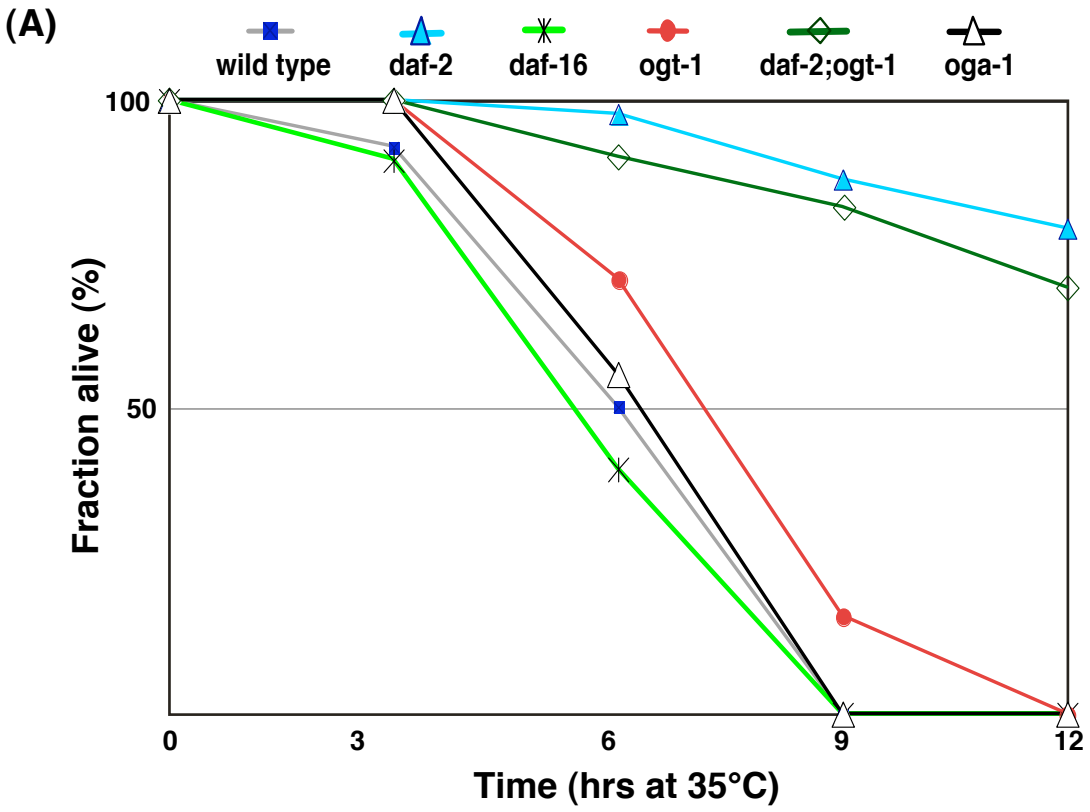

(B)
vector RNAi
age-1 RNAi
oga-1 RNAi
sod-3::GFP level
vector only
age-1 RNAi
oga-1 RNAi

Figure 3.5: Inactivation of O-GlcNAc cycling enzymes does not affect thermal stress resistance in adult animals controlled by the insulin-signaling pathway. A) The long-lived *oga-1(ok1207)* mutant is sensitive to heat stress (35°C) similar to wild type and the *daf-16(mu86)* mutant. B) Quantification of the *hsp-16-2*::GFP (driven by *hsp-16-2* heat-shock promoter) upon inactivation of OGA-1. Inactivation of the insulin pathway effector kinase AGE-1 by feeding RNAi induces *hsp-16::*GFP expression, while OGA-1 inactivation does not induce *hsp-16::*GFP expression under similar experimental conditions. Bar graphs show relative GFP signal intensity (deducted from the background) with arbitrary units in the Y-axis. Asterisks denote statistically significant differences from the control (* $p < 0.05$ and ** $p < 0.01$).


(A)
wild type
daf-2
daf-16
ogt-1
daf-2;ogt-1
oga-1
100
50
Fraction alive (%)
0
3
6
9
12
Time (hrs at 35°C)

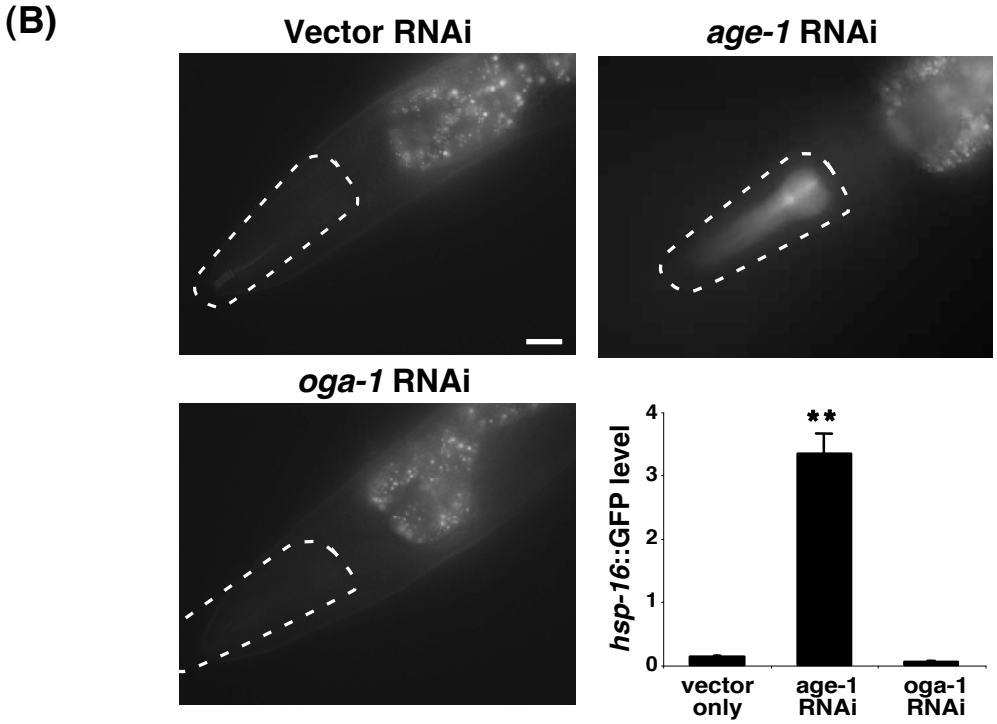

(B)
Vector RNAi
age-1 RNAi
oga-1 RNAi
hsp-16::GFP level
**
4
3
2
1
0
vector only
age-1 RNAi
oga-1 RNAi

Figure 3.6: Inactivation of O-GlcNAc cycling enzymes does not affect postembryonic development and fecundity controlled by the insulin-signaling pathway. A) Lack of O-GlcNAc addition or removal functions does not affect postembryonic developmental timing [from hatch to fourth larval molt] in either *ogt-1(ok1474)* or *oga-1(ok1207)* mutants. Delayed postembryonic development is prominent in the long-lived *daf-2(e1370)* mutant, and is unaffected by loss of protein O-GlcNAcylation in a *daf-2(e1370);ogt-1 (ok1474)* double mutant. B) Lack of O-GlcNAc addition or removal functions does not affect the number viable offspring in either *ogt-1(ok1474)* or *oga-1(ok1207)* mutant. Reduced insulin signaling reduces fecundity in long-lived *daf-2(e1370)* mutant animals, and reduced fecundity level is not rescued by loss of protein O-GlcNAcylation in a *daf-2(e1370);ogt-1(ok1474)* double mutant. Asterisks above bars denote statistically significant differences from wild type (* $p < 0.05$ and ** $p < 0.01$) while asterisks above the solid lines connecting two genotypes refer to statistically significant difference between the genotypes.

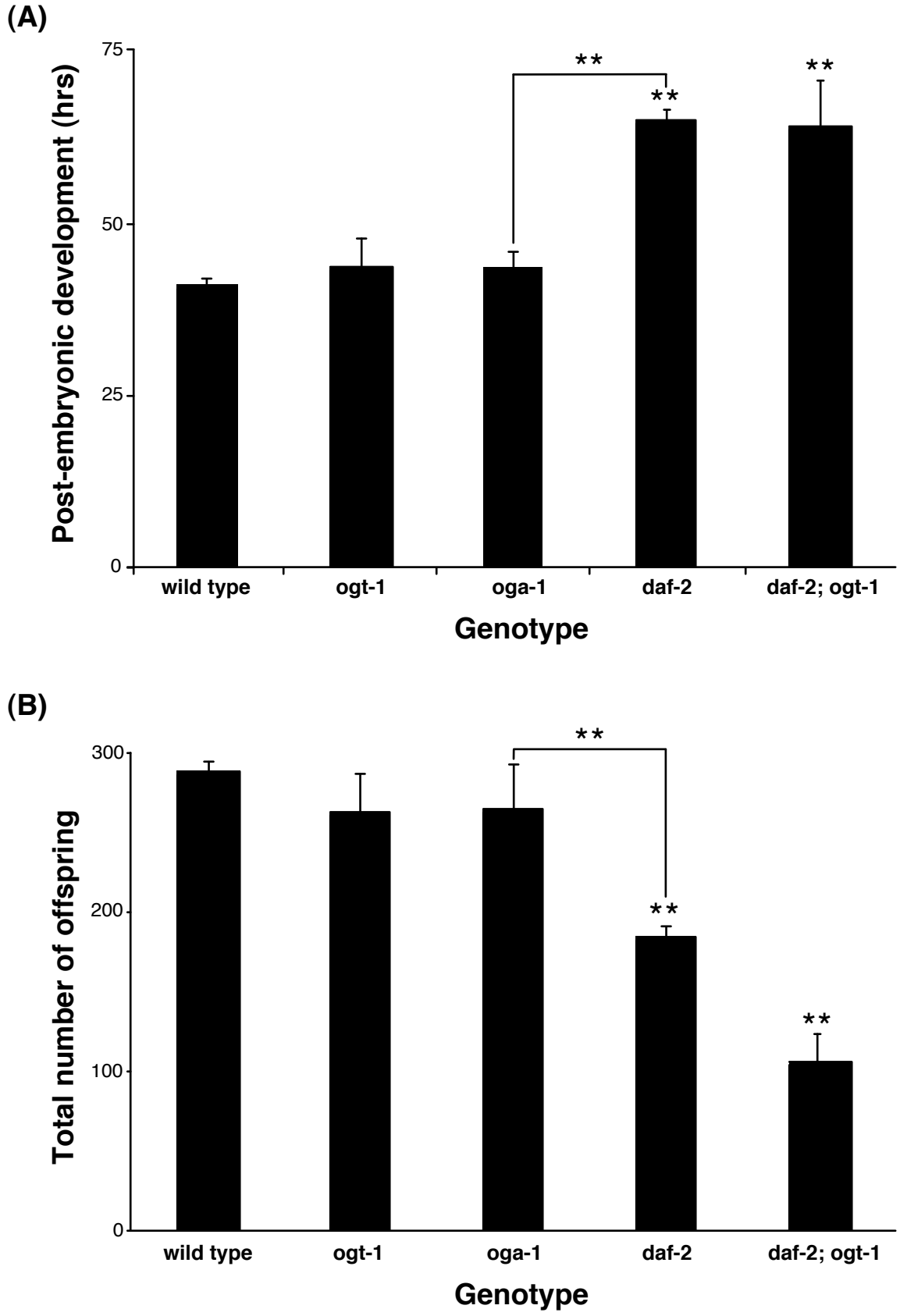
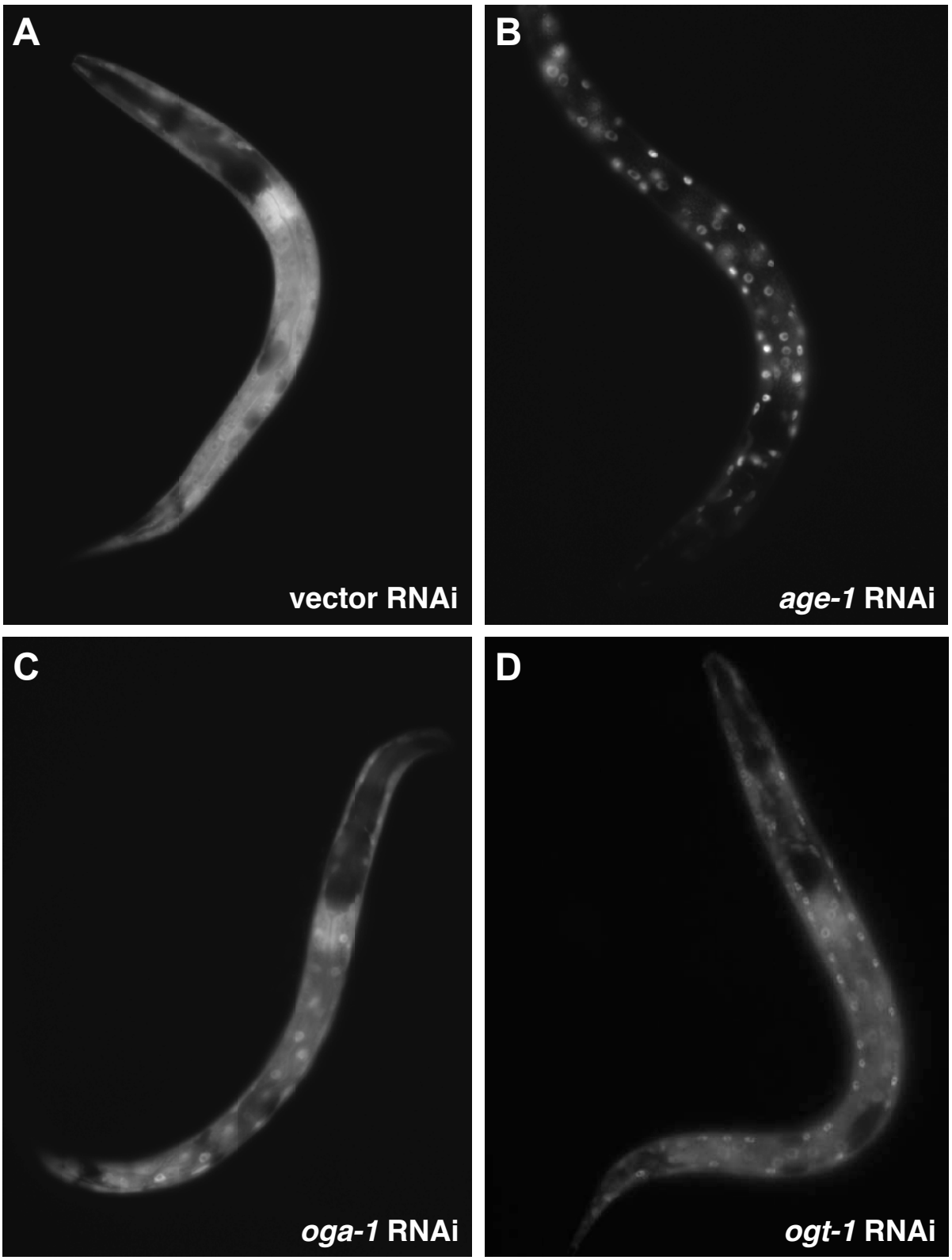

(A)
Post-embryonic development (hrs)
75
50
25
0
**
**
**
wild type
ogt-1
oga-1
daf-2
daf-2; ogt-1
Genotype
(B)
Total number of offspring
300
200
100
0
**
**
**
wild type
ogt-1
oga-1
daf-2
daf-2; ogt-1
Genotype

Figure 3.7: Changes in protein O-GlcNAcylation levels modestly affect DAF-16 nuclear localization. A-D. DAF-16 nuclear localization in *oga-1(RNAi)* and *ogt-1(RNAi)* animals is modestly increased relative to control (vector RNAi) animals but is much less than in *age-1(RNAi)* treated animals. Quantification of the DAF-16::GFP (translational fusion driven by daf-16 promoter) staining pattern is shown in 3.7E.


A
vector RNAi
B
age-1 RNAi
C
oga-1 RNAi
D
ogt-1 RNAi

E.

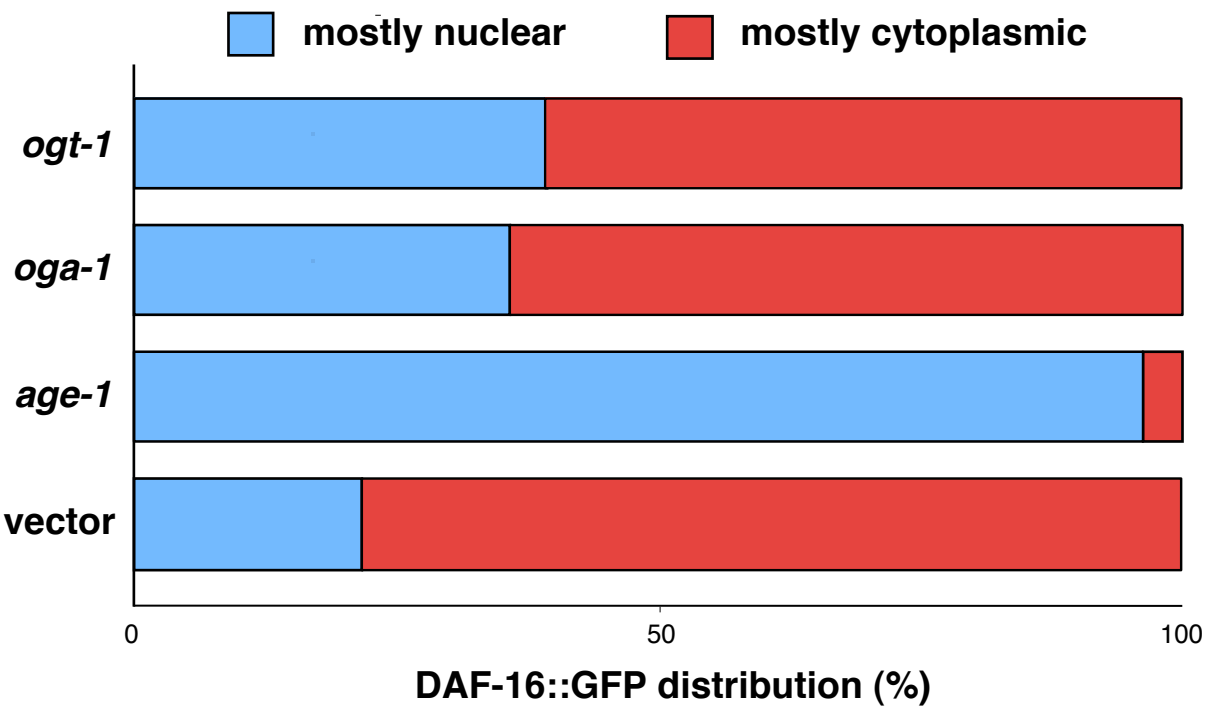

mostly nuclear
mostly cytoplasmic
ogt-1
oga-1
age-1
vector
0
50
100
DAF-16::GFP distribution (%)

Figure 3.8: CUL-1 function is essential for lifespan extension in long-lived OGA-1 mutant. Inactivation of CUL-1 function dramatically reduces lifespan extension in *oga-1 (ok1207)* mutant. CUL-1 protein function is also essential for wild-type lifespan as seen in the lifespan curves. L3/L4 larvae were transferred to *cul-1* feeding RNAi plates as inactivation of *cul-1* in embryos or early larvae severely arrest development. Control animals were fed with vector RNAi in similar experimental conditions (n=85-125 animals per genotype per assay).

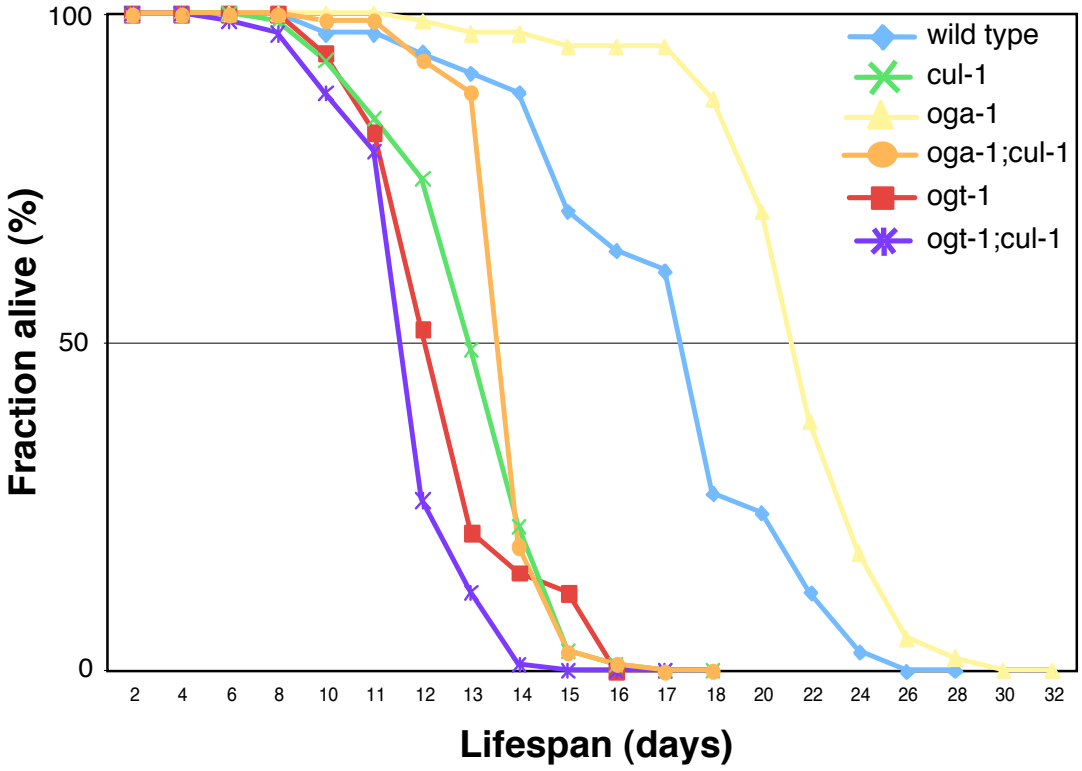
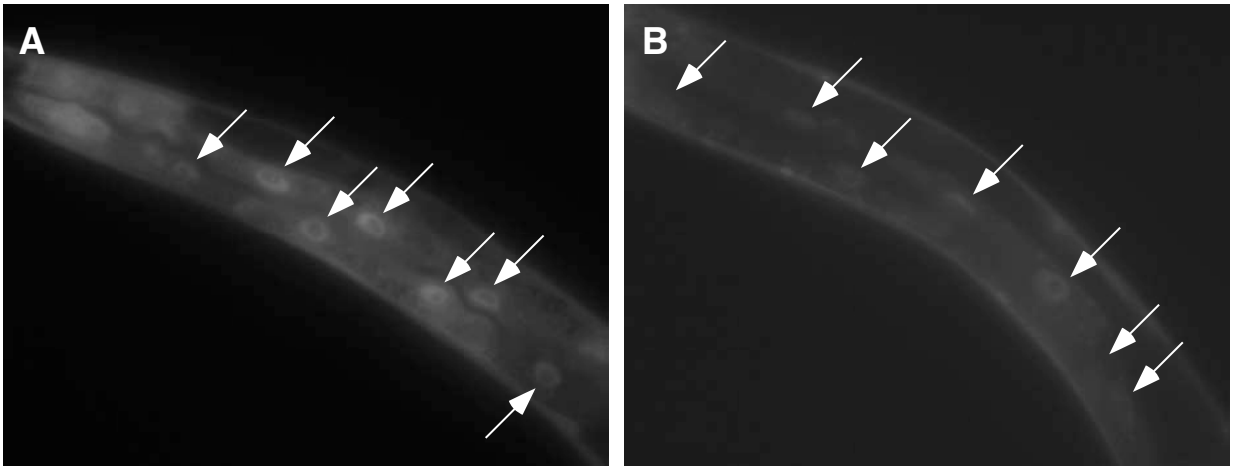

100
50
0
Fraction alive (%)
2
4
6
8
10
11
12
13
14
15
16
17
18
20
22
24
26
28
30
32
Lifespan (days)
wild type
cul-1
oga-1
oga-1;cul-1
ogt-1
ogt-1;cul-1

Figure 3.9: CUL-1 function is essential for DAF-16 protein stability in aging wild type animals. Inactivation of CUL-1 function reduces cellular DAF-16 to undetectable levels (12 day-old adult animals assayed at 20°C). White arrows indicate location of intestinal cells. Figure 3.9b shows quantification of DAF-16::GFP levels that demonstrates a significant reduction upon *cul-1* inactivation. Bar graph shows relative GFP signal intensity (deducted from the background) with arbitrary units in the Y-axis. Asterisks denote statistically significant differences from control (* $p < 0.05$ and ** $p < 0.01$).

Intestinal cell DAF-16::GFP expression



C.

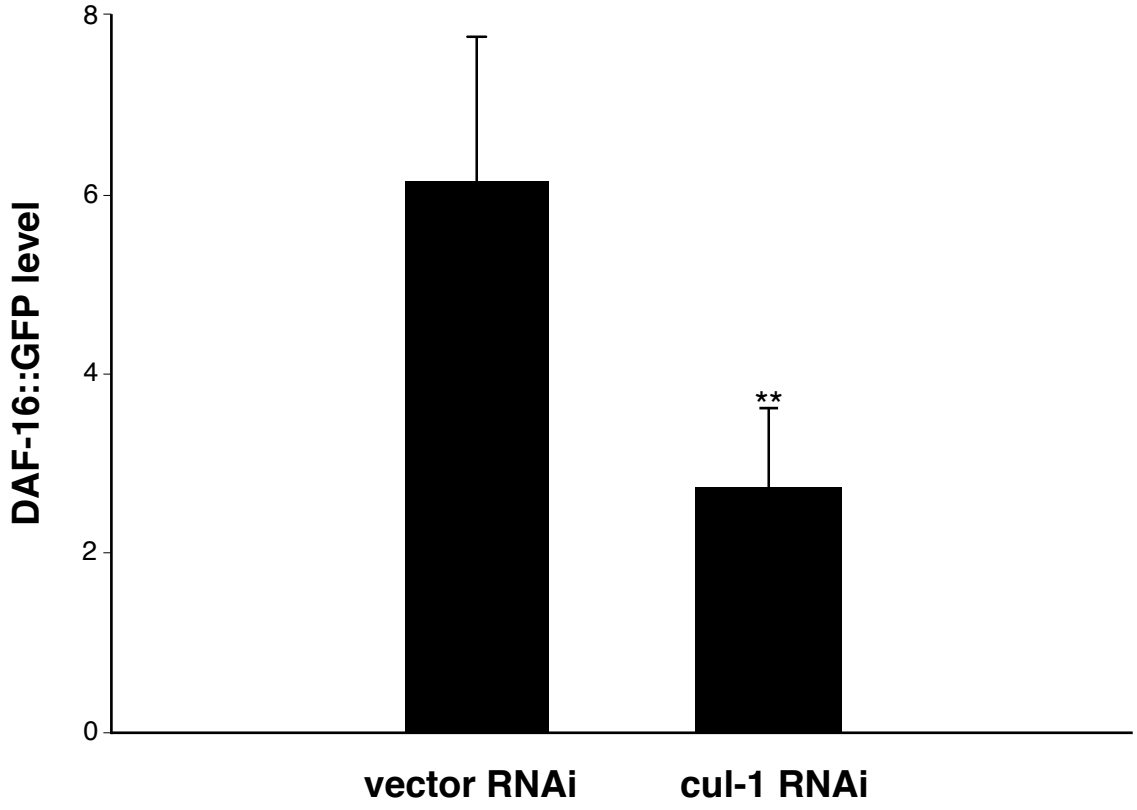
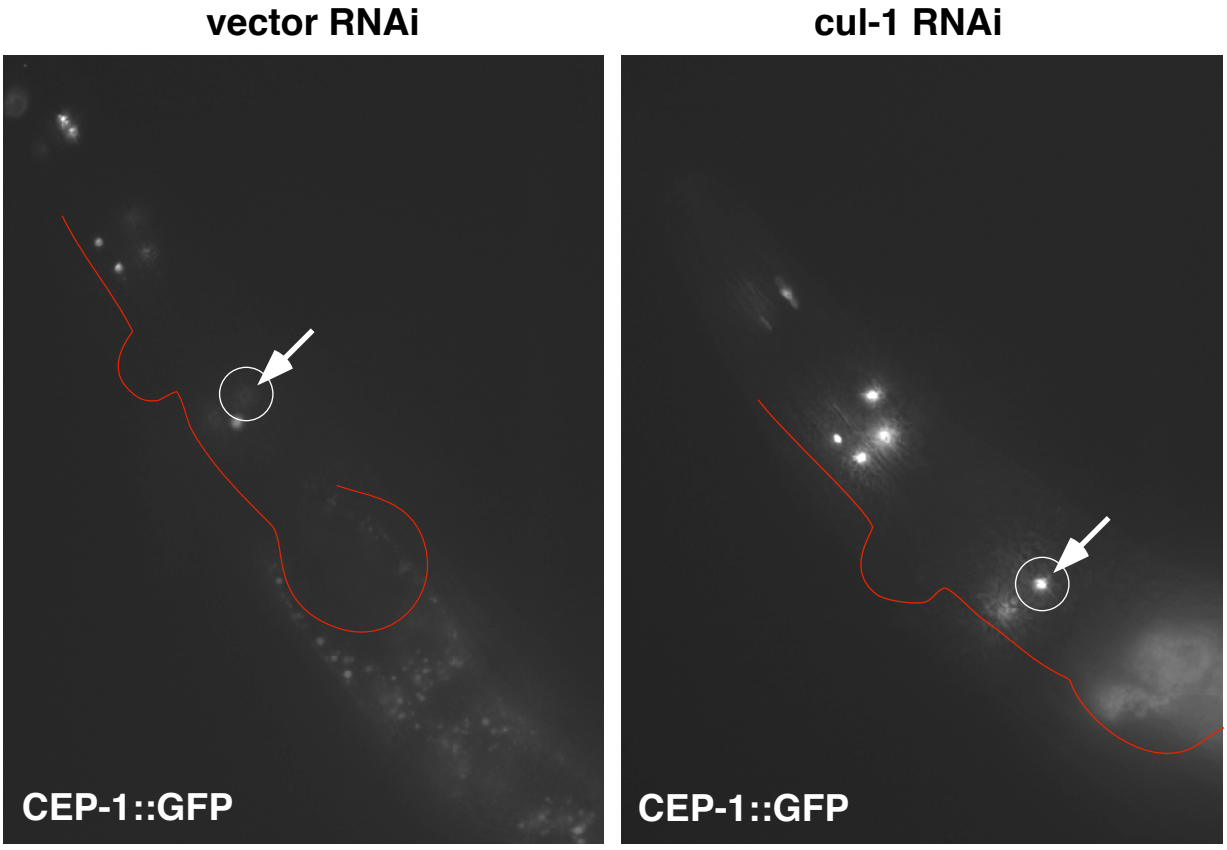
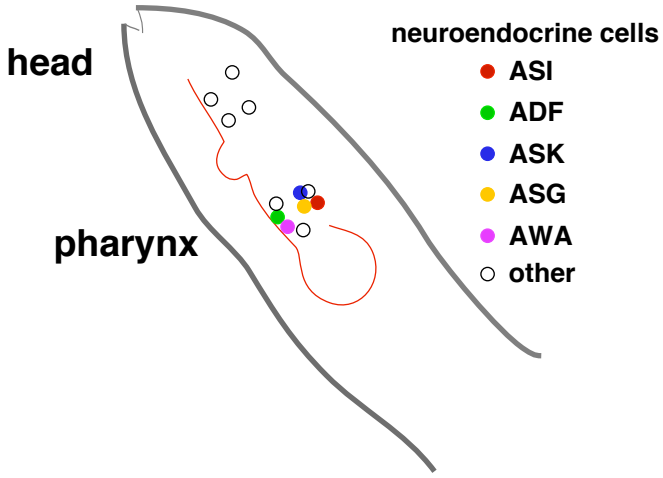
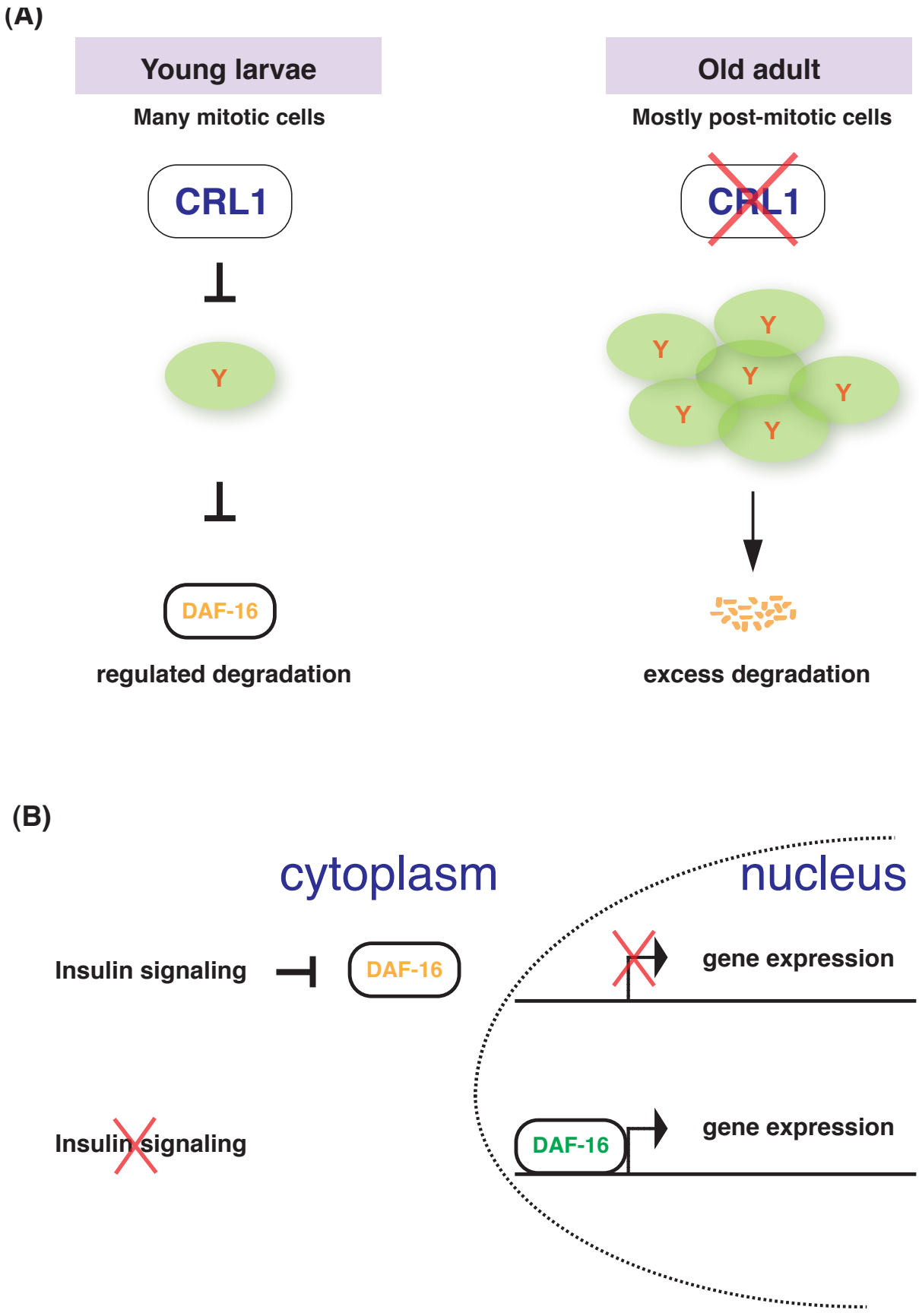


Figure 3.10: CUL-1 negatively regulates CEP-1 protein level in neuro-endocrine and other neuronal cells. Inactivation of CUL-1 function leads to an abnormally high accumulation of CEP-1 protein (GFP translational fusion driven by *cep-1* promoter) protein in unidentified neuro-endocrine cells (equivalent cells at the center of the circle with pointed arrow). In the pharynx region, there are several neuro-endocrine cells that are critical for the integration of environmental cues, such as nutrient availability (colored dots). CEP-1 accumulation is also prominent in other neuronal cells (black circles) in the head region. Cartoon depicts location of the neuroendocrine (next to pharynx), and other neuronal cells.


vector RNAi
cul-1 RNAi
CEP-1::GFP
CEP-1::GFP


head
pharynx
neuroendocrine cells
ASI
ADF
ASK
ASG
AWA
other

Model 3.B: *C. elegans* DAF-16 in the crossroad of multiple-pathway regulation. CUL-1 mediated E3 ubiquitin ligase complex negatively regulates currently unknown negative regulator(s) of DAF-16 protein responsible for its stability in aging tissues in adult *C. elegans* (upper panel A). In metazoa, the insulin pathway is known to regulate numerous cellular processes through DAF-16/FoxO transcription factor by restricting its sub-cellular localization in response to environmental cues, such as availability of nutrients.


(A)
Young larvae
Many mitotic cells
CRL1
Y
DAF-16
regulated degradation
Old adult
Mostly post-mitotic cells
CRL1
Y
Y
Y
Y
Y
Y
excess degradation
(B)
cytoplasm
nucleus
Insulin signaling
DAF-16
gene expression
Insulin signaling
DAF-16
gene expression

CHAPTER 4

THE CRL4^{DCAF1} UBIQUITIN LIGASE IS CRITICAL FOR RIBOSOME SYNTHESIS AND NUCLEOLUS MORPHOLOGY IN THE *C. ELEGANS* GERMLINE

Introduction

Cullin4 RING ubiquitin Ligase (CRL4) complexes play key roles in fundamental cellular processes like transcription, cell cycle, and embryonic development (Cang, Zhang et al. 2006; Jackson and Xiong 2009). The CRL4 complex recruits target substrates for ubiquitination, and subsequent degradation by the 26S proteosome. DDB1 (damaged DNA binding) protein serves as the CRL4 adapter component (He, McCall et al. 2006). CRL4/DDB1 function is essential for genomic integrity and viability of proliferating cells by regulating ubiquitin-mediated degradation of CDT1, c-Jun, STAT, p27^{Kip1}, p21^{cip1}, and cyclin E (Wertz, O'Rourke et al. 2004; Precious, Childs et al. 2005; Bondar, Kalinina et al. 2006; Higa, Banks et al. 2006; Higa, Yang et al. 2006; Kim, Starostina et al. 2008). Loss of CUL4 or DDB1 causes failure in progression through meiosis in yeast, massive DNA re-replication in nematodes, third instar larval lethality in fruit flies, and embryonic lethality at day E12.5 in mice (Li, Ruiz et al. 2002; Zhong, Feng et al. 2003; Takata, Yoshida et al. 2004; Holmberg, Fleck et al. 2005; Cang, Zhang et al. 2006). DDB1 brings distinct substrate recognition subunit (SRS) proteins, many belong to the WD40 repeat domain protein family, to the CRL4 complex to recruit target substrates for ubiquitination (Jackson and Xiong 2009). The WD40 repeat SRS proteins share the same signature WDXR motif from yeast to human and are collectively known as DCAF (DDB1 Cullin4 associated factor). DCAF proteins interact physically with DDB1 acting as SRSs in distinct CRL4 complexes for ubiquitin-mediated degradation of target substrates; although the cellular

functions of most DCAF proteins are unknown (Lee and Zhou 2007; Choe, Przybysz et al. 2009; Jackson and Xiong 2009).

The most prominent nuclear compartment, the nucleolus, is formed around the ribosomal DNA (rDNA) repeats, and is the central site for ribosome biogenesis in animal cells (Boisvert, van Koningsbruggen et al. 2007). The total number of ribosomes produced in a cell is related to its metabolic activity; and increased ribosome production precedes the initiation of DNA synthesis and mitosis (Reddan and Unakar 1976). Besides harboring proteins involved in ribosome biogenesis, the nucleolus also serves as a major repository for dormant RNA and proteins otherwise involved in cell cycle progression as well as DNA replication and repair. Sequestration and confinement of the cell cycle regulatory components, e.g., Cdc14 phosphatases, Protein phosphaste 1, telomerase reverse transcriptase within the nucleolus actively contribute to ensure proper cell cycle progression [reviewed in (Boisvert, van Koningsbruggen et al. 2007)]. Disruption in nucleolar function or 'nucleolar stress' impairs ribosome synthesis and halts the cell cycle in cultured mammalian cells (Lindstrom, Deisenroth et al. 2007; Dai, Sun et al. 2008). Despite the increasing appreciation of the role of the nucleolus in cell survival, growth, and proliferation; the molecular mechanisms that limit ribosome number in response to nutrient availability or growth factors in a growing cell prior to cell division are poorly understood. The nematode *C. elegans* germline provides an excellent model system to investigate how cellular growth and division are regulated in metazoa. The *C. elegans* hermaphrodite gonad employs cell divisions to generate daughter cells that can differentiate into both sperm and oocyte in the proximal region while permitting self-renewal of germ stem cells (GSC) in the distal niche. Here we report for the first time that DCAF-1, a novel SRS for a CRL4 E3 ubiquitin ligase complex is essential for ribosome synthesis coupled to germ cell growth and proper cell cycle progression in *C. elegans*. The corresponding human homolog VprBP, which physically binds to human DDB1, is a key cellular target upon human

immunodeficiency virus (HIV) infection as viral Vpr protein hijacks cellular CRL4 complexes through its interaction with VprBP to facilitate virus propagation in host cells (Le Rouzic, Belaidouni et al. 2007; Casey, Wen et al. 2010). CRL4 function is crucial for host cell survival, growth, and proliferation but cellular targets of VprBP in absence of viral Vpr protein are still largely unknown (Casey, Wen et al. 2010).

In the study we report that DCAF-1, a WDXR repeat domain protein, negatively regulates FOG-1 protein level in the *C. elegans* germline to ensure proper germ cell growth and cell cycle progression. Atypical accumulation of FOG-1, a cytoplasmic poly-A element binding (CPEB) protein, is linked to the abnormal nucleolus morphology, and germ cell growth arrest in the *dcaf-1* mutant gonad. Selective inactivation of FOG-1 function completely rescues the nucleolus morphology defect in *dcaf-1* mutant germ cells. Taken together, our work has uncovered FOG-1, a CPEB protein linked to nucleolus integrity in the *C. elegans* germline. Furthermore, we demonstrate that DCAF-1 protein function could mediate a novel regulatory step in the initiation of ribosome synthesis.

Genetic Methods and Materials

Strains and Maintenance

Worms were handled according to standard procedures and maintained at 20°C with the exception of temperature sensitive (ts) mutants that were maintained at 16°C (Brenner 1974). The following mutant strains were used in the study provided were provided by CGC unless mentioned otherwise: **LGI:** *atm-1(gk186), rrf-1(ok589), cep-1(w40), cep-1(gk138), cep-1(ep347), lin-35(n745), fog-1(q253), fog-1(e2121), fog-1(q325)/hT2, fog-3(q470)unc-29(e1072)/sys-1(q544), fog-3(q540)/hT2*, **LGII:** *cki-1(gk132)/mIn1[dpy-10(e128)mIs14], fbf-1(ok90), fbf-1(ok90) fbf-2(q704)/mIn1, daz-1(tj3)/mIn1, gld-3(q741)/mIn1, gld-3(ok308)/mIn1, gld-3(q730)/mIn1; him-5(e1490),* **LGIII:** *ncl-1(e1865), gmn-1(ok1708)/hT2[bli-4(e937)let-*

?(q782)qIs48], tra-1(e2006), **LGIV:** *atl-1(tm853)/nT1[unc-?(n754)let-?qIs50], ced-3(n1286), ddb-1(tm1769)/dpy-20(e2017), dcaf-1(ok1867)/nT1[qIs51](IV;V), fem-3(e2006ts), fem-3(e1996); him-5(e1490) dpy-21(e428), fem-3(q96ts), him-8(e1849)* **LGV:** *chk-1(tm938), him-5(e1490)*; the wild type strain was Bristol N2. GFP/YFP reporter transgenics used in our current study (from CGC): WS2072 *opIs76[Pcyb-1::cyb-1::yfp; unc-119(+)]*, JH2107 *axIs1720[pie-1 prom:gfp:pgl-1 ORF:pgl-1 3' , unc-119(+)]*,AV280 *meIs5[unc-119(+) + him-17::GFP],* and JH2119 *axIs1533[pie-1 prom:gfp:daz-1 ORF:daz-1 3' , unc-119(+)]*.

DCAF-1 Deletion Mutant

To identify *dcaf-1(ok1867)* mutant allele we used following primer sets as described in *C. elegans* Knock-out Consortium webpage. External left primer: ACGTTCCGACAATTCTTTGC. External right primer: TCGAACTCACCGAAGAACAA. Internal left primer: AGCGGATAGTGGACGAGAGA. Internal right primer: CAGAGCATGAAGCCGTATGA. Internal WT amp icon: 3213 bp. Deletion size: 1162 bp. Deletion left flank: TCATCTGTTGAATGAGAACGTGTAGCCATT. Deletion right flank: TTTTTCGGATAGAATCCGCTTCTGTCAGTG. [http://www.celeganskoconsortium.omrf.org]

RNA interference (RNAi)

All RNAi experiments were performed at 25°C unless otherwise mentioned with *E. coli* strain HT115 transformed with pPD129.36 variant clones as described previously (Timmons, Court et al. 2001). For control RNAi experiment, HT115 transformed with empty vector pPD129.36 was used under similar experimental conditions. All RNAi clones used in different experiments were from the Ahringer library unless otherwise mentioned.

Immunofluorescence

Antibodies against the following proteins were used as described: anti-FOG-1 [1:50], anti-CYE-1 [1:300], anti-CKI-1 [1:50], anti-CDT-1 [1:250], and anti-H3K9me2 [1:200].

Immunofluorescence on dissected gonad was carried out as described previously (Kim and Kipreos 2007). Images were taken on a Zeiss Axioscope microscope using OpenLab5.5 software. Images were processed similarly using Photoshop CS3 and Canvas8 for analysis. Secondary antibodies were coupled to AlexaFluor 433, 540, and 633 (Molecular Probes).

Results

While *cul-4* homozygous mutants arrest as early larvae, homozygous *ddb-1* mutant animals born from heterozygous parents develop to become adults, presumably because of a greater perdurance of maternal product (Kim and Kipreos 2007). *ddb-1* mutant adults demonstrate a 100% penetrant germline defect that produces sterility (Figure 4.1B & 4.1D). Similar germline defect leading to sterility in adults is also observed upon *ddb-1* or *cul-4* RNAi treatment in wild-type animals. *ddb-1* mutant germ cells do not exhibit an increased amount of DNA content, unlike somatic blast cells in *cul-4* or *ddb-1* mutants, which undergo massive levels of DNA re-replication (Zhong, Feng et al. 2003) (*ddb-1* mutant germ cells have 2.6 ± 0.6 C DNA content vs. 2.2 ± 0.4 for wild type, $p>0.02$, n=19) (Figure 4.1E-H). Previous work from our laboratory established that the *C. elegans* $CRL4^{CDT-2}$ complex negatively regulates the DNA replication licensing factors CDT-1 (via direct ubiquitin-mediated degradation), and CDC-6 (by controlling its nuclear localization indirectly through ubiquitin-mediated degradation of CKI-1 protein) (Kim, Feng et al. 2007; Kim and Kipreos 2007). We failed to detect any increase in CDT-1 or CKI-1 protein levels in *ddb-1* mutant germline (Figure 4.2A and 4.2B respectively). Consistently, we also failed to rescue *ddb-1* mutant germline defect by reducing *cdt-1* or *cki-1* levels in *ddb-1* mutant animals (Table 4.1). We also failed to detect previously reported DDB1 substrates, such as, CYE-1 to accumulate significantly in the *ddb-1* mutant germline (Figure 4.2C). Taken together our current experimental data undoubtedly suggests the existence of distinct CRL4/DDB1 substrate(s) in the *C. elegans* germline regulated through a CRL4 complex with an SRS other than CDT-2.

Recent literature suggested a growing list of WD repeat domain proteins that can serve as SRSs for CRL4 dependent cellular processes (He, McCall et al. 2006; Lee and Zhou 2007). From such analysis about 36 WDXR repeat coding ORFs are predicted in *C. elegans* genome, none of which have been studied to-date except WDR-23, which physically binds DDB-1 and functions as an SRS for a CRL4 E3 ubiquitin ligase (Choe, Przybysz et al. 2009). We systematically screened WDXR repeat domain ORFs by feeding RNAi (see method section), and discovered that RNAi depletion of the ZK1251.9 gene phenocopied the *ddb-1* mutant germline defect and produced 100% sterility (Table 4.2 & Figure 4.3D). Mutant *ddb-1* animals on ZK1251.9 RNAi did not demonstrate further enhancement of the germline defect suggesting that DDB-1 and ZK1251.9 function in the same genetic pathway in the *C. elegans* germline (data not shown). *ZK1251.9* encodes a well-conserved protein that is the ortholog of mammalian VprBP/DCAF1 protein, which physically binds to human DDB1 protein (Le Rouzic, Belaidouni et al. 2007; McCall, Miliani de Marval et al. 2008). We renamed ZK1251.9 as *C. elegans* DCAF-1 to correspond to the name of its mammalian counterpart (Figure 4.3B).

To understand DCAF-1 function in germ cell growth and proliferation we analyzed the *ok1867* deletion allele provided by The *C. elegans* Gene Knockout Consortium, which we out-crossed six times prior to experimental analysis. The *ok1867* allele is a large 1.2 kb deletion spanning exons 12 to 15 that removes 184 amino acids and eliminates the 'LisH' domain of DCAF-1 (Figure 4.3A). The deletion leads to two stop codon after a few more amino acids residues following the deletion start site. In humans, mutation of the LisH domain in proteins results in diminished protein-protein dimerization, reduced half-life, and altered cellular localization (Gerlitz, Darhin et al. 2005). The LisH domain protein TBL1 (Transducin beta-like) has been reported to serve as an adaptor to recruit target protein (CtBP) for ubiquitin-mediated degradation (Perissi, Scafoglio et al. 2008). The homozygous *dcaf-1(ok1867)* mutant demonstrates the same germline phenotype as *dcaf-1* RNAi (Figure 4.3E & 4.3D respectively).

The *ok1867* allele is likely a functional null as *dcaf-1(ok1867)* germline phenotypes remain unchanged upon *dcaf-1* feeding RNAi. Similar to *ddb-1* mutants, *dcaf-1* homozygous mutants generated from heterozygous parents develop into sterile adults. The *dcaf-1(ok1867)* mutant animals are active, and appear normal (data not shown).

dcaf-1 mRNA is enriched in the hermaphrodite germline (Reinke, Gil et al. 2004; Wang, Zhao et al. 2009). We asked whether DCAF-1 functions cell autonomously in germ cells to allow normal germ cell development. To test this, we inactivated *dcaf-1* by RNAi in the *rrf-1* mutant, which abolishes RNAi efficacy in the soma remains RNAi competent in the germline (Sijen, Fleenor et al. 2001). In *rrf-1* mutant animals, *dcaf-1* RNAi conferred a 100% penetrant nucleolus morphology defect in the germ cells, and produced sterile adults (Table 4.1). Therefore, we assume that DCAF-1 acts in the *C. elegans* germline to promote normal germ cell growth and proliferation.

We observed that *ncl-2(e1896)* mutant has a similar germ cell nucleolar morphology defect to that of the *dcaf-1(ok1867)* mutant (Figure 4.3F). The *ncl-2* gene was not previously cloned, but was mapped to the same arm of chromosome IV as the *dcaf-1* gene (by EM Hedgecock). A functional complementation test between the two mutant alleles revealed that *dcaf-1(ok1867)* does not complement *ncl-2(e1896)*. We sequenced the *dcaf-1* genomic region from the *ncl-2(e1896)* mutant and identified a point mutation (G1333A) in the *dcaf-1* coding region (Figure 4.3A). The mutation changes amino acid 445 of the DCAF-1 protein from an acidic Glutamic acid (E), which is conserved in mouse and human VprBP, to a basic Lysine (K). These results suggest that *ncl-2* and *dcaf-1* are the same gene.

In *dcaf-1* homozygous mutant animals, the germ cell nucleolus is abnormal in size, multi-lobed, and irregular in shape (Figure 4.3D-F). We utilized a nucleolus specific reporter (nucleostemin::GFP translational fusion) to confirm the nucleolus defect in the mutant germ cells (Kudron and Reinke 2008). Nucleostemin, which is essential for germ cell survival, growth, and

proliferation in *C. elegans*, is not inappropriately accumulated or localized in the *dcaf-1* mutant germ cells (Figure 4.3G-J). We also analyzed the highly conserved nucleolar enzyme Fibrillarin, which is essential for ribosome biogenesis and germ stem cell proliferation (Jansen, Hurt et al. 1991; Newton, Petfalski et al. 2003). We similarly did not observe abnormal accumulation or localization of fibrillarin in *dcaf-1* mutant germline (data not shown).

Transmission electron micrograph of the distal germline of *dcaf-1* mutant demonstrates a significant decrease in ribosome number relative to wild-type germ cells (58±7.7 vs. 20±5.4 average number per arbitrary square units in wild type and mutant animals, respectively, $p < 0.0001$, $n = 20$ and 40, respectively; Figure 4.4 panel G&H and panel E). No previous studies reported similar nucleolus morphology defect linked to any known disorders that would consistently lead to ribosome synthesis defect. Inhibition of RNA pol I mediated transcription in mouse embryonic fibroblast cells causes nucleolus disruption, a ribosome biogenesis defect, and reduction in assembled ribosomes leading to cell growth arrest (Yuan, Zhou et al. 2005). We tested whether increased RNA pol I mediated transcription of rRNA would rescue the nucleolus morphology defect in the mutant germline. Mutations in the *ncl-1* gene, which encodes a repressor of RNA Pol I- and RNA Pol III-mediated transcription, results in excessive rRNA and 5S RNA transcription and enlargement of nucleoli in *C. elegans* (Frank and Roth 1998). Similarly, LIN-35/Rb is also reported to negatively regulate rRNA transcription in *C. elegans* (Voutev, Killian et al. 2006). If the nucleolus morphology defect were due to reduced level of rRNA production then a global increase in rRNA transcription would rescue the typical nucleolus defect observed upon *dcaf-1* inactivation. We inactivated DCAF-1 in *ncl-1(e1865)* and *lin-35(n745)* mutants separately, and observed that in both instances, the animals had 100% penetrant germline nucleoli morphology defect (Table 4.3). The data implies that DCAF-1 acts at a different level than NCL-1- and LIN-35/Rb-mediated RNA Pol I/III transcription regulation.

To determine whether DCAF-1 protein function is vital in embryogenesis, we placed *dcaf-1(ok1867)* heterozygous L3/L4-stage larvae on *dcaf-1* feeding RNAi, and analyzed the F1 generation for viability. No embryonic lethality was observed, and all F1 animals hatched on *dcaf-1* RNAi developed into sterile adults with the same germline nucleolus defect (data not shown). Similar results were also obtained with wild-type animals fed *dcaf-1* RNAi (data not shown). Therefore we conclude that DCAF-1 is critical for reproductive development but not essential for early embryonic development.

Next we examined the importance of DCAF-1 protein function in germ cell proliferation. We analyzed the brood size of heterozygous *dcaf-1(ok1867)* mutant animals, and found that the brood size is significantly smaller at 20°C (79 ± 4.4 for *dcaf-1* mutant vs. 292 ± 10.1 for wild type, $p < 0.00001$, $n = 10$). When animals were grown at a higher temperature, e.g., 26°C, we observed a significant reduction in brood size in *dcaf-1* heterozygous mutant animals (35 ± 3.4 at 26°C vs. 79 ± 4.4 at 20°C, $p < 0.0001$, $n = 20$) similar to the brood size change in wild type (150±6.7 at 26°C vs. 292±10.1 at 20°C, $p < 0.00001$, $n = 10$). Surprisingly we observed sterility in ~50% of the F2 heterozygous *dcaf-1* mutant progeny when maintained at 26°C while no sterility was observed in wild-type animals (n=20). Hence we conclude that DCAF-1 is essential for sustained germline proliferation to achieve normal brood size in *C. elegans*.

GLD-1 is an RNA binding protein that is essential for germ cell meiotic entry and progression through meiosis to differentiate into sperm and oocytes (Lee and Schedl 2001). *gld-1(q485)* null mutants have germline tumors, as germ cells fail to exit meiosis properly and re-enter mitosis from the meiotic state in the promixal gonad (Francis, Barton et al. 1995). In the wild type, GLD-1 expression is low in the distal mitotic region of the gonad (which has proliferating mitotic cells). GLD-1 protein levels increase dramatically in the transition zone (as germ cells enter meiosis), and then decrease to a basal level in differentiated oocyte and sperm (Suh, Crittenden et al. 2009). We utilized a GLD-1::GFP translational fusion reporter that has

the wild-type expression pattern (Lee and Schedl 2001; Suh, Crittenden et al. 2009). Interestingly, upon inactivation of DCAF-1 function by RNAi we observed that GLD-1::GFP expression is repressed to an undetectable level throughout the proximal gonad when compared to the control RNAi-treated group (Figure 4.5B). The loss of GLD-1 expression in wild type produces germline tumors, yet the absence of GLD-1 expression in *dcaf-1* mutant is accompanied by a reduction in germ cells, demonstrating an inherent growth defect in the *dcaf-1* mutant germ cells. To further confirm the disarray in proper germ cell growth and normal cell cycle progression in the *dcaf-1* mutant gonad we utilized another *C. elegans* germline specific protein HIM-17. HIM-17 is expressed in all germ cells but its proper sub-cellular distribution is critical for systematic cell cycle progression, as shown in *him-17* mutants that exhibit complete sterility at 25°C (Reddy and Villeneuve 2004; Bessler, Reddy et al. 2007). We utilized a HIM-17::GFP reporter (translational fusion) as a read-out to follow normal germ cell growth in the *dcaf-1* mutant gonad. Our experimental data showed an atypical alteration in HIM-17 distribution pattern in the *dcaf-1* mutant germ cells (Figure 4.6). Taken together, in absence of optimal DCAF-1 protein 'sick' or 'defective' germ cells in the distal gonad fail to grow properly, and subsequently fail to generate sperm and oocyte leading to complete sterility in homozygous mutant adults, and reduced brood size in the heterozygote animals.

The distal tip cell (DTC) is the primary regulator of the germ cell transition from mitosis to meiosis. DTCs govern germ stem cell proliferation in the distal gonad via the GLP/Notch signaling pathway (Hansen and Schedl 2006). Entry into Meiosis, and differentiation (spermatogenesis and oogenesis) occur in the region of the gonad proximal to the DTC. We asked whether GLP/Notch pathway components are epistatic to DCAF-1 in the germline. Systematic RNAi screening of GLP/Notch pathway mutants revealed that DCAF-1 protein functions independently of the GLP-1-mediated germline proliferation pathway, as neither loss-of-function *glp-1(e2141)* nor gain-of-function *glp-1(ar202)* mutant could revert or rescue the

dcaf-1 germline nucleolus defect (Table 4.3). Additionally we also observed a severe reduction in germline tumor formation in *glp-1(ar202)gf* mutants (data not shown) bolstering our previous observation of DCAF-1's critical role in germ cell growth and proliferation. Inactivation of further downstream effectors, *e.g.,* FBF or GLD proteins also did not rescue the *dcaf-1* germ cell nucleolus morphology defect (Table 4.3).

Physiological apoptosis in the *C. elegans* adult germline is mediated by the CED-3-dependent core apoptotic machinery and eliminates almost half of the oogenic germ cells (Gumienny, Lambie et al. 1999). We wanted to determine whether inappropriate apoptosis contributes to the reduction in germ cell numbers in *dcaf-1* mutants. In the wild-type adult gonad, apoptotic germ cells are clearly visible as 'button-shaped' cells using DIC microscopy (Gartner, Boag et al. 2008). We never observed the characteristic 'button-shaped' apoptotic germ cells in *dcaf-1* mutant gonads (data not shown). Inactivation of *ced-3* by RNAi, which would block apoptosis, did not rescue the germ cell defect in *dcaf-1* mutant; and a similar result was obtained by reciprocal inactivation of *dcaf-1* by RNAi in *ced-3* mutants (Table 4.1). Finally we utilized a well-established *ced-1* driven GFP reporter that marks apoptotic germ cells in the adult gonad (Lu, Yu et al. 2009), and did not observe any *Pced-1*::GFP reporter expression in *dcaf-1* mutant germ cells (Figure 4.7). Taken together we conclude that the nucleolus morphology defect in *dcaf-1* mutant animals is not linked to inappropriate activation of CED-3 dependent apoptosis.

Previous studies reported WDR12, a WD-repeat domain protein is linked to nucleolar stress, ribosome biogenesis defect, and p53-induced cell cycle arrest in mammalian cells (Holzel, Rohrmoser et al. 2005; Rohrmoser, Holzel et al. 2007). *C. elegans* has a clear p53 ortholog CEP-1, which is known to mediate apoptosis and meiotic chromosome segregation in the wild-type germline (Derry, Putzke et al. 2001) but it is not known whether nematode p53/CEP-1 is implicated in germ cell nucleolus integrity. In our current study we observed that

co-inactivation of *cep-1* and *dcaf-1* do not rescue the *dcaf-1* germ cell nucleolus morphology defect (Table 4.1). Our current data suggests that the *dcaf-1* germ cell nucleolus defect is independent of CEP-1 mediated functions in the *C. elegans* germline.

In *C. elegans*, the inappropriate activation of the DNA damage response (DDR) checkpoint proteins arrests cell cycle progression both at G1 and G2 cell cycle phases (Boulton, Gartner et al. 2002; Stergiou and Hengartner 2004). To rule out the possibility that the inappropriate activation of checkpoint proteins, *e.g.*, CHK-1, ATL-1, and ATM-1 kinases, would cause the germ cell nucleolus morphology defect in *dcaf-1* mutant animal, we inactivated the *dcaf-1* gene function by RNAi in *chk-1*, *atl-1*, and *atm-1* mutants. The *dcaf-1* RNAi produced a 100% penetrant nucleoli defect with reduced germ cell numbers in the DDR pathway mutants, implying that the *dcaf-1* germ cell mutant phenotype is not caused by inappropriate checkpoint activation (Table 4.1). Hence we conclude that inappropriate initiation of apoptosis or activation/accumulation of CED-3, CEP-1, CHK-1, ATL-1, and ATM-1 is not critical for the nucleolus morphology defect and reduced germ cell numbers in *dcaf-1* mutants.

In *C. elegans,* the Pumilio family FBF proteins are key regulators of the mitotosis/meiosis decision in the *C. elegans* germline. FBF proteins function to downregulate GLD-1 expression to promote mitosis in the distal gonad. Our experimental finding that repression of GLD-1 expression upon inactivation of DCAF-1 function by RNAi prompted us to investigate whether FBF proteins are implicated in the germline nucleolus morphology defect. Although we did not observe *fbf-1* or *fbf-2* mutants to rescue germ cell nucleolus defect upon inactivation of DCAF-1 function, surprisingly we found that a selective reduction in FOG-1 function could completely rescue *dcaf-1* nucleolus morphology defect (Figure 4.8B & 4.8C). FOG-1, which is also actively regulated by FBF proteins, is known to play a key role in sperm/oocyte switch in the *C. elegans* gonad (Luitjens, Gallegos et al. 2000) but has not been reported to play any role in germ cell survival, growth and proliferation. Interestingly, it appears that only a partial reduction in FOG-1

activity can rescue the *dcaf-1* mutant phenotype. A complete absence of FOG-1 in a *fog-1 (q350)* null mutant could not rescue the *dcaf-1* germline defect while a heterozygous could rescue the defect partially suggesting a minimal FOG-1 function is essential for normal germ cell growth (Table 4.3). In contrast, a temperature-sensitive allele *fog-1(q253)ts* completely rescues the *dcaf-1* mutant germ cell nucleolus morphology defect. In addition to its critical role in sperm/oocyte switch reported earlier, here we would like to propose a novel FOG-1 function essential for germ cell nucleolus integrity previously unrecognized.

FEM proteins, which are the master regulators of sex determination pathway actively regulate FOG-1 protein function through TRA proteins in the *C. elegans* adult gonad (Ellis and Schedl 2007). Loss of FEM protein function results in feminization of the germline, and *fem-3* mutant animals generate only oocytes similar to *fog-1* mutants (Hodgkin 1986). However, in contrast to *fog-1* mutants, *fem-3(e2006)* mutants failed to rescue the *dcaf-1* nucleolus defect (Table 4.3). Similarly, neither *tra-1* loss-of-function or gain-of-function mutants could rescue *dcaf-1* nucleolus defect. Our experimental findings strongly advocates for a novel FOG-1 function in maintaining proper germ cell growth and cell cycle progression independent of its known role in spermatogenesis in the *C. elegans* gonad (Luitjens, Gallegos et al. 2000). We investigated further to explore whether germ cell sex is linked to the nucleolus morphology defect in the *dcaf-1* mutant. In *C. elegans* GLP-4 function is required for normal germ cell proliferation but is also key to differentiation of germ cells into sperm and oocytes. In *glp-4(bn2)* mutants, germ cells arrest at mitotic prophase and do not differentiate into sperm or oocytes (Beanan and Strome 1992). Upon inactivation of the *dcaf-1* gene in *glp-4(ts)* mutant animals we observed a penetrant germ cell nucleolus defect in the RNAi treated animals at the restrictive temperature (26°C for *glp-4* mutants) (Table 4.3). Our current experimental data indicates that DCAF-1 protein acts prior to germ cell sex determination in the *C. elegans* gonad.

Next we asked whether DCAF-1 function is linked to FOG-1 protein level in the mutant gonad. Immunostaining with anti-FOG-1 antibody revealed that FOG-1 protein accumulated in the *dcaf-1* mutant gonad (Figure 4.8F). DCAF-1 might act as a putative SRS in the CRL4 E3 ubiquitin ligase complex to target ubiquitin-mediated protein degradation. We asked, whether DCAF-1 physically interact with FOG-1 to recruit FOG-1 as a substrate for CRL4 complex. In yeast two-hybrid assay FOG-1 failed to interact with DCAF-1 (data not shown) suggests that FOG-1 might be an indirect target of CRL4/DCAF1 E3 ubiquitin ligase. Several attempts to express FOG-1 in a transgenic animal line for further biochemical analysis to determine whether FOG-1 is ubiquitinated *in vivo* in a CRL4/DCAF-1 dependent manner were unsuccessful due to lethality. Even FOG-1 transgenics generated through bombardment or the MoSci method (both introduce low copy numbers) led to sterility making further analysis technically challenging.

Our laboratory previously reported that inactivation of CRL1 function causes excessive cell proliferation or hyperplasia in numerous tissues including the germline in *C. elegans* (Kipreos, Lander et al. 1996). Interestingly, inactivation of *cul-1* function completely rescues germ cell nucleolus defect in the *dcaf-1* mutant germline (data now shown). Our current experimental data indicates a novel CRL1 E3 ubiquitin ligase mediated regulation of nucleolus integrity linked to germ cell growth and proliferation defect in the *C. elegans* germline (Figure 4.9).

Discussion

Recently, numerous WD-repeat domain (DCAF) proteins have been suggested to act as SRS to recruit substrates for CRL4 E3 complexes, although their physiological roles with CRL4 complexes are largely unspecified (He, McCall et al. 2006; Lee and Zhou 2007; Jackson and Xiong 2009). Our current study provides insight into the essential role of the conserved WDXR domain protein DCAF-1 in maintaining nucleoli morphology in the *C. elegans* germline. The

experimental findings indicate that the $CRL4^{DCAF-1}$ E3 complex is required for normal levels of ribosome synthesis in germ cells. In mammalian cells, impaired ribosome biogenesis can be caused by nucleolar dysfunction, which is reported to prevent cells from re-entering the cell cycle (Fumagalli, Di Cara et al. 2009). The ubiquitin proteosome system is known to play an important role in ribosome biogenesis, and changes in nucleolar morphology is observed upon proteosome inhibition in mammalian tissue culture cells (Stavreva, Kawasaki et al. 2006).

Our current data suggests that $CRL4^{DCAF1}$ is required for proper ribosome synthesis, which is linked to proper germ cell growth in *C. elegans.* In line with the current literature we would like to investigate the possibility of ribosome biogenesis as a key defect linked to abnormal nucleolus morphology in *C. elegans dcaf-1* mutant leading causing sterility (Figure 4.1). *C. elegans* RBD-1 is a conserved nucleolar rRNA binding protein detrimental to 38S and 18S rRNA processing, and subsequent ribosome assembly critical for germ cell growth and proliferation (Bjork, Bauren et al. 2002; Saijou, Fujiwara et al. 2004). However, the same study demonstrated no obvious nucleolus morphology defect in the *rbd-1* mutant germ cells although there was a profound defect in ribosome biogenesis leading to a significant reduction in the assembled ribosome pool causing slow-growth and sterility. A recent mammalian study also reported similar findings suggesting that disruption of 40S ribosome biogenesis had no effect on nucleolus integrity (Fumagalli, Di Cara et al. 2009). Additionally, a mutation in nucleolar protein nucleostemin *nst-1(vr6)* severely decreased ribosome biogenesis but did not produce an obvious defect in nucleolar morphology (Kudron and Reinke 2008). In *C. elegans,* inactivation of the key ribosomal protein components causes severe developmental defects making it technically challenging to study the importance of individual ribosomal protein components *in vivo*. For *in vivo* studies we are instead dependent on available mutant alleles that exemplify germline phenotypes. A large ribosomal subunit L11 protein is encoded by the *rpl-11.1* gene is critical for germline proliferation in *C. elegans* (Maciejowski, Ahn et al. 2005). Loss of *rpl-11.1*

function was reported to be embryonic lethal and maternal sterile (Piano, Schetter et al. 2000), but we did not observe a nucleolus morphology defect in the *rpl-11.1* mutant germline (data not shown). The rps-15 gene encodes the small ribosomal subunit S15 protein, and *rps-15(ok1750)* mutants have a small brood size (Qi, Missiuro et al. 2006). We observed an impenetrant germline defect in *rps-15* mutant, with germ cells arrested in the pachytene region, which was not reported previously, however, the distal germ cell nucleolus morphology was normal (data not shown). Finally, increasing rRNA transcription, by inactivating the *ncl-1*, which increases the volume of the nucleolus significantly, also failed to rescue the *dcaf-1* mutant germline defect (Table 4.3).

dcaf-1 mutant germ cells have DNA content indicative of cells in G1 or Go phase (quiescence) rather than arrested in G2 or M phase, which would have at least twice the level of DNA (Figure 4.1). Additionally, our observation of reduced nematode cyclins in the mutant germ cells suggests germ cells are arrested in G1 or Go phase (Figure 4.2 for cyclin E, and Figure 4.5 for cyclin B levels respectively). Unfortunately we could not determine the exact cell cycle phase in the mutant germ cells due to lack of *in vivo* phase specific markers (G1/Go) available for *C. elegans* germline. Our current assumption of germ cell arrest in *dcaf-1* mutant is in line with the current literature as *in vitro* studies also demonstrated that human VprBP knock-down by shRNAi can arrest cell cycle progression (McCall, Miliani de Marval et al. 2008).

We demonstrated that $CRL4^{DCAF\text{-}1}$ negatively regulates FOG-1 protein levels in the *C. elegans* germline. A reduction in FOG-1 function completely rescues the *dcaf-1* germline nucleolus morphology defect, and also allows germ cells to progress through meiosis normally and generate oocytes. FOG-1 loss-of-function mutants generate only oocytes, and lack sperms (Luitjens, Gallegos et al. 2000). A reduction of FOG-1 function similarly rescue *ddb-1* mutant germline nucleolus morphology defect, supporting the notion that DCAF-1 regulates the process as part of CRL4/DCAF-1 E3 complex in the *C. elegans* germline. Previous studies confirmed

physical interaction between DDB1 and VprBP, the mammalian homolog of *C. elegans* DCAF-1 protein that function in a CRL4 complex (Le Rouzic, Belaidouni et al. 2007).

Inactivation of FEM protein function, which also generates oocytes only failed to rescue *dcaf-1* mutant germline defect (Table 4.3). Neither TRA-1 gain nor loss of function could rescue the *dcaf-1* mutant germline defect. Taken together we assume that the FOG-1 function linked to germline nucleolus morphology maintenance is independent of its regulation through FEM/TRA proteins in the *C. elegans* germline. We also observed that FOG-1 protein contains a consensus 'RRXR' nucleolar localization signal (NoLS) motif near its C-terminal, previously unrecognized in current literature that reported FOG-1 as a cytoplasmic protein (Thompson, Bernstein et al. 2005). In mammalian cells, 'RRXR' NoLS motif is sufficient for localization of any EGFP reporter construct into the nucleolus (Muller, Bremer et al. 2010). FOG-1 belongs to the mammalian CPEB protein family, which predominantly regulate mRNA translation (Luitjens, Gallegos et al. 2000). Mammalian CPEB1 protein is also reported to localize into the nucleolus but never investigated for ribosome biogenesis defect when deregulated (Ernoult-Lange, Wilczynska et al. 2009). Right now we do not know whether *C. elegans* FOG-1 can be nucleolar localized, and such localization could be critical for nucleolus integrity and ribosome synthesis in the *dcaf-1* mutant germline. Undue FOG-1 accumulation in dcaf-1 mutant germ cells is presumed to repress its targets, which are currently unknown. As deregulation of negative regulators of rRNA transcription did not rescue *dcaf-1* nucleolus defect, we would rather speculate translation from one or more critical mRNA (encoding a ribosomal or nucleolar component) is repressed when DCAF-1 is missing.

Finally we demonstrated that inactivation of CRL1 function rescues the *dcaf-1* mutant germ cell nucleolus morphology defect. The *cul-1* mutant is associated with a defect in cell cycle exit leading to hyperplasia in numerous tissues including the germline in *C. elegans* (Kipreos, Lander et al. 1996). Terminal differentiation coupled to cell cycle exit is critical for

organogenesis in animals (Buttitta and Edgar 2007). Cell cycle exit upon terminal differentiation has many things in common with quiescent cells, like prolonged cell cycle arrest with G1 DNA content. Unlike terminally differentiated cells, quiescent cells retain the potential to enter the cell cycle again (Shou, Seol et al. 1999; Buttitta and Edgar 2007). In our current experiments, we demonstrated a critical role of a CUL-1-mediated function in the maintenance of nucleolus morphology in *C. elegans* germ cells. It is not well understood, how *cul-1* inactivation could link to nucleolus morphology in the *C. elegans* germline. For now we can only speculate that inactivation of *cul-1* function might lead to accumulation of currently unknown target proteins, and thereby rescue the nucleolus morphology defect in *dcaf-1* mutant germ cells.

It's not known whether mammalian VprBP (aka DCAF1) regulates ribosome synthesis. It would be interesting to know whether DCAF-1 has a conserved role in modulating cell growth and proliferation by regulating ribosome synthesis, or if it has diverged to adapt to the rapidly proliferating germ cells in nematodes. DCAF1 might play a key role in linking the process of translational regulation by regulating the rates of ribosome synthesis thus regulating cell growth. Given the substantial number of predicted WDXR domain containing DCAF proteins, and their possible interaction with DDB1, we anticipate a large number of CRL4 substrates identified in the near future. The level of conservation among the CRL complexes likely will allow *C. elegans* studies to shed light into similar steps in higher organisms in the future.

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References

Beanan, M. J. and S. Strome (1992). "Characterization of a germ-line proliferation mutation in C. elegans." Development **116**(3): 755-766.

Bessler, J. B., K. C. Reddy, et al. (2007). "A role for Caenorhabditis elegans chromatin-associated protein HIM-17 in the proliferation vs. meiotic entry decision." Genetics **175**(4): 2029-2037.

Bjork, P., G. Bauren, et al. (2002). "A novel conserved RNA-binding domain protein, RBD-1, is essential for ribosome biogenesis." Mol Biol Cell **13**(10): 3683-3695.

Boisvert, F. M., S. van Koningsbruggen, et al. (2007). "The multifunctional nucleolus." Nat Rev Mol Cell Biol **8**(7): 574-585.

Bondar, T., A. Kalinina, et al. (2006). "Cul4A and DDB1 associate with Skp2 to target p27Kip1 for proteolysis involving the COP9 signalosome." Mol Cell Biol **26**(7): 2531-2539.

Boulton, S. J., A. Gartner, et al. (2002). "Combined functional genomic maps of the C. elegans DNA damage response." Science **295**(5552): 127-131.

Brenner, S. (1974). "The genetics of Caenorhabditis elegans." Genetics **77**(1): 71-94.

Buttitta, L. A. and B. A. Edgar (2007). "Mechanisms controlling cell cycle exit upon terminal differentiation." Curr Opin Cell Biol **19**(6): 697-704.

Cang, Y., J. Zhang, et al. (2006). "Deletion of DDB1 in mouse brain and lens leads to p53-dependent elimination of proliferating cells." Cell **127**(5): 929-940.

Casey, L., X. Wen, et al. (2010). "The functions of the HIV1 protein Vpr and its action through the DCAF1.DDB1.Cullin4 ubiquitin ligase." Cytokine **51**(1): 1-9.

Choe, K. P., A. J. Przybysz, et al. (2009). "The WD40 repeat protein WDR-23 functions with the CUL4/DDB1 ubiquitin ligase to regulate nuclear abundance and activity of SKN-1 in Caenorhabditis elegans." Mol Cell Biol **29**(10): 2704-2715.

Dai, M. S., X. X. Sun, et al. (2008). "Aberrant expression of nucleostemin activates p53 and induces cell cycle arrest via inhibition of MDM2." Mol Cell Biol **28**(13): 4365-4376.

Derry, W. B., A. P. Putzke, et al. (2001). "Caenorhabditis elegans p53: role in apoptosis, meiosis, and stress resistance." Science **294**(5542): 591-595.

Ellis, R. and T. Schedl (2007). "Sex determination in the germ line." WormBook: 1-13.

Ernoult-Lange, M., A. Wilczynska, et al. (2009). "Nucleocytoplasmic traffic of CPEB1 and accumulation in Crm1 nucleolar bodies." Mol Biol Cell **20**(1): 176-187.

Francis, R., M. K. Barton, et al. (1995). "gld-1, a tumor suppressor gene required for oocyte development in Caenorhabditis elegans." Genetics **139**(2): 579-606.

Frank, D. J. and M. B. Roth (1998). "ncl-1 is required for the regulation of cell size and ribosomal RNA synthesis in Caenorhabditis elegans." J Cell Biol **140**(6): 1321-1329.

Fumagalli, S., A. Di Cara, et al. (2009). "Absence of nucleolar disruption after impairment of 40S ribosome biogenesis reveals an rpL11-translation-dependent mechanism of p53 induction." Nat Cell Biol **11**(4): 501-508.

Gartner, A., P. R. Boag, et al. (2008). "Germline survival and apoptosis." WormBook: 1-20.

Gerlitz, G., E. Darhin, et al. (2005). "Novel functional features of the Lis-H domain: role in protein dimerization, half-life and cellular localization." Cell Cycle **4**(11): 1632-1640.

Gumienny, T. L., E. Lambie, et al. (1999). "Genetic control of programmed cell death in the Caenorhabditis elegans hermaphrodite germline." Development **126**(5): 1011-1022.

Hansen, D. and T. Schedl (2006). "The regulatory network controlling the proliferation-meiotic entry decision in the Caenorhabditis elegans germ line." Curr Top Dev Biol **76**: 185-215.

He, Y. J., C. M. McCall, et al. (2006). "DDB1 functions as a linker to recruit receptor WD40 proteins to CUL4-ROC1 ubiquitin ligases." Genes Dev **20**(21): 2949-2954.

Higa, L. A., D. Banks, et al. (2006). "L2DTL/CDT2 interacts with the CUL4/DDB1 complex and PCNA and regulates CDT1 proteolysis in response to DNA damage." Cell Cycle **5**(15): 1675-1680.

Higa, L. A., X. Yang, et al. (2006). "Involvement of CUL4 ubiquitin E3 ligases in regulating CDK inhibitors Dacapo/p27Kip1 and cyclin E degradation." Cell Cycle **5**(1): 71-77.

Hodgkin, J. (1986). "Sex determination in the nematode C. elegans: analysis of tra-3 suppressors and characterization of fem genes." Genetics **114**(1): 15-52.

Holmberg, C., O. Fleck, et al. (2005). "Ddb1 controls genome stability and meiosis in fission yeast." Genes Dev **19**(7): 853-862.

Holzel, M., M. Rohrmoser, et al. (2005). "Mammalian WDR12 is a novel member of the Pes1-Bop1 complex and is required for ribosome biogenesis and cell proliferation." J Cell Biol **170**(3): 367-378.

Jackson, S. and Y. Xiong (2009). "CRL4s: the CUL4-RING E3 ubiquitin ligases." Trends Biochem Sci **34**(11): 562-570.

Jansen, R. P., E. C. Hurt, et al. (1991). "Evolutionary conservation of the human nucleolar protein fibrillarin and its functional expression in yeast." J Cell Biol **113**(4): 715-729.

Kim, J., H. Feng, et al. (2007). "C. elegans CUL-4 prevents rereplication by promoting the nuclear export of CDC-6 via a CKI-1-dependent pathway." Curr Biol **17**(11): 966-972.

Kim, Y. and E. T. Kipreos (2007). "The Caenorhabditis elegans replication licensing factor CDT-1 is targeted for degradation by the CUL-4/DDB-1 complex." Mol Cell Biol **27**(4): 1394-1406.

Kim, Y., N. G. Starostina, et al. (2008). "The CRL4Cdt2 ubiquitin ligase targets the degradation of p21Cip1 to control replication licensing." Genes Dev **22**(18): 2507-2519.

Kipreos, E. T., L. E. Lander, et al. (1996). "cul-1 is required for cell cycle exit in C. elegans and identifies a novel gene family." Cell **85**(6): 829-839.

Kudron, M. M. and V. Reinke (2008). "C. elegans nucleostemin is required for larval growth and germline stem cell division." PLoS Genet **4**(8): e1000181.

Le Rouzic, E., N. Belaidouni, et al. (2007). "HIV1 Vpr arrests the cell cycle by recruiting DCAF1/VprBP, a receptor of the Cul4-DDB1 ubiquitin ligase." Cell Cycle **6**(2): 182-188.

Lee, J. and P. Zhou (2007). "DCAFs, the missing link of the CUL4-DDB1 ubiquitin ligase." Mol Cell **26**(6): 775-780.

Lee, M. H. and T. Schedl (2001). "Identification of in vivo mRNA targets of GLD-1, a maxi-KH motif containing protein required for C. elegans germ cell development." Genes Dev **15**(18): 2408-2420.

Li, B., J. C. Ruiz, et al. (2002). "CUL-4A is critical for early embryonic development." Mol Cell Biol **22**(14): 4997-5005.

Lindstrom, M. S., C. Deisenroth, et al. (2007). "Putting a finger on growth surveillance: insight into MDM2 zinc finger-ribosomal protein interactions." Cell Cycle **6**(4): 434-437.

Lu, N., X. Yu, et al. (2009). "Detecting apoptotic cells and monitoring their clearance in the nematode Caenorhabditis elegans." Methods Mol Biol **559**: 357-370.

Luitjens, C., M. Gallegos, et al. (2000). "CPEB proteins control two key steps in spermatogenesis in C. elegans." Genes Dev **14**(20): 2596-2609.

Maciejowski, J., J. H. Ahn, et al. (2005). "Autosomal genes of autosomal/X-linked duplicated gene pairs and germ-line proliferation in Caenorhabditis elegans." Genetics **169**(4): 1997-2011.

McCall, C. M., P. L. Miliani de Marval, et al. (2008). "Human immunodeficiency virus type 1 Vpr-binding protein VprBP, a WD40 protein associated with the DDB1-CUL4 E3 ubiquitin ligase, is essential for DNA replication and embryonic development." Mol Cell Biol **28**(18): 5621-5633.

Muller, C., A. Bremer, et al. (2010). "Nucleolar retention of a translational C/EBPalpha isoform stimulates rDNA transcription and cell size." EMBO J **29**(5): 897-909.

Newton, K., E. Petfalski, et al. (2003). "Fibrillarin is essential for early development and required for accumulation of an intron-encoded small nucleolar RNA in the mouse." Mol Cell Biol **23**(23): 8519-8527.

Perissi, V., C. Scafoglio, et al. (2008). "TBL1 and TBLR1 phosphorylation on regulated gene promoters overcomes dual CtBP and NCoR/SMRT transcriptional repression checkpoints." Mol Cell **29**(6): 755-766.

Piano, F., A. J. Schetter, et al. (2000). "RNAi analysis of genes expressed in the ovary of Caenorhabditis elegans." Curr Biol **10**(24): 1619-1622.

Precious, B., K. Childs, et al. (2005). "Simian virus 5 V protein acts as an adaptor, linking DDB1 to STAT2, to facilitate the ubiquitination of STAT1." J Virol **79**(21): 13434-13441.

Qi, Y., P. E. Missiuro, et al. (2006). "Semi-supervised analysis of gene expression profiles for lineage-specific development in the Caenorhabditis elegans embryo." Bioinformatics **22**(14): e417-423.

Reddan, J. R. and N. J. Unakar (1976). "Electron microscopy of cultured mammalian lenses. II. Changes preceding and accompanying insulin-induced mitosis." Invest Ophthalmol **15**(5): 411-417.

Reddy, K. C. and A. M. Villeneuve (2004). "C. elegans HIM-17 links chromatin modification and competence for initiation of meiotic recombination." Cell **118**(4): 439-452.

Reinke, V., I. S. Gil, et al. (2004). "Genome-wide germline-enriched and sex-biased expression profiles in Caenorhabditis elegans." Development **131**(2): 311-323.

Rohrmoser, M., M. Holzel, et al. (2007). "Interdependence of Pes1, Bop1, and WDR12 controls nucleolar localization and assembly of the PeBoW complex required for maturation of the 60S ribosomal subunit." Mol Cell Biol **27**(10): 3682-3694.

Saijou, E., T. Fujiwara, et al. (2004). "RBD-1, a nucleolar RNA-binding protein, is essential for Caenorhabditis elegans early development through 18S ribosomal RNA processing." Nucleic Acids Res **32**(3): 1028-1036.

Shou, W., J. H. Seol, et al. (1999). "Exit from mitosis is triggered by Tem1-dependent release of the protein phosphatase Cdc14 from nucleolar RENT complex." Cell **97**(2): 233-244.

Sijen, T., J. Fleenor, et al. (2001). "On the role of RNA amplification in dsRNA-triggered gene silencing." Cell **107**(4): 465-476.

Stavreva, D. A., M. Kawasaki, et al. (2006). "Potential roles for ubiquitin and the proteasome during ribosome biogenesis." Mol Cell Biol **26**(13): 5131-5145.

Stergiou, L. and M. O. Hengartner (2004). "Death and more: DNA damage response pathways in the nematode C. elegans." Cell Death Differ **11**(1): 21-28.

Suh, N., S. L. Crittenden, et al. (2009). "FBF and its dual control of gld-1 expression in the Caenorhabditis elegans germline." Genetics **181**(4): 1249-1260.

Takata, K., H. Yoshida, et al. (2004). "Drosophila damaged DNA-binding protein 1 is an essential factor for development." Genetics **168**(2): 855-865.

Thompson, B. E., D. S. Bernstein, et al. (2005). "Dose-dependent control of proliferation and sperm specification by FOG-1/CPEB." Development **132**(15): 3471-3481.

Timmons, L., D. L. Court, et al. (2001). "Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in Caenorhabditis elegans." Gene **263**(1-2): 103-112.

Voutev, R., D. J. Killian, et al. (2006). "Alterations in ribosome biogenesis cause specific defects in C. elegans hermaphrodite gonadogenesis." Dev Biol **298**(1): 45-58.

Wang, X., Y. Zhao, et al. (2009). "Identification of genes expressed in the hermaphrodite germ line of C. elegans using SAGE." BMC Genomics **10**: 213.

Wertz, I. E., K. M. O'Rourke, et al. (2004). "Human De-etiolated-1 regulates c-Jun by assembling a CUL4A ubiquitin ligase." Science **303**(5662): 1371-1374.

Yuan, X., Y. Zhou, et al. (2005). "Genetic inactivation of the transcription factor TIF-IA leads to nucleolar disruption, cell cycle arrest, and p53-mediated apoptosis." Mol Cell **19**(1): 77-87.

Zhong, W., H. Feng, et al. (2003). "CUL-4 ubiquitin ligase maintains genome stability by restraining DNA-replication licensing." Nature **423**(6942): 885-889.

Table 4.1

Analysis of germline nucleolus morphology defect

Strain	RNAi	Germline defect	n
ddb-1(tm1769)	vector	100%	20
ddb-1(tm1769)	*cki-1*	100%	20
ddb-1(tm1769)	*cdt-1*	100%	20
cki-1(gk132)	*ddb-1*	100%	15
gmn-1(tm2212)	*ddb-1*	100%	15
N2 wild type	vector	0%	20
N2 wild type	*ddb-1*	100%	20
dcaf-1(ok1867)	vector	100%	15
N2 wild type	vector	0%	
N2 wild type	*dcaf-1*	100%	20
dcaf-1(ok1867)	*cki-1*	100%	15
dcaf-1(ok1867)	*ddt-1*	100%	15
rrf-1(ok589)	*dcaf-1*	100%	20
Checkpoint			
chk-1(tm938)	*dcaf-1*	100%	15
atl-1(tm853)	*dcaf-1*	100%	15
atm-1(gk186)	*dcaf-1*	100%	15
Cell death			
cep-1(w40)	*dcaf-1*	100%	15
cep-1(gk138)	*dcaf-1*	100%	15
cep-1(ep347)	*dcaf-1*	100%	15
ced-3(n1286)	*dcaf-1*	100%	15
ced-3(n717)	*dcaf-1*	100%	15

Table 4.2

Identification of CRL4/DDB1 putative SRS proteins in *C. elegans*

WD40 proteins		**RNAi phenotype**	**Phenocopy *ddb-1* mutant**
Mammalian	***C. elegans***	**observed in *C. elegans***	**germ cell defect**
CDT2	T01C3.1	Large seam cell	No
WDSOF1	ZK430.9	Lva, large germ cell	No
WDR21A	F47D12.9	Lva, Let, Gro	No
WDR22	R11D1.1B	-	No
WDR42A	Y73E7A.9	-	No
WDR68	F53C11.7	-	No
VprBP	ZK1251.9	Ste	**Yes**
WDR23	D2030.9	Egl, Gro	No
WDR26	Y39H10A.6	Ste, Emb	No

Table 4.3

Analysis of *dcaf-1* germline nucleolus defect suppression

Strain	Mutant phenotype	Suppression of germline defect	n
ncl-1(e1865)	Large nucleoli	No	15
lin-35(n745)	RNAi enhanced	No	15
glp-1(e2141)	Ste	No	15
glp-1(ar202)gf	Tum	No	15
gld-3(q741)	Tum	No	10
gld-3(q730)	Tum	No	10
gld-3(ok308)	Tum	No	10
gld-3(q730);nos-3(q650)	Tum	No	15
fbf-1(ok91)	-	No	15
fbf-2(q704)	-	No	15
daz-1(tj3)	Ste	No	15
glp-4(bn2)	Ste	No	15
fem-3(e2006)	Fem	No	15
tra-1(e1488)	Tra	No	15
tra-1(e1099)	Tra	No	10
tra-1(e1575)	Gro	No	20
fog-1(q325)	Fog	Partial*	15
fog-1(q372)	Fog	No	10
fog-1(e2121)	Fog	Partial*	15
fog-1(q253)ts	Fog	Yes	15
fog-1(q241)	Fog	Partial*	10
dcaf-1(ok1867);fog-1(RNAi)	Fog	Yes	10

*Partial suppression of nucleolar morphology defect observed only in few germ cells in the distal region while typical defective germ cells were observed all through the gonad.

Figure 4.1. Germline phenotype in *ddb-1(tm1769)* mutant. Wild-type gonad with normal germ cells (A&C), and defective germ cells observed in *ddb-1(tm1769)* homozygous mutant gonad (B&D). Animals were stained with DAPI to visualize germ cell nuclei. One gonad arm of each wild type (E&G), and *ddb-1(tm1769)* homozygous mutant (F&H) demonstrates no difference in the germ cell DNA content.

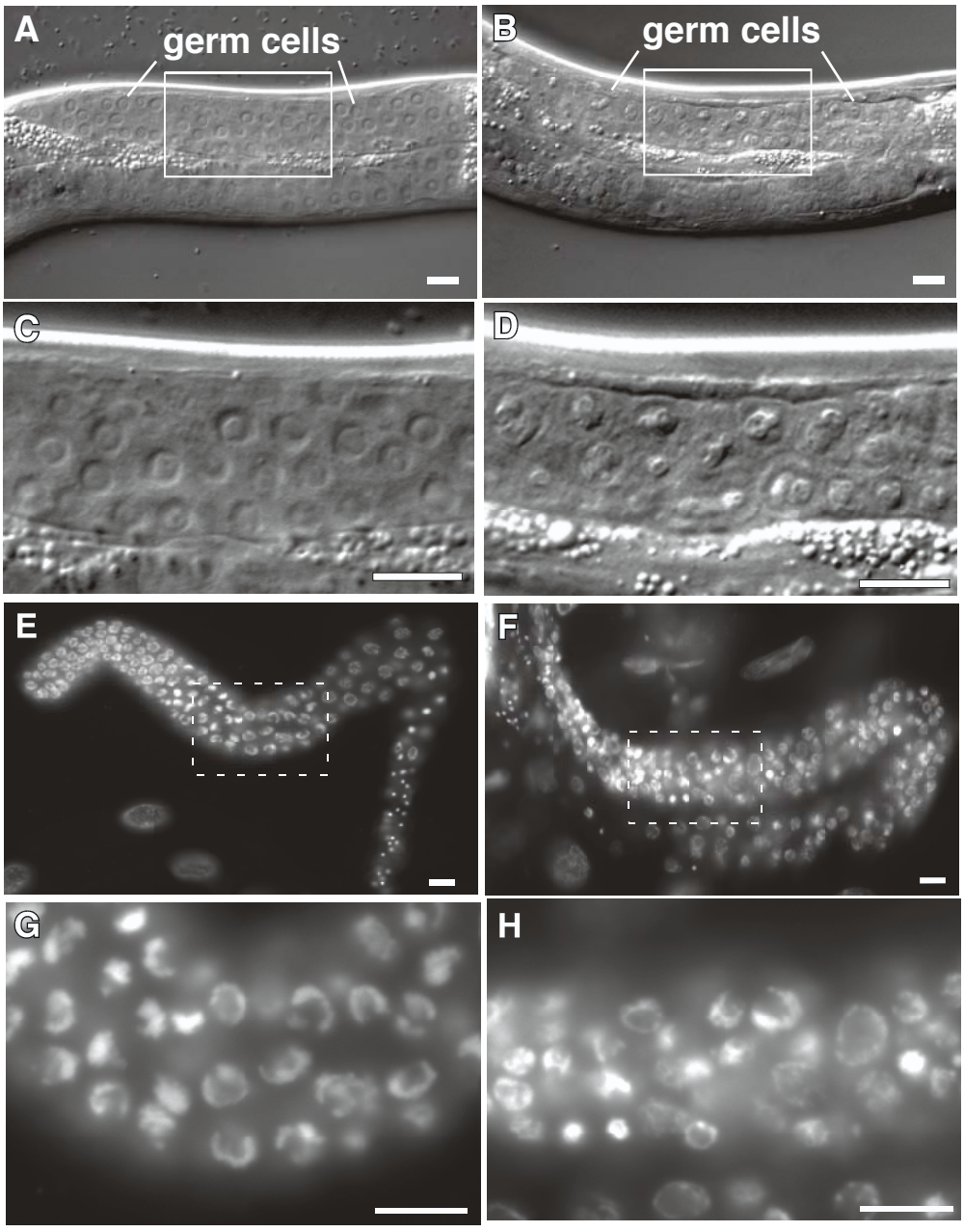
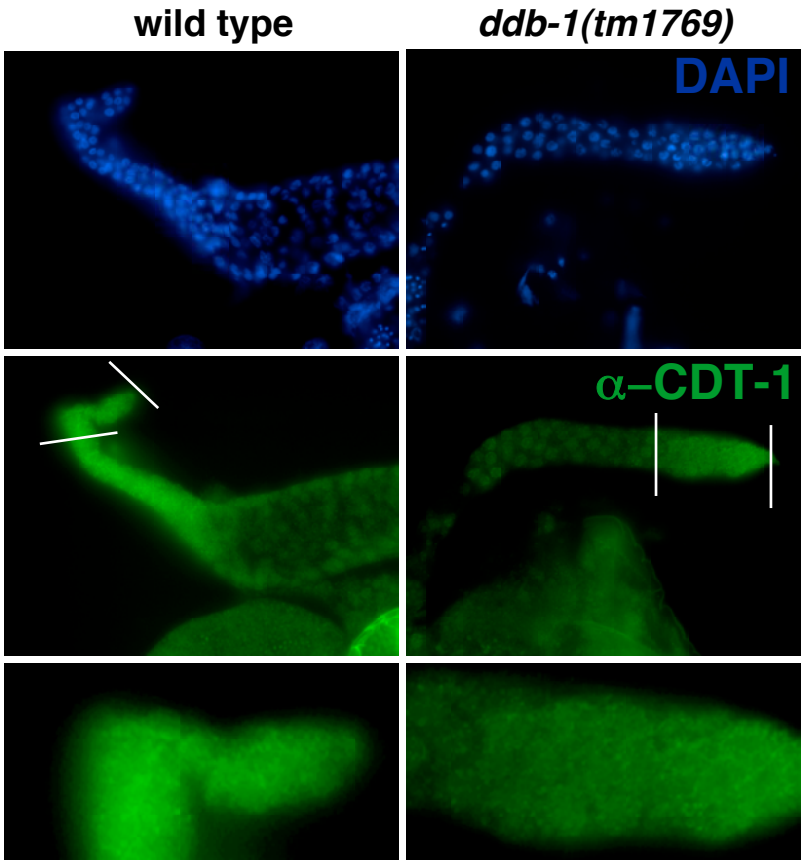
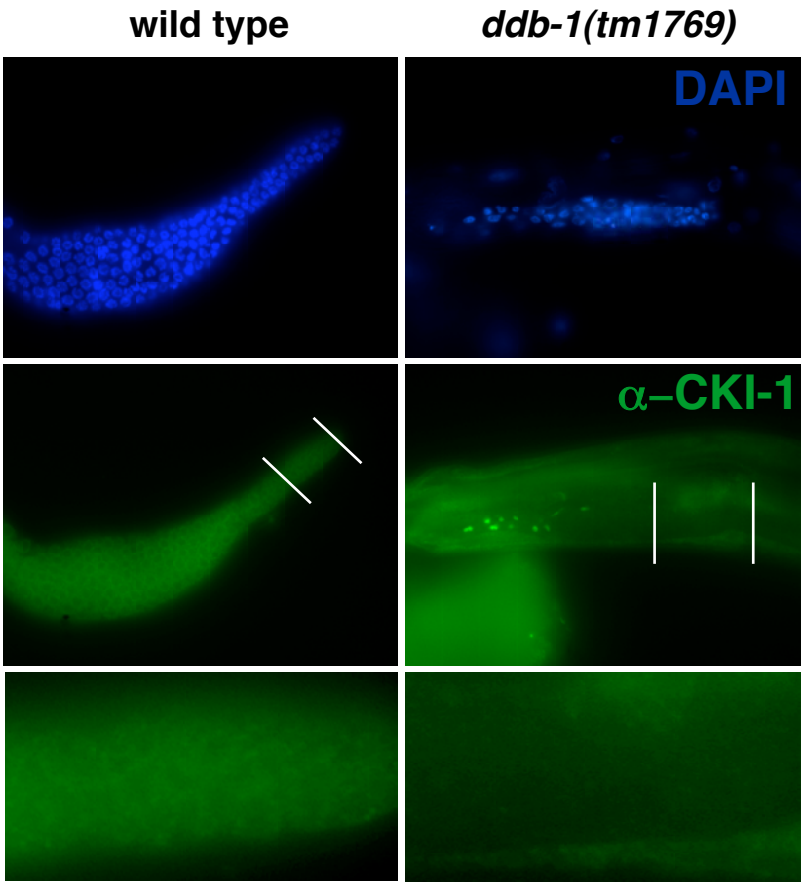

A
germ cells
B
germ cells
C
D
E
F
G
H
wild type
ddb-1(tm1769)

Figure 4.2. Substantial accumulation of known DDB-1 substrates not observed in *ddb-1* germline. Top panels show DAPI staining to visualize the germ cell nuclei while bottom panels show enlarged section of distal region of the germline upon immunostaining with anti-CDT-1 (2A), anti-CKI-1 (2B), and anti-CYE-1 (2C) specific antibodies. No substantial accumulation of the respective proteins is observed in *ddb-1(tm1769)* mutant germ cells.

2A.



2B.



2C.

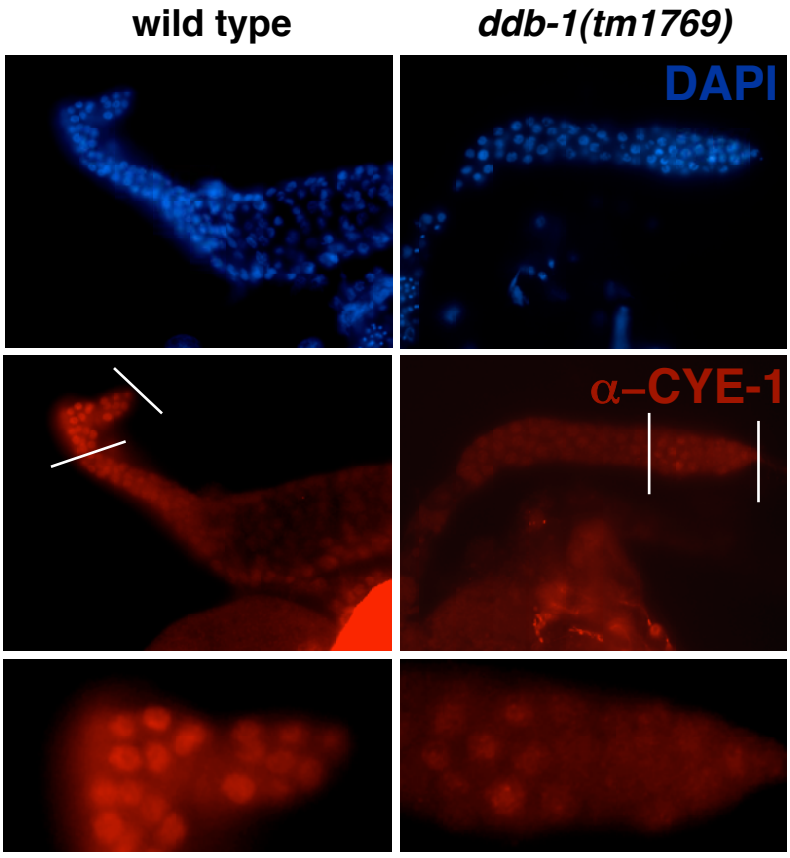
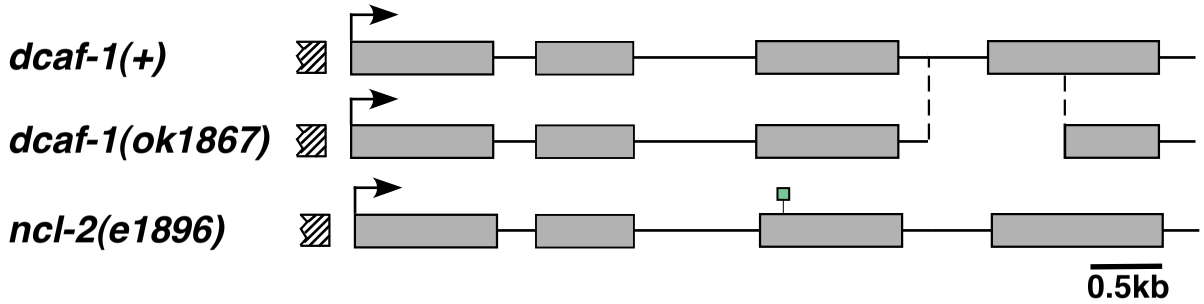
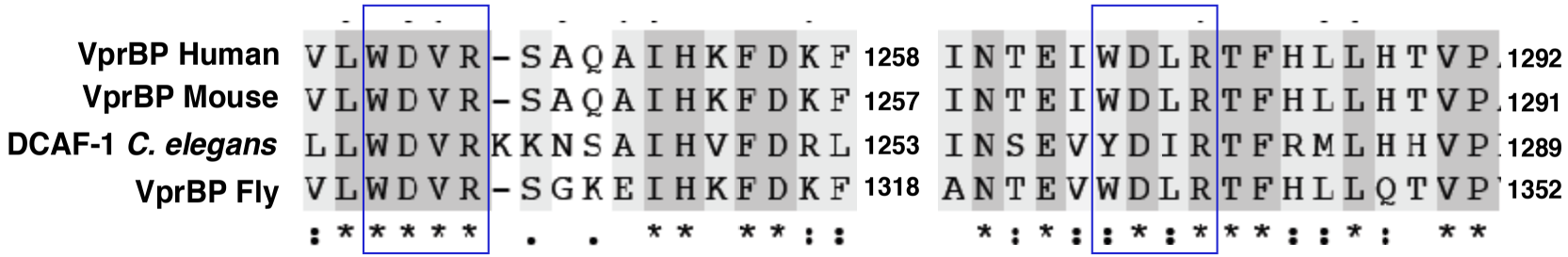


Figure 4.3. *dcaf-1* locus and germline mutant phenotype. (A) Schematic of the *dcaf-1 (ok1867)* deletion locus, and *ncl-2(e1896)* mutation. The deleted region is marked by dotted lines [grey boxes stand for exons and solid lines for introns]. (B) Sequence comparison of the WD'X'R repeats in DCAF-1 protein with VprBP family of proteins reported in higher organisms [***** stands for conserved, and **:** for similar amino acids at the same position]. (C-F) Inactivation of ZK1251.9 by RNAi results in similar germline defect observed in both *dcaf-1* and *ncl-2* mutant animals. (G-J) A conserved nucleolar protein NST-1 attached to GFP reporter (translation reporter) in *C. elegans* shows that germ cell nucleolus is primarily affected upon inactivation of DCAF-1 function although its expression level or localization remains unaltered.

A


dcaf-1(+)
dcaf-1(ok1867)
ncl-2(e1896)
0.5kb

B


VprBP Human VLWDVR-SAQAIHKFDKF 1258 INTEIWDLRTFHLLHTVP 1292
VprBP Mouse VLWDVR-SAQAIHKFDKF 1257 INTEIWDLRTFHLLHTVP 1291
DCAF-1 C. elegans LLWDVRKKNSAIHVFDRL 1253 INSEVYDIRTFRMLHHVP 1289
VprBP Fly VLWDVR-SGKEIHKFDKF 1318 ANTEVWDLRTFHLLQTVP 1352

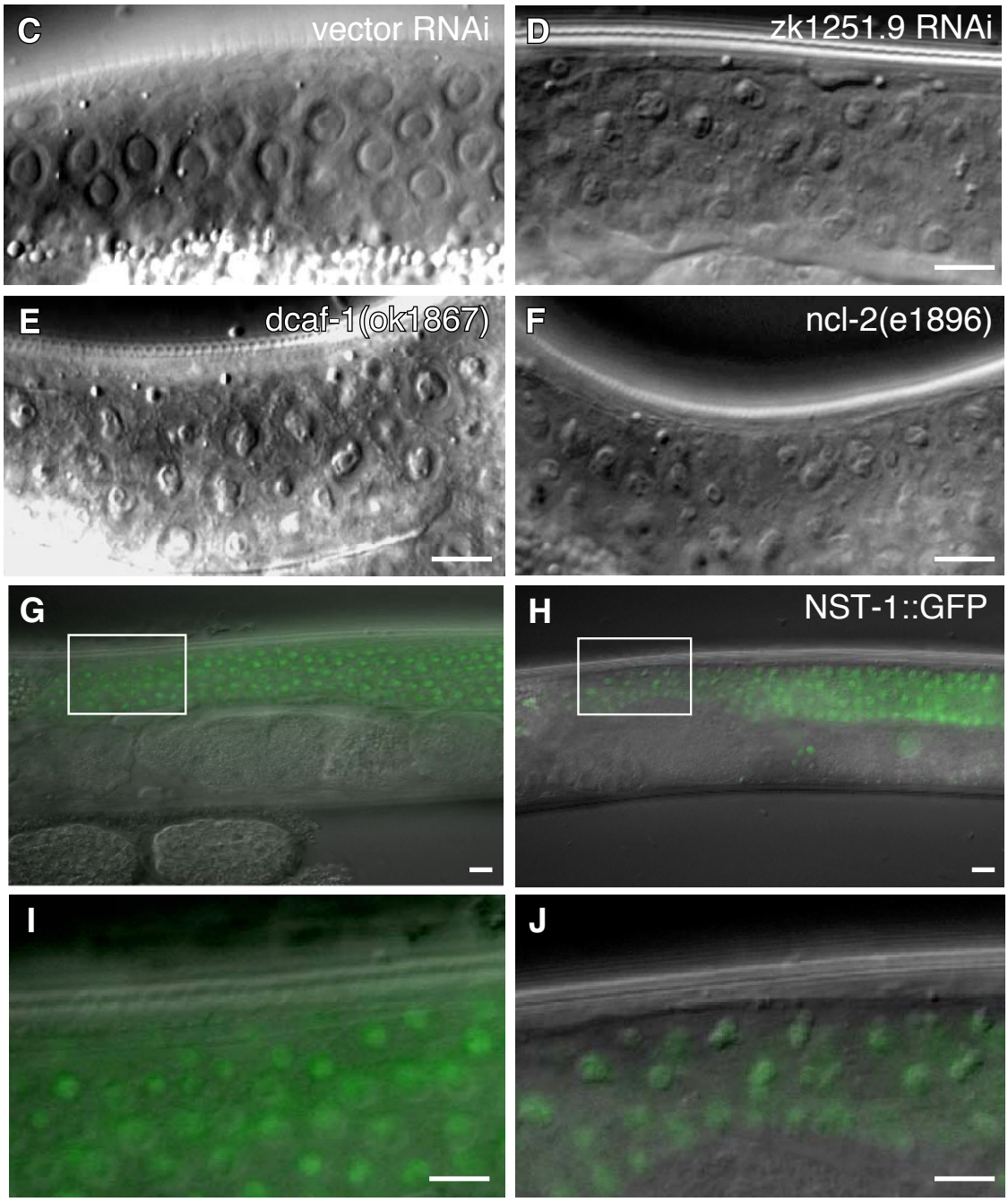
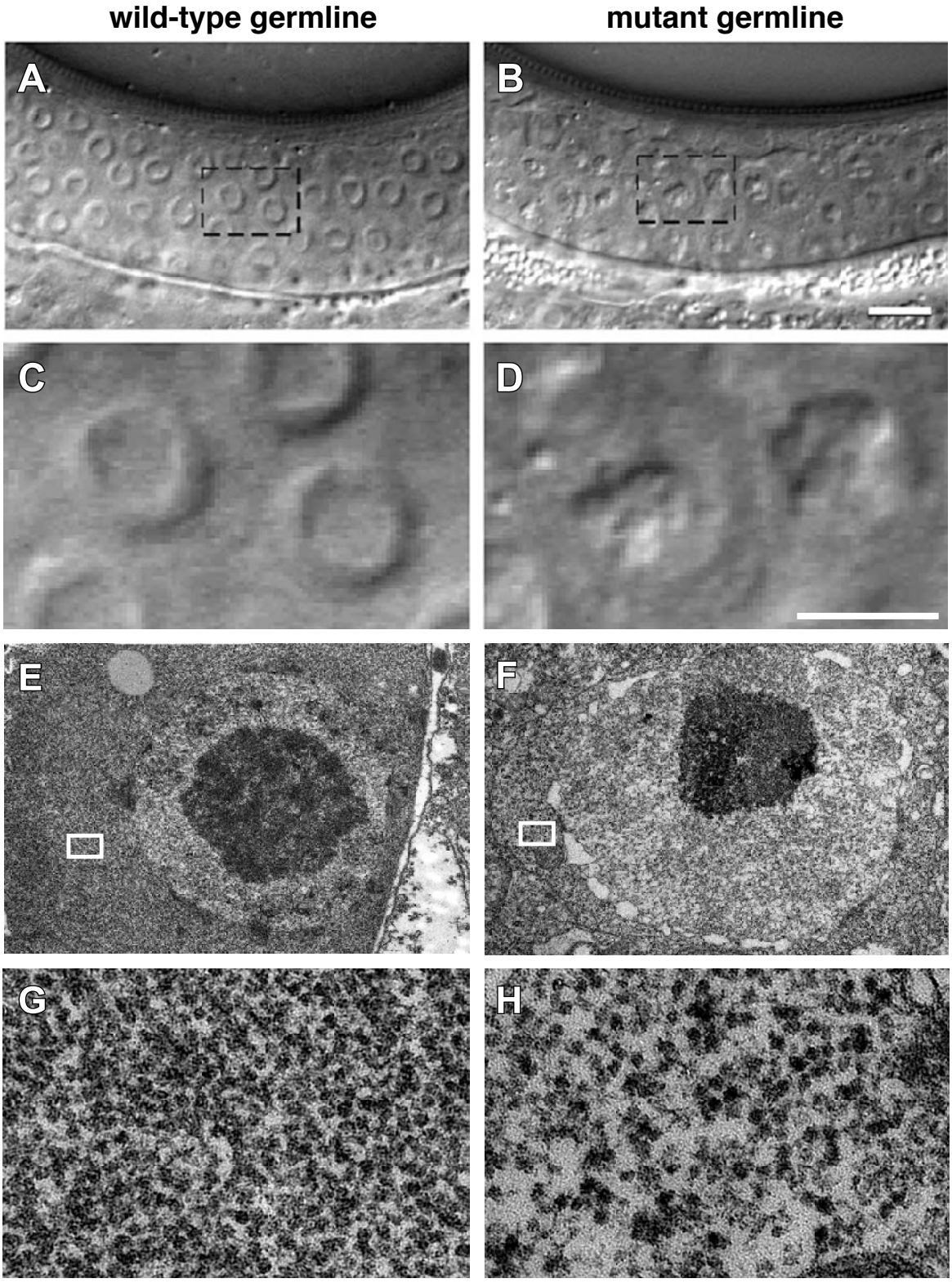
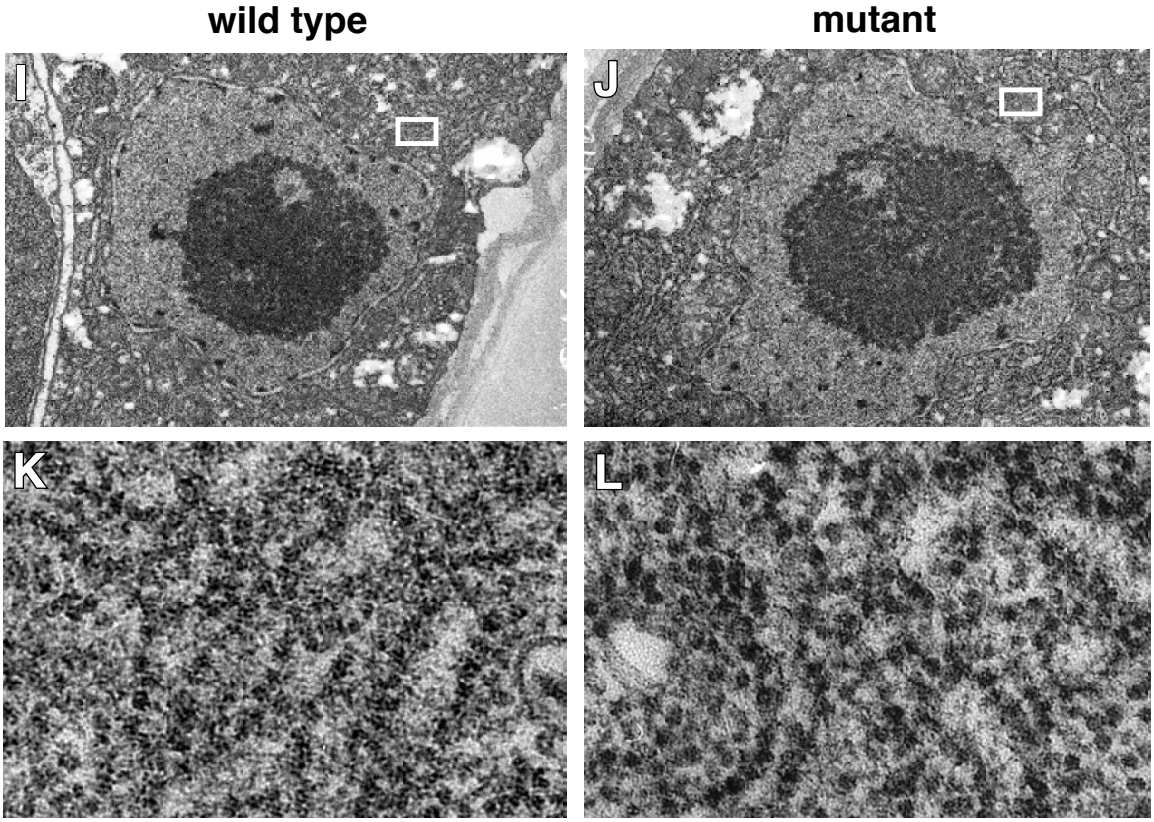

C
vector RNAi
D
zk1251.9 RNAi
E
dcaf-1(ok1867)
F
ncl-2(e1896)
G
H
NST-1::GFP
I
J

Figure 4.4. DCAF-1 germline nucleolus morphology defect is associated with reduction in ribosome number in the mutant germ cells. Panels A-D demonstrate the germline nucleolus morphology defect in *dcaf-1* mutant animals compared to the wild-type germline. Transmission election micrograph of the *C. elegans* distal germline (E-H) demonstrates the difference in ribosome numbers between wild type and mutant germ cells. Ribosome number in non-germ cells, *e.g.,* in post-mitotic neurons remains same in the mutant animals (B&D) compared to wild-type animals (A&C). Panels E-F represent quantitative analysis of the average ribosome number from electron micrographs. It shows a significant decrease ($p<0.0001$) in ribosome number in the *ncl-2* germ cells (E) while the ribosome number remains unchanged ($p=0.449$) in non-germ cells in the *ncl-2* mutant animals (F).


wild-type germline
mutant germline
A
B
C
D
E
F
G
H


wild type
mutant
I
J
K
L

Comparison of ribosome number

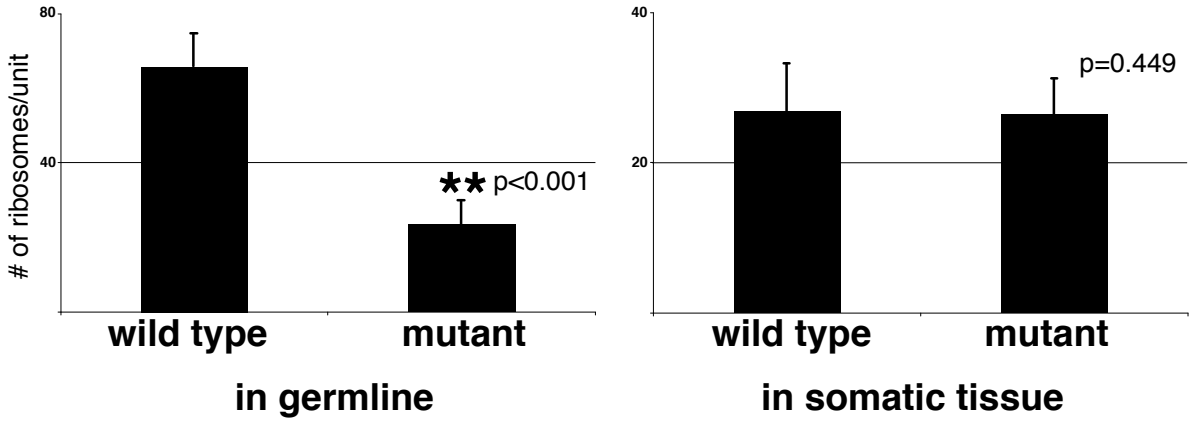


Figure 4.5. Inactivation of DCAF-1 negatively regulates GLD-1 levels. (A-B) Inactivation of *dcaf-1* results in loss of GLD-1 protein expression in *dcaf-1* RNAi treated animals whereas vector RNAi treated animals express GLD-1::GFP normally at 25°C. (C-F) Germline specific proteins, *e.g.*, DAZ-1 or PGL-1 levels remains unchanged as seen in tagged GFP expression between vector and *dcaf-1* RNAi treatment groups at 25°C.

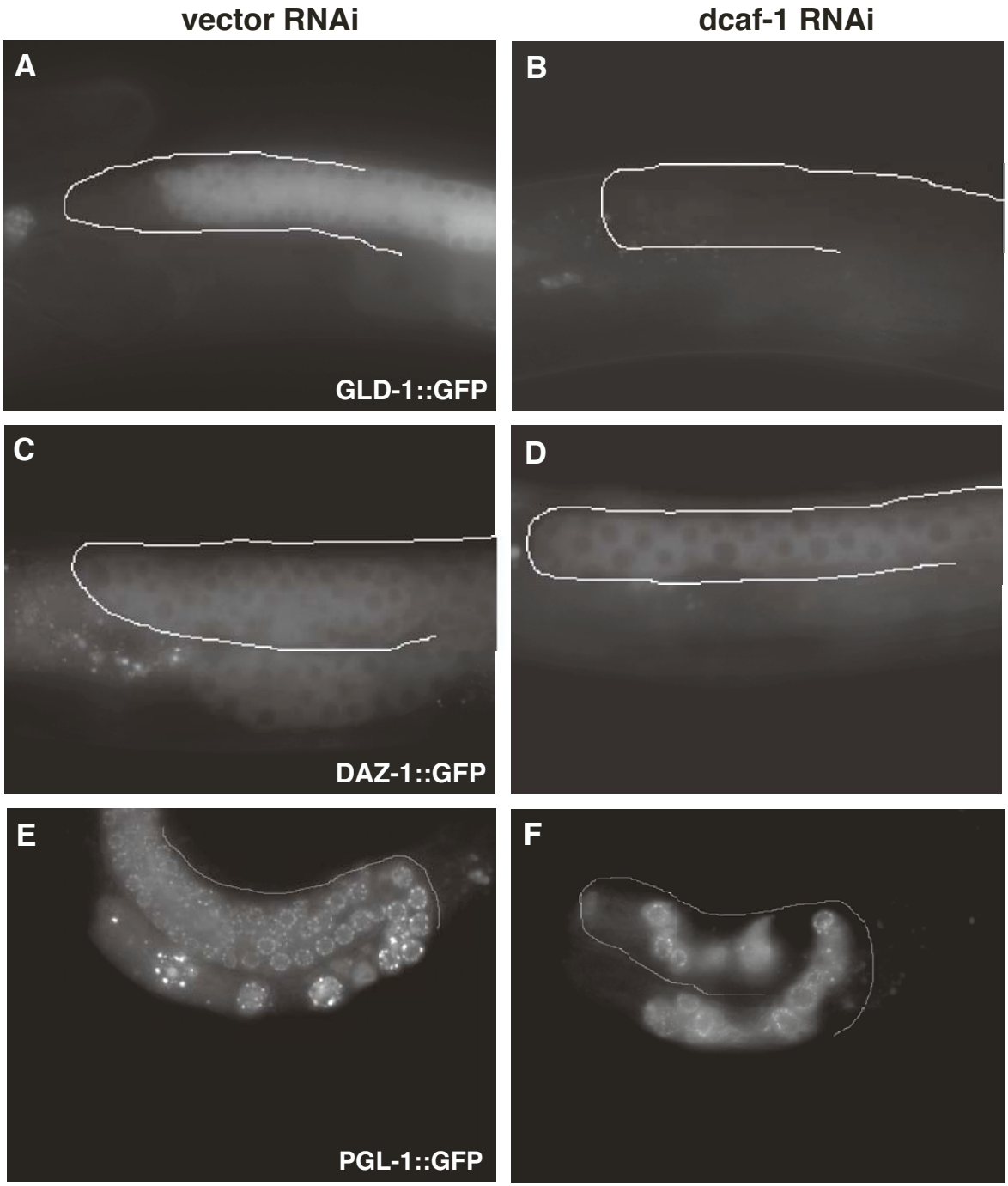

vector RNAi
dcaf-1 RNAi
A
GLD-1::GFP
B
C
DAZ-1::GFP
D
E
PGL-1::GFP
F

Figure 4.6. DCAF-1 function is essential for normal HIM-17 protein distribution. HIM-17 protein is expressed in all germ cells and localizes to the chromatin critical for germ cell cycle progression (Bessler, Reddy et al. 2007). Upon inactivation of the *dcaf-1* gene, we observed diffused localization of HIM-17 protein in the mutant germ cells (D) when compared to the discrete localization of HIM-17 protein in the vector RNAi treated germ cells (B). Inset images are magnification of selected region in the distal gonad.

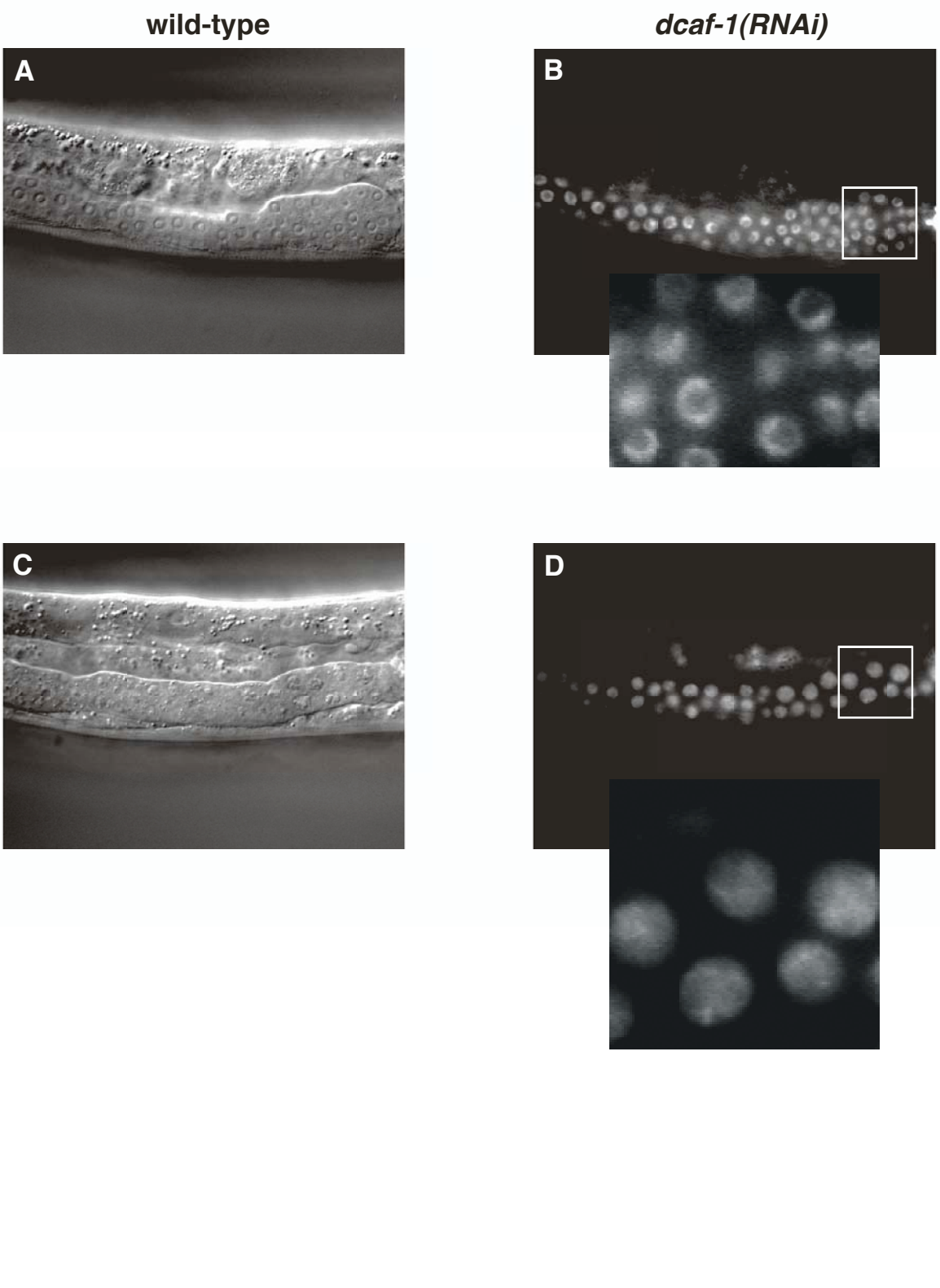

wild-type
dcaf-1(RNAi)
A
B
C
D

Figure 4.7. Analysis of mitotic cyclin B and apoptosis reporter expression upon inactivation of DCAF-1 function. (A-B) Similar to G1 Cyclin E (Figure 4.2C) we also observed repression of Cyclin B1 expression in the distal germ cells upon inactivation of *dcaf-1*. (C-E) In the wild-type germline, apoptotic cells are easily visible near the gonad bend (marked by the white rings of *P*ced-1::2XFYVE::GFP reporter) while in the *dcaf-1* mutant germ cells apoptotic markers are not expressed.

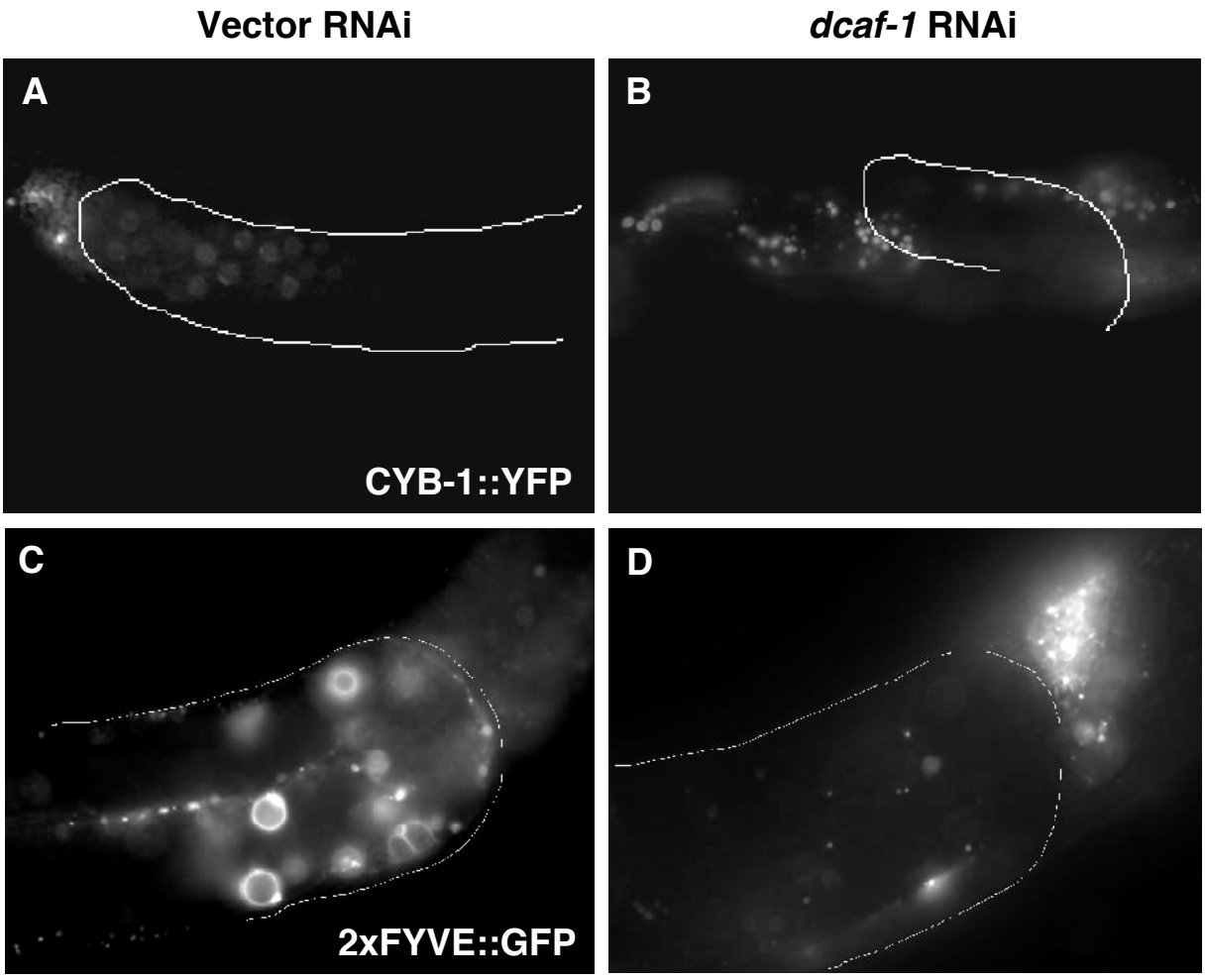

Vector RNAi
dcaf-1 RNAi
A
B
CYB-1::YFP
C
D
2xFYVE::GFP

Figure 4.8. FOG-1 as a putative target for DCAF-1 in the *C. elegans* germline. (A-C) Inactivation of FOG-1 function in the *fog-1(q253)* mutant completely rescues the germ cell nucleolus morphology defect in both *dcaf-1* (B) and *ddb-1* (C) mutants assayed at 25°C. (D-F) Immunostaining with anti-FOG-1 antibody of cut gonads demonstrates accumulation of FOG-1 protein in the *dcaf-1* mutant germline (F) when compared to the wild-type hermaphrodite germline (E). Wild-type males were used as positive control for high FOG-1 expression normally observed in the male germline (D). (G-H) Cartoon depicting the current model: FOG-1 as a putative substrate for CRL4/DCAF-1 E3 ubiquitin ligase complex. In this model, inactivation of DCAF-1 function causes accumulation of its substrate FOG-1 in the target tissue.

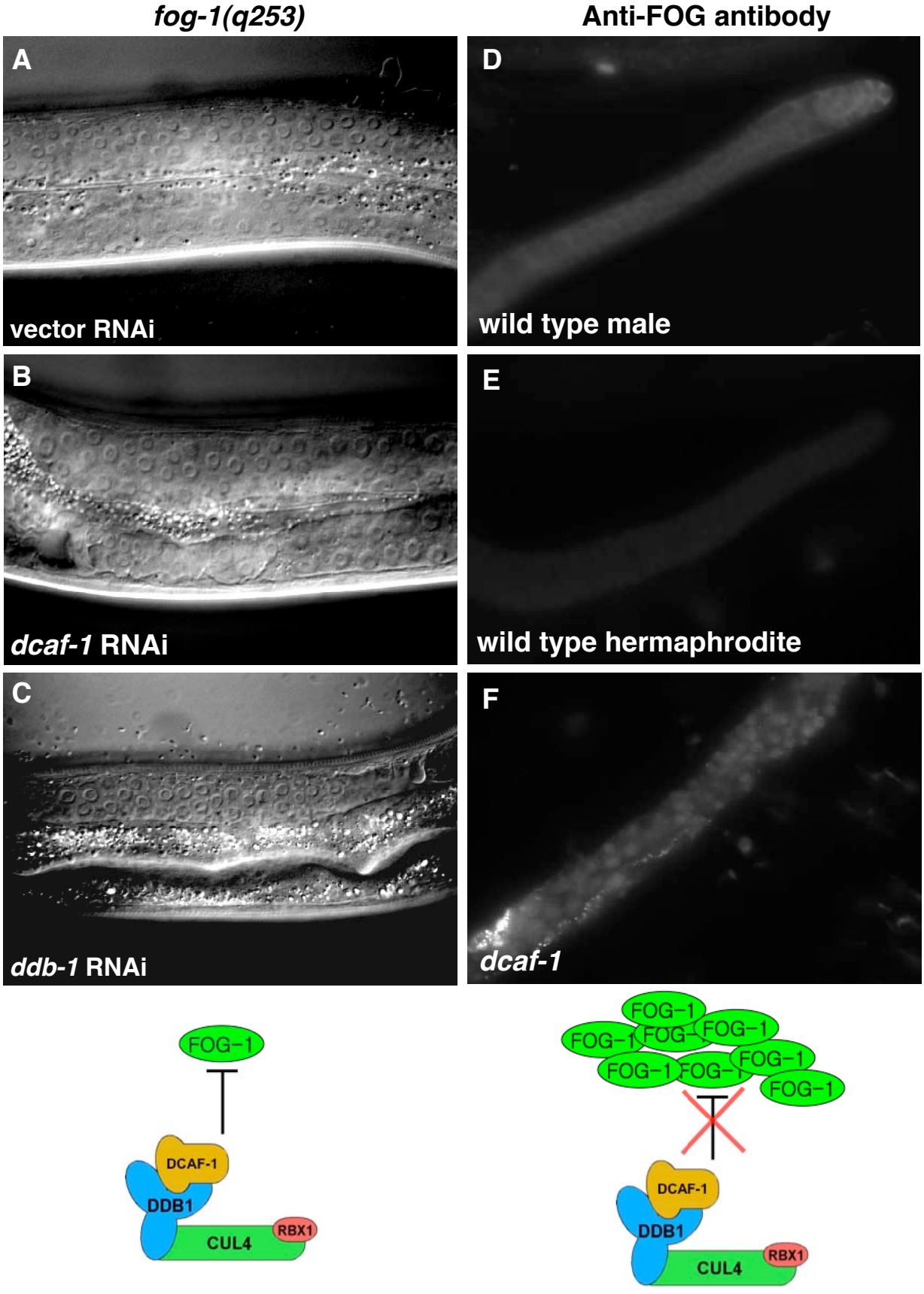
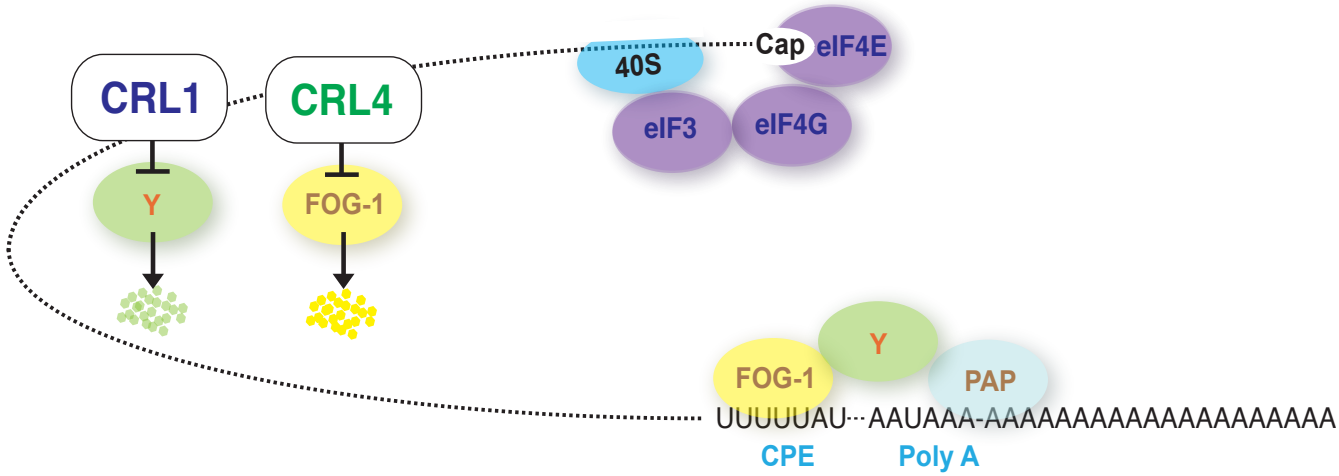
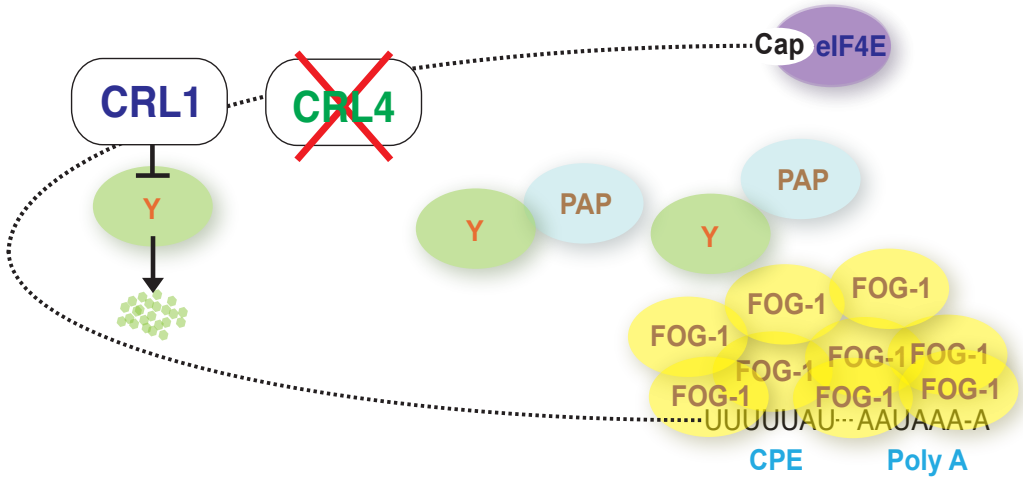

fog-1(q253)
Anti-FOG antibody
A
vector RNAi
D
wild type male
B
dcaf-1 RNAi
E
wild type hermaphrodite
C
ddb-1 RNAi
F
dcaf-1
FOG−1
DCAF-1
DDB1
CUL4
RBX1
FOG−1
FOG−1
FOG−1
FOG−1
FOG−1
FOG−1
FOG−1
DCAF-1
DDB1
CUL4
RBX1

Figure 4.9. Co-operative regulation by distinct CRL complexes is essential for normal germ cell cycle progression during reproductive development in the *C. elegans* germline. Schematic diagram depicts our proposed model where we expect FOG-1 to repress its cellular targets inappropriately when deregulated upon loss of CRL4 mediated control to cause growth arrest in target tissue. Basal level of FOG-1 is detrimental for normal growth. On the other hand, inactivation of distinct CRL1 complexes linked to cell cycle exit regulation either blocks or dilutes out FOG-1 mediated translation repression in the same cell to allow limited cellular growth and enable cells re-enter the cell cycle.

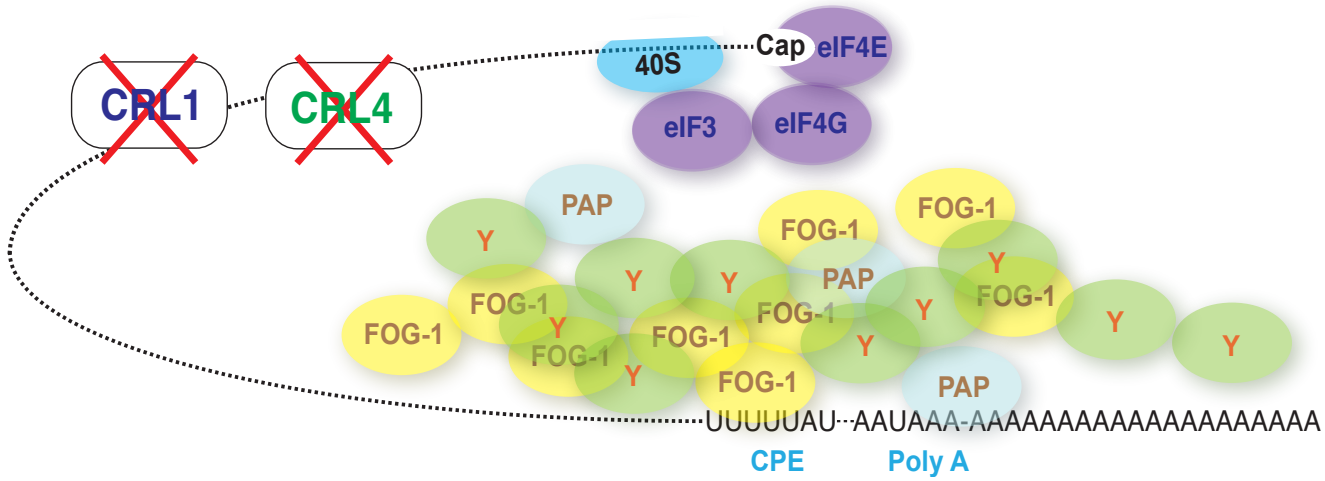
A. Translationally active mRNA



B. Translationally dormant mRNA



C. Translationally active mRNA (partial)



CHAPTER 5

GENERAL DISCUSSION

In this dissertation, I have presented my current research findings, which contribute to our basic understanding of cellular growth and development by providing a comprehensive analysis of Cullin-RING E3 ubiquitin ligase (CRL) mediated by protein degradation pathways in model organism *C. elegans*. In metazoa, E3 ubiquitin ligase complexes play vital roles in gene expression regulation by degrading critical regulatory proteins. Many of the E3 complexes are known but hundreds of them remain unidentified. Identification of novel E3 ubiquitin ligase complexes that are decisive in distinct cellular processes would allow us to develop novel interventions. Here I briefly summarize the major conclusions of my research work, and discuss possible future avenues.

My first dissertation project was to establish an *in vitro* cell culture line from *C. elegans* tissues. We planned to derive cell lines from embryo, germline, and adult somatic tissues. We successfully established protocols to isolate embryonic cells, germ cells, and hypodermal cells for *in vitro* culture. We also successfully established *in vitro* cell culture media based on a modification of published embryo suspension media formulation (Edgar 1995; Strange, Christensen et al. 2007). In our modified cell culture media, isolated embryonic cells survived for at least six months but cell division became very slow following a week after isolation. All previous attempts to establish *C. elegans in vitro* embryonic cell lines failed due to two primary reasons: first, within a few days following isolation, cell death was imminent without any cell proliferation in the culture media; and secondly, isolated embryonic cells, which could evade initial cell death in culture eventually differentiated into neurons (Christensen, Estevez et al. 2002; Strange, Christensen et al. 2007). We were able to overcome both of these hurdles with

our improved media formulation (see Methods section) and the use of cell cycle exit mutant (*cul-1*) animals as the primary source of embryonic cells in culture. In *C. elegans*, a conserved CUL1 protein, the scaffold component of the CRL1 E3 ubiquitin ligase complexes, is essential for cell cycle exit in all mitotic cells. Inactivation of CUL1 function leads to hyperplasia in numerous tissues in *C. elegans* (Kipreos, Lander et al. 1996). The major achievements of our current study were that we established a protocol to isolate embryonic cells from CUL-1 mutant animals that allowed proliferation for a long period albeit at a slow pace in our modified cell culture media, and significantly the CUL-1 mutant embryonic cells in culture media do not differentiate into neurons.

We also established a successful protocol to isolate germ cells from adult animals in culture media for the first time. Although isolated germ cells survive for a few weeks in our modified culture media, the germ cells never proliferated *in vitro* under the current conditions. We isolated germ cells from all published tumorous germline mutant animals (Beanan and Strome 1992) but none of the 'tumor' germ cells ever proliferated, even briefly in the culture media. Later we also learned that the tumorous germline reported in different *C. elegans* mutants might not retain its proliferative potential even *in vivo* as most of the germ cells that aberrantly accumulated in the distal gonad, express various types of differentiation markers, like muscle and skin (Ciosk, DePalma et al. 2006). In the past, *C. elegans* literature partially differentiated, previously unrecognized, non-proliferative germ cells in the proximal gonad was termed as a germline 'tumor' (Ciosk, DePalma et al. 2006). Finally, we were able to deregulate adult hypodermal cell division regulation, and force adult hypodermal stem cells to divide *in vivo* in *cul-1* loss of function mutant animals. Similar to germ cells, we were able to isolate the adult hypodermal cells, and place them in to culture media but failed to sustain cell proliferation *in vitro*. Future studies can identify the critical mitogenic growth factors required for continuous cell proliferation, for both germline and somatic cell *in vitro* culture. Although isolated embryonic

cells proliferate *in vitro* but at a very slow pace following a week after isolation in the current culture media. It needs further optimization to sustain cell proliferation at a moderate rate, which would make the embryonic cell lines suitable for further manipulation in various biochemical assays.

Following months in culture, we observed that larger cell aggregates in *cul-1;cki-1* double mutant derived embryonic cells often acquire a rounded smooth exterior that in part resembles the embryoid bodies (EB) seen to form in mammalian embryonic stem (ES) cell cultures when ES cells cultured in media lacking appropriate growth factors essential to maintain pluripotency. Characterization of spheroid bodies derived from *C. elegans* embryonic cells *in vitro* might allow us to examine the tissue organization and differentiation pattern frequently masked by developmental events in large mutant screens. Cell-cell communication, invariant cell number and cell positioning are critical in lineage selection during the *C. elegans* embryogenesis *in vivo*, and spheroid bodies might offer a functional insight of the differentiation events with limitations. It would also provide a useful alternative to investigate the impact of null mutations when gene inactivation turns into embryonic lethality. Spheroid bodies derived from various mutant lines would allow us to identify novel differentiation patterns *in vitro,* and will shed further light on the mechanisms of lineage selection in *C. elegans*. In future studies, identification of cell lineages in these spheroid structures *in vitro* would also allow a better understanding of the signaling pathways involved in the differentiation process in *C. elegans*.

The project to establish *in vitro* cell culture lines from various *C. elegans* tissues allowed us to better understand the intrinsic regulation of cell proliferation vs. cell fate determination in early embryos. The switch from proliferation to differentiation is a critical decision for pluripotent and multi-potent cells in order to initiate differentiation steps at the right moment during development to generate tissues, and organs in an animal correctly (Buttitta and Edgar 2007). Our current experimental observations suggested that in *C. elegans,* deregulation of only cell

cycle or cell fate determination individually does not lead to indefinite proliferation in target tissues. The *cul-1* mutant cells are forced in target tissues to divide only a few more times suggesting the existence of inherent mechanisms that limit indefinite cell division in *C. elegans* tissues remains unknown. Taken together we were unable to generate a mutant animal, which would allow uncontrolled proliferation of cells in any tissue *in vivo*. Considering many similarities between nematode and vertebrate signaling pathways are regulated in development, the vertebrate paradigm of cancerous tissue growth from uncontrolled cell proliferation (Nakayama and Nakayama 2006) seems to be not realistic in the nematode *C. elegans*.

A second dissertation project revealed the role of O-GlcNAc modification, a post-translational modification (PTM), of proteins critical in governing the insulin signaling pathway to connect it to other key cellular processes like protein degradation pathways in the aging process. Both structural proteins and enzymes are modified post-translationally through numerous pathways at different time points in development (Huber and Hardin 2004). Post-translational modifications of proteins allow cells to regulate cellular functions at multiple levels, and adjust more efficiently to abrupt or minute changes in the environment (Huber and Hardin 2004). We chose to study the importance of dynamic O-GlcNAc modification of proteins in development. O-GlcNAcylation is the only known nucleo-cytoplasmic dynamic protein glycosylation event compared to other static and mostly irreversible protein glycosylations that occur in the golgi and endoplasmic reticulum (ER) (Hart, Housley et al. 2007).

In our study we demonstrated that O-GlcNAc cycling negatively modulates insulin signaling in *C. elegans* but only affects a small subset of downstream functional outcomes involving oxidative stress and adult lifespan extension. In *C. elegans* O-GlcNAc cycling enzyme mutants are viable and overtly wild type. While inactivation of protein O-GlcNAcylation is deleterious in other animals, our results gave us a unique opportunity to understand how changes in protein O-GlcNAcylation *in vivo* can modulate insulin signaling pathway functional

outcomes. Dynamic O-GlcNAcylation of proteins help attain transient changes in the extracellular environment adapted into cellular processes without deleterious changes, such as slow down glucose uptake in insulin-sensitive tissues to protect the cells from glucose toxicity (Hart, Housley et al. 2007). In our current study, for the first time, we have identified a novel set of O-GlcNAc modified proteins in *C. elegans* through affinity purification coupled to tandem mass spectrometry. Most of the identified proteins that are putative O-GlcNAc targets are downstream of the insulin-signaling pathway (Table 3.4). Many of the proteins identified in our current affinity purification are also O-GlcNAc modified in mammalian cells. Future studies can determine the sites of O-GlcNAc modifications in the specific proteins, and how such modifications affect the function of the individual proteins. Further investigations are needed to reveal how such modifications help integrate changes in insulin signaling to growth and development. Our study also indicated a novel CRL1 mediated function in aging tissues, previously not recognized, in the crossroad of insulin regulated developmental processes. Further experimental evidence is required to elucidate the role of insulin signaling mediated developmental pathways in regulating CRL1 E3 ubiquitin ligase complexes key to ubiquitin-mediated protein degradation in terminally differentiated post-mitotic cells in aging adults.

My third project led to the identification of a novel CRL4/DCAF-1 E3 ubiquitin ligase complex essential for germ cell growth and development in *C. elegans*. Proper cellular growth and cell cycle progression in the germline is critical for metazoan reproductive development to regenerate itself through the production of healthy progeny (Hubbard and Greenstein 2005). Environmental cues, such as, availability of nutrients and growth factors couples cell cycle to organismal development. It plays a key role in the initiation of cellular growth at G1 phase of the cell cycle to initiate DNA replication, and subsequent mitotic cell division to generate two equivalent daughter cells (Budirahardja and Gonczy 2009). In germ cells, the cell division pathway is unique in the sense that it not only leads to an increase in the number of germ cells

by mitotic divisions but also through meiotic cell divisions generates haploid daughter cells, which will ultimately differentiate into sperm and oocytes to make gametes (Kimble and Crittenden 2007). In metazoa, CRL4 E3 ubiquitin ligase complexes are critical in DNA replication licensing, which restricts cells to faithfully duplicate its entire genome only once per cell division (Kim, Feng et al. 2007). Our current study provides the first evidence that a cullin RING finger protein CUL-4, the scaffold for CRL4 E3 ubiquitin ligase complexes is a novel regulator of ribosome synthesis, nucleolus integrity, cellular growth, and cell division in the *C. elegans* germline. We have also identified FOG-1, a CPEB protein as a putative substrate for the novel CRL4/DCAF-1 complex that is linked to germ cell nucleolus integrity, and subsequent germ cell growth arrest. During our study we also found that inactivation of CRL1 can rescue the germline nucleolus defect, and allow growth-arrested germ cells to re-enter into cell cycle in the *dcaf-1* mutant germline. Previously CRL1 was only known to regulate proteins linked to the cell cycle exit regulation in *C. elegans* (Kipreos, Lander et al. 1996). Future studies can determine how CRL1-mediated E3 ubiquitin ligase complex impinges on CRL4 E3 ubiquitin ligase complex mediated regulation in nucleolus integrity and maintenance in the germline. Future studies will also needed to identify CPEB/FOG-1 mRNA targets to reveal the molecular mechanism of cellular growth regulation linked to the nucleolus morphology defect. The total number of ribosome in a cell is directly linked to its metabolic activity (Boisvert, van Koningsbruggen et al. 2007). In our current study we also observed a high number of ribosomes in the rapidly proliferating germ cells compared to post-mitotic somatic cells in *C. elegans*. Further recognition of nutrient sensing and other developmental pathways linked to both CRL1 and CRL4-mediated protein degradation pathways would give us a better understanding how growth signals, such as, nutrient availability are being translated into the initiation of ribosome synthesis critical for protein translation in growing cells prior to the initiation of DNA replication and cell division events.

C. elegans DCAF-1 belongs to the mammalian VprBp family of proteins, which is critical in human HIV infection and pathogenesis. Upon HIV infection, viral Vpr protein binds to mammalian VprBp/DCAF-1 to hijack the CRL4 complex for viral propagation, and evade cellular surveillance mechanisms (McCall, Miliani de Marval et al. 2008). Similar to HIV, other RNA viruses also target the nucleolus, and recruit nucleolar proteins for viral propagation (Greco 2009). The putative CRL4/DCAF1 substrate FOG-1 contains a consensus nucleolar localization signal (our current study). In future studies, when the role of DCAF-1 in nucleolar integrity is revealed it might help us better understand the importance of nucleolus in RNA virus propagation, which is not well addressed in the current literature (McCall, Miliani de Marval et al. 2008; Greco 2009).

References

Beanan, M. J. and S. Strome (1992). "Characterization of a germ-line proliferation mutation in C. elegans." Development **116**(3): 755-766.

Boisvert, F. M., S. van Koningsbruggen, et al. (2007). "The multifunctional nucleolus." Nat Rev Mol Cell Biol **8**(7): 574-585.

Budirahardja, Y. and P. Gonczy (2009). "Coupling the cell cycle to development." Development **136**(17): 2861-2872.

Buttitta, L. A. and B. A. Edgar (2007). "Mechanisms controlling cell cycle exit upon terminal differentiation." Curr Opin Cell Biol **19**(6): 697-704.

Christensen, M., A. Estevez, et al. (2002). "A primary culture system for functional analysis of C. elegans neurons and muscle cells." Neuron **33**(4): 503-514.

Ciosk, R., M. DePalma, et al. (2006). "Translational regulators maintain totipotency in the Caenorhabditis elegans germline." Science **311**(5762): 851-853.

Edgar, L. G. (1995). "Blastomere culture and analysis." Methods Cell Biol **48**: 303-321.

Greco, A. (2009). "Involvement of the nucleolus in replication of human viruses." Rev Med Virol **19**(4): 201-214.

Hart, G. W., M. P. Housley, et al. (2007). "Cycling of O-linked beta-N-acetylglucosamine on nucleocytoplasmic proteins." Nature **446**(7139): 1017-1022.

Hubbard, E. J. and D. Greenstein (2005). "Introduction to the germ line." WormBook: 1-4.

Huber, S. C. and S. C. Hardin (2004). "Numerous posttranslational modifications provide opportunities for the intricate regulation of metabolic enzymes at multiple levels." Curr Opin Plant Biol **7**(3): 318-322.

Kim, J., H. Feng, et al. (2007). "C. elegans CUL-4 prevents rereplication by promoting the nuclear export of CDC-6 via a CKI-1-dependent pathway." Curr Biol **17**(11): 966-972.

Kimble, J. and S. L. Crittenden (2007). "Controls of germline stem cells, entry into meiosis, and the sperm/oocyte decision in Caenorhabditis elegans." Annu Rev Cell Dev Biol **23**: 405-433.

Kipreos, E. T., L. E. Lander, et al. (1996). "cul-1 is required for cell cycle exit in C. elegans and identifies a novel gene family." Cell **85**(6): 829-839.

McCall, C. M., P. L. Miliani de Marval, et al. (2008). "Human immunodeficiency virus type 1 Vpr-binding protein VprBP, a WD40 protein associated with the DDB1-CUL4 E3 ubiquitin ligase, is essential for DNA replication and embryonic development." Mol Cell Biol **28**(18): 5621-5633.

Nakayama, K. I. and K. Nakayama (2006). "Ubiquitin ligases: cell-cycle control and cancer." Nat Rev Cancer **6**(5): 369-381.

Strange, K., M. Christensen, et al. (2007). "Primary culture of Caenorhabditis elegans developing embryo cells for electrophysiological, cell biological and molecular studies." Nat Protoc **2**(4): 1003-1012.