Reduction of Derlin activity suppresses Notch-dependent tumours in the *C. elegans* germ line

Short Title: Derlin proteins and Notch signalling

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ABSTRACT

Regulating the balance between self-renewal (proliferation) and differentiation is key to the long-term functioning of all stem cell pools. In the Caenorhabditis elegans germline, the primary signal controlling this balance is the conserved Notch signaling pathway. Gain-of-function mutations in the GLP-1/Notch receptor cause increased stem cell self-renewal, resulting in a tumor of proliferating germline stem cells. Notch gain-of-function mutations activate the receptor, even in the presence of little or no ligand, and have been associated with many human diseases, including cancers. We demonstrate that reduction in CUP-2 and DER-2 function, which are Derlin family proteins that function in endoplasmic-reticulum-associated degradation (ERAD), suppresses the C. elegans germline over-proliferation phenotype associated with glp-1(gain-of-function) mutations. We further demonstrate that their reduction does not suppress other mutations that cause over-proliferation, suggesting that over-proliferation suppression due to loss of Derlin activity is specific to glp-1/Notch (gain-of-function) mutations. Reduction of CUP-2 Derlin activity reduces the expression of a read-out of GLP-1/Notch signaling, suggesting that the suppression of over-proliferation in Derlin loss-of-function mutants is due to a reduction in the activity of the mutated GLP-1/Notch(GF) receptor. Over-proliferation suppression in cup-2 mutants is only seen when the Unfolded Protein Response (UPR) is functioning properly, suggesting that the suppression, and reduction in GLP-1/Notch signaling levels, observed in Derlin mutants may be the result of activation of the UPR. Chemically inducing ER stress also suppress glp-1(gf) over-proliferation but not other mutations that cause over-proliferation. Therefore, ER stress and activation of the UPR may help correct
for increased GLP-1/Notch signaling levels, and associated over-proliferation, in the *C. elegans* germline.
AUTHOR SUMMARY

Notch signaling is a highly conserved signaling pathway that is utilized in many cell fate decisions in many organisms. In the *C. elegans* germline, Notch signaling is the primary signal that regulates the balance between stem cell proliferation and differentiation. Notch gain-of-function mutations cause the receptor to be active, even when a signal that is normally needed to activate the receptor is absent. In the germline of *C. elegans*, gain-of-function mutations in GLP-1, a Notch receptor, results in over-proliferation of the stem cells and tumor formation. Here we demonstrate that a reduction or loss of Derlin activity, which is a conserved family of proteins involved in endoplasmic-reticulum-associated degradation (ERAD), suppresses over-proliferation due to GLP-1/Notch gain-of-function mutations. Furthermore, we demonstrate that a surveillance mechanism utilized in cells to monitor and react to proteins that are not folded properly (Unfolded Protein Response-UPR) must be functioning well in order for the loss of Derlin activity to suppress over-proliferation caused by *glp-1/Notch* gain-of-function mutations. This suggests that activation of the UPR may be the mechanism at work for suppressing this type of over-proliferation, when Derlin activity is reduced. Therefore, decreasing Derlin activity may be a means of reducing the impact of phenotypes and diseases due to certain Notch gain-of-function mutations.
INTRODUCTION

Stem cell populations provide the source material for future tissue generation and play an important role in the development and maintenance of many tissues. A defining feature of stem cells, their ability to both self-renew and differentiate, is key to their function. Stem cells must maintain a balance between self-renewal and differentiation as excessive self-renewal can lead to tumour formation while too much differentiation leads to a depleted stem cell pool. The decision to self-renew or differentiate is essential for the proper development of their tissues. Critical systems like this require many layers of redundancy in order to have a high level of robustness. This way, if pressure is applied to one layer, other layers are able to ensure proper decision-making. These layers of redundancy can also allow external inputs to impinge on the system, giving it the ability to adapt. Understanding these layers of redundancy will aid in research in stem cell-related diseases and in using stem cells as therapeutic agents.

An indirect mechanism to modulate stem cell systems is through the regulation of protein folding and protein quality control. For example, recently it has been shown that increasing the genesis of misfolded proteins in Hematopoietic stem cells (HSCs) impairs self-renewal of HSCs (1). In the case of muscle stem cells, impairment of autophagy, the lysosomal degradation of long-lived proteins and damaged organelles, leads to senescence and stem cell exhaustion (2). As another example, the transcription factor NRF3 is significantly mutated across twelve cancer cell lines and promotes cancer cell proliferation (3,4). NRF3 is regulated by ER retention and endoplasmic-reticulum-associated degradation (ERAD), two cellular mechanisms responsible for surveillance of protein folding (5,6). ERAD is a multi-step process in which misfolded proteins are recognized, retrotranslocated into the cytoplasm and targeted for
degradation by the proteasome (6–8). Recognition of misfolded proteins involves lectins and chaperone proteins in the ER (6). Retrotranslocation occurs in protein complexes containing E3 ubiquitin ligases that also ubiquitinate the misfolded protein. In yeast, the Doa10/Ubc7 complex retrotranslocates and ubiquitinates proteins with misfolded cytosolic domains (ERAD-C pathway), while the Hrd1/Hrd3/Der1 complex acts on proteins with misfolded ER luminal domains (ERAD-L pathway) (9,10). This distinction between different ERAD pathways is less clear in mammalian systems (7,11). In both yeast and mammals, a p97 (Cdc48)/Npl4/Ufd1 complex extracts many of the targeted proteins in an ATPase-dependent manner allowing them to be degraded by the proteasome (Carvalho et al. 2006; Brodsky and Skach 2011).

The housekeeping function of ERAD has important physiological implications for protein homeostasis. For example, as much as 75% of the wild type cystic fibrosis transmembrane conductance regulator (CFTR) protein is targeted for degradation through ERAD (13–17). Single amino acid mutations in the 140 kDa, twelve transmembrane domain CFTR protein disrupt its proper folding such that all CFTR protein is degraded by ERAD leading to cystic fibrosis (14)(18). Dysregulation of ERAD can lead to the accumulation of misfolded proteins in the ER, which induces ER stress (Kadowaki and Nishitoh 2013). In response to ER stress, a series of protective cellular events are triggered to deal with the accumulation of misfolded proteins. Translation is attenuated to limit protein folding burden, the ER expands and becomes more elaborate to increase protein folding capacity, expression of chaperone proteins is increased and expression of ERAD components are increased to assist in protein folding. Collectively this response is called the Unfolded Protein Response (UPR)(Walter and Ron 2011; Kadowaki and Nishitoh 2013). If the UPR fails, apoptosis can be triggered to eliminate the stressed cell in both animal
models and human disease (Shore et al. 2011; Kadowaki and Nishitoh 2013). The increased levels of protein synthesis required for overproliferation in cancer cells is thought to increase basal levels of ER stress and the UPR (21). This increase in ER stress is thought to either make cancer cells more resilient, or more susceptible to artificially inducing ER stress (22,23). Understanding how cancer cells (and all stem cells) regulate and respond to ER stress is crucial in order to be able to understand how therapeutics act on them (24).

The adult *C. elegans* germline harbours stem cells whose self-renewal is regulated by the GLP-1/Notch signalling pathway. The gonads of the *C. elegans* hermaphrodite comprise of two U-shaped tubes that meet at a common uterus (25,26). Germ cells are born at the distal end of each arm and mature as they move proximally towards the vulva. The most distal population of germ cells are mitotically dividing stem cells (Kimble and White 1981). As cells move proximally, they enter into meiosis and mature in an assembly line like manner to produce gametes. The pool of distal stem cells is maintained by GLP-1/Notch signalling and loss of GLP-1/Notch signalling results in a loss of the stem cell population, while increased GLP-1/Notch signalling leads to tumour formation (27–29). Two redundant pathways comprising of GLD-1 and GLD-2 function downstream of GLP-1/Notch signaling to promote differentiation. If the activities of both these pathways is reduced or eliminated a germline tumor results, similar to that due to increased GLP-1/Notch signaling (Kadyk and Kimble 1998; Eckmann et al. 2004; Hansen et al. 2004). Many other regulatory controls ranging from factors controlling cell division such as CYE-1/CDK-2, subunits of the DNA polymerase alpha-primase complex, or proteasomal activity to signalling pathways such as MPK-1 ERK, Insulin, TGF-β, and TOR have been identified that modulate the balance between self-renewal vs differentiation to provide
robust control (30–36). They also add a layer of modulatory control necessary for the germline to adapt. Disruption of any one of these modulatory controls have weak effects on the balance between self-renewal and differentiation in an otherwise wildtype genetic background under ideal conditions; however, the redundancy of these pathways combine to create a robust system necessary for the balance between germline stem cell self-renewal and differentiation.

CUP-2 is a member of the Derlin (degradation in the ER) family of proteins, that function in ERAD (11,37–39). Derlins were initially discovered in yeast with Der1 and Dfm1 (39,40). Mammals have three Derlin family members, Derlin-1, Derlin-2 and Derlin-3 (41). CUP-2 is most similar to human Derlin-1 and a second C. elegans Derlin, DER-2, is most similar to human Derlin-2 and Derlin-3 (37,41,42). As expected for proteins functioning in ERAD, loss of cup-2 and der-2 result in activation of the UPR (37,38). Further support for cup-2 and der-2’s role in ERAD is provided by the fact that as is the case with yeast der1, cup-2 is also synthetically lethal with ire-1, a sensor for the UPR and overexpression of der-2 in Δder1 Δire1 yeast strains partially suppresses the conditional lethality associated with the strain (37,40,43).

CUP-2 (coelomocyte uptake defective) was first identified in C. elegans as being required for endocytosis by the scavenger-like cells, the coelomocytes (44). Later, CUP-2 was found to bind SNX-1 (sorting nexin), a component of the retromer complex in early endosomes (45,46). This interaction was also observed with human Derlins and Sorting Nexins (46). Similar to its role in ERAD, in endocytosis CUP-2 is thought to aid in recognition of misfolded plasma membrane proteins and their transportation to the ER for degradation (46).

As part of another study to identify potential mRNA targets of PUF-8 (to be published elsewhere), we found that loss of cup-2 activity strongly suppressed the overproliferation
phenotype observed in \textit{puf-8(0); glp-1(gf)} animals (see below). Although \textit{cup-2} does not appear to be a direct target of PUF-8 (Supp Fig 1), the loss of \textit{cup-2} suppressing overproliferation provides an inroad into studying the role of ERAD in affecting GLP-1/Notch signaling and the proliferation vs. differentiation decision. Previous studies have shown that worms mutant for the ERAD component CUP-2, have increased expression of HSP-4::GFP, a hallmark of ER stress, as well as activation of the Unfolded Protein Response (UPR) (37,38). These studies have also shown that \textit{der-2}, the other worm Derlin, is partially redundant with \textit{cup-2} in the activation of the UPR. Here we investigate the effect that these Derlin mutants and ER stress have on the balance between stem cell proliferation and differentiation in the \textit{C. elegans} germ line.

Recently, studies have highlighted an emerging link between Notch signalling, ER stress and the UPR. Disruptions to ER zinc homeostasis affect Notch trafficking and activity in human cancer cell lines and \textit{Drosophila} imaginal wing discs (47,48). Mutations in p97, a key ERAD component, also disrupt Notch signalling in \textit{Drosophila} wing development (49). Inducing ER stress in human cell culture induces expression of the Notch ligand DLL4 (50). Whether ERAD plays a role in physiological Notch signalling, or whether Notch signalling is modulated only in response to ER stress is unclear from these latest studies.

Here we describe the effect that ERAD and the UPR have on the Notch-dependent control of stem cell proliferation in the \textit{C. elegans} germline. We show that germline tumour formation resulting from increased GLP-1/Notch signalling is suppressed by mutations in \textit{cup-2} and \textit{der-2}, encoding Derlin proteins which are components of ERAD. We also show that ectopically induced ER stress suppresses germline stem cell over-proliferation caused by increased GLP-1/Notch signaling and that this suppression requires the UPR. Both loss of \textit{cup-2}
and induced ER stress can only suppress GLP-1/Notch-dependent tumours, suggesting they act directly on the Notch pathway, in a context-specific manner that suppresses excess overproliferation. We propose that ER stress and the UPR have a protective role, correcting for aberrant over-proliferation caused by increased GLP-1/Notch signaling levels in the *C. elegans* germline and restoring proper balance between stem cell proliferation and differentiation. Therefore, this study contributes to our understanding of how affecting protein folding capacity by modulating ER stress, can regulate the balance between stem cell self-renewal and differentiation.

**RESULTS**

**Loss of *cup-2* suppress *glp-1(gf)* tumours in correlation with the strength of GLP-1/Notch signalling**

As mentioned above, our initial observation leading us to investigate the role of ERAD in the proliferation vs. differentiation decision was the partial suppression of overproliferation in *puf-8(0); glp-1(gf)* animals through the loss of *cup-2* activity. As we have previously reported, loss of *puf-8* strongly enhances the overproliferation phenotype of *glp-1(gf)* alleles (51). To distinguish proliferating stem cells from differentiating cells, we analyzed germline phenotypes using antibodies against REC-8 (marker for proliferating cells in mitosis) and HIM-3 (marker for differentiating cells entering meiosis) (52–54) (Fig 1A). We found that while 95% of *puf-8(q725); glp-1(oz264gf)* gonads were completely tumorous, with no evidence of cells entering meiosis as measured by anti-REC-8 and anti-HIM-3 staining, only 14% of gonads were completely tumorous when *cup-2* activity was also removed (Fig 1B) (Table 1). Indeed, while no
phenotypically wild-type *puf-8(q725); glp-1(oz264gf)* gonads were observed, 22% of *cup-2(tm2838); puf-8(q725); glp-1(oz264)* were wild-type with no evidence of overproliferation. Therefore, the loss of *cup-2* activity strongly suppresses the overproliferation observed in *puf-8(q725); glp-1(oz264gf)* animals.

We first more fully characterized the suppression of *puf-8(0); glp-1(gf)* and asked whether the suppression by loss of *cup-2* activity correlated with the strength of the *glp-1* gain-of-function allele used. Loss of *puf-8* in *glp-1(gf)* mutants results in overproliferation, with the degree of overproliferation appearing to be dependent on the strength of the *glp-1* gain-of-function allele (51). We analyzed three different *glp-1(gf)* alleles, *ar202, oz264* and *ar224*, which in a *puf-8(0)* mutant background results in 100%, 95% and 86% gonads being fully tumorous at 20°C, respectively (Fig 1B) (Table 1). When *cup-2* activity was also removed in these backgrounds the percentage of fully tumorous gonads was reduced to 92%, 14% and 41%, respectively. Therefore, the ability of *cup-2(0)* to suppress overproliferation is not *glp-1(gf)* allele-specific, and the degree of suppression is at least somewhat correlated with the relative strength of the *glp-1(gf)* allele.

To determine if the suppression by *cup-2(0)* is dependent on loss of *puf-8* activity, we tested whether *cup-2(0)* could suppress other overproliferation mutants that are due to increased GLP-1/Notch signaling, but wild-type for *puf-8*. We found that *cup-2(0)* partially suppresses overproliferation observed in an *rfp-1(ok572); glp-1(oz264gf)* double mutant background. RFP-1 is an E3 ligase that promotes proteasomal degradation of proliferation promoting proteins in order to allow germ cells to differentiate (55). In *rfp-1(ok572); glp-1(oz264gf)* 61% of gonads contain an incomplete tumour, while 39% of the gonads appear wild-
type for proliferation (Fig 1B) (Table 1). Loss of cup-2 strongly suppresses this overproliferation phenotype, with 93% of the gonads appearing wild-type and only 7% having an incomplete tumour when cup-2 activity is also removed (Fig 1B) (Table 1). cup-2(0) also suppresses overproliferation in glp-1(gf) single mutants. For this analysis we counted the number of cells in the distal proliferative zone at 20°C (Fig 2A). Wild-type gonads contain ~221 cells in this region, while glp-1(ar202gf) and glp-1(oz264gf) gonads contain ~610 and ~384 cells respectively (Fig 2A and Fig 2B) (Table 2), demonstrating significant overproliferation. The cup-2(tm2838) single mutant contains ~193 cells, slightly fewer than in wild-type gonads (Table 2). Removal of cup-2 strongly suppressed glp-1(ar202gf) and glp-1(oz264gf) overproliferation, with only ~195 and ~226 cells in the respective double mutants (Table 2). We noticed that although glp-1(ar224gf) single mutants do not show an overtly large proliferative zone size at 20°C, removal of cup-2, was also able to slightly suppress the proliferative zone size in this genetic background. Therefore, cup-2(0)’s suppression of germline overproliferation is not dependent on the loss of puf-8.

We have demonstrated that loss of cup-2 activity suppresses glp-1(gf) mediated overproliferation. We reasoned that if cup-2 interacts with the GLP-1/Notch signaling pathway then its loss may also enhance the temperature sensitive partial loss-of-function glp-1(bn18) allele (56). We found that at 20°C, cup-2(0); glp-1(bn18) gonads have ~95 cells in the proliferative zone, while cup-2(0) and glp-1(bn18) have ~195 and ~138, respectively (Fig 2B) (Table 2). While it does appear that cup-2(0) enhances the smaller proliferative zone phenotype of glp-1(bn18), we do not consider this to be a strong enhancement because enhancement is not to the point of causing a Glp (germ line proliferation defective) phenotype.
in which no proliferative cells are present (27), which is observed with other enhancers of glp-1(bn18) (57). Even at 22.5°C we do not observe enhancement resulting in Glp animals (Supp Table 1). This suggests that loss of cup-2 has a stronger effect on gain-of-function alleles of glp-1 than loss-of-function alleles.

Loss of the cup-2 paralog, der-2, also suppress glp-1(gf) mediated overproliferation

cup-2 encodes a Derlin protein that has previously been shown to be involved in ERAD (37,38), functioning partially redundantly with DER-2, the other C. elegans Derlin protein (Schaheen et al. 2009). DER-2 is thought to be the functional ortholog of yeast Der1p since overexpression of C. elegans DER-2 in yeast Δder1 Δire1 strains partially restores degradation of an Der1p associated ERAD substrate and partially suppresses the conditional lethality phenotype of the double mutant (40). If disruption of ERAD is responsible for cup-2(0)’s ability to suppress germline overproliferation, then we would expect loss of der-2 to likewise suppress overproliferation. We found that loss of der-2 does decrease the size of the distal proliferative zone in glp-1(ar202gf) animals from ~610 cells in glp-1(ar202gf) to ~405 in der-2(tm6098) glp-1(ar202gf) double mutants (Fig 2C) (Table 2). Importantly, the size of the distal proliferative zone in der-2(tm6098) single mutants is similar to that in wild-type animals (~227 and ~221 respectively), suggesting that the suppression of glp-1(ar202gf) is not simply due to an overall reduced rate of proliferation. The suppression of glp-1(ar202gf) is most pronounced when the activities of both cup-2 and der-2 are removed (Fig 2A and 2C) (Table 2), reducing the size of the proliferative zone from ~610 to ~122. This suggests that cup-2 and der-2 may have some redundant function. Indeed, the size of the distal proliferative zone in the cup-2(tm2838); der-
2(tm6098) double mutant (~142) is smaller than either single mutant (~195 for cup-2 and ~227 for der-2), or the wild-type proliferative zone (~221)(Fig 2C) (Table 2). Moreover, while loss of cup-2 suppresses the complete tumour phenotype from 100% tumourous gonads in puf-8(q725); glp-1(ar202gf) double mutants to 92% tumourous animals in cup-2(tm2838); puf-8(q725); glp-1(ar202) triple mutants, also eliminating der-2 function in cup-2(tm2838); puf-8(q725); der-2(tm6098) glp-1(ar202) quadruple mutants reduces the percentage of completely tumourous animals to 67% (Fig 1B) (Table 1). Therefore, the suppression of glp-1(ar202gf) (proliferative zone counts), and the suppression of puf-8(0); glp-1(ar202gf) by loss of cup-2 and der-2 suggests that cup-2 and der-2 function redundantly in promoting robust germline proliferation.

**Derlin loss-of-function mutants have smaller proliferative zone sizes**

In order to ascertain whether Derlin mutants affect cell proliferation, we first asked whether the size of the proliferative zone is altered in Derlin mutants, cup-2 and der-2. We found that cup-2 mutant worms have a slightly smaller proliferative zone than wild-type, whereas der-2 single mutants do not significantly alter the proliferative zone size (Fig 2C) (Table 2). Moreover, cup-2; der-2 double mutants have a statistically significantly smaller proliferative zone size than wild-type (p = 2.182 X 10^-5, t-test independent samples with Bonferroni correction) and cup-2 single mutants (p = 2.014 X 10^-4, t-test independent samples with Bonferroni correction) (Fig 2C) (Table 2). This implies that cup-2 and der-2 may have overlapping but partially redundant roles in regulating cell proliferation.
Of the two Derlins, cup-2’s interaction with glp-1 gain-of-function alleles is stronger; however, cup-2 and der-2 additively have the strongest effect. We conclude that Derlin’s interaction with the GLP-1/Notch signalling pathway could be proportional to the strength of excessive GLP-1/Notch signalling and is most pronounced with strong glp-1 gain-of-function alleles.

**Derlin loss-of-function mutants do not suppress Notch-independent tumours**

We have demonstrated that loss of both cup-2 and der-2 suppress germline overproliferation due to increased GLP-1/Notch signaling. This suppression could be achieved by lowering GLP-1/Notch signaling levels; alternatively, it could be achieved by affecting signaling downstream or parallel to GLP-1/Notch signaling, or by directly inhibiting proliferation. To differentiate between these possibilities, we tested whether Derlin mutants could suppress germline overproliferation mutants that do not increase GLP-1/Notch signalling. The GLD-1 and GLD-2 pathways function downstream of GLP-1/Notch signaling. If the activity of just one of these two pathways is eliminated germ cells proliferate and enter meiosis similar to wild-type (52,58,59). However, if the activities of both pathways are reduced or eliminated a germline tumor results, similar to the tumour due to increased GLP-1/Notch signalling (52,58,60,61). We tested overproliferation mutants that have reduced or no GLD-1 and GLD-2 pathway activities to see if loss of Derlin activity could suppress the overproliferation.

We first tested the gld-3(0) nos-3(0) animals, with gld-3(0) reducing GLD-2 pathway activity and nos-3(0) reducing GLD-1 pathway activity, which form a robust GLP-1/Notch signaling independent tumor (60). We found that no gld-3(q730) nos-3(q650) (n=102) or cup-


2(tm2838); gld-3(q730) nos-3(q650) (n=111) gonads showed evidence of meiotic cells, as measured by the presence of REC-8(-) HIM-3(+) cells, suggesting that loss of cup-2 does not suppress this overproliferation (Fig 3A and 3B).

Since our analyses of Notch-dependent tumours suggest that loss of cup-2 only weakly suppresses more robust tumorous backgrounds, we were concerned that the gld-3(0) nos-3(0) tumour was perhaps too strong for cup-2(0) to suppress. Therefore, we also analyzed gld-2(0) gld-1(0) animals. In these double mutants all gonads are over-proliferative (58); however, anti-REC-8 and anti-HIM-3 staining reveals that many cells in the distal arm of the gonad and/or around the loop region enter meiosis (REC-8(-) HIM-3(+)), while all cells in the proximal arm appear proliferative in most gonads (Fig 3C and 3D) (Table 3)(52). We reasoned that since many cells enter meiosis in gld-2(0) gld-1(0) animals, this genetic background would be more sensitized for suppression of overproliferation. We found that neither loss of cup-2 nor der-2 suppressed gld-2(0) gld-1(0) tumours, as measured by an expansion of REC-8(-) HIM-3(+) cells into the proximal arm (Fig 3D) (Table 3). Even simultaneous loss of cup-2 and der-2 did not suppress overproliferation in gld-2(0) gld-1(0) double mutants (Fig 3C and 3D) (Table 3). Taken together, we conclude that loss of Derlin activity is unable to suppress overproliferation that is due to loss of GLD-1 and GLD-2 pathway genes. This suggests that Derlin mutants do not suppress proliferation in general, but rather, specifically suppress GLP-1/Notch signalling.

**cup-2** loss-of-function mutant reduces the expression of SYGL-1, a readout of GLP-1/Notch signalling, in *glp-1(gf)* gonads
We have demonstrated that loss of Derlin activity suppresses overproliferation due to increased GLP-1/Notch signalling, but not overproliferation in GLD-1/GLD-2 pathway mutants. This raises the possibility that loss of Derlin activity reduces the amount of GLP-1/Notch signalling in glp-1(gf) mutants. sygl-1 is a downstream transcriptional target of GLP-1/Notch signalling in the germline, functioning redundantly with lst-1, and is expressed in the distal proliferating cells in the germline (62–66). To determine the effect of loss of Derlin activity on GLP-1/Notch signalling, we analyzed SYGL-1 expression in the relative mutants (Fig 4A).

Consistent with previous reports of SYGL-1 expression levels in glp-1(gf) mutants, we found that the zone of SYGL-1 expression expanded proximally in glp-1(ar202gf) gonads as compared to wild-type (Supp Fig 1B)(62,63). In wild-type gonads, SYGL-1 expression peaks around five cell diameters from the distal end, then decreases gradually until plateauing around 20 cell diameters from the distal end (Fig 4B). In glp-1(ar202gf) gonads the peak is around seven cell diameters and the plateau is around 24 cell diameters (Fig 4B). In cup-2(tm2838); glp-1(ar202gf) double mutants the expansion of SYGL-1 expression is suppressed, with the pattern of SYGL-1 expression being very similar to wild-type (Fig 4B) (Supp Fig 1C).

Interestingly, the SYGL-1 pattern in cup-2 single mutants is also shifted ~two cell diameters distally as compared to wild-type (Fig 4B) (Supp Fig 1A). Since the data shown in Fig 4B was generated by comparing genotypes imaged on different slides and normalizing against SYGL-1 intensity of a wild-type background as an internal control (see Materials and Methods) we wanted to more directly compare the effect of loss of cup-2 activity on SYGL-1 expression in glp-1(ar202gf) animals, without normalization. Therefore, we compared SYGL-1 expression in glp-1(ar202gf) animals with cup-2(tm2838); glp-1(ar202gf) animals on the same slide (Supp Fig
1D). This experiment yielded similar results to those obtained with normalization; the loss of cup-2 activity suppresses the expansion of SYGL-1 expression along the distal-proximal axis in glp-1(ar202gf) animals. The distal movement of the SYGL-1 expression pattern in cup-2(0) as compared to wild-type, and in cup-2(tm2838); glp-1(ar202gf) as compared to glp-1(ar202gf), suggests that reduction of Derlin activity results in a decrease in GLP-1/Notch signaling, and that this reduction in GLP-1/Notch signaling is the likely cause of the suppression of the glp-1(gf) overproliferation by the loss/reduction of Derlin activity.

**CUP-2 functions in the germline to affect proliferation**

Our genetic results suggest that loss of Derlin protein function, particularly CUP-2, results in reduction of excessive GLP-1/Notch signalling levels in the germline. To gain more insight into how CUP-2 may interact with GLP-1/Notch signaling we sought to determine its expression pattern. For this we tagged the endogenous cup-2 locus with a C-terminal v5::2xflag epitope tag using CRISPR/Cas9 editing (67–71). We created three independent cup-2::v5::2Xflag alleles (cup-2(ug1), cup-2(ug2) and cup-2(ug3)), all expressing CUP-2::V5::2FLAG as detected by an anti-FLAG western blot (data not shown). We found that CUP-2 is expressed throughout the germline (Fig 5A). The expression levels of CUP-2 are lower in the distal end of the gonad and increase proximally. Although CUP-2 levels are low in the distal region of the gonad, where GLP-1/Notch signalling is known to be active, it is above background (Fig 5B and C).

Derlin proteins are known components of the ERAD protein quality control mechanism (38–41,72,73). Therefore, if CUP-2 is involved in ERAD in the germline, we would expect to find CUP-2 localized to the endoplasmic reticulum (ER). We found that CUP-2 expression within the germline partially co-localizes with SP12, a signal peptidase that is a commonly used ER marker
The expression of CUP-2 in the ER is consistent with previous studies of CUP-2 expression in coelomocytes using transgenes (37,46). We conclude that a proportion of CUP-2 is localized to the ER in germ cells.

To better understand the mechanism by which *cup-2* participates in regulating stem cell proliferation, we asked whether *cup-2* activity is required within the germline or the somatic gonad for robust proliferation in *glp-1(gf)* animals. In *rrf-1(pk1417)* mutants RNAi is not efficiently processed in the somatic gonad but is efficiently processed in the germline (75,76). We found that when *cup-2* was knocked down by RNAi in *rrf-1(pk1417); puf-8(q725); glp-1(oz264gf) animals, 63% of animals contained complete tumors as compared to 97% in the *gfp(RNAi)* negative control animals (Fig 6A) (Table 4). Moreover, 16% of the gonads were wild-type (no overproliferation) as compared to no wild-type gonads in the negative control (Fig 6A) (Table 4). Therefore, the overproliferation suppression by *cup-2* RNAi in a *rrf-1* mutant background suggests that *cup-2*’s role in stem cell proliferation takes place in the germline and not the somatic gonad. Although the level of suppression observed with *cup-2* RNAi was less as compared to when using *cup-2* mutants, this is likely due to RNAi only partially reducing CUP-2 activity (Fig 6A) (Table 4).

*cup-2*’s role in retrograde transport may partially contribute to its function in promoting germ cell proliferation

Apart from functioning in ERAD, Derlins also function with SNX-1, a sorting nexin, in the retrograde transport of integral membrane proteins from the endosomes to the Golgi apparatus (37,46). Our analysis of CUP-2’s expression pattern revealed that some CUP-2 was
present outside the ER, in the cytoplasm of germ cells (Fig 5C). It is possible that some of this cytoplasmic CUP-2 associates with the endosomal compartments for retrograde transport and may be responsible for cup-2’s role in promoting stem cell proliferation. Therefore, we asked whether CUP-2’s role in retrograde transport could account for its function in promoting germ cell proliferation. We found that snx-1(tm847) suppresses the tumorous phenotype in some puf-8(q725); glp-1(oz264gf) animals. While 95% of the gonads of puf-8(q725); glp-1(oz264gf) were completely tumorous, 74% of puf-8(q725); glp-1(oz264); snx-1(tm847) were completely tumorous, although none were wild-type (Fig 6B) (Table 5). This level of suppression was much weaker than that observed when cup-2 activity is absent, in which only 14% of the germlines are completely tumorous and 22% were wild-type (Fig 6B) (Table 5). Therefore, we conclude that although retrograde transport may partially contribute to the loss of cup-2 suppressing glp-1(gf) mediated overproliferation, it is unlikely to be the main mechanism by which cup-2 acts in this role. There is an emerging link being established between the retromer complex and Notch signalling in other developmental contexts and the slight tumor suppression that we see in snx-1 mutants could be due to the retromer complex directly affecting the trafficking of the Notch receptor (77–79).

Chemical induction of ER stress suppresses GLP-1/Notch overproliferation, and the ability of cup-2(0) to suppress over-proliferation, requires xbp-1, a key player in the Unfolded Protein Response

We have demonstrated that loss of Derlin activity suppresses glp-1(gf) mediated overproliferation in the C. elegans germ line. Derlin proteins are known to have important
functions in mediating ERAD (38,39,41,72,73,80,81). In the absence of CUP-2 activity misfolded proteins accumulate, resulting in UPR induction (37,38). Previous studies have demonstrated that cup-2 mutants express high levels of hsp-4::gfp, a reporter for XBP-1-dependent UPR activation (37,38). We hypothesized that the induction of ER stress and the UPR could be responsible for the tumor suppression seen in cup-2 mutants. Therefore, we first asked whether inducing ER stress by another means (chemical induction) is sufficient to suppress Notch-dependent overproliferation in a manner similar to when Derlin function is reduced/eliminated.

To induce ER stress, we used two commonly used chemicals, DTT and Thapsigargin (TG), at doses known to induce ER stress in worms (82,83). TG is a specific inhibitor of an ER membrane Ca^{2+}-ATPase. By depleting the calcium stores of the ER, it alters the protein folding environment thereby inducing ER stress. DTT inhibits disulfide bridge formation in proteins, preventing proteins from folding properly and thereby inducing ER stress. We found that both DTT and TG suppress puf-8(q725); glp-1(oz264gf) overproliferation in a dose-dependent manner (Fig 7A), (Table 6) and (Supp Fig 3A), (Supp Table 2). Of the two chemicals, DTT was the stronger suppressor as fewer complete tumors were seen at high DTT doses and some wild-type germlines were also observed, as compared to high TG doses where the reduction in complete tumors was smaller and no wild-type germlines were observed (Fig 7A) (Table 6).

We have demonstrated that Derlin mutants partially suppress GLP-1/Notch-dependent tumours, such as puf-8(q725); glp-1(oz264gf), but not GLP-1/Notch-independent tumours, such as gld-2(0) gld-1(0) tumours (Fig 2 and Fig 3). We tested whether chemical induction of ER stress also specifically suppressed GLP-1/Notch-dependent tumours. We found that DTT
treatment suppressed \textit{glp-1(oz264gf)} and \textit{glp-1(ar202gf)} in a dose-dependent manner at 25°C (Supp Fig 3B)(Supp Table 2). While 28% of the gonads of 0 mM DTT treated \textit{glp-1(oz264gf)} worms were completely tumourous, 17% of 2 mM DTT treated and only 11% of 5 mM DTT treated were completely tumourous (Supp Fig 3B)(Supp Table 2). Similarly, DTT treatment of \textit{glp-1(ar202gf)} worms at 25°C resulted in dose-dependent suppression of tumours with 0 mM DTT treatment producing 26% completely tumourous gonads, while only 15% and 10% completely tumourous gonads were observed for the 2 mM and 5 mM DTT treatments, respectively (Supp Fig 3B)(Supp Table 2). We found that DTT treatment of worms carrying the weaker gain-of-function \textit{glp-1(oz264gf)} allele resulted in more wild-type looking gonads (69%, 82% and 88% wild-type with 0 mM, 2 mM and 5 mM DTT treatments, respectively) rather than protumourous gonads (Supp Fig 3B)(Supp Table 2). The nature of the tumour suppression of the stronger gain-of-function \textit{glp-1(ar202gf)} allele with DTT treatment was more variable and favoured the production of more wild-type gonads at the intermediate 2 mM dose (19% and 34% protumours for 0 and 2 mM DTT, respectively), but at the higher 5 mM dose more protumourous gonads were observed (55%, 51%, 79% Protumours with 0 mM, 2 mM and 5 mM DTT treatments, respectively) background, suggesting that the tumour suppression was weaker in the stronger gain-of-function \textit{glp-1(ar202gf)} DTT treated worms as compared to weaker gain-of-function \textit{glp-1(oz264gf)} DTT treated worms (Supp Fig 3B)(Supp Table 2). However, DTT treatment was unable to suppress GLP-1/Notch-independent \textit{gld-2(0) gld-1(0)} tumours at all (Supp Fig 3C) (Supp Table 3). Therefore, chemical induction of ER stress mimicked the effect of loss of Derlin activity in suppressing overproliferation, suggesting that ER stress and the
consequent induction of the UPR may be the mechanism by which Derlin mutants are able to suppress Notch-dependent tumours.

Previous studies demonstrated that cup-2 mutants are synthetically lethal with ire-1, the sensor of the IRE-1/XBP-1 branch of the UPR (37,43), supporting the suggested role of cup-2 in ERAD. Additionally, other ERAD; ire-1 and ERAD; xbp-1 double mutants fail to induce the UPR (Sasagawa et al. 2007), suggesting dependence on the IRE-1/XBP-1 pathway. To further our understanding of how induction of the UPR suppresses GLP-1/Notch mediated overproliferation we asked if XBP-1 is necessary for the cup-2 mediated suppression of overproliferation. We found that RNAi against xbp-1 in cup-2(tm2838); puf-8(q725); glp-1(oz264) increased the proportion of tumorous worms (Fig 7B) (Table 7). Therefore, this suggests that the suppression of GLP-1/Notch mediated overproliferation by reducing cup-2 activity requires xbp-1 activity and UPR induction.

We have demonstrated that cup-2(0); der-2(0) double mutants suppress GLP-1/Notch tumours more strongly than either single mutant. In addition, a previous study has demonstrated that cup-2(0); der-2(0) mutants more strongly induce the UPR than either single mutant (37). Therefore, we wondered whether further activating the UPR by increasing ER stress in a cup-2(tm2838); puf-8(q725); glp-1(oz264) background could increase the tumour suppression even further. We found that inducing ER stress by DTT treatment in the cup-2(tm2838); puf-8(q725); glp-1(oz264) background increased the tumour suppression to such an extent such that no tumorous worms were observed at the highest DTT dose of 5 mM tested (Fig 7C)(Table 6). This suggests that in cup-2 mutants, although the UPR is activated and is able to suppress GLP-1/Notch dependent tumours, increasing the level of UPR activation even
further results in stronger suppression of overproliferation. Therefore, the UPR is capable of being induced more than that achieved through loss of cup-2 activity, and increasing UPR induction increases the suppression of glp-1(gf) induced overproliferation (Fig 7C).

We wanted to test whether chemical induction of ER stress alone, without disturbing cup-2 activity, was sufficient to suppress GLP-1/Notch signalling levels. We therefore measured the protein levels of the readout for GLP-1/Notch signalling, SYGL-1, in worms treated with DTT. We found that in both wild-type and glp-1(ar202) worms, increasing doses of DTT lead to a more proximal shift of the peak of SYGL-1 expression curve in the germline, accompanied by shortening of the average position of the transition zone (Fig 8) (Supp Fig 4). This proximal shift of the SYGL-1 expression curve is reminiscent of the effect of removing cup-2 activity in glp-1(ar202) gonads (Fig 4B). Taken together, this supports the possibility that induction of ER stress by removal of cup-2 activity and the consequent induction of the UPR may be responsible for suppressing GLP-1/Notch signalling levels and Notch-dependent overproliferation. We further found that knockdown of xbp-1 activity reverses the proximal shift of the SYGL-1 expression curve in cup-2(0); glp-1(ar202) gonads, suggesting that a functional UPR is indeed needed to suppress excessive GLP-1/Notch signalling levels when cup-2 activity is removed (Supp Fig 5).

DISCUSSION

We have shown that Derlin mutants suppress germline tumours caused by gain-of-function mutations affecting the Negative Regulatory Region (NRR) in GLP-1/Notch’s extracellular domain (29,84) and in proportion to the relative strength of the gain-of-function
mutation. The suppression of these tumourous phenotypes is strongest in animals lacking both cup-2 and der-2 function. Derlin mutants do not suppress Notch-independent tumours, even in mutants that have some cells that enter into meiosis, such as the gld-2(0) gld-1(0) Notch-independent tumour, even loss of both cup-2 and der-2 function fail to suppress gld-2(0) gld-1(0) Notch-independent tumours, suggesting that tumorous suppression is specific to Notch signaling. By measuring the expression levels and extent of SYGL-1, a readout for GLP-1/Notch signalling, we found that the extent/region of SYGL-1 expression is compressed in cup-2; glp-1(ar202gf) germlines compared to that of glp-1(ar202gf) germlines, suggesting that the suppression of overproliferation in glp-1(gf) mutants by the reduction of Derlin function is likely achieved by reducing GLP-1/Notch signaling levels.

We found that many of the key features of germline tumour suppression seen in Derlin mutants are phenocopied by chemically inducing ER stress. Consistent with the suppression observed with the reduction of Derlin activity, inducing ER stress suppresses Notch-dependent tumours but not Notch-independent tumours, and reduces the extent of the SYGL-1 expression in glp-1(ar202gf) germlines. We further found that cup-2’s suppression of a puf-8(0); glp-1(oz264gf) Notch-dependent tumour requires xbp-1 activity, which encodes a component of the IRE-1/XBP-1 branch of the Unfolded Protein Response. Moreover, we found that increasing ER stress even further by chemical treatment in cup-2(0); puf-8(0); glp-1(oz264gf) worms results in increased tumour suppression. Our results support a model in which ER stress, in general, counteracts the development of Notch-dependent germline tumours and suggest that the IRE-1/XBP-1 branch of UPR may be one of the arms involved in this counterbalance.
Reduction of Derlin activity reduces Notch signaling in gain-of-function mutants

We have demonstrated that a reduction in Derlin function reduces the amount of overproliferation observed in various glp-1 gain-of-function mutants, but fails to suppress overproliferation due to a loss of gld-1 and gld-2 pathway genes, even in mutants that have a number of cells that enter meiotic prophase. Derlins function, in part, by shuttling misfolded proteins from the ER to the cytosol for degradation, as part of ERAD (39,41,72,81,85–89). Therefore, the suppression of germline overproliferation in cup-2 and der-2 mutants is likely the result of an accumulation of unfolded or misfolded proteins. There are likely many models as to how this accumulation could reduce the level of GLP-1/Notch signaling and the level of overproliferation. For example, this accumulation could cause a core member of the Notch signaling pathway, or a positive regulator of Notch signaling, to not function properly. However, if a reduction in Derlin function were to interfere with a core member or a positive regulator of GLP-1/Notch signaling, then we would expect to have seen a reduction in GLP-1/Notch signaling, as determined by SYGL-1 accumulation, when Derlin function was reduced in an otherwise wild-type background. However, we only see dramatic reduction of Notch signaling in glp-1 gain-of-function mutants.

It is intriguing that proper protein folding of the Notch receptor has been shown to be important for the function of the NRR (Negative Regulator Region), where many of the known Notch gain-of-function mutations cluster (28,29,90–92). Folding in this region is thought to protect the S2 cleavage site, allowing it only to be accessed upon ligand binding (93–95). Improper folding caused by mutations in this region are thought to allow for ligand-independent cleavage and activation (94,96). Could reduction in ERAD affect the folding of the
GLP-1/Notch receptor, including the NRR region, and thereby affect the level of Notch signaling? We have demonstrated that a reduction in Derlin activity reduces the increased level of Notch signaling achieved by glp-1 gain-of-function mutants. Obviously, GLP-1/Notch proteins harbouring gain-of-function mutations must not be misfolded to a degree in which all such proteins are detected by the ERAD system and degraded, otherwise they would not be available for ligand independent signaling. However, perhaps some proportion of the gain-of-function proteins are misfolded to the point of being detected by the ERAD system and targeted for degradation. When Derlin activity is reduced, these and other misfolded proteins may accumulate in the ER, causing ER stress and the activation of UPR. UPR activation causes the initiation of signaling pathways meant to reduce ER stress, including enhancing protein folding and degradation (97). Therefore, it is possible that activation of UPR could result in enhanced protein folding of key factors in the Notch signaling pathway, or increased degradation of misfolded proteins, perhaps including the aberrant gain-of-function form of the GLP-1/Notch receptor itself. Indeed, enhanced protein folding could result in the NRR region properly folding, even if harbouring a gain-of-function mutation, thereby mimicking a more wild-type form of the protein in terms of activity. Indeed, the glp-1(ar202) gain-of-function mutation that we demonstrated was suppressed most strongly by a reduction in Derlin activity does still have some ligand-dependence (98), suggesting that either the NRR domain in these proteins is not as misfolded as in other gain-of-function mutants, or that the proportion of misfolded proteins is lower. In either situation, enhancement of proper NRR folding would suppress the increase in GLP-1/Notch signaling levels. Alternatively, it is possible that UPR induction enhances the recognition of GLP-1 gain-of-function proteins as aberrantly folded, leading to enhanced
degradation of the receptor. Enhanced degradation would reduce the overall levels of GLP-1 receptors, thereby resulting in a decrease in signalling levels. It is also possible that the models of enhanced degradation and/or enhanced folding could apply to a regulator of GLP-1/Notch signalling that interacts with the NRR of the GLP-1 receptor; however, we consider this less likely given our understanding of how other components of the ERAD machinery interact with Notch signalling, as explained below.

ERAD, UPR and Notch signaling

The Derlins are not the first components of the ERAD machinery to be identified as interacting with Notch signalling in *C. elegans*, perhaps hinting at some inherent sensitivity of this signalling pathway to ERAD-driven measures for protein quality control. SEL-11 and SEL-1, homologs of the mammalian HRD1 and SEL1 ERAD components (99,100), were initially identified in a screen for suppressors of the egg-laying defective phenotype (Egl) of a partial loss-of-function allele of *lin-12* (101), which, like *glp-1*, encodes a homologue of the Notch protein (102–104). SEL-11/HRD1/Hrd1p is an E3 ubiquitin ligase that ubiquitinates misfolded or unfolded proteins, while SEL-1/SEL1L/Hrd3p interacts with SEL-11/HRD1/Hrd1 (10,72,88,105–108). In *C. elegans*, *sel-1* and *sel-11* mutations were also found to suppress the maternal effect embryonic lethality phenotype of *glp-1* (*e2142*), a partial loss-of-function allele, suggesting that *sel-1* and *sel-11* interact with Notch signalling rather than with processes regulated by Notch (101,109). Importantly, loss of *sel-1* and *sel-11* strongly suppresses *lin-12* partial-loss-of-function alleles, but do not suppress a *lin-12* null, suggesting that the LIN-12/Notch receptor must be present, even in a mutant form, in order for suppression to occur.
While sel-1 and sel-11 suppress glp-1 and lin-12 partial loss-of-function alleles, and therefore are thought to be negative regulators of Notch signaling, we have demonstrated that reduction in Derlin activity, through cup-2 and/or der-2 mutations, results in suppression of glp-1 gain-of-function alleles, and has little if any effect on glp-1 partial loss-of-function alleles, suggesting that the Derlins are positive regulators of Notch signaling.

Derlins are thought to complex with SEL-11/HRD1 and SEL-1/SEL1L to facilitate transport of misfolded proteins from the ER to the cytosol, where they would be degraded by the proteasome (72,110–112). A recent study in yeast, suggests that Der1 and Hrd1 form two “half-channels” to allow ERAD substrates to move through the ER membrane (112). Therefore, it would be anticipated that a reduction of Derlin function would have the same overall consequence as a reduction in SEL-1/SEL1 and SEL-11/HRD1 function in reducing the ability of unfolded or misfolded proteins to be degraded. However, we have demonstrated that reduction of Derlin activity has an opposite effect on Notch signalling as that described for SEL-1/SEL1 and SEL-11/HRD1 (100,101). This may reveal that, at least in some contexts, the SEL-11/HRD1 complex and Derlins may have opposite effects on ERAD, or perhaps that removing individual components of the complex leads to different compensatory mechanisms with opposing outcomes on Notch as a substrate.

However, we believe this apparent contradiction may be resolved by considering the folding properties of the mutant Notch alleles in which sel-1 and sel-11 have been characterized. Perhaps the glp-1 and lin-12 partial loss-of-function mutants used in these studies are also recognized by the ERAD machinery as misfolded. If so, then upon the loss of ERAD factors, the UPR would be induced and these mistakenly folded receptors could be
restored to wild-type receptor activity levels through the enhanced degradation and/or enhanced folding mechanisms proposed above. Such a model also accommodates the need for some mutant form of the Notch receptor to be present for the sel-1 and sel-11 mutants to be able to exert their influence on Notch signalling (101,109). Therefore, we speculate that loss of ERAD factors leads to correction of aberrantly folded mutant Notch receptors, since the UPR does not differentiate between whether a receptor is gain-of-function or loss-of-function in activity, but instead simply recognizes them as being misfolded and needing correction. Some support for such a model in which the UPR serves as a quality control mechanism for aberrant Notch folding and signalling, exists amongst studies using Drosophila. While ectopic induction of ER stress alone has no effect on wildtype Notch receptor localization or levels, upregulation of the UPR or overexpression of a Notch-specific chaperone in a Notch-deficient mutant can restore Notch signalling levels in Drosophila (113,114).

The extracellular portion of the GLP-1/Notch receptor may be more sensitive to ERAD defects

We recognize that the proposed model of the UPR as a quality control mechanism to correct aberrant Notch receptors would not apply to all mutant forms of Notch receptors, as we were unable to see enhancement of the glp-1(bn18) loss-of-function Glp phenotype in cup-2 mutants, and the sel-1 and sel-11 mutants similarly fail to suppress the two reduction-of-function alleles glp-1(q231) and glp-1(e2144) (101). However, it is intriguing that glp-1(bn18), glp-1(q231) and glp-1(e2144) mutations all affect the intracellular portion of the GLP-1 receptor (56), while the glp-1(ar202), glp-1(ar224), glp-1(oz264) mutations, with which cup-2 interacts, all affect the extracellular portion of the GLP-1 receptor (29,84). Similarly, glp-1(e2142), the
partial loss-of-function allele with which \textit{sel-1} and \textit{sel-11} interact, also affects the extracellular portion (56,101). Therefore, we speculate that the extracellular portion of the GLP-1/Notch receptor may be more sensitive to defects in components of the ERAD machinery than the intracellular portion. This may suggest that ERAD/UPR specifically acts at the level of GLP-1/Notch receptor activation.

**Derlins as a Therapeutic Target**

Dysregulation of Notch signaling has been implicated in the development of many diseases, including certain cancers (115). While some cancers are associated with loss-of-function mutations in the Notch receptor, others, such as Triple-Negative Breast Cancer, correlate with gain-of-function mutations, including those due to mutations in the NRR (115,116). In fact, more than 50\% of cases of human T cell acute lymphoblastic leukemia (T-ALL) are associated with activating mutations in NOTCH1, with the majority of these affecting the NRR (91). Therefore, inhibition of Notch signaling is being actively pursued as a possible treatment for certain cancers (117). However, since the Notch signaling pathway is utilized in the development and maintenance of so many tissues throughout the body, Notch inhibitors can have detrimental effects, such as intestinal toxicity (117). Here we demonstrate that reduction in Derlin activity suppresses GLP-1/Notch gain-of-function mutants, but has little effect on normal Notch signaling as measured by SYGL-1 expression in \textit{cup-2} mutants. Therefore, targeting Derlin activity in individuals suffering from diseases caused by Notch gain-of-function mutations, perhaps particularly those mutations that affect the extracellular portion of Notch,
may provide a means to specifically target the aberrant Notch gain-of-function allele without causing adverse side effects associated with reducing systemic Notch signalling levels.

MATERIALS AND METHODS

Strains and genetics

All the strains used in this study were maintained at 20°C unless otherwise indicated. The wildtype strain used was the N2 Bristol strain. Supplemental Table 4 lists all the strains and alleles used in this study.

Worm synchronization

Gravid adults were washed off of plates with PBS and washed three times with PBS to remove excess bacteria. Samples were then incubated with freshly prepared bleach solution (20% sodium hypochlorite, 50mM NaOH) for 4-5 minutes with vigorous vortexing. Excess bleach was washed off by washing the egg pellet with PBS three times. Eggs were allowed to hatch in PBS at 20°C for 1-2 nights to obtain a synchronized population of L1 larvae.

Dissections and immunostaining

Unless indicated otherwise in the figure legend, all phenotypes were analyzed in dissected gonads. About 150 synchronized adults were picked for dissections and dissected as previously described (59). Dissected gonads were fixed in 3% paraformaldehyde for 10 minutes at room temperature, post-fixed using 100% methanol and kept at -20°C for at least one night. Samples were then rehydrated by washing with PBT (PBS + 0.1% Tween-20) three times and
blocked in 3% BSA at 4°C for at least one hour. Primary antibodies used were α-REC-8 rat (1:200) (53), α-HIM-3 rabbit (1:750) (54), α-FLAG mouse M2 (1:1000) Sigma #F1804, α-V5 mouse (1:2000) Invitrogen #R960-25, α-V5 rabbit (1:1000) Cell Signaling Technology #D3H8Q and α-GFP mouse (3E6) (1:750) Molecular Probes #A11120. Primary antibodies were diluted in 3% BSA and incubated for at least 1 hour at room temperature. Secondary antibodies used were: α-Rat Alexa 488 (1:200) Molecular Probes #A21208, α-Rabbit Alexa 594 (1:500) Molecular Probes #A21207 and α-mouse Alexa 488 (1:200) Molecular Probes #A21202. Secondary antibodies were diluted in 3% BSA and incubated for at least 2 hours at room temperature. Samples were washed three times with PBT with 5 min incubations, after primary and secondary antibody incubations to remove excess unbound antibodies. For visualizing nuclei, samples were incubated with 100ng/mL DAPI (4’, 6- diamidino-2- phenylindole dihydrochloride) diluted 1:1000 in PBS for 5 minutes. Stained gonads were visualized by mounting on a 1% agarose pad.

Image Acquisition

All images were acquired on a Zeiss Imager Z.1 microscope fitted with an AxioCam MRm camera using AxioVision 4.8.2.0 software. Z stacks were taken at 1µm intervals. Confocal images were taken on a Leica SP5 laser confocal microscope. For experiments measuring SYGL-1 levels, distal ends of gonads were focused in the middle focal plane, as much as possible, for image acquisition.

For determining CUP-2 localization by comparing α-V5 staining intensity in XB681 cup-2(ug1) vs N2 dissected gonads, the two strains were fixed, permeabilized and blocked
independently and in parallel. The XB681 sample was incubated with $\alpha$-HIM-3 antibody while the N2 sample was kept in block overnight. This helped mark the XB681 gonads so that they can be easily distinguished when mixed with the N2 sample. The next day, the XB681 sample was washed three times with PBT and mixed with the N2 sample so that subsequent processing on the two samples could be done in the same tube to enable a direct comparison. The mixed sample was incubated with $\alpha$-V5 mouse overnight. The next day the sample was processed with secondary antibodies as described above and mounted on the same pad. Exposure measurements were taken in three positions across the slide and the average exposure was set as the exposure for acquisition of images across the whole slide. Therefore, both XB681 and N2 samples were imaged with the same exposure settings.

**Image Processing and Analysis**

Fiji (Fiji is Just ImageJ) was used to process and analyze images (118,119). Brightness and contrast adjustments, and the addition of scale bars was done in Fiji. Images were cropped and organized into figure panels using Adobe Illustrator. For stitching a complete gonad picture, the stitching plugin in Fiji was used (120). For counting the total number of cells in the proliferative zone, Z stack images of the distal ends of gonads were manually analyzed using the Cell Counter plugin (121).

Statistical analysis and plotting was done in Python using Seaborn, a visualization library based on the 2D graphics package, Matplotlib (122,123). t-tests (independent) were performed using the statannot package (124).
**SYGL-1 Intensity Measurements**

For an experiment, the strain of interest was dissected in parallel with WU1770 *sygl-1*(*am307*) as an internal positive control. The two strains were fixed, permeabilized and blocked independently. The WU1770 dissected samples were incubated with α-HIM-3 antibody while the other sample was left in block overnight. The next day, the WU1770 sample was washed three times with PBT and the two samples were pooled for subsequent processing with α-FLAG antibody, secondary antibody, DAPI and mounting.

For each experiment, the ideal exposure settings for each channel was determined by sampling three germlines of each genotype across the slide using autoexposure and then using the average exposure measurement as the setting for acquiring images for that slide. Germlines were focussed in the central plane, through the middle of the rachis for acquiring images. For analyses, pictures of 15 gonads of each genotype were analyzed. The images were opened on FIJI and a segmented line was drawn in the middle of the germline starting from the distal-most germ cell to the most proximal cell in the field of view to generate a plot profile for each germline. The values from the plot profile were copied into excel, subsequent analyses i.e., calculation of the average intensity of each genotype, standard deviation, normalization and plotting were done using Seaborn. For comparing multiple genotypes against each other, normalization was performed to the average WU1770 plot profiles for these experiments. For example, when comparing WU1770, XB709, XB710 and XB711 genotypes against each other, the ‘scale average’ was calculated as the average WU1770 plot profile of these three experiments (namely XB709 vs WU1770, XB710 vs WU1770 and XB711 vs WU1770). To obtain the scaling factor for XB709, the ‘scale average’ was divided by the average WU1770 plot profile.
for the XB709 experiment. The scaling factor for XB709 was then multiplied by the plot profile for XB709 to normalize it. The same was repeated for the XB710 and XB711 experiments to normalize their plot profiles. The normalized plot profiles of XB709, XB710 and XB711 were plotted on the same graph along with the ‘scale average’ to represent the average WU1770 intensity across these three experiments. In order to more easily contrast the plot profiles against each other, the plot profiles were fitted to a sixth-order polynomial to generate the smoothened plots seen in Figure 4B. The raw, non-normalized plots for the three experiments is shown in Supplemental Figure 1A-C. Since standard deviations could not be normalized, the standard deviation shown in Figure 4B is the same as that shown in Supplemental Figure 1A-C.

For determining the distance to the transition zone, a new line was drawn from the distal end to the first transition zone cells which were discernable by their crescent-shaped DAPI stained nuclei. Since the pictures were acquired in the central Z plane which has fewer nuclei, transition zones for all gonad pictures was difficult to determine. However, transition zones for at least seven images for each genotype was determined. The average distance to transition zone for each genotype was calculated in Excel and an arrowhead was drawn in Adobe Illustrator to indicate the average position on the plot. By measuring the distance to transition zone and using the cell counter plug in in FIJI for counting the number of germ cell diameters to transition zone for one WU1770 image, it was determined that one germ cell diameter (g.c.d.) approximately corresponded to 2.833 microns. This formula was applied to the distance in microns axes in the plots in Seaborn to convert the distance in microns to g.c.d. and a second X axis indicating the approximate distance in g.c.d. was generated as a reference.
CRISPR/Cas9 editing

Two guide RNA sequences towards the 3’end of the cup-2 coding region were selected manually by scanning for nearby NGG sites and running the seed sequence through NCBI BLAST to check that it was not predicted to match any other region of the genome. MfeI and XbaI restriction enzyme sites flanked 320bp fragments containing the sgRNA sequence were synthesized by Eurofins MWG Operon as per a previous study (67). The plasmid was cut by MfeI and XbaI restriction enzymes and the 320bp fragment was cloned into a PU6::sgRNA vector derived from Addgene plasmid #46169, as per a previous study (67). This generated PU6::cup-2_sgRNA#4 (pDH386) and PU6::cup-2_sgRNA#5 (pDH387) vectors for injections. These sgRNA plasmids were each used at the recommended 50ng/µl final concentration for injections (67).

For the repair template, a 258bp DNA fragment was designed containing a 90bp sequence corresponding to a codon optimized V5::2XFLAG tag placed upstream of the endogenous cup-2 stop codon and flanked by homology sequences, based on recommendations for PCR directed repair templates by a previous study (68). A silent point mutation (CAA to CAg) was introduced one base pair upstream of the PAM sequence recognized by sgRNA#5 to prevent potential re-cutting as advised by previous studies (68–70). This repair template was synthesized by Eurofins MWG Operon to generate plasmid pDH373. For injections, PCR using E10 (5’-ATCAGAGGACGCAGCA-3’) and E11 (5’-AGGAAAAAGGAATAAATTA-3’) primers on pDH373 as a template generated a smaller fragment that was cleaned up by QIAGEN MinElute PCR Purification Kit (#28004). The PCR fragment was used at 72ng/µl final concentration for injections. All other components of the injection mixture were used as previously described for co-conversions (69). All plasmids used
for injections were purified using QIAGEN Plasmid Midi Kit #12143. The *dpy-10* targeting sgRNA, pJA58 (Addgene #59933), was used at a final concentration of 25ng/µl. The *dpy-10*(cn64) oligo repair template was synthesized by IDT as 4nm Ulramer DNA Oligo and was used at 0.5µM final concentration for injections. The Cas9 expressing plasmid, pDD162 (Addgene #47549), was used at a final concentration of 50ng/µl (71). Injected worms were placed on individual plates after injections and Dpy and/or Roller progeny were cloned individually and screened by PCR for insertions.

**dsRNA injections for RNAi**

1000ng of a miniprep of full-length *cup*-2 cDNA cloned into pL4440 and GFP cloned into pL4440 was used to PCR amplify dsDNA using a T7 primer named HB61 (5’ TAATACGACTCACTATAGG 3’) and NEB OneTaq DNA Polymerase. 3.8µg of dsDNA was used as a template for *in vitro* transcription using NEB T7 polymerase and RNAseOUT inhibitor at 37°C overnight as per manufacturer’s instructions. This yielded ~1000ng/µL of *cup*-2 dsRNA and ~850ng/µL of *gfp* dsRNA that was used for RNAi injections after checking integrity of the RNA product by running it on a gel. One day past L4 XB737 worms were injected and their non-green progeny were analyzed by dissections and immunostaining.

**Feeding RNAi and whole mount DAPI**

Feeding RNAi was performed as per standard procedures (125). NGM plates supplemented with 100µg/mL Ampicillin and 1mM IPTG were prepared as per standard procedures (126). The bacterial clones obtained from the Ahringer RNAi library were verified by
sequencing and working stocks of verified clones were used for seeding RNAi plates. The bacteria were grown overnight in LB supplemented with 100µg/mL Ampicillin, seeded on to RNAi plates and allowed to grow for 2 days at room temperature. For each batch of RNAi experiments, a positive and negative control (empty vector) were included for comparison.

Synchronized L1s were obtained as above and were placed on plates at the desired temperature. Adult animals were analyzed 72 hours later by whole mount DAPI. Briefly, adult animals were washed off plates in PBS and fixed in methanol at -20°C overnight. Fixed animals were washed once in PBS then stained in 100ng/mL DAPI in PBS for 5 minutes, washed twice more, then mounted on 1% agarose pads for microscopy.

Drug treatment assays

Worm preparation and analyses for DTT and Thapsigargin (TG) experiments were performed as for RNAi experiments described above. NGM plates containing the respective concentrations of drugs were made by adding drugs (or an equivalent amount of DMSO for no drug control plates) to the standard NGM agar recipe. DTT and Thapsigargin concentrations used were as previously described (82,83).

ACKNOWLEDGEMENTS

The original strain containing the *glp-1(ar224)* allele was a gift from Jane Hubbard. We thank Tim Schedl for the strain containing *sygl-1(am307)*. Strains FX02838 *cup-2(tm2838)* and FX06098 *der-2(tm6098)* were provided by the National BioResource Project (NBRP), Japan. Some strains were provided by the CGC, which is funded by NIH Office of Research
Infrastructure Programs (P40 OD010440). The HIM-3 antibody was a gift from Monique Zetka. The REC-8 antibody was a gift from Pavel Pasierbek and Joseph Loidl. We thank Marcus Samuel for use of the confocal microscope. We thank members of the Hansen lab, Paul Mains, Jim McGhee and Tim Schedl for helpful discussions. This work was funded by grants from Canadian Institutes of Health Research (CIHR) (https://cihr-irsc.gc.ca) and the Natural Sciences and Engineering Research Council of Canada (NSERC) (https://www.nserc-crsng.gc.ca) to Dave Hansen. Hilary Racher was partially funded by a Queen Elizabeth II graduate scholarship and a CIHR training grant in Genetics, Child Development & Health.

AUTHOR CONTRIBUTIONS


COMPETING INTERESTS

The authors declare no competing interests.
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Fig 1. Derlin mutants suppress Notch-dependent tumours in correlation with the strength of GLP-1/Notch signalling
A. Representative images of wild-type (WT), protumorous (Pro) and completely tumorous (Tum) phenotypes as visualized by α -REC-8(green) and α -HIM-3(red) immunostaining. DAPI staining was used to visualize nuclei. Wild-type (WT) is defined as a gonad arm with distal anti-REC-8(+) cells followed by anti-HIM-3(+) cells and presence of both sperm and oocytes in the proximal arm of the gonad. A protumour is defined as a gonad arm containing both anti-REC-8(+) and anti-HIM-3(+) cells, but not differentiated sperm and oocytes. Proximal tumourous gonad arms with only sperm but no oocytes were counted as protumours. Gonad arms with mostly only anti-REC-8(+) and a few anti-HIM-3(+) positive cells were also counted as protumourous. Complete tumour is defined as a gonad arm that contains only anti-REC-8(+) cells and no anti-HIM-3(+) cells. Both tumorous phenotypes are of the cup-2(tm2838); puf-8(q725); der-2(tm6098) glp-1(ar202) genetic background. Asterisk, distal tip. Scale bar = 20µm.
B. Quantification of phenotypic analysis of the indicated genotypes. In this and all other bar graphs, numbers on the top of the bars indicate the number of dissected gonads analyzed by α -REC-8(green) and α -HIM-3(red) immunostaining.

Fig 2. Derlin mutants have smaller proliferative zone sizes
A. Representative images of distal germlines of wildtype(WT), cup-2(tm2838); der-2(tm6098), glp-1(ar202) and cup-2(tm2838); der-2(tm6098) glp-1(ar202) worms stained by DAPI to visualize nuclei. Asterisk, distal tip. Dashed line, transition zone. Scale bar = 10µm.
B. cup-2(tm2838) suppresses the proliferative zone sizes of glp-1(bn18), glp-1(ar224) and glp-1(oz264) germlines. Total number of cells in the proliferative zone of the indicated genotypes.
C. Derlin mutants additively suppress the size of the proliferative zone of WT and glp-1(ar202) germlines. Total number of cells in the proliferative zone of the indicated genotypes. In this and subsequent scatter plots, thick line = mean; error bars = standard deviation; ns = 0.05 < p ≤ 1 ; * = 0.01 < p ≤ 0.05, ** = 0.001 < p ≤ 0.01, *** = 0.0001 < p ≤ 0.001, **** = p ≤ 0.0001.

Fig 3. Derlin mutants do not suppress Notch-independent tumours
A. Representative images of germlines of indicated genotypes visualized by α -REC-8(green) and α -HIM-3(red) immunostaining. DAPI staining was used to visualize nuclei. No nuclei stained with meiotic entry marker HIM-3, were detected in both genotypes. Asterisk, distal tip. Scale bar = 20µm.
B. Quantification of phenotypic analysis of the indicated genotypes analyzed by α-REC-8 and α-HIM-3 immunostaining.

C. Representative images of germlines of indicated genotypes visualized by α-REC-8 (green) and α-HIM-3 (red) immunostaining. DAPI staining was used to visualize nuclei. Nuclei stained with meiotic entry marker HIM-3, were detected in the distal arm in both genotypes but not in the proximal arm of the gonad. Asterisk, distal tip. Scale bar = 20µm

D. Quantification of phenotypic analysis of the indicated genotypes analyzed by α-REC-8 and α-HIM-3 immunostaining.

Fig 4. Loss of cup-2 decreases SYGL-1 protein levels in glp-1(ar202) germlines

A. Representative images of SYGL-1 protein expression observed in sygl-1(am307); glp-1(ar202) and cup-2(tm2838) sygl-1(am307); glp-1(ar202) genetic backgrounds by α-FLAG immunostaining. The sygl-1(am307) allele represents a 3XFLAG tagged version of endogenous SYGL-1. DAPI staining was used to visualize nuclei. Asterisk, distal tip. Scale bar = 10µm

B. Normalized, fitted average SYGL-1 intensities measured by α-FLAG immunostaining of the indicated genotypes, each harbouring the sygl-1(am307) allele. Shaded areas indicate unscaled fitted standard deviation of the intensity measurements for each genotype. Average normalized intensities and standard deviations were fit to a sixth order polynomial. Fifteen gonads were analyzed for intensity measurements. Arrowheads point to the average location of the transition zone measured in at least seven gonads of each genotype. Distances from distal end (DE) were measured in microns and converted to germ cell diameters (g.c.d) as a reference, by assuming 1 g.c.d. = 2.833 microns. WT intensity measurement shown is the average sygl-1(am307) intensity across the three experiments used for scaling.

Fig 5. CUP-2 is expressed in the proliferative zone of the germline and co-localizes with SP12, an ER marker

A. CUP-2 expression visualized by α-V5 mouse immunostaining in the germline of cup-2(ug1), a V5::2XFLAG tagged version of cup-2. DAPI staining was used to visualize nuclei. Asterisk, distal tip. Scale bar = 20µm

B. CUP-2 expression in the distal region of germline of cup-2(ug1) visualized by α-V5 mouse immunostaining in comparison to wild type untagged worms. Both genotypes were processed together and imaged on the same slide at the same intensity to enable comparison, except that cup-2(ug1) were preincubated α-HIM-3 antibodies to distinguish them from wild-type germlines. DAPI staining was used to visualize nuclei. Asterisk, distal tip. Scale bar = 10µm.

C. Confocal images of CUP-2 expression in cup-2(ug1); ojis23[GFP::SP12] germline visualized by α-V5 rabbit immunostaining in distal and loop regions of the germline. Expression of ER marker SP12 was visualized by α-GFP mouse immunostaining. Images
were acquired using a confocal microscope. Arrowheads point to regions of colocalization. Asterisk, distal tip. Scale bar = 10µm

Fig 6. *cup*-2 activity partially requires retrograde transport and is required mainly within the germline to promote germ cell proliferation

A. Quantification of phenotypic analysis of the effect of loss of *snx*-1, *cup*-2's partner in retrograde transport, on suppression of *puf*-8(*q725)*; *glp*-1(*oz264)* tumours. Phenotypes were analyzed by α-*REC*-8 and α-*HIM*-3 immunostaining.

B. Quantification of phenotypic analysis of the effect of knockdown of *cup*-2 activity within the germline by using a *rrf*-1(*pk1417)* mutant background which largely restricts RNAi efficacy to the germline. *cup*-2 RNAi and *gfp* RNAi was performed by injection of dsRNA. *puf*-8(*q725)*; *glp*-1(*oz264)* and *cup*-2(*tm2838)*; *puf*-8(*q725)*; *glp*-1(*oz264)* phenotypic analysis is included for reference. Phenotypes were analyzed by α-*REC*-8 and α-*HIM*-3 immunostaining.

Fig 7. Chemical induction of ER stress also suppresses Notch-dependent tumours and suppression of tumours by loss of *cup*-2 activity requires *xbp*-1, a key player in the Unfolded Protein Response

A. Quantification of phenotypic analysis of the effect of increasing doses of DTT and Thapsigargin (TG) on suppression of *puf*-8(*q725)*; *glp*-1(*oz264)* tumours. We hypothesize that if ER stress induced by abrogating *cup*-2 activity is responsible for the suppression of the tumour, then inducing ER stress by chemical means should also produce a similar effect. Phenotypes were analyzed by whole mount DAPI staining.

B. Quantification of phenotypic analysis of the effect of *xbp*-1 knockdown by RNAi on *puf*-8(*q725)*; *glp*-1(*oz264)* tumours. We hypothesize that if activation of the branch of the UPR is involved in the response to ER stress and suppression of tumours in *cup*-2(*tm2838)* worms, then knocking down *xbp*-1 function in *cup*-2(*tm2838)*; *puf*-8(*q725)*; *glp*-1(*oz264)* would be expected to reverse the tumour suppression seen back to more tumourous gonads. RNAi was performed by feeding. RNAi on *puf*-8(*q725)*; *glp*-1(*oz264)* was performed as a control. Phenotypes were analyzed by whole mount DAPI staining.

C. Quantification of phenotypic analysis of the effect of inducing ER stress by DTT treatment on *cup*-2(*tm2838)*; *puf*-8(*q725)*; *glp*-1(*oz264)* tumours. We hypothesize that if either DTT or loss of *cup*-2 function can induce ER stress and suppress the tumours, then the combination of the two would enhance the suppression. Phenotypes were analyzed by whole mount DAPI staining.

Fig 8. Chemical induction of ER stress by DTT reduces the spread of SYGL-1, a readout for GLP-1/Notch signalling in *glp*-1(*ar202)* germlines

A. Normalized, fitted average SYGL-1 intensities of otherwise wild type gonads harbouring the *sygl*-1(*am307)* allele grown on DTT. Shaded areas indicate unscaled fitted standard deviation of the intensity measurements for each genotype. Average normalized intensities and standard deviations were fit to a sixth order polynomial. Fifteen gonads were analyzed for intensity measurements. Arrowheads point to the average location of...
the transition zone measured in at least seven gonads of each genotype. Distances from distal end (DE) were measured in microns and converted to germ cell diameters (g.c.d) as a reference, by assuming 1 g.c.d. = 2.833 microns.

B. As in (A) but including the glp-1(ar202) allele.

Supplementary Figures

Supp Fig 1. Raw, average CUP-2 intensity measurements to compare CUP-2 levels in WT vs. puf-8(q725) genetic backgrounds at 25°C. (A). CUP-2 immunostaining (by α-V5) in wild-type (WT) and puf-8(q725) dissected gonads. Both strains contain the cup-2(ug1[V5::2XFLAG::CUP-2]) allele. Scale bar = 10µm. (B) CUP-2 intensities measured by α - V5 immunostaining by drawing a line through the center of the germline from the distal end along the distal-proximal axis. Shaded area represents the standard deviation of average intensity measurements of each genotype. Fifteen germlines were analyzed for CUP-2 intensity measurements of each genotype. Arrowheads point to the average location of the transition zone measured in at least seven gonads of each genotype. Dashed line represents the predicted peak of PUF-8 expression, based on previous work that found that PUF-8’s expression pattern is a bell-shaped curve centered around the transition zone with low expression levels in the distal end (Racher and Hansen 2012). While we find that the overall CUP-2 expression levels are higher in puf-8(q725) gonads compared to wild type gonads, since this increase does not correlate with the known expression pattern of PUF-8 in wild type gonads, CUP-2 levels are unlikely to be directly regulated by PUF-8.

Supp Fig 2. Raw, unscaled average SYGL-1 intensity measurements of individual experiments to compare SYGL-1 intensity in glp-1(ar202) genetic backgrounds. SYGL-1 intensities were measured by α - FLAG immunostaining by drawing a line through the center of the germline from the distal end along the distal-proximal axis of the indicated genotypes. Each subfigure indicates an individual experiment comparing two genotypes that were processed together and imaged on the same slide with the same exposure setting. A-C measurements were used to generate scaled, fitted intensity curves shown in Fig 4B. Shaded area represents the standard deviation of average intensity measurements of each genotype. Fifteen germlines were analyzed for SYGL-1 intensity measurements of each genotype. Arrowheads point to the average location of the transition zone measured in at least seven gonads of each genotype.

A. Average SYGL-1 intensity comparison of sygl-1(am307) against cup-2(tm2838) sygl-1(am307) germlines
B. Average SYGL-1 intensity comparison of sygl-1(am307) against sygl-1(am307); glp-1(ar202) germlines
C. Average SYGL-1 intensity comparison of sygl-1(am307) against cup-2(tm2838) sygl-1(am307); glp-1(ar202) germlines
D. Average SYGL-1 intensity comparison of sygl-1(am307); glp-1(ar202) against cup-2(tm2838) sygl-1(am307); glp-1(ar202) germlines

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**Supp Fig 3. Effect of DTT treatment on weak Notch-dependent tumours and Notch-independent tumours**

A. Suppression of puf-8(q725); glp-1(oz264) tumours by DTT treatment. Phenotypes were analyzed by dissections followed by REC-8/HIM-3 staining.

B. Quantification of phenotypic analysis of the effect of increasing doses of DTT on glp-1(oz264) and glp-1(ar202) tumours at 25°C. Phenotypes were analyzed by whole mount DAPI.

C. Quantification of phenotypic analysis of the effect of increasing doses of DTT on Notch-independent gld-2(q497) gld-1(q485) tumours. Phenotypes were analyzed by whole mount DAPI.

**Supp Fig 4. Raw, unscaled average SYGL-1 intensity measurements of individual experiments to compare SYGL-1 intensity in wildtype and glp-1(ar202) genetic backgrounds with induction of ER stress by DTT treatment.** SYGL-1 intensities were measured by α-FLAG immunostaining by drawing a line through the center of the germline from the distal end along the distal-proximal axis of the indicated treatments. Each subfigure indicates an individual experiment comparing two treatments that were processed together and imaged on the same slide with the same exposure setting. A-B measurements were used to generate scaled, fitted intensity curves shown in Fig 8A. C-D measurements were used to generate scaled, fitted intensity curves shown in Fig 8B. Shaded area represents the standard deviation of average intensity measurements of each treatment. Fifteen germlines were analyzed for SYGL-1 intensity measurements of each treatment. Arrowheads point to the average location of the transition zone measured in at least seven gonads of each treatment.

A. Average SYGL-1 intensity comparison of 0 DTT treated against 2 DTT treated sygl-1(am307) germlines

B. Average SYGL-1 intensity comparison of 0 DTT treated against 5 DTT treated sygl-1(am307) germlines

C. Average SYGL-1 intensity comparison of 0 DTT treated against 2 DTT treated sygl-1(am307); glp-1(ar202) germlines

D. Average SYGL-1 intensity comparison of 0 DTT treated against 5 DTT treated sygl-1(am307); glp-1(ar202) germlines

**Supp Fig 5. Raw, unscaled average SYGL-1 intensity measurements comparing xbp-1 RNAi treated RNAi germlines against empty vector control RNAi treated germlines of cup-2(tm2838) sygl-1(am307); glp-1(ar202).** SYGL-1 intensities were measured by α-FLAG immunostaining by drawing a line through the center of the germline from the distal end along the distal-proximal axis of the indicated treatments. Shaded area represents the standard
deviation of average intensity measurements of each treatment. At least twelve gonads were analyzed for SYGL-1 intensity measurements of each treatment.
Figure 1

A

WT

Protumour

Complete Tumour

α-REC-8
α-HIM-3

DAPI

B

% Phenotypes

>50 50-90 100

0 20 40 60 80

WT

cup-2(tm2388)
cup-2(tm2388); pul-8(q725); pul-8(q725)
cup-2(tm2388); pul-8(q725); pul-8(q725); pul-8(q725)
cup-2(tm2388); pul-8(q725)
Figure 3

A

\[ \text{gld-3(q730) nos-3(q650)} \quad \text{cup-2(tm2838); gld-3(q730) nos-3(q650)} \]

\( \alpha \text{-REC-8} \quad \alpha \text{-HIM-3} \)

B

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<th>REC-8(+), HIM-3(+)</th>
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<td></td>
</tr>
<tr>
<td>111</td>
<td>[Green bar]</td>
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</table>

\[ \text{gld-3(q730) nos-3(q650)} \quad \text{cup-2(tm2838); gld-3(q730) nos-3(q650)} \]

C

\[ \text{gld-2(q497) gld-1(q485)} \quad \text{gld-2(q497) gld-1(q485) cup-2(tm2838); der-2(tm6098)} \]

\( \alpha \text{-REC-8} \quad \alpha \text{-HIM-3} \)

D

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</table>
Figure 4
Figure 8

A

WT
g.c.d.

Intensity (Arbitrary Units)

Distance from DE (Microns)

0  7   14  21  28

0mM DTT
2mM DTT
5mM DTT

B

glp-1(ar202)
g.c.d.

Intensity (Arbitrary Units)

Distance from DE (Microns)

0  7   14  21  28

0mM DTT
2mM DTT
5mM DTT
Supplementary Figure 1
Supplementary Figure 3

A. *puf-8(q725); glp-1(oz264)*

B. *glp-1(oz264) at 25°C*  
   *glp-1(ar202) at 25°C*

C. *gld-2(q497) gld-1(q485)*
Supplementary Figure 4

A WT

B WT

C glp-1(ar202)

D glp-1(ar202)
Supplementary Figure 5

cup-2(tm2838); glp-1(ar202)

Intensity (Arbitrary Units)

Distance from DE (Microns)

empty vector RNAi
xdr-1 RNAi
Table 1 – Loss of Derlin activity supresses *glp-1(gf)* overproliferation

<table>
<thead>
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<th>Complete Tumour</th>
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<td>&gt; 50</td>
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<td>0%</td>
<td>0%</td>
<td>96</td>
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<td>61%</td>
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<td>163</td>
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<td>3%</td>
<td>97%</td>
<td>259</td>
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</table>

^1**Wild-type (WT)** is defined as a gonad arm with distal anti-REC-8(+) cells followed by anti-HIM-3(+) cells and presence of both sperm and oocytes in the proximal arm of the gonad.

^2**A protumour** is defined as a gonad arm containing both anti-REC-8(+) and anti-HIM-3(+) cells, but not differentiated sperm and oocytes. Proximal tumourous gonad arms with only sperm but no oocytes were counted as protumours. Gonad arms with mostly only anti-REC-8(+) and a few anti-HIM-3(+) positive cells were also counted as protumourous.

^3**Complete tumour** is defined as a gonad arm that contains only anti-REC-8(+) cells and no anti-HIM-3(+) cells

^4**Number of gonad arms**

^5**N2**

^6**cup-2(tm2838)**

^7**der-2(tm6098)**

^8**rfp-1(ok572)**

^9**puf-8(q725)**
| cup-2(0); puf-8(0); der-2(0) glp-1(ar202gf) | 0% | 33% | 67% | 234 |
Table 2 – Loss of Derlin activity suppress *glp-1(gf)* overproliferation in the proliferative zone

<table>
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<th>Genotype</th>
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<th>S.D.</th>
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<th>max</th>
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<td>186</td>
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<td>12</td>
</tr>
<tr>
<td><em>der-2(0)&lt;sup&gt;14&lt;/sup&gt;</em></td>
<td>227</td>
<td>19</td>
<td>194</td>
<td>255</td>
<td>10</td>
</tr>
<tr>
<td><em>cup-2(0); der-2(0)</em></td>
<td>142</td>
<td>30</td>
<td>92</td>
<td>184</td>
<td>10</td>
</tr>
<tr>
<td><em>glp-1(ar202gf)</em></td>
<td>610</td>
<td>106</td>
<td>394</td>
<td>731</td>
<td>10</td>
</tr>
<tr>
<td><em>cup-2(0); glp-1(ar202gf)</em></td>
<td>196</td>
<td>24</td>
<td>163</td>
<td>237</td>
<td>10</td>
</tr>
<tr>
<td><em>der-2(0) glp-1(ar202gf)</em></td>
<td>405</td>
<td>69</td>
<td>246</td>
<td>505</td>
<td>10</td>
</tr>
<tr>
<td><em>cup-2(0); der-2(0) glp-1(ar202gf)</em></td>
<td>122</td>
<td>28</td>
<td>80</td>
<td>166</td>
<td>10</td>
</tr>
<tr>
<td><em>glp-1(bn18ts)</em></td>
<td>138</td>
<td>16</td>
<td>114</td>
<td>171</td>
<td>10</td>
</tr>
<tr>
<td><em>cup-2(0); glp-1(bn18ts)</em></td>
<td>95</td>
<td>16</td>
<td>77</td>
<td>113</td>
<td>10</td>
</tr>
<tr>
<td><em>glp-1(ar224gf)</em></td>
<td>202</td>
<td>48</td>
<td>126</td>
<td>261</td>
<td>10</td>
</tr>
<tr>
<td><em>cup-2(0); glp-1(ar224gf)</em></td>
<td>143</td>
<td>18</td>
<td>125</td>
<td>176</td>
<td>10</td>
</tr>
<tr>
<td><em>glp-1(oz264gf)</em></td>
<td>384</td>
<td>26</td>
<td>336</td>
<td>430</td>
<td>10</td>
</tr>
<tr>
<td><em>cup-2(0); glp-1(oz264gf)</em></td>
<td>226</td>
<td>49</td>
<td>172</td>
<td>312</td>
<td>10</td>
</tr>
</tbody>
</table>

<sup>10</sup> Proliferative cells defined as cells distal to the transition zone as identified by crescent-shaped nuclei stained by DAPI

<sup>11</sup> Number of gonad arms for which the total number of cells in the proliferative zone were counted

<sup>12</sup> N2

<sup>13</sup> *cup-2(tm2838)*

<sup>14</sup> *der-2(tm6098)*
Table 3 – Loss of Derlin activity does not supress \textit{gld-2} \textit{gld-1} Notch-independent tumours

<table>
<thead>
<tr>
<th>Genotype</th>
<th>HIM-3(-) in proximal germline</th>
<th>HIM-3(+) in proximal germline</th>
<th>n \textsuperscript{15}</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{gld-2(0) gld-1(0)} \textsuperscript{16}</td>
<td>96%</td>
<td>4%</td>
<td>45</td>
</tr>
<tr>
<td>\textit{gld-2(0) gld-1(0) cup-2(0)} \textsuperscript{17}</td>
<td>94%</td>
<td>6%</td>
<td>53</td>
</tr>
<tr>
<td>\textit{gld-2(0) gld-1(0); der-2(0)} \textsuperscript{18}</td>
<td>96%</td>
<td>4%</td>
<td>51</td>
</tr>
<tr>
<td>\textit{gld-2(0) gld-1(0) cup-2(0); der-2(0)} \textsuperscript{e}</td>
<td>98%</td>
<td>2%</td>
<td>51</td>
</tr>
</tbody>
</table>

\textsuperscript{15} Number of gonad arms
\textsuperscript{16} Complete genotype \textit{gld-2(q497) gld-1(q485)}
\textsuperscript{17} Complete genotype \textit{gld-2(q497) gld-1(q485) cup-2(tm2838)}
\textsuperscript{18} Complete genotype \textit{gld-2(q497) gld-1(q485); der-2(tm6098)}
\textsuperscript{e} Complete genotype \textit{gld-2(q497) gld-1(q485) cup-2(tm2838); der-2(tm6098)}
Table 4 – Loss of *cup-2* activity in the germline is likely responsible for tumour suppression

<table>
<thead>
<tr>
<th>Genotype$^{19}$</th>
<th>RNAi</th>
<th>WT$^{20}$</th>
<th>Protumour$^{21}$</th>
<th>Complete Tumour$^{22}$</th>
<th>n$^{23}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>puf-8</em>(0); <em>glp-1</em>(gf)</td>
<td>none</td>
<td>0%</td>
<td>5%</td>
<td>95%</td>
<td>127</td>
</tr>
<tr>
<td><em>cup-2</em>(0); <em>puf-8</em>(0); <em>glp-1</em>(gf)</td>
<td>none</td>
<td>22%</td>
<td>64%</td>
<td>14%</td>
<td>182</td>
</tr>
<tr>
<td><em>rrf-1</em>(0); <em>puf-8</em>(0); <em>glp-1</em>(gf)</td>
<td><em>gfp</em></td>
<td>0%</td>
<td>3%</td>
<td>97%</td>
<td>190</td>
</tr>
<tr>
<td><em>rrf-1</em>(0); <em>puf-8</em>(0); <em>glp-1</em>(gf)</td>
<td><em>cup-2</em></td>
<td>16%</td>
<td>21%</td>
<td>63%</td>
<td>219</td>
</tr>
</tbody>
</table>

$^{19}$ Alleles used *puf-8*(q725), *cup-2*(tm2838), *rrf-1*(pk1417) and *glp-1*(oz264gf)

$^{20}$ Wild-type (WT) is defined as a gonad arm with distal anti-REC-8(+) cells followed by anti-HIM-3(+) cells and presence of both sperm and oocytes in the proximal arm of the gonad

$^{21}$ A protumour is defined as a gonad arm containing both anti-REC-8(+) and anti-HIM-3(+) cells, but not differentiated sperm and oocytes. Proximal tumorous gonad arms with only sperm but no oocytes were counted as protumours. Gonad arms with mostly only anti-REC-8(+) and a few anti-HIM-3(+) positive cells were also counted as protumours.

$^{22}$ Complete tumour is defined as a gonad arm that contains only anti-REC-8(+) cells and no anti-HIM-3(+) cells

$^{23}$ Number of gonad arms
Table 5 – Loss of snx-1 weakly suppresses tumours

<table>
<thead>
<tr>
<th>Genotype</th>
<th>WT</th>
<th>Protumour</th>
<th>Complete Tumour</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>puf-8(0); glp-1(gf)</td>
<td>0%</td>
<td>5%</td>
<td>95%</td>
<td>127</td>
</tr>
<tr>
<td>puf-8(0); glp-1(gf); snx-1(0)</td>
<td>0%</td>
<td>26%</td>
<td>74%</td>
<td>543</td>
</tr>
<tr>
<td>cup-2(0); puf-8(0); glp-1(gf)</td>
<td>22%</td>
<td>64%</td>
<td>14%</td>
<td>182</td>
</tr>
</tbody>
</table>

24 Alleles used puf-8(q725), cup-2(tm2838), snx-1(tm847) and glp-1(oz264gf)
25 Wild-type (WT) is defined as a gonad arm with distal anti-REC-8(+) cells followed by anti-HIM-3(+) cells and presence of both sperm and oocytes in the proximal arm of the gonad
26 A protumour is defined as a gonad arm containing both anti-REC-8(+) and anti-HIM-3(+) cells, but not differentiated sperm and oocytes. Proximal tumourous gonad arms with only sperm but no oocytes were counted as protumours. Gonad arms with mostly only anti-REC-8(+) and a few anti-HIM-3(+) positive cells were also counted as protumourous.
27 Complete tumour is defined as a gonad arm that contains only anti-REC-8(+) cells and no anti-HIM-3(+) cells
28 Number of gonad arms
Table 6 – DTT and Thapsigargin (TG) suppress *glp-1(gf)* overproliferation in a dose-dependent manner

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Genotype 29</th>
<th>WT 30</th>
<th>Protumour 31</th>
<th>Complete Tumour 32</th>
<th>n 33</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM DTT</td>
<td><em>puf-8(0); glp-1(gf)</em></td>
<td>0%</td>
<td>1%</td>
<td>99%</td>
<td>525</td>
</tr>
<tr>
<td>2 mM DTT</td>
<td><em>puf-8(0); glp-1(gf)</em></td>
<td>0%</td>
<td>19%</td>
<td>81%</td>
<td>186</td>
</tr>
<tr>
<td>5 mM DTT</td>
<td><em>puf-8(0); glp-1(gf)</em></td>
<td>4%</td>
<td>62%</td>
<td>33%</td>
<td>471</td>
</tr>
<tr>
<td>10 mM DTT</td>
<td><em>puf-8(0); glp-1(gf)</em></td>
<td>7%</td>
<td>89%</td>
<td>4%</td>
<td>350</td>
</tr>
<tr>
<td>0 µM TG</td>
<td><em>puf-8(0); glp-1(gf)</em></td>
<td>0%</td>
<td>1%</td>
<td>99%</td>
<td>465</td>
</tr>
<tr>
<td>1 µM TG</td>
<td><em>puf-8(0); glp-1(gf)</em></td>
<td>0%</td>
<td>9%</td>
<td>91%</td>
<td>471</td>
</tr>
<tr>
<td>5 µM TG</td>
<td><em>puf-8(0); glp-1(gf)</em></td>
<td>0%</td>
<td>26%</td>
<td>74%</td>
<td>391</td>
</tr>
<tr>
<td>10 µM TG</td>
<td><em>puf-8(0); glp-1(gf)</em></td>
<td>0%</td>
<td>29%</td>
<td>71%</td>
<td>441</td>
</tr>
<tr>
<td>0 mM DTT</td>
<td><em>cup-2(0); puf-8(0); glp-1(gf)</em></td>
<td>22%</td>
<td>71%</td>
<td>7%</td>
<td>278</td>
</tr>
<tr>
<td>2 mM DTT</td>
<td><em>cup-2(0); puf-8(0); glp-1(gf)</em></td>
<td>51%</td>
<td>47%</td>
<td>2%</td>
<td>246</td>
</tr>
<tr>
<td>5 mM DTT</td>
<td><em>cup-2(0); puf-8(0); glp-1(gf)</em></td>
<td>81%</td>
<td>19%</td>
<td>0%</td>
<td>217</td>
</tr>
</tbody>
</table>

29 Complete genotypes *puf-8(q725); glp-1(oz264)* and *cup-2(tm2838); puf-8(q725); glp-1(oz264)*
30 Wild-type (WT) is defined as a gonad arm with presence of both sperm and oocytes in the proximal arm of the gonad as seen by whole mount DAPI staining
31 A protumour is defined as a gonad arm with a mass of proliferative cells in the proximal end preceded more distally by presence of sperm and/or eggs as seen by whole mount DAPI staining
32 Complete tumour is defined as a gonad arm that contains only proliferative cells as seen by whole mount DAPI staining
33 Number of gonad arms
Table 7 – Reduction in xbp-1 function partially enhances glp-1(gf) overproliferation when also lacking cup-2 activity

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Treatment</th>
<th>WT</th>
<th>Protumour</th>
<th>Complete Tumour</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>puf-8(0); glp-1(gf)</td>
<td>vector(RNAi)</td>
<td>0%</td>
<td>1%</td>
<td>99%</td>
<td>398</td>
</tr>
<tr>
<td></td>
<td>xbp-1(RNAi)</td>
<td>0%</td>
<td>0%</td>
<td>100%</td>
<td>410</td>
</tr>
<tr>
<td>cup-2(0); puf-8(0); glp-1(gf)</td>
<td>vector(RNAi)</td>
<td>0%</td>
<td>46%</td>
<td>54%</td>
<td>146</td>
</tr>
<tr>
<td></td>
<td>xbp-1(RNAi)</td>
<td>0%</td>
<td>13%</td>
<td>87%</td>
<td>252</td>
</tr>
</tbody>
</table>

Supplementary Table 1 - Phenotypic analysis of the effect of loss of cup-2 on expression of the Glp phenotype. Individual gonads were analyzed at 22.5°C by whole mount DAPI.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>% Glp</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>glp-1(bn18)</td>
<td>23.5%</td>
<td>294</td>
</tr>
<tr>
<td>cup-2(tm2838); glp-1(bn18)</td>
<td>20.35%</td>
<td>314</td>
</tr>
</tbody>
</table>

34 Wild-type (WT) is defined as a gonad arm with distal anti-REC-8(+) cells followed by anti-HIM-3(+) cells and presence of both sperm and oocytes in the proximal arm of the gonad  
35 A protumour is defined as a gonad arm containing both anti-REC-8(+) and anti-HIM-3(+) cells, but not differentiated sperm and oocytes. Proximal tumourous gonad arms with only sperm but no oocytes were counted as protumours. Gonad arms with mostly only anti-REC-8(+) and a few anti-HIM-3(+) positive cells were also counted as protumourous. 
36 Complete tumour is defined as a gonad arm that contains only anti-REC-8(+) cells and no anti-HIM-3(+) cells  
37 Number of gonad arms  
38 Complete genotype puf-8(q725); glp-1(oz264)  
39 Complete genotype cup-2(tm2838); puf-8(q725); glp-1(oz264)
Supplementary Table 2- Phenotypic analysis of the effect of ER stress induced by DTT treatment on suppression of Notch-dependent tumorous phenotypes. *puf-8(q725); glp-1(oz264)* was analyzed by dissections followed by REC-8/HIM-3 staining while the *glp-1(oz264)* and *glp-1(ar202)* phenotypes was analyzed by whole mount DAPI.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Temperature</th>
<th>Treatment</th>
<th>WT</th>
<th>Protumour</th>
<th>Complete Tumour</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>puf-8(q725); glp-1(oz264)</td>
<td>20°C</td>
<td>0 mM DTT</td>
<td>0%</td>
<td>25%</td>
<td>75%</td>
<td>122</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 mM DTT</td>
<td>2%</td>
<td>90%</td>
<td>8%</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 mM DTT</td>
<td>37%</td>
<td>49%</td>
<td>14%</td>
<td>65</td>
</tr>
<tr>
<td>glp-1(oz264)</td>
<td>25°C</td>
<td>0 mM DTT</td>
<td>69%</td>
<td>3%</td>
<td>28%</td>
<td>280</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 mM DTT</td>
<td>82%</td>
<td>1%</td>
<td>17%</td>
<td>316</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 mM DTT</td>
<td>88%</td>
<td>1%</td>
<td>11%</td>
<td>103</td>
</tr>
<tr>
<td>glp-1(ar202)</td>
<td>25°C</td>
<td>0 mM DTT</td>
<td>19%</td>
<td>55%</td>
<td>26%</td>
<td>319</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 mM DTT</td>
<td>34%</td>
<td>51%</td>
<td>15%</td>
<td>327</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 mM DTT</td>
<td>11%</td>
<td>79%</td>
<td>10%</td>
<td>251</td>
</tr>
</tbody>
</table>

Supplementary Table 3- Phenotypic analysis of the effect of ER stress induced by DTT treatment on suppression of a *gld-2 gld-1* Notch-independent tumours. Phenotypes were analyzed by dissections followed by REC-8/HIM-3 staining.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>Treatment</th>
<th>HIM-3(-)</th>
<th>HIM-3(+) in proximal germline</th>
<th>HIM-3(+) in proximal and distal germline</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>gld-2(q497) gld-1(q485)</td>
<td>0 mM DTT</td>
<td>6%</td>
<td>75%</td>
<td>19%</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>2 mM DTT</td>
<td>59%</td>
<td>38%</td>
<td>3%</td>
<td>39</td>
</tr>
</tbody>
</table>

**Supplementary Table 4: List of strains used**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2</td>
<td>Wild type</td>
</tr>
<tr>
<td>FX02838</td>
<td><em>cup-2(tm3838)</em> I</td>
</tr>
<tr>
<td>FX06098</td>
<td><em>der-2(tm6098)</em> II</td>
</tr>
<tr>
<td>XB671</td>
<td><em>cup-2(tm2838)/hT2g</em> I; <em>der-2(tm6098)/hT2g</em> III</td>
</tr>
<tr>
<td>BS3148</td>
<td><em>glp-1(ar202)</em> III</td>
</tr>
<tr>
<td>XB590</td>
<td><em>cup-2(tm2838)</em> I; <em>glp-1(ar202)</em> III</td>
</tr>
<tr>
<td>XB655</td>
<td><em>der-2(tm6098)</em> <em>glp-1(ar202)</em> III</td>
</tr>
<tr>
<td>XB659</td>
<td><em>cup-2(tm2838)/hT2g</em> I; <em>der-2(tm6098)</em> <em>glp-1(ar202)/hT2g</em> III</td>
</tr>
<tr>
<td>BS121</td>
<td><em>glp-1(bn18)</em> III</td>
</tr>
<tr>
<td>XB663</td>
<td><em>cup-2(tm2838)</em> I; <em>glp-1(bn18)</em> III</td>
</tr>
<tr>
<td>XB739</td>
<td><em>cup-2(tm2838)/hT2g</em> I; <em>unc-32(e189)</em> <em>glp-1(ar224)/hT2g</em> III</td>
</tr>
<tr>
<td>XB740</td>
<td><em>unc-32(e189)</em> <em>glp-1(ar224) / hT2g</em> III</td>
</tr>
<tr>
<td>Genotype</td>
<td>Phenotype</td>
</tr>
<tr>
<td>------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>XB184</td>
<td>glp-1(oz264) III</td>
</tr>
<tr>
<td>XB492</td>
<td>cup-2(tm2838) I; glp-1(oz264) III</td>
</tr>
<tr>
<td>XB445</td>
<td>rfp-1(ok572) glp-1(oz264)/hT2g III</td>
</tr>
<tr>
<td>XB661</td>
<td>cup-2(tm2838)/hT2g I; rfp-1(ok572) glp-1(oz264)/hT2g III</td>
</tr>
<tr>
<td>XB745</td>
<td>puf-8(q725)/mc6g II; unc-32(189) glp-1(ar224) III</td>
</tr>
<tr>
<td>XB744</td>
<td>cup-2(tm2838) I; puf-8(q725)/mc6g II; unc-32(e189) glp-1(ar224) III</td>
</tr>
<tr>
<td>XB306</td>
<td>puf-8(q725)/mc6g II; glp-1(oz264) III</td>
</tr>
<tr>
<td>XB506</td>
<td>cup-2(tm2838) I; puf-8(q725)/mc6g II; glp-1(oz264) III</td>
</tr>
<tr>
<td>XB269</td>
<td>puf-8(q725)/mc6g II; unc-32(e189) glp-1(ar202)/hT2 III</td>
</tr>
<tr>
<td>XB591</td>
<td>cup-2(tm2838) I; puf-8(q725)/mc6g II; glp-1(ar202) III</td>
</tr>
<tr>
<td>XB658</td>
<td>puf-8(q725)/mc6g II; der-2(tm6098) glp-1(ar202) III</td>
</tr>
<tr>
<td>XB724</td>
<td>cup-2(tm2838)/hT2 I; puf-8(q725)/mc6g II; der-2(tm6098) glp-1(ar202)/hT2 III</td>
</tr>
<tr>
<td>JK3182</td>
<td>gld-3(q730) nos-3(q650)/mIn1[mIs4 dpy-10(e128)] II</td>
</tr>
<tr>
<td>XB686</td>
<td>cup-2(tm2838) I; gld-3(q730) nos-3(q650)/mIn1 [mIs14 dpy-10(e128)] II</td>
</tr>
<tr>
<td>XB487</td>
<td>gld-2(q497) gld-1(q485)/hT2g I</td>
</tr>
<tr>
<td>XB648</td>
<td>gld-2(q497) gld-1(q485) cup-2(tm2838)/hT2g I</td>
</tr>
<tr>
<td>XB706</td>
<td>gld-2(q497) gld-1(q485)/hT2g I; der-2(tm6098)/hT2g III</td>
</tr>
<tr>
<td>XB705</td>
<td>gld-2(q497) gld-1(q485) cup-2(tm2838)/hT2g I; der-2(tm6098)/hT2g III</td>
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<td>WU1770</td>
<td>sygl-1(am307) I</td>
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<td>XB710</td>
<td>sygl-1(am307)/hT2g I; glp-1(ar202)/hT2g III</td>
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<td>XB711</td>
<td>cup-2(tm2838) sygl-1(am307)/hT2g I</td>
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<td>Strain</td>
<td>Mutation Details</td>
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<td>XB709</td>
<td><em>cup-2(tm2838) sygl-1(am307)/hT2g I; glp-1(ar202)/hT2g III</em></td>
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<td>XB681</td>
<td><em>cup-2(ug1) I</em></td>
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<td><em>cup-2(ug1) I; ojls23[pie-1p::GFP::C34B2.10]</em></td>
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<td><em>puf-8(q725)/mc6g II; glp-1(oz264) III; snx-1(tm847) X</em></td>
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<td><em>rrf-1(pk1417) I; puf-8(q725)/mc6g II; glp-1(oz264) III</em></td>
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