Spring 5-2017

UBR-5, a conserved HECT-Type E3 ubiquitin ligase, negatively regulates notch-type signaling in Caenorhabditis elegans

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UBR-5, a Conserved HECT-Type E3 Ubiquitin Ligase, Negatively Regulates Notch-Type Signaling in *Caenorhabditis elegans*

A Capstone Project Submitted in Partial Fulfillment of the
Requirements of the Renée Crown University Honors Program at
Syracuse University

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and Renée Crown University Honors
Spring 2017

Honors Capstone Project in Biochemistry

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Abstract

Germline stem cell proliferation in *C. elegans* is regulated by the distal tip cell (DTC) located at the distal end of each gonad arm. The DTC signals germ cells, through a Notch-type signaling pathway, to proliferate. As germ cells move farther away from the DTC, they transition into meiosis and produce gametes. There are two Notch-type receptors in *C. elegans,* GLP-1 and LIN-12, which play a role in regulating cell fate decisions. Whenever GLP-1 function is lost, all the germ cells exit mitosis and enter meiosis. If this happens early in development, then the animal is sterile because it does not have a large enough germ cell population to maintain gamete production. On the other hand, if there is a *glp-1* gain-of-function (gf) mutation, then the animal develops a distal tumor because the germ cells over-proliferate. In normal development, a healthy balance between mitosis and meiosis is needed for proper germline function. Defects in Notch signaling have been implicated in human developmental disorders and cancers underlining the importance of better understanding how cells regulate this pathway.

Our work identified UBR-5, a HECT-type E3 ubiquitin ligase, as a negative regulator of *glp-1* signaling.[1] E3 ligases are enzymes involved in attaching ubiquitin to target proteins, marking them for degradation. UBR-5 was shown to negatively regulate GLP-1/Notch signaling in the adult gonad.[1] Furthermore, knocking down expression of *ubr-5* in a weak, temperature-sensitive, *glp-1(ar202ts)* gain-of-function background enhanced the gain-of-function phenotype. *ubr-5(RNAi)* in a *rrf-1(0);glp-1(ar202ts)* background, where RNAi is defective in the soma, also enhanced the gain-of-function phenotype. This suggests that *ubr-5* acts in the germ line to regulate turnover of the Notch receptor rather than in the signaling cell to regulate turnover of the ligand. Redundancy tests with a second E3-ligase, SEL-10*,* provided evidence that these two E3 ligases act synergistically in the early embryo.

Currently we are aiming to identify specific targets of UBR-5 in the GLP-1/Notch signaling pathway. Evidence suggests that SEL-10, which negatively regulates LIN-12/Notch signaling in *C. elegans*, is involved in proteasomal degradation of the Notch intracellular domain (ICD)[2]. We hypothesize that UBR-5 is regulating GLP-1 signaling in an analogous manner by binding to the ICD. Preliminary protein blot data with 3xFLAG-tagged GLP-1 ICD supports this hypothesis. In addition, we are attempting to tag UBR-5 with the 3xFLAG epitope using the CRISPR/Cas9 genome editing system. This reagent will be used for immunoprecipitation and mass spectrometry analysis of UBR-5 binding partners on a more global scale.

Executive Summary

Stem cells are cells capable of differentiating into any mature cells in the body. As such, organisms have many mechanisms by which this process is regulated as improper regulation of differentiation can lead to developmental disorders and cancer. One of the mechanisms by which cells regulate differentiation decisions is the Notch signaling pathway. In a general sense, Notch signaling is one way for two adjacent cells to communicate with each other and coordinate development. It is a highly conserved signaling pathway found in many different organisms, and misregulation of the Notch pathway has been linked to diseases such as cancer in humans.

Notch activation requires interaction between a protein ligand on one cell and its receptor on an adjacent cell. This interaction between ligand and receptor activates a cascade of events that ultimately result in gene expression changes in the receiving cell. In some tissues, Notch signaling regulates stem cell behavior. Depending on the tissue type, these gene expression changes either cause the stem cell to differentiate into its mature cell type or keeps the stem cell in its naïve state. We use the nematode, *C. elegans,* as a model organism to study how the Notch pathway is regulated. *C. elegans* is a good model organism to study these processes because it is easy to manipulate or maintain in the laboratory. Specifically, we are interested in how Notch signaling mediates signaling between one somatic gonadal cell and the pool of germline stem cells, a process that is crucial for fertility.

This project focuses on UBR-5, a potential regulator of the Notch pathway involved in turning off signaling when it is no longer needed. UBR-5 is an E3 ligase, an enzyme that is involved in protein turnover via ubiquitin-mediated degradation pathways. Cells use ubiquitin as a marker to indicate which proteins need to be destroyed. E3 ligases, such as UBR-5, are crucial enzymes in this pathway responsible for finding and attaching ubiquitin to target proteins.

Here we show that UBR-5 is negatively regulating Notch signaling because loss of UBR-5 activity results in hallmarks of increased Notch signaling. We report evidence that suggests UBR-5 is active in the

receiving cells in the germ line. Of all the components of the Notch pathway, we currently hypothesize that UBR-5 is targeting a component of the Notch receptor for degradation. Finally, we provide updates on our attempts to add an epitope tag to the UBR-5 protein, which will allow us to perform future assays to identify the multitude of UBR-5 targets.

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Acknowledgements

I want to give thanks to the Coronat Scholars and the Renee Crown University Honors Program, both of which have contributed funding for this project. I would especially like to thank the Coronat Scholars Program, without which I would not have found my way to Syracuse University. I'm especially grateful to my advisor, Dr. Eleanor Maine, for invaluable mentorship and advice over the years. Thank you for welcoming an inexperienced freshman into your lab and giving her the opportunity to learn and develop a passion for research. This project wouldn't have been possible without contributions from the wonderful members of the Maine Lab: former lab members Komal Safdar and Xia Xu, who laid the groundwork for this project, Dr. Yiqing Guo and Dr. Matt Sullenberger. I would especially like to thank current graduate students, Bing Yang and Yini Li. Thank you for taking me under your wing and answering my countless questions. I would also like to thank my Honors Reader, Dr. Sarah Hall and my academic advisor, Dr. Sam Chan, for their advice and input. Finally, I would like to thank my friends and family at Syracuse University for giving me their constant support during my undergraduate years.

Introduction

Introduction to *Caenorhabditis elegans* as a model organism

Caenorhabditis elegans (C. elegans) is a soil-dwelling nematode (round worm) species first developed as a model organism by Sydney Brenner in 1963.[3] Many characteristics of this nematode make them especially amenable to model organism studies. Adult worms are approximately a millimeter in size and easily maintained in the lab. They have a rapid life cycle of approximately 3 days from fertilized egg to egg-laying adult. *C. elegans* exist primarily as self-fertilizing hermaphrodites that produce both sperm and oocytes with a lower percentage of the population existing as males, which produce only sperm. Because these worms are transparent, they are a good system to study developmental processes as researchers can follow the fate of every cell from fertilization to adulthood in live animals. *C. elegans* was also the first multicellular organism to have its genome completely sequenced (in 1998), and approximately 38% of its protein-coding genes have similarity to humans making it a powerful model system for genetic studies.[3]

C. elegans has five autosomal chromosomes and an X chromosome. Hermaphrodite worms possess two X chromosomes (XX) and males possess only one (XO). Hermaphrodites can produce male offspring from self-fertilization through a rare meiotic non-disjunction event that occurs in approximately 0.1% of progeny. Because hermaphrodites are capable of self-fertilization, maintaining homozygous mutant lines is relatively simple. It is also easy to set up matings in *C. elegans* between males and hermaphrodites.[3]

After hatching, *C. elegans* proceeds through four developmental larval stages (L1-L4) before reaching adulthood. However, if these nematodes are exposed to environmental stressors (starvation, high temperature, or overcrowding) during late L1 stage, they can enter an alternative developmental stage called dauer. Dauer worms have increased resistance to stressors and can survive for many

months. Once environmental conditions become more favorable, worms exit the dauer stage and continue to develop into post-dauer adults.[4]

Their short reproductive cycle and large brood size makes *C. elegans* easy to use for large-scale genetic screens. One common genetic approach that makes them such a powerful model organism to work with is RNA interference (RNAi). When *C. elegans* is exposed to double-stranded RNA (dsRNA), an endogenous defense system is activated, which initiates a cascade of events that result in knock down of mRNA sequences complimentary to the dsRNA sequence. In nature, this defense system protects the nematode from viruses and regulate endogenous genes. However, researchers can take advantage of this mechanism to silence expression of target genes. Furthermore, the use of a *rrf-1* null mutation, which renders RNAi deficient in many somatic tissue types, allows researchers to eliminate RNAi activity in the somatic gonad.[5]

The hermaphrodite germ line is made up of two identical U-shaped gonad arms where the sperm and oocytes develop (Figure 1). In contrast, males have a single U-shaped gonad.[3] The *C. elegans* germ line develops from a solitary primordial germ cell early in development.[6] In the adult worm, the germ line has a pool of mitotically dividing stem cells in the distal end which produce the gametes.[6] The distal tip cell (DTC), located at the distal end of each gonad arm, promotes germline stem cell proliferation (Figure 1). Ablating the DTC with a laser causes germline stem cells to enter meiosis whereas moving the DTC to a new location promotes germline stem cell development ectopically. These results indicate that the DTC is both necessary and sufficient for germ cell proliferation.[6] The DTC signals germ cells via the Notch-type signaling pathway.[5]

Notch Signaling in *C. elegans*

The Notch signaling pathway is a highly conserved signaling pathway found in many animal species that regulates cell fate specification decisions in multiple tissues throughout development. Notch signaling is also important for stem cell maintenance in many adults tissues.[7] Notch signaling is a

very localized type of signaling where receiving cells expressing the Notch receptor respond to interactions with the Delta-Serrate-Lag2 (DSL) family of ligands expressed on the surface of adjacent signaling cells (Figure 2). After ligand-receptor binding, an ADAM family protease cleaves the ectodomain of the receptor. A second protease, γ-secretase, then cleaves within the transmembrane domain of the receptor, releasing the intracellular domain (ICD).[8] The Notch ICD then enters the nucleus where it functions as a transcription factor, interacting with a DNA-binding protein from the CSL family of transcription factors to assemble a protein complex and regulate gene transcription. Many components of the Notch signaling pathway have been implicated in various human cancers including breast cancer, melanoma, pancreatic cancer, and leukemia.[7,9]

In *C. elegans* there are two Notch receptor homologues, GLP-1 (abnormal germline proliferation) and LIN-12 (abnormal cell lineage). GLP-1 is the primary Notch receptor active in the germ line to mediate DTC-germline signaling. In the absence of GLP-1, germ cells exit mitosis and enter meiosis to differentiate into gametes. If GLP-1 is inactivated early in development, the subsequent adult worm is sterile since it does not have a large enough germ cell population to produce sperm and oocytes. In contrast, when there is a *glp-1 gain-of-function (gf)* mutation, the animal develops a tumor because the germ cells over-proliferate. In normal development, a balance of proliferation and differentiation is needed for proper germline function.

Identification of UBR-5

A genetic screen for suppressors of *glp-1(ts)* defects identified 14 recessive suppressors that rescued embryonic lethality and germline proliferation defects of *glp-1 (q231ts)* allele.[10] These *glp-1(ts)* mutants produce viable progeny at permissive temperatures (15°C), but at restrictive temperatures (20-25°C) their germ cells enter meiosis prematurely and the animals produce fewer progeny than normal. None of the progeny are viable due to defective GLP-1 during early embryogenesis. These suppressors were named *sog* genes for suppressor of *glp-1* and rescued embryonic lethality phenotypes at varying

degrees.[10] Whole genome sequencing was performed to identify the location of the genetic mutation in one of these *sog* alleles, *sog-1.* By comparing the sequence of *sog-1* to the standard *C. elegans* reference genome sequence, it was determined that *sog-1* mutants contained mutations in the open reading frame, F36A2.13. F36A2.13 is predicted to encode a HECT-Type E3 Ubiquitin Ligase, UBR-5.[1]

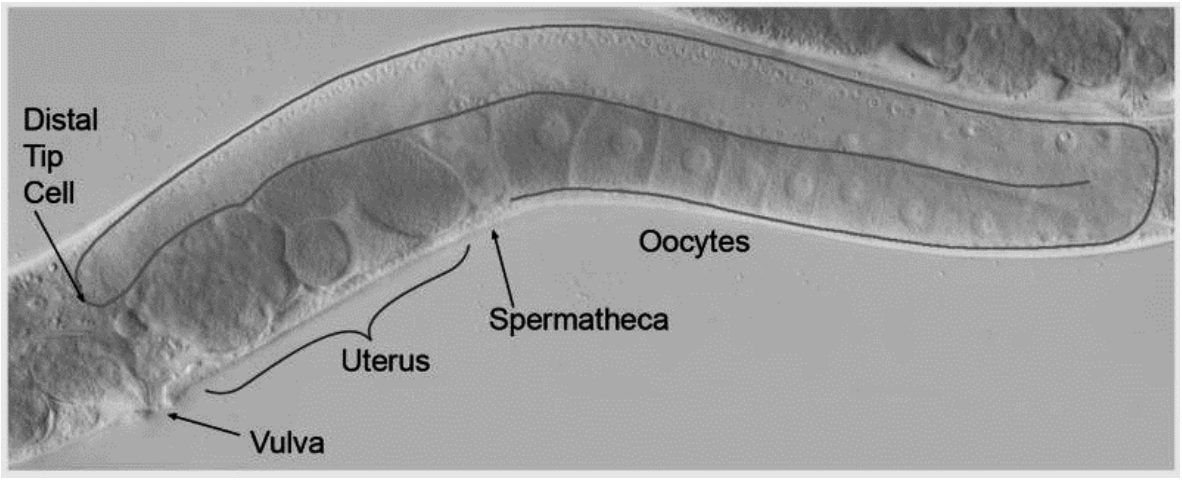
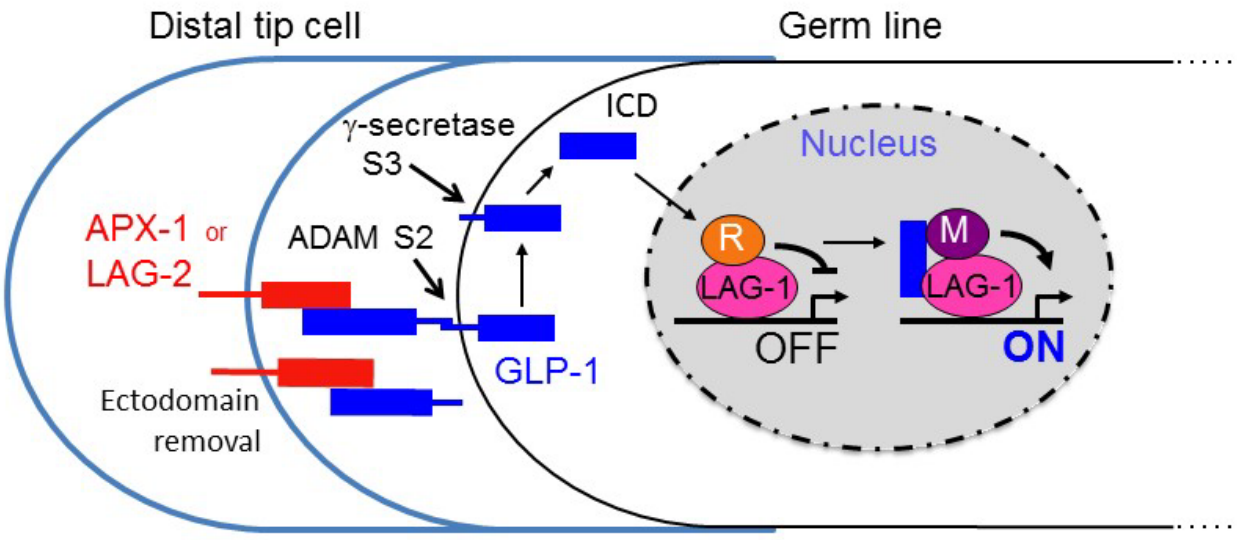


Figure 1. Differential Interference Contrast (DIC) Microscopy of one gonad arm in *C. elegans* hermaphrodite worm (Image from Pazdernik & Schedl 2013). Notch signaling from the Distal Tip Cell (DTC) to germline stem cells maintains the stem cell population. In hermaphrodites, as germ cells travel around the bend in the gonad arm, they develop first into sperm (in L4 stage) and then switch to making oocytes (as adults). Maturing oocytes are fertilized by sperm in the spermatheca and developing embryos are ejected from the worm through the vulva.



LAG-2, APX-1	ligand
GLP-1	receptor heterodimer
ADAM	protease
γ-secretase	protease complex
LAG-1	CSL-1 family transcription factor
R	co-repressor complex
M	SEL-8/LAG-3 Mastermind equivalent

Figure 2. Notch Signaling Pathway in *C. elegans* (Image from Safdar et al 2016). Activation of the Notch signaling pathway in the *C. elegans* germ line begins with ligand-receptor interaction. The ligand, LAG-2, is produced by the DTC. The Notch receptor in the receiving germ cells is GLP-1. Binding of ligand to receptor initiates proteolytic cleavage of the receptor by ADAM and γ-secretases. The GLP-1 ICD is released and enters the nucleus where it interacts with a CSL-1 family transcription factor, LAG-1. The ICD displaces a co-repressive complex and recruits an activator complex to initiate gene transcription. (Figure not drawn to scale to emphasize protein interactions.)

E3 Ligases and Notch Signaling

Ubiquitination is a mechanism that cells use to regulate protein turnover where attaching a ubiquitin molecule to a protein marks it for degradation by the proteasome complex. Before ubiquitin can be attached to target proteins, it must first be activated by the E1 enzyme through an ATP-dependent process. It is then transferred to the E2 enzyme followed by the E3 ubiquitin ligase, which attaches ubiquitin to target molecules marking them for cellular degradation. In this pathway, E3 ligases are the enzymes that contribute substrate recognition and specificity.[7,11] Because organisms depend upon E3 ligases for substrate recognition, it is common for there to be many E3 ligases within an individual species relative to E1 and E2 enzymes. For example, humans are estimated to have two E1 enzymes and thirty E2 enzymes, but over 600 E3 ligases.[11] There are two main families of E3 ligases: the RING and HECT-type E3 ligases. HECT-type E3 ligases are so named because they contain a conserved HECT (homologous to E6-AP carboxyl terminus) domain usually located at the C-terminus.[7,11,12]

Previous work has implicated E3 ligases in Notch regulation in *C. elegans* and other animal species. For example, SEL-10/Fbw7, a component of the Skp1/Cul1/F-box protein-Rbx1-type ubiquitin ligase, has been implicated in LIN-12/Notch signaling. Evidence suggests that SEL-10 ubiquitinates the Notch intracellular domain and is involved in proteasomal degradation.[2,7,13] Ubiquitination has been implicated in many different aspects of the Notch signaling pathway from regulating endocytosis of both the Delta ligand and the Notch receptor to proteasomal degradation of the Notch intracellular domain.[7]

Here we provide evidence that UBR-5, an E3 ubiquitin ligase, negatively regulates Notch-type signaling in *C. elegans* germ line and that it interacts synergistically with SEL-10 in early embryonic development. We also provide evidence suggesting that UBR-5 is active in the germ line to regulate downstream components of the Notch pathway in the receiving cell. Finally, we provide preliminary

evidence supporting the hypothesis that UBR-5 negatively regulates Notch-type signaling by targeting the Notch intracellular domain for proteasome degradation.

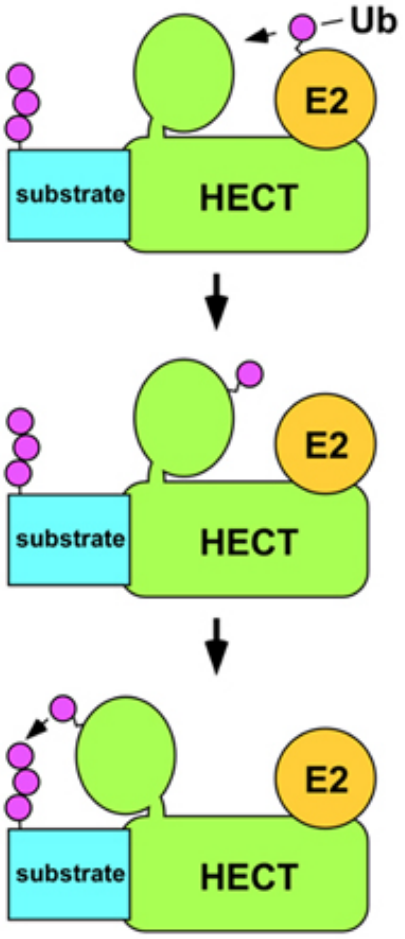


Figure 3. E3 Ligases are Crucial Components of Ubiquitin-Mediated Protein Degradation Pathways (Image from Kipreos 2005). Before ubiquitin can be attached to substrate proteins, they must first be activated by the activity of the E1 enzyme. From there, ubiquitin is passed to the E2 enzyme and finally to the E3 ligase. E3 ligases are the enzymes that attach ubiquitin to target proteins marking them for proteasomal degradation.

Methods

Mutant Strains Used

Standard culture methods were used as described in Epstein and Shakes 1995[14]. Gene nomenclature is as described in Wormbase.

LGI: *sog-1* alleles *om2* (Safdar et al) and *ok1108* (OMRF Knockout Group, see wormbase.org), *rrf-1(pk1417)*

LGIII: *glp-1(q231ts)*, *glp-1(q175)*, *glp-1(ar202ts)*, *unc-32(e189)*, *tnIs39[glp-1::gfp::3xFLAG];glp-1(q175)* (personal communication, David Greenstein and Tim Schedl)

LGIV: *hsp-1::3xFLAG* (Endogenous gene tagged by CRISPR/Cas9 genome editing from Bing Yang)

LGV: *sel-10(ok1632)*.

RNAi

Worms were placed at the L4 stage onto plates containing HT115 *E. coli* expressing *ubr-5* double-stranded RNA. The adult progeny were then assessed for a tumorous germline sterile phenotype. Worms were removed from the plate as they were counted. Controls were grown on regular OP50 feeding bacteria. Empty vector controls were performed using the L4440 vector. See Safdar et al for further details.

CRISPR

For epitope tagging UBR-5 with CRISPR/Cas9, guide RNAs (sgRNAs) were designed near the start codon of UBR-5 using the CRISPR design tool at http://crispr.mit.edu. sgRNAs were inserted into pGEM-T vector and transformed into DH5α cells. Plasmids were isolated using an isopropanol precipitation protocol and confirmed for successful insertion via restriction enzyme digestion with SacII and NdeI. Samples with correct restriction enzyme digestion pattern were sent for sequencing to confirm the insertion. Sequenced plasmids were then retransformed into DH5α cells and isolated using E.Z.N.A. Plasmid DNA Mini Kit I (Omega). An injection mix consisting of 50ng/µl *Cas-9* plasmid, 25 ng/µl *dpy-10*

sgRNA plasmid, 25 ng/µl *dpy-10* ssDNA, 25 ng/µl *ubr-5* sgRNA plasmid, and 50 ng/µl *ubr-5* repair DNA template was injected into N2 adult hermaphrodites. *ubr-5* repair template 1 was generated by PCR and purified either by gel extraction or using a PCR DNA clean-up kit (New England Biolabs). Due to difficulties with PCR yield and quality, *ubr-5* repair template 2 was ligated into pGEM-T vector and injected as a plasmid. *dpy-10* acted as a co-conversion marker as discussed in Arribere et al.[15] Progeny of injected worms were screened for the *dpy-10 (cn64)* roller (Rol) phenotypes and Rol animals were picked onto individual plates. After laying eggs, these Rol worms were screened for successful 3xFLAG insertion using PCR and enzyme digestion with ClaI or EcoRV as indicated.

Primers used for detection:

1. F: GTTCGCTCGCTTTTACTTGT (flanking insert region)

   R: CATCGTCATCCTTGTAATCG (within 3xFLAG sequence)
2. F: TCATACTTCGCGCTCAGTTC

   R: GTGCGTAAGCGTGTGATTTC
3. F: GTTCGCTCGCTTTTACTTGT

   R: CTTGAACTGGTCGACAATTC

Protein Blot

Predominantly adult staged worms were washed with M9 buffer and flash frozen in liquid Nitrogen. An equivalent volume of glass beads were added to the samples, which were then subjected to Fastprep to lyse the worms open. I µl protease inhibitor was added for every 50µl sample. After adding 6xSDS sample buffer and 1:20 dilution β-mercaptoethanol, samples were boiled for ten minutes at 95°C with vortexing after five minutes. Samples were centrifuged for ten minutes at 13,200 rpm to pellet cellular debris. The worm lysate (30-80µl) was loaded onto a 6% polyacrylamide gel and run at 100V for approximately 1-1.5 hours. Protein samples were transferred to nitrocellulose membrane. The membrane was blocked with 5% Milk-PBS for 1 hour at room temperature. The membrane was

incubated with the primary antibody at 4°C overnight and washed 3-4x with 1xPBS for 5-10min. each. The membrane was then incubated with the secondary antibody for 2 hours at room temperature. Before visualization, the membrane was washed 3-4x with 1xPBS for 5-10min. Blots were detected by Thermo Scientific SuperSignal West Femto or West Pico Chemiluminescent substrate as indicated.

List of antibodies used: anti-flag (monoclonal mouse from Sigma 1:500), anti-β-tubulin (E7 monoclonal mouse from the Developmental Studies Hybridoma Bank 1:2000), and anti-mouse (HRP conjugated from Pierce 1:2000). Blots were imaged using X-ray film or BioRad ChemiDoc XRS+ Imaging System and quantified via ImageJ.

Phenotypic Analysis

See Safdar et al for further details.

Results

UBR-5 Negatively Regulates GLP-1 Signaling

In *glp-1(q231ts)* mutants, GLP-1 activity is reduced at restrictive culture temperatures. This treatment results in embryonic lethality due to defects in Notch signaling during early embryonic development. We show through phenotypic analysis that knocking out expression of *ubr-5* in the *glp-1* mutant background partially rescues the embryonic lethality phenotype. Two *ubr-5* alleles were used in this assay. The one allele, *ubr-5(om2),* contains a deletion that shifts the open reading frame and inserts a stop codon. It is expected to encode a truncated protein lacking a substantial portion of the protein, including the highly conserved HECT-domain (Figure 4). Because the HECT-domain is so critical for E3 ligase activity, this allele is expected to be a null mutation. The second allele, *ubr-5(ok1108)*, also contains a deletion that removes a portion of the HECT-domain.

At 20°C, both mutations in *ubr-5* rescue brood size and embryonic lethality phenotypes of *glp-1(q231ts)* mutants (Figure 5). This is consistent with previous data characterizing the ability of different point mutations of *ubr-5* to suppress *glp-1(q231ts)* phenotypes to varying degrees.[10]

The Impact of UBR-5 on Mitotic Zone Size is Dependent Upon a *glp-1* Mutant Background

To determine if the increased brood size observed in *ubr-5(-);glp-1(q231ts)* mutants is the result of a larger mitotic zone, we treated adult worms with DAPI to visualize individual nuclei in the germ line and scored the size of each mitotic zone (Table 1). We used the number of cell rows in the mitotic zone as a measure of proliferative zone size. Interestingly, *ubr-5* mutants alone do not have a significantly larger mitotic zone compared to wild-type (N2). However, *ubr-5;glp-1* mutants do have over twice as large a mitotic zone compared to *glp-1* mutants alone, suggesting that the increased brood size observed in these mutants is the result of increased *glp-1* signaling and a subsequently larger proliferative zone.

UBR-5 Acts Primarily in the Germ Line

Our data suggest that UBR-5, as an E3 ubiquitin ligase, is involved in negatively regulating GLP-1 activity. We hypothesize that UBR-5 is involved in degradation of some component of the Notch signaling pathway (Figure 2). However, whether it was active primarily in the receiving cells in the germline or in the DTC as part of the somatic gonad was unclear. To better understand what component of the Notch signaling pathway is being targeted by UBR-5, we assessed the ability of *ubr-5* RNAi to enhance GLP-1 signaling in a *rrf-1(0);glp-1(ar202)* mutant background. *glp-1(ar202)* is a weak temperature-sensitive gain-of-function allele where at elevated temperatures, a certain percentage of the population displays tumorous germline phenotypes due to increased Notch signaling. *rrf-1(0)* mutants are RNAi deficient in many somatic tissues including the somatic gonad. If UBR-5 activity is required in the germ line to regulate turnover of GLP-1 signaling components in the receiving cell, then we would expect to see similar enhancement of the *glp-1(gf)* phenotype in *rrf-1(+) and rrf-1(0)* backgrounds. However, if UBR-5 is primarily required in the DTC as part of the somatic gonad, then we would not expect treatment with *ubr-5* RNAi to have any effect as *rrf-1(0)* mutants are RNAi deficient in the DTC.

First, we confirmed that RNAi against *ubr-5* successfully increased GLP-1 activity in a *glp-1(ar202)* gain-of-function background (Table 2). Using RNAi to knock down expression of *ubr-5* in this mutant background did indeed enhance the tumorous sterility phenotype as almost 80% of the population developed tumorous germ lines compared to 20% in OP50 controls (Table 2). *ubr-5(RNAi)* enhanced the sterility phenotype in a *rrf-1(0)* background as well, comparable to *rrf-1(+),* suggesting that UBR-5 activity in the somatic gonad is not necessary for regulating GLP-1 signaling (Table 2). Therefore, we conclude that UBR-5 acts predominantly in the germ line, i.e. the receiving cells, to regulate turnover of either the Notch receptor or other downstream components of the signaling pathway.

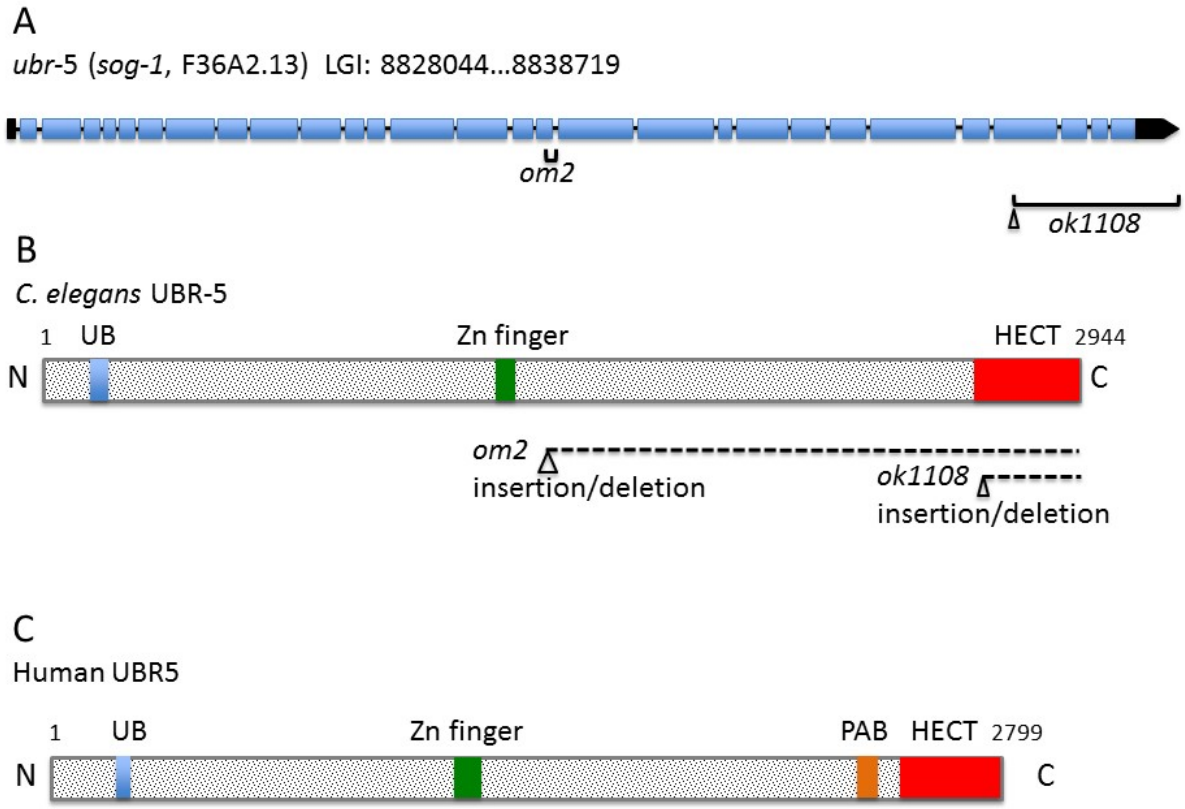


Figure 4. Schematic of *ubr-5* gene and protein (Image from Safdar et al). **(A)** *ubr-5* gene locus with predicted *ok1108* and *om2* mutation locations. **(B)** *om2* and *ok1108* are alleles expected to lack portions of C-terminal domains including highly conserved HECT domain. **(C)** *C. elegans* UBR-5 protein contains similarly conserved domains with the human ortholog.

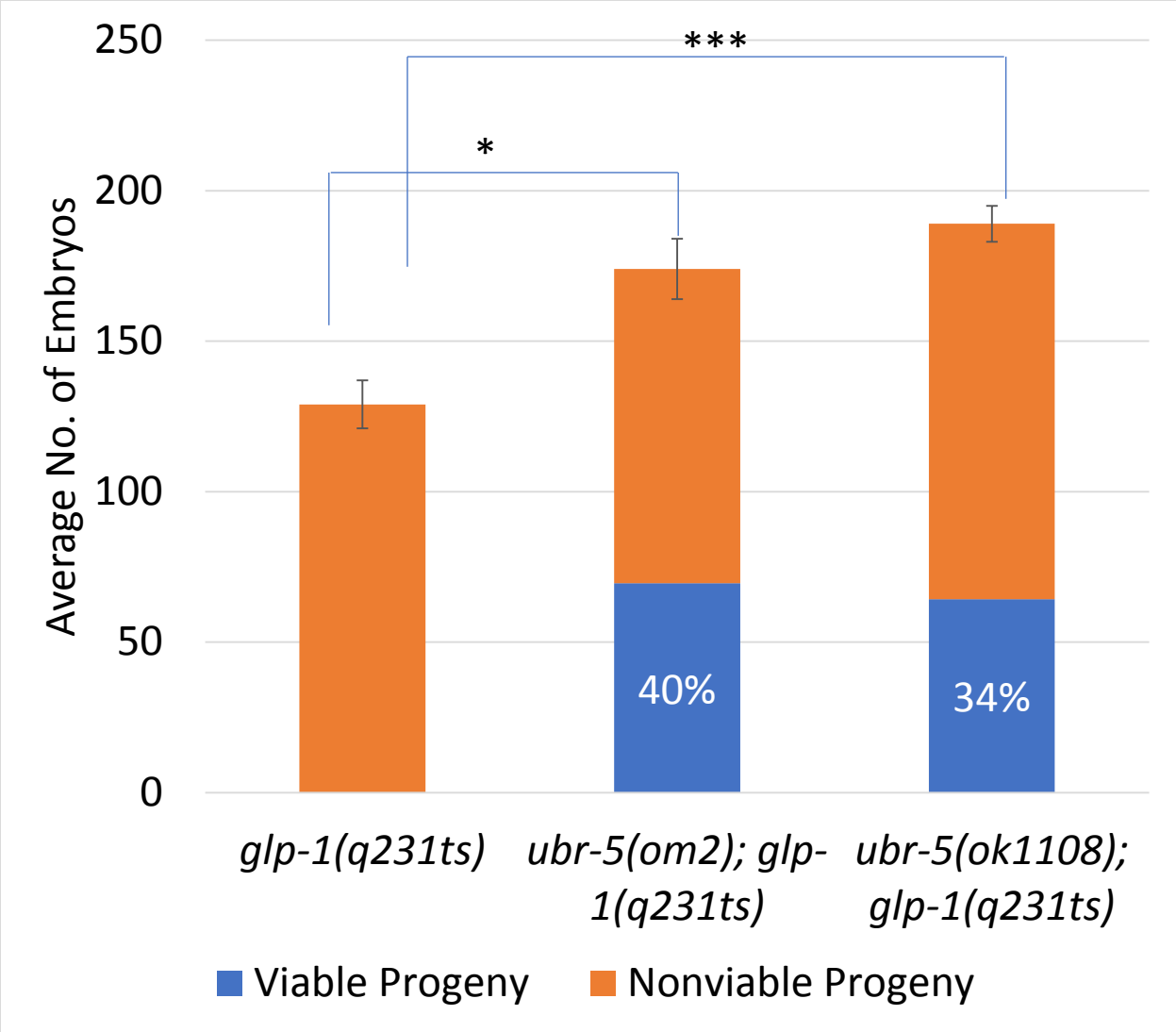


Figure 5. Mutations in *ubr-5* partially rescue *glp-1(q231ts)* brood size and embryonic lethality phenotypes at 20°C. Plotted here is the average number of viable and nonviable progeny as a proportion of the mean total number of embryos produced. Each category represents two independent trials. N is the number of independent broods assayed. *glp-1(q231ts)* N=16. *ubr-5(om2);glp-1(q231ts)* N=10. *ubr-5(ok1108);glp-1(q231ts)* N=14. Error bars represent standard error of the mean of brood size. P values for brood size calculated from two-tailed student t-test. *indicates $p<0.05$ ***indicates $p<0.0005$

Table 1. *ubr-5 (om2)* suppresses germline proliferation defect of *glp-1(q231ts)*

Genotype	**Average Mitotic Rows ± Standard Error**
N2 (wild-type)	21±0.6*
ubr-5(om2)	21±0.8*
glp-1(q231)	4±0.7
ubr-5(om2);glp-1(q231)	11±0.4*
sel-10(ok1632)	15±0.6*
glp-1(q231);sel-10(ok1632)	6±0.9
ubr-5(om2);sel-10(ok1632)	19±1.2*
ubr-5(om2);glp-1(q231);sel-10(ok1632)	12±0.6*

*****indicates p value < 0.0005 with respect to *glp-1(q231).* Although *sel-10(ok1632)* appears to have a smaller mitotic zone compared to *ubr-5(om2);sel-10(ok1632)* or *ubr-5(om2)* mutants, it should be noted that these mitotic zones were counted before outcrossing of the *sel-10(ok1632)* strain. As such there could potentially be mutations in the background that are contributing to the reduced proliferative zone observed. *ubr-5(om2);sel-10(ok1632),* which was essentially outcrossed in the making of the strain, does not have a significantly smaller mitotic zone compared to either *N2* or *ubr-5(om2).*

Table 2. UBR-5 activity is important for normal germline proliferation

Genotype and RNAi treatment	**% *glp-1(gf)* sterile**	**N**
glp-1(ar202) control	20	108
ubr-5(RNAi);glp-1(ar202)	79	96
rrf-1;glp-1(ar202) control	10	110
rrf-1 ubr-5(RNAi);glp-1(ar202)	54	100

A paired t-test indicates there is no significant difference between enhancement in a *rrf-1(+)* background compared to *rrf-1(0).*

Redundancy Tests Between SEL-10 and UBR-5

Because *ubr-5* mutants alone have no distinguishable phenotype, we hypothesized that a second E3 ligase was capable of compensating for *ubr-5* activity in a wild-type background. We identified SEL-10 as an E3 ligase with potential redundancy to UBR-5 because SEL-10 has been implicated in Notch signaling regulation previously[2,13]. To determine if there was any redundancy between SEL-10 and UBR-5, we characterized the phenotype of *ubr-5;glp-1;sel-10* triple mutants (Figure 6). *ubr-5(om2)* mutants do not have a significantly larger brood size than what is expected of wild-type worms. They also demonstrate nearly 100% viability. The *sel-10(ok1632)* strain, RB1432, that we used was acquired from the *Caenorhabditis* Genetics Center. Initial examination found that this strain contained significantly decreased embryonic viability and smaller brood size phenotypes not previously reported in the literature[16]. These defects were not observed in *ubr-5;sel-10* double mutants generated from the RB1432 strain. To determine if this observation was the result of *ubr-5;sel-10* interaction or simply the presence of background mutations in the RB1432 strain, we re-isolated *sel-10(ok1632)* from *ubr-5;sel-10.* We did observe brood size and embryonic lethality defects to the same extent as before. We therefore concluded that some background mutations in the RB1432 strain contributed to the unique phenotypes described. The newly re-isolated *sel-10* strain demonstrates nearly 100% embryonic viability and a larger brood size compared to what was observed prior to re-isolation (Figure 6).

The double mutant, *ubr-5;sel-10,* also displays similar embryonic viability to each of the single mutants. Although the interaction between SEL-10 and LIN-12 is better studied, loss of *sel-10* function has been shown to weakly suppress embryonic lethality phenotypes in *glp-1* mutants[17]. Consistent with these findings, we observed that *sel-10(ok1632)* can weakly suppress embryonic lethality in *glp-1(q231ts)* background with 1-2 worms hatching per brood. However, *glp-1(q231ts);sel-10(ok1632)* does not seem to have a significantly larger brood size compared to *glp-1(q231ts)* mutants alone (Figure 6B).

Surprisingly, *ubr-5(om2);glp-1(q231ts);sel-10(ok1632)* triple mutants display drastically higher embryonic viability compared to either of the double mutants (Figure 6A). The effect is substantially larger than what we would expect to observe if both E3 ligases had an additive effect on embryonic viability. We therefore conclude that UBR-5 and SEL-10 are interacting synergistically in the early embryo to result in the significantly higher percentage viable progeny observed. However, we do not see an interaction between UBR-5 and SEL-10 with respect to the number of progeny produced, as triple mutants do not display a substantially larger brood size compared to either of the double mutants (Figure 6B). Staining of the mitotic zone of these worms supports the brood size observation as triple mutants and *glp-1(q231ts);ubr-5(om2)* double mutants have a similar number of mitotic rows (Table 1). It should be noted that there does appear to be a slight proliferation defect in *sel-10(ok1632)* mutants even after outcrossing as they have a slightly smaller brood size compared to *ubr-5(om2);sel-10(ok1632)* double mutants or *ubr-5(om2)* single mutants (Figure 6). This same trend is observed in the size of the mitotic zone (Table 1). However, the mitotic zone was calculated with a *sel-10(ok1632)* stain that had not been outcrossed and as such, it is difficult to say for certain if the smaller brood size observed in *sel-10(ok1632)* mutants is due to a smaller mitotic zone size or some other flaw such as defective sperm. *ubr-5* mutations appear to rescue this phenotype to some extent as *ubr-5(om2);sel-10(ok1632)* double mutants have an intermediate brood size although this trend is not observed in the mitotic zone (Table 1). Follow-up experiments using an outcrossed *sel-10* strain should be performed to confirm if this phenomenon is due to a proliferation defect.

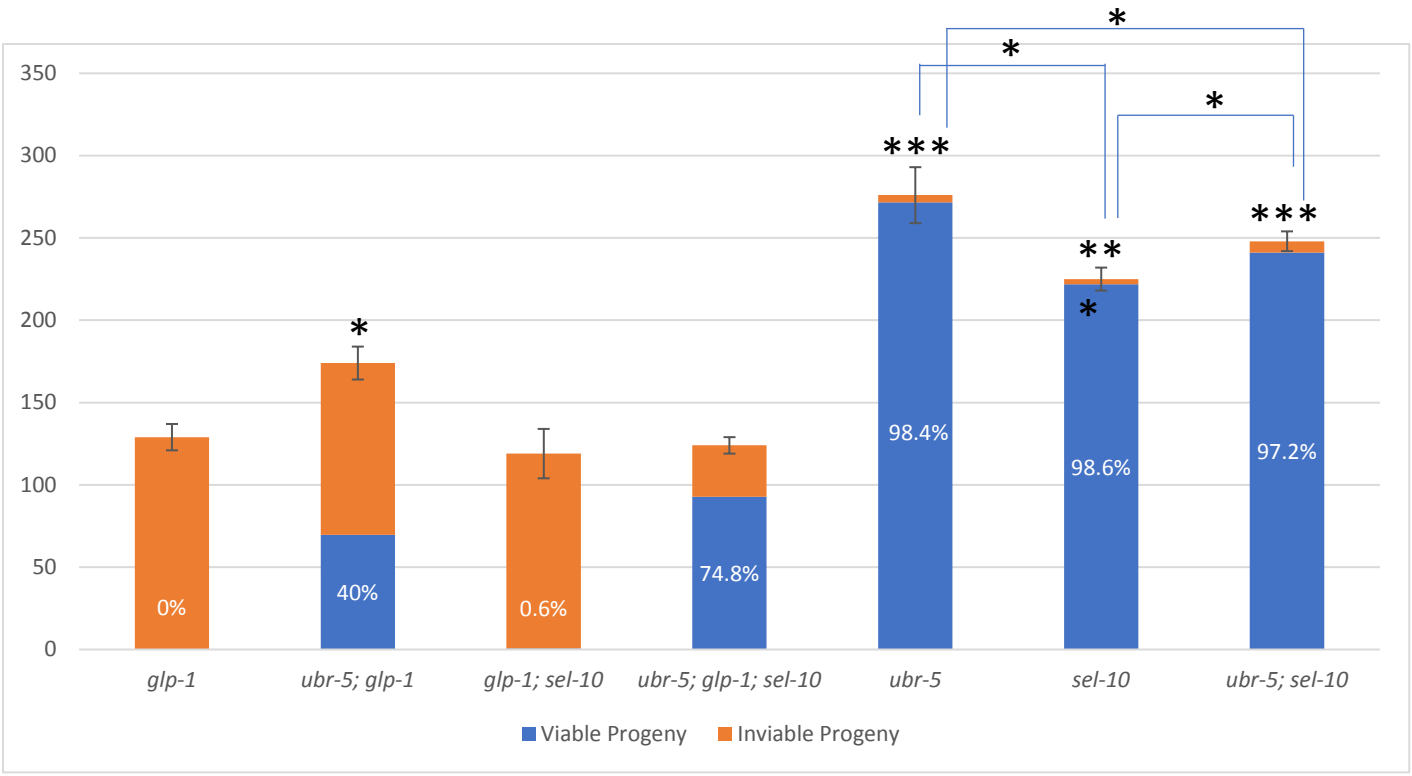


Figure 6. UBR-5 and SEL-10 act synergistically to regulate embryonic viability. Plotted here is the average number of viable and nonviable progeny as a proportion of the mean total number of embryos produced. *glp-1* (2 trials, N=16). *ubr-5;glp-1* (2 trials, N=10). *glp-1;sel-10* (2 trials, N=12). *ubr-5;glp-1;sel-10* (1 trial, N=10). *ubr-5* (1 trial, N=7). *sel-10* (1 trial, N=5). *ubr-5;sel-10* (2 trials, N=12). Error bars represent standard error of the mean of brood size.

GLP-1 Intracellular Domain is more abundant in UBR-5 mutant background

Because SEL-10 has previously been shown to interact with the intracellular domain (ICD) of LIN-12[13], we hypothesized that UBR-5 might regulate GLP-1 signaling in an equivalent manner. Failure to suppress *lag-1(om13ts)* defects also supports this hypothesis.[1] To determine if UBR-5 is involved in proteasomal degradation of the GLP-1 ICD, we looked to see if the amount of GLP-1 protein present was greater in a *ubr-5* mutant background. For this assay, we generated strains containing a *glp-1::gfp::3xFLAG* transgene in *ubr-5(om2)* background by crossing the *tnIs39;glp-1(q175)* strain obtained from the Schedl lab into our *ubr-5(om2)* strain. The 3xFLAG tag in the *tnIs39;glp-1(q175)* strain is located on the ICD of the GLP-1 receptor. We assayed GLP-1 levels by protein blot (Figure 7A). If UBR-5 activity primarily serves to regulate turnover of the GLP-1 ICD, we might expect to see more GLP-1 ICD present in *ubr-5(-)* mutants. Preliminary results support this hypothesis as we observed that the GLP-1 ICD is approximately 2.5 times more abundant in a *ubr-5(om2)* mutant background than in wild-type (Figure 7B). Although the location of the 3xFLAG tag should allow for visualization of both the full-length receptor and the cleaved ICD portion, only the ICD was observed (Figure 7A). This is not entirely surprising as the full-length receptor is present at an even lower level *in vivo* than the ICD.[18]

Epitope Tagging UBR-5 using CRISPR/Cas9

To identify UBR-5 targets on a more global scale, we attempted to tag the endogenous protein with a 3xFLAG epitope tag using the CRISPR/Cas9 genome editing system. This reagent would be used for immunoprecipitation assays. We identified two different sgRNA sequences that might be appropriate for inserting the 3xFLAG sequence in-frame into *ubr-5* (Figure 8A). We initially used sgRNA 1 as a guide RNA and attempted to screen for successful insertions using “In and Out” PCR with one primer in the flanking region and one primer within the 3xFLAG insert sequence (see Methods). Lines without a successful insertion should not have an amplification product after PCR. However, the large majority of our samples demonstrated non-specific amplification at the expected size. We concluded

these DNA products were false positives and did not represent successful inserts. We next tested two different flanking PCR approaches coupled with restriction enzyme digestion. The restriction enzymes selected are expected to cut only in the 3xFLAG sequence. The first set of flanking primers (#2, see Methods) produced an amplified product of approximately 710bp. After ClaI restriction enzyme digestion, the two resulting products are expected to be 615bp and 95bp. Faint non-specific bands at approximately 500bp make detecting the presence of the 615bp product difficult. The 95bp product is potentially too faint to visualize on an agarose gel. Therefore, we designed a second set of flanking primers (#3, see Methods) for all future screening using EcoRV restriction enzyme digestion. In total, approximately 152 Rol worms were screened from "jackpot broods". A jackpot brood refers to many Rol worms being obtained from one injected hermaphrodite and jackpot broods are expected to have a higher frequency of successful edits.[19] All Rol worms screened were negative for successful insertion. We therefore tried using sgRNA2 sequence, which would put the insert site closer to the Cas9 cut site. To minimize the likelihood of disrupting protein function, we aligned the UBR-5 amino acid sequence in many closely related nematodes and identified one area near the N-terminus that possessed some variability between species (Figure 8B). Approximately 162 Rol worms were screened from jackpot broods and all were negative for successful insertion. These experiments are currently on-going.

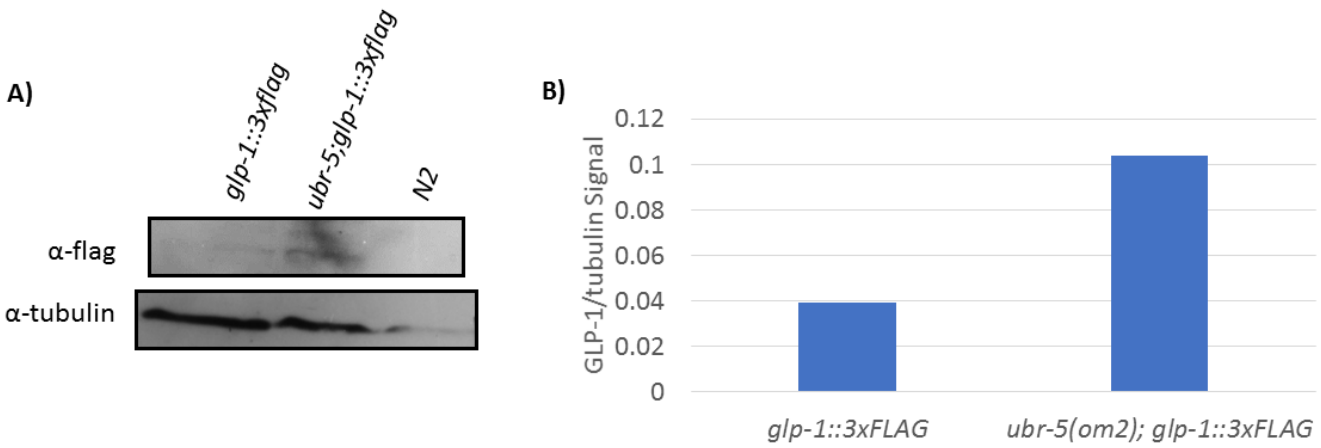


Figure 7. GLP-1 intracellular domain is more abundant in a *ubr-5* mutant background. (A) Western blot using antibodies against 3xFLAG and β-tubulin. Less β-tubulin present in N2 lane could be the result of fewer N2 worms being used and the sample being diluted more during the preparation process. **(B)** α-flag and α-β-tubulin signal were quantified using ImageJ and normalized to background. N2 not quantified as no α-flag signal was observed.

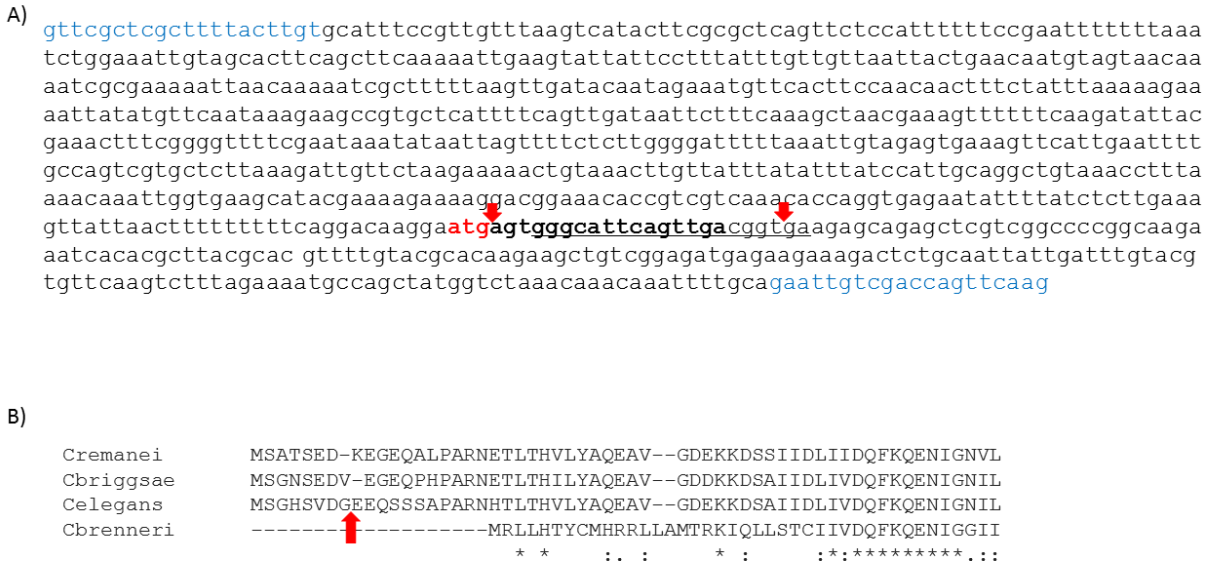


Figure 8. CRISPR/Cas9 Design. (A) Red letters denote start codon. Arrows indicate two different sites for insertion of the 3xFLAG sequence. sgRNA 1 sequence is bolded. sgRNA 2 sequence is underlined. Blue letters denote primers used to detect insert. After PCR amplification, DNA was treated with EcoRV restriction enzyme. Insert site 1 restriction enzyme digest pattern: wildtype (818bp). Expected insert (650bp + 234bp). Insert site 2 restriction enzyme digest pattern: wildtype (818bp). Expected insert (671bp + 213bp). **(B)** Alignment of UBR-5 amino acid sequence in closely related nematodes (*C. remanei, C. briggsae, C. elegans, and C. brenneri*). Red arrow indicates less conserved sequence near N-terminus where second CRISPR insert site was selected.

Discussion

Here we show that UBR-5, a HECT-type E3 ubiquitin ligase negatively regulates GLP-1/Notch signaling in *C. elegans.* We also provide RNAi data suggesting that UBR-5 is acting primarily in the germ line to regulate turnover of the Notch receptor or other downstream components of the signaling pathway. Failure to suppress *lag-1(om13ts)* suggests the most likely target of UBR-5 is the Notch receptor and not the downstream transcription factor, LAG-1.[1] Our current hypothesis is that UBR-5 is regulating turnover of the GLP-1 ICD. This is consistent with our preliminary western blots where the GLP-1 ICD is more abundant in a *ubr-5* mutant background, suggesting that UBR-5 may play a role in turnover of the Notch ICD. Previous research on the relationship between E3 ligases and Notch has implicated SEL-10, a component of the SCF E3 ubiquitin complex as a negative regulator of LIN-12/Notch signaling in *C. elegans*.[2] SEL-10 has been shown to physically interact with the ICD of LIN-12 in *C. elegans* as well as the ICD of Notch 1 and Notch 4 in mammals.[2,20] Our data supports the hypothesis that UBR-5 regulates GLP-1 signaling in an analogous manner by targeting the ICD for degradation.

Our data seems to suggest a complicated relationship between UBR-5 and SEL-10. The two E3 ligases appear to have a synergistic interaction with respect to embryonic viability since *ubr-5(om2); glp-1(q231ts); sel-10(ok1632)* progeny have a much higher survival rate than might be expected if the two E3 ligases simply had an additive effect (Figure 6A). One explanation is that SEL-10 is primarily involved in later embryogenesis to regulate turnover of LIN-12 whereas UBR-5 is involved in early development to regulate turnover of GLP-1. GLP-1 is important for the early embryonic cell cleavages whereas LIN-12 plays a more prominent role in later stages of embryonic development.[21] Both receptors are capable of compensating for each other in specific circumstances.[22] This could explain why triple mutants seem to have higher embryonic viability as upregulating both LIN-12 and GLP-1 might lead to an overall increase of Notch signaling above the threshold needed for survival.

Notch receptors are known to have PEST domains, which are associated with proteins that have a short half-life and are rapidly degraded.[23,24] As such, it seems logical that ubiquitination would be a potential mechanism by which Notch signaling is regulated. Furthermore, mutations in Notch PEST domains that lead to improper activation of the pathway have been linked to the development of specific types of cancer, such as breast cancer.[25] Mammalian UBR-5 itself has also been implicated in cancer development as mutations that disrupt a conserved cysteine residue in the HECT domain have been associated with 18% of Mantel Cell Lymphoma cases.[26] In general, dysregulation of UBR-5 has been found in a variety of human cancer cases with ovarian, breast, and bladder being the most common.[26] However, null mutations of UBR-5 in mouse models are embryonic lethal, making it difficult to use as a cancer model.[27] Given that null mutations in *C. elegans* seem to have no developmental defects to date, understanding the mechanisms behind this lack of phenotype could be enlightening for UBR-5's importance in mammalian systems. Additionally, using *C. elegans* to identify and characterize UBR-5 targets could give us a better understanding of how UBR-5 mutations lead to cancer development in humans. It is possible that dysregulation of UBR-5 in human cancers is at least in part due to the indirect misregulation of Notch signaling pathways. These discoveries underscore the importance of proper Notch regulation for human health and emphasize the importance of better understanding the role of E3 ligases such as UBR-5 in Notch regulation.

Future Work

Generating a successful transgenic line with UBR-5::3xFLAG is our primary goal. Once this line has been successfully obtained, we aim to better understand UBR-5 targets on a more global scale using immunoprecipitation and mass spectrometry analysis to identify proteins associating with UBR-5. Of those proteins, we expect to see the two Notch receptors, LIN-12 and GLP-1, as well as other components of the ubiquitin pathway including E2 enzymes and ubiquitin. However, it would be

especially intriguing to identify new proteins via the immunoprecipitation that could shed some light on other interactors of UBR-5 in *C. elegans* that participate in Notch signaling regulation or other targets of UBR-5 outside of the Notch signaling pathway.

Furthermore, we can use co-immunoprecipitation to confirm that UBR-5 and GLP-1 are physically interacting. Additionally, we can analyze the expression patterns of UBR-5 using immunofluorescence to see which tissues the E3 ligase is localizing to. Evidence in the literature reports that mammalian SEL-10 (mSEL-10) is primarily localized in the nucleus.[20] It is a possibility that UBR-5 might also be localized in the nucleus, potentially in germ cells, which would be consistent with our RNAi analyses.

Finally, due to the difficulty visualizing GLP-1 receptor on a western blot, it might be worth repeating the experiment with a proteasome inhibitor such as MG132. A previous biochemical study looking at mSEL-10 noted that ubiquitination of the Notch 1 ICD could only be observed in the presence of mSEL-10 and a proteasome inhibitor MG132, suggesting that ubiquitinated Notch ICD is rapidly shunted to the proteasome.[20] Treatment with MG132 might make levels of the GLP-1 ICD more abundant and therefore easier to visualize.

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