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

Komal Safdar,*1 Anniya Gu,*1 Xia Xu,* Vinci Au,*† Jon Taylor,*† Stephane Flibotte,*† Donald G. Moerman,*† and Eleanor M. Maine*,2

*Department of Biology, Syracuse University, New York 13244 and †Department of Zoology, University of British Columbia, Vancouver, British Columbia V6T 1Z4, Canada

ABSTRACT Notch-type signaling mediates cell–cell interactions important for animal development. In humans, reduced or inappropriate Notch signaling activity is associated with various developmental defects and disease states, including cancers. Caenorhabditis elegans expresses two Notch-type receptors, GLP-1 and LIN-12. GLP-1 mediates several cell-signaling events in the embryo and promotes germline proliferation in the developing and adult gonad. LIN-12 acts redundantly with GLP-1 in certain inductive events in the embryo and mediates several cell–cell interactions during larval development. Recovery of genetic suppressors and enhancers of glp-1 or lin-12 loss- or gain-of-function mutations has identified numerous regulators of GLP-1 and LIN-12 signaling activity. Here, we report the molecular identification of sog-1, a gene identified in screens for recessive suppressors of conditional glp-1 loss-of-function mutations. The sog-1 gene encodes UBR-5, the sole C. elegans member of the UBR5/Hyd family of HECT-type E3 ubiquitin ligases. Molecular and genetic analyses indicate that the loss of ubr-5 function suppresses defects caused by reduced signaling via GLP-1 or LIN-12. In contrast, ubr-5 mutations do not suppress embryonic or larval lethality associated with mutations in a downstream transcription factor, LAG-1. In the gonad, ubr-5 acts in the receiving cells (germ cells) to limit GLP-1 signaling activity. SEL-10 is the F-box component of SCFSEL-10 E3 ubiquitin–ligase complex that promotes turnover of Notch intracellular domain. UBR-5 acts redundantly with SEL-10 to limit Notch signaling in certain tissues. We hypothesize that UBR-5 activity limits Notch-type signaling by promoting turnover of receptor or limiting its interaction with pathway components.

KEYWORDS Notch germ cell GLP-1 LIN-12 HECT domain

The highly conserved Notch-type signaling process mediates inductive cell interactions during animal development (see reviews by Greenwald and Koval 2013; Koch et al. 2013; Suresh and Irvine 2015; Yamamoto et al. 2014). Notch signaling is active in many different tissues in any given species, and defective Notch signaling is associated with many human disease conditions, including developmental syndromes and certain cancers (see reviews by Louvi and Artavanis-Tsakonas 2012; Ntziachristos et al. 2014; Penton et al. 2012; Suresh and Irvine 2015). Notch-type signaling is unusual compared with other developmentally important signaling mechanisms in that it is limited to adjacent cells, involves cleavage of the receptor to release a transcription factor, and acts in a relatively diverse set of developmental and physiological contexts. During canonical Notch signaling, summarized in Figure 1A, the DSL (Delta, Serrate, LAG-2) -type ligand on the signaling cell binds membrane-associated Notch-type receptor on the receiving cell, and this interaction triggers proteolytic cleavage of the receptor. Sequential cleavage events, accomplished by ADAM protease (the S2 cleavage) and γ-secretase (the S3 cleavage), release the Notch intracellular domain (NICD) for transport to the nucleus where it interacts with a CSL (CBF1/Su(H)/LAG-1)-type DNA binding protein and a conserved co-activator protein (Mastermind family in mammals and Drosophila, SEL-8/LAG-3 in nematodes), and displaces a corepressor complex.
The NICD/CSL activator complex up-regulates transcription of target genes whose identity depends on cell type.

Notch-type signaling is modulated by a variety of mechanisms (Louvi and Artavanis-Tsakonas 2012; Yamamoto et al. 2014). In the signaling cell, endocytosis of the ligand–receptor complex causes an essential conformational change that allows the S2 cleavage to occur. This process requires components of the endosomal trafficking machinery and mono-ubiquination of DSL intracellular domain by E3 ubiquitin ligases. In some signaling contexts, endocytosis also plays an earlier role in activation of ligand. In the receiving cell, newly synthesized Notch-type receptor receives a variety of post-translational modifications that modulate its activity prior to reaching the cell surface. As is the case for signaling activity in general, it is important that Notch-type signaling not be continuous, and mechanisms are in place to down-regulate NICD activity (reviewed by Baron 2012; Barth and Kohler 2014; Le Bras et al. 2011; Kandachar and Roegiers 2012; Weinmaster and Fischer 2011). For example, NICD activity is limited by ubiquitin-mediated targeting to the proteasome for degradation (reviewed by Lai 2002; Wang et al. 2011). In this process, ubiquitin is covalently linked to ubiquitin-activating enzyme (E1), transferred to ubiquitin-conjugating enzyme (E2), and finally transferred to the protein substrate (e.g., NICD) via the activity of a ubiquitin–protein ligase (E3) (reviewed by Kipreos 2005; Hershko and Ciechanover 1998). Target specificity is conferred by the E3 ligase, and SEL-10/Fbw7 is a conserved E3 ligase component that limits Notch signaling activity in many species (Hubbard et al. 1997; see reviews by Lai 2002; Wang et al. 2011). In addition to promoting protein turnover, E3-mediated ubiquitination can modulate protein–protein interactions and impact processes such as nuclear import (Rodriguez 2014). Therefore, E3 ligase activity may also regulate Notch signaling by modulating interactions between signaling components.

Two Notch isoforms are present in Caenorhabditis elegans: GLP-1 (germline proliferation defective-1) and LIN-12 (lineage defective-12) (Greenwald and Kovall 2013). GLP-1 and/or LIN-12 mediate numerous cell-signaling events throughout development and in the adult gonad (reviewed by Priess 2005; Sternberg 2005; Greenwald and Kovall 2013). GLP-1 is active in cell–cell interactions in the embryo and in soma-to-germline signaling in the larval and adult gonad. LIN-12 is redundant with GLP-1 for some signaling events in the late embryo and later mediates signaling events in many different somatic cells during development.
larval development. Numerous modulators of Notch signaling – as well as core components of the pathway – have been identified in extensive genetic screens for suppressors or enhancers of loss- or gain-of-function mutations in glp-1, lin-12, or components of the γ-secretase complex (reviewed by Greenwald and Koval 2013). Most of these modulators also regulate additional developmental processes not known to involve Notch signaling.

GLP-1-mediated inductive signaling from the somatic gonad to the germline is essential for germ cell proliferation in the larva and adult (reviewed by Hansen and Schedl 2013; Kimble and Seidel 2013). All germ cells are proliferative in early larval development; later, proximal germ cells enter meiosis, and germline proliferation becomes restricted to the distal region of the gonad arm. During the early proliferative phase, several somatic gonadal cells signal to the germline via GLP-1; in the later phase, only the distal tip cells signal to the germline via GLP-1. Proliferative germ cells are not maintained if signaling is reduced or abolished by mutation in a GLP-1 pathway component or by ablation of somatic signaling cells; instead, germ cells prematurely exit mitosis, enter meiosis, and form gametes. Additional factors promote a wild-type level of germ cell proliferation, including the distal sheath cells (Killian and Hubbard 2005), gap junctions between the somatic gonad and germline (Starich et al. 2014), and nutritional factors, among others (Hubbard et al. 2013).

We previously recovered suppressor of glp-1 (sog) mutations in genetic screens for suppressors of the glp-1 temperature-sensitive (ts) phenotype (Maine and Kimble 1993). The sog mutations partially suppressed glp-1 maternal effect embryonic lethality and germline proliferation defects. Here, we report the molecular characterization of sog-1. We demonstrate that sog-1 encodes UBR-5, a member of the HECT (homologous to the E6-AP carboxyl terminus)-type E3 protein–ubiquitin ligase family whose closest mammalian relative is UBR5 (Ubiquitin protein ligase E3 component n-recognin 5; also called EDD, E3 identified by differential display). We find that loss of UBR-5 activity causes an increase in both GLP-1 and LIN-12 signaling activity in sensitized genetic backgrounds where the receptor carries a conditional mutation. Genetic analysis suggests that UBR-5 acts in the receiving cell. The SCPSEL-10 E3 ubiquitin–ligase complex is known to limit LIN-12 activity and, to a minor extent, GLP-1 activity by binding to and promoting turnover of the intracellular domain (Sundaram and Greenwald 1993; Hubbard et al. 1997). Our genetic analysis suggests that UBR-5 and SEL-10 function in concert to limit Notch signaling in some tissues.

### MATERIALS AND METHODS

#### Mutant strains

*C. elegans* strains were cultured using standard methods (Epstein and Shakes 1995). All strains were derived from the Bristol strain, N2. Mutations used in this study are described in [www.wormbase.org](http://www.wormbase.org) unless otherwise noted and include the following:

**LGII:** sog-1 alleles q295, q298, q303, q305, q308, q345 (all described in Maine and Kimble 1993), om2 (this study), ok1108 (OMRF Knockout Group, see wormbase.org), rrf-1(pk1417), unc-13(e51), ndf25, ccbs4251 [myo-3p::GFP(NLS):LacZ (pSAK2) + myo-3p::GFP (mitochondrially targeted) (pSAK4) + dpy-20(+)].

**LGIII:** glp-1(q231ts), glp-1(ar202ts), lin-12(ar170), unc-32(e189).

**LGIV:** lag-1(om3ts), nT1 [qIs51].

**LGV:** him-5(e1467), him-5(e1490), sel-10(ok1632).

In addition, syIs50 [cdh-3::GFP + dpy-2(+)] served as an anchor cell marker (Pettit et al. 1996; Inoue et al. 2002).

The *om2* deletion allele was isolated in a noncomplementation screen as follows. L4 *unc-13(s51); glp-1(q231)* hermaphrodites were treated with 20–30 µg/ml trimethylpsoralen (TMP) in M9 medium, irradiated for 20 sec at a distance of 10 cm with a long-wave UV power source, allowed to recover for several hr, and mated with sog-1(q298); glp-1(q231);him-5(e1490) males at 15°C. Mating plates were shifted to 20°C, after a substantial number of F1 embryos had been produced, and screened 2–3 d later for the presence of fertile non-Unc cross-progeny, which were presumed to be genotype *unc-13(-) sog-1(q298)/unc-13 sog-1(omx); glp-1(q231); him-5(e1490+).* Homozygous *unc-13 sog-1(omx); glp-1(q231)* animals were recovered and out-crossed to confirm that the new sog allele was linked to *unc-13.*

#### Whole genome sequencing

Genomic DNA isolation from strains JK946 [carrying sog-1(q303)] and JK952 [carrying sog-1(q308)], library construction, whole genome sequencing (WGS), and bioinformatics analysis were performed as described (Filbotte et al. 2010; Thompson et al. 2013). For analysis of other sog-1 strains, the F36A2.13 gene region was recovered by DNA amplification and sequenced using standard methods. The sog-1 mutations were originally mapped to the cluster on LGI, and we particularly focused on WGS data from this region.

In the course of this study, we also performed WGS analysis of JK953, a strain previously reported to carry a sog-1 mutation called *q309.* Unlike other sog-1 alleles, which were recovered in *f2* screens for recessive suppressors of glp-1, *q309* was recovered in the course of a dominant suppressor screen as described (Maine and Kimble 1993). WGS analysis revealed that JK953 is wild-type for glp-1 and does not contain a mutation in F36A2.13. We conclude that the reduced brood size and temperature sensitivity associated with JK953 result from a combination of other mutations in the genetic background.

Our molecular data led us to revise how we interpret earlier gene dosage data. Genetic mapping had placed sog-1 mutations in a region uncovered by the deficiencies *ozDf5 and ndf25* (Maine and Kimble 1993). Neither *sog-1(-)/ozDf5 nor sog-1(-)/ndf25 suppressed glp-1(q231); therefore we speculated that the sog-1 alleles isolated in our glp-1 suppression screen were recessive gain-of-function mutations. Now, based on our molecular identification of sog-1 as located outside the region uncovered by *ozDf5,* we would not expect *sog-1(-)/ozDf5* to suppress glp-1(ts) (Figure 2). In contrast, *ndf25* uncovers genes that flank sog-1, suggesting it should uncover sog-1 (Figure 2). To investigate further, we generated an *ndf25/unc-13 ccds4251; glp-1(q231ts)* strain and mated hermaphrodites of this genotype with *sog-1(om2);glp-1(q231ts)* animals. This assay is straightforward compared with our earlier test because use of a GFP-tagged chromosome obviates the need for other marker mutations and allows us to identify unambiguously the very slow growing *ndf25/sog-1(om2)* cross-progeny. Matings were conducted at 15°C, and progeny were shifted to 20°C after hatching. *ndf25/sog-1(om2); glp-1(q231ts)* hermaphrodites were picked to a separate plate and observed to segregate viable embryos, indicating that *ndf25/sog-1(om2)* suppresses glp-1(q231ts).* Hence, *ndf25* indeed appears to uncover sog-1. *ndf25/sog-1(om2); glp-1(q231ts)* hermaphrodites also segregated non-viable embryos, presumably *ndf25* homozygotes.

#### Phenotypic analysis

Brood size assays were carried out using standard methods as follows. All sog-1 unc-32 glp-1(231ts) strains were maintained at 20°C. The *unc-32 glp-1(q231ts)* control strain was maintained at 15°C, and animals to be used for brood size experiments were shifted to 20°C as late-stage embryos or
newly hatched L1 larvae. Broods were assayed by placing individual L4 larvae onto single plates; once they became gravid adults, they were moved to a fresh plate daily until they no longer produced embryos. Embryos were counted immediately after the mother was moved; viable L4 larvae were counted 2–3 d later.

The anchor cell (AC)-ventral uterine (VU) precursor phenotype was assayed by examining late L2 stage larvae with differential interference contrast and epifluorescence microscopy using a Zeiss Axioscope. Anchor cells were identified based on morphology, position within the gonad primordium, and expression of a cdh-3:gfp transgene included in the strain.

Proliferative zone size was analyzed in adults labeled with DAPI using standard methods (e.g., Qiao et al. 1995). L4 stage hermaphrodites were picked to a fresh culture plate, aged 24–25 h, fixed for ~15 min with −20° methanol, stained for ~15 min with 0.2 μg/ml DAPI, and mounted in Vectashield (Vector Laboratories) for epifluorescence analysis. To assay the proliferative region of each specimen, we counted the number of rows of germ cells distal to the leptotene–zygotene “transition” zone.

RNAi assays

RNA interference (RNAi) was performed by the feeding method (Timmons et al. 2001). L4 larvae were placed onto culture plates seeded with *E. coli* expressing sog-1 double-stranded RNA (dsRNA), and their F1 progeny were assayed in the first day of adulthood for the presence/absence of embryos. Animals were removed from culture plates as they were counted. Non-RNAi controls were performed by culturing each strain [glp-1(ar202) or rrf-1(pk1417);glp-1(ar202)] on the standard OP50 *E. coli* food source. Presence of the rrf-1(pk1417) deletion did not affect DAPI nuclear staining. In addition, the sensitivity of rrf-1(pk1417);glp-1(ar202) animals to RNAi of various somatic genes was tested to confirm the presence of the RNAi defect as described by Kistler and Hansen (2012). As a control for an effect of RNAi per se on the glp-1(ar202) phenotype, RNAi was performed with empty L4440 vector, which produces a short dsRNA that does not correspond to *C. elegans* genomic sequence.

Data availability

Strains are available upon request. Whole genome sequence data are provided in Supplemental Material, Table S1.

RESULTS

sog-1 encodes a HECT-type E3 ubiquitin ligase

Mutations in sog-1 were recovered in a genetic screen for recessive suppressors of glp-1(q224ts) and glp-1(q231ts) (Maine and Kimble 1993). Both of these glp-1(ts) alleles have a Glp-1 null phenotype at 25° and a partial loss-of-function (fl) phenotype at 20° (Maine and Kimble 1989). Consequently, when L1 stage glp-1(ts) larvae are shifted from permissive temperature to 25°, their germ cells exit mitosis, enter meiosis, and undergo spermatogenesis. In contrast, when L1 stage glp-1(ts) larvae are shifted from permissive temperature to 20°, their germ cells proliferate for a period of time before prematurely entering meiosis and undergoing gametogenesis; in most cases, a full complement of sperm and some oocytes form and some embryos are generated. These embryos die due to defects in GLP-1 signaling during embryogenesis. The sog-1 mutations partially suppress the Glp-1(ts) defects at 20°, but not at 25°, and therefore do not bypass the requirement for GLP-1 activity (Maine and Kimble 1993).

We initiated a molecular study of sog-1 by performing whole genome sequence analysis of two sog-1 mutant strains: JK946, containing sog-1(q303); and JK952, containing sog-1(q308) (see Materials and Methods). When we compared the JK946 and JK952 sequence data to the reference *C. elegans* genome, we identified numerous common mutations that presumably were present in the original unc-32(e189) glp-1(q231ts) strain prior to mutagenesis (Table S1). In addition, we identified a number of mutations unique to either JK946 or JK952. Of note, JK946 and JK952 contain distinct mutations in a common open reading frame (ORF), F36A2.13 (Table S1, Figure 1B). F36A2.13 is predicted to encode a member of the HECT family of E3 ubiquitin ligases (Figure 1). Its closest mammalian relative is UBR5/EDD (Callaghan et al. 1998; Tasaki et al. 2005), and consequently F36A2.13 is listed in Wormbase as ubr-5 (UBR E3 ubiquitin ligase homolog – 5) (www.wormbase.org).

We confirmed that ubr-5 and sog-1 are the same gene by amplifying and sequencing the F36A2.13 genomic region from five additional strains carrying sog-1 alleles recovered following mutagenesis with EMS (q295, q298, q305, q345; Maine and Kimble 1993) or UV (om2; see Materials and Methods). Each strain contained a mutation in F36A2.13 (Figure 1, Table S2). In addition, we obtained an F36A2.13 deletion allele, ok1108, from the Caenorhabditis Genetics Center and tested its ability to suppress glp-1(q231ts). At 20°, ok1108 partially suppresses glp-1(q231ts) (Table 1). We conclude that sog-1 and F36A2.13 are the same gene. Although sog-1 is the original published gene name, “ubr-5” better denotes the gene product. Therefore, we will refer to F36A2.13 as ubr-5 for the rest of this article.

Previous analysis of ubr-5 mutants indicated that they were superficially normal (Maine and Kimble 1993). We reevaluated this question with the deletion alleles, ubr-5(om2) and ubr-5(ok1108). These mutants likewise do not have obvious developmental defects in a glp-1(+)-background under laboratory conditions. In particular, we considered that ubr-5 mutants might impact the germline stem cell pool. As a measure of proliferative zone size, we counted the number of rows of germ cells nuclei from the distal end of the somatic gonad to the start of the lepoteone/zygotene region in animals raised at 20°. When we compared the number of rows of nuclei in the mitotic zone in glp-1(q231ts) and ubr-5(om2); glp-1(q231ts) animals at 24 hr post-L4 stage, we observed an increase in mitotic zone size from an average of four rows in glp-1(q231ts) to an average of 11 rows in ubr-5(om2); glp-1(q231ts) (Table 2). Therefore, the loss of UBR-5 activity leads to increased germ cell proliferation in the sensitized GLP-1(ts) background. In contrast, we did not observe an increase in the number of rows of mitotic germ cells in ubr-5 mutants compared with wild-type at 24 hr post-L4 (Table 2). Hence, the loss of UBR-5 activity did not impact the length of the mitotic region in germlines with wild-type GLP-1 function.
UBR-5 negatively regulates GLP-1 activity

The predicted ubr-5 product is a 2944 amino acid protein with domain architecture characteristic of the UBR5/EDD HECT protein subfamily. It contains three conserved motifs: a UB (also called E3) domain located near the N terminus, a zinc-finger domain located in the middle of the protein (the UBR motif), and a C-terminal HECTc domain (Figure 1C). Many members of this protein family also contain a PABP (poly A binding protein; also called MLLE) domain within ~100 amino acids of the HECT domain (Callaghan et al. 1998; Tasaki et al. 2005; Scheffner and Kumar 2014) (Figure 1D). C. elegans UBR-5 appears to lack this domain. Among the eight ubr-5 alleles we characterized, three contain a premature stop codon. The ubr-5(om2) allele is likely to be null; it contains a deletion of 105 nucleotides in the center of the gene just upstream of the zinc-finger domain that is predicted to shift the ORF, inserting 45 amino acids and deleting the C-terminal half of the protein, including the entire HECT domain (Figure 1, Table S2). Similarly, ubr-5(q345) may be null as it contains a single nucleotide change that converts residue 1026 to a stop codon (Figure 1, Table S2). It is predicted to encode a truncated protein lacking the zinc-finger and HECT domains. ubr-5(ok1108) is a complex mutation with a 1360 nucleotide deletion and 73 nucleotide insertion; most of the HECT domain is deleted as well as the 3′ UTR and some downstream sequence (Figure 1, Table S2). The net result of the mutation is to insert nine amino acids downstream of residue 2631. This allele is expected to lack E3 ligase activity, as well. The nature of these alleles suggests that a change that converts residue 1026 to a stop codon (Figure 1, Table S2). We hypothesize that some of these mutations may influence the degree of suppression by ubr-5 in one or more tissues. Indeed, it was noted during previous three-factor mapping experiments that suppression by ubr-5 is abrogated to a large extent in the presence of certain marker mutations (Maine and Kimble 1993). As detailed in the Materials and Methods, data obtained in the course of our studies allow us to make corrections to the literature with respect to (i) gene dosage requirements and (ii) the identity of a previously reported allele, q309.

To confirm further that the ubr-5 mutations are loss-of-function, we knocked down UBR-5 protein in a glp-1 gain-of-function background using RNAi and examined the consequences for GLP-1 signaling. Gain-of-function glp-1 mutations have elevated GLP-1 signaling, resulting in germline over-proliferation and eventual formation of a germline tumor (Berry et al. 1997; Pepper et al. 2003). The glp-1(ar202gf) allele is temperature sensitive, producing a more elevated level of GLP-1 signaling at higher temperatures (Pepper et al. 2003). At 25°C, germ cells form a tumor relatively early in development, and the animal does not produce oocytes. At lower (semipermissive) temperatures, overproliferation occurs more slowly, and a sizable proportion of glp-1(ar202) mutants are fertile (Pepper et al. 2003).

We tested the extent to which reducing ubr-5 activity would increase the level of GLP-1 signaling in the glp-1(ar202) background at the semipermissive temperature, 22°C. We assayed the increase in GLP-1

Table 2 Suppression of the glp-1(q231ts) germline proliferation defect by ubr-5(om2)

| Genotype           | No. Rows of Nuclei in Proliferative Zone* (Range) | n  |
|--------------------|--------------------------------------------------|----|
| Wild type (N2)     | 21 ± 0.6 (16–24)                                | 18 |
| ubr-5(om2)         | 21 ± 0.8 (16–25)                                | 13 |
| glp-1(q231ts)      | 4 ± 0.7 (0–11)                                  | 36 |
| ubr-5(om2);glp-1(q231ts) | 11 ± 0.4 (5–16)                  | 32 |
| sel-10(ok1632)     | 15 ± 0.6 (11–20)                                | 14 |
| glp-1(q231ts); sel-10(ok1632) | 6 ± 0.9 (0–14)                  | 24 |
| ubr-5(om2);glp-1(q231ts);sel-10(ok1632) | 12 ± 0.6 (8–19)                  | 21 |
| ubr-5(om2);sel-10(ok1632) | 19 ± 1.2 (13–28)                  | 16 |

Assays were conducted at 20°C. L4 stage larvae were picked to a fresh plate and DAPI-stained 24 hr later. n, number of gonad arms evaluated.
*± represents standard error of the mean. The number of rows of proliferative nuclei was rounded to the nearest whole number.

Table 1 Suppression of glp-1(q231ts) by ubr-5 deletion alleles at 20°C

| Genotype                       | Avg. No. Embryos Produced per Brood | % Viable | n  |
|--------------------------------|------------------------------------|----------|----|
| unc-32 glp-1(q231ts)           | 129 ± 8*                           | 0        | 16 |
| ubr-5(om2);unc-32 glp-1(q231ts) | 174 ± 10                           | 40       | 10 |
| ubr-5(ok1108);unc-32 glp-1(q231ts) | 189 ± 6                           | 34       | 14 |

Full broods were counted for the indicated ("n") number of hermaphrodites, including both viable and nonviable embryos.

a The baseline No. of embryos produced by unc-32 glp-1(q231ts) controls in these experiments was substantially higher than previously reported (e.g., Maine and Kimble 1989, 1993). Controls were performed with two strains, both of which had been frozen since the early 1990s and were thawed specifically for these assays (see Materials and Methods). As described in Maine and Kimble (1993), ~98% of unc-32 glp-1(q231ts) controls produced some (nonviable) embryos, and only ~2% were Glp-1 sterile. >99.9% of ubr-5(om2);glp-1(q231ts) animals produced embryos.

b We note that the sel-10(ok1632) strain, RB1432, contains additional mutations that may reduce mitotic zone size. See text.
signaling by quantifying the increase in % tumorous sterility in the population at ~24 hr post-L4 stage. Under conditions used in our assay, on average ~20% of glp-1(ar202) control animals were tumorous and lacked embryos, and this phenotype increased nearly fourfold to ~79% in ubr-5(RNAi); glp-1(ar202) animals (Figure 3). The tumorous glp-1(ar202) controls and ubr-5(RNAi); glp-1(ar202) double mutants contained mitotic nuclei in the proximal germline (Figure 3B). We conclude that ubr-5(RNAi) significantly increases the level of GLP-1 signaling in glp-1(ar202) mutants. This result supports the conclusion that reduced UBR-5 activity leads to elevated GLP-1 signaling activity.

**UBR-5 acts in the germ cells to limit GLP-1 pathway activity in the gonad**

To evaluate whether UBR-5 activity is important in the signaling and/or receiving cell, we assayed the ability of ubr-5(RNAi) to enhance GLP-1 signaling activity in an rrf-1(0) mutant background. In rrf-1(pk1417) mutants, RNAi is severely impaired in many somatic tissues, including the somatic gonad (Kumsta and Hansen 2012; Sijen et al. 2001). We performed ubr-5 RNAi in parallel in rrf-1(pk1417);glp-1(ar202) and rrf-1(+);glp-1(ar202) hermaphrodites and assayed enhancement of the glp-1(ar202gf) sterile phenotype (Figure 3). GLP-1(ar202gf) sterility was enhanced significantly in rrf-1(pk1417) animals (Figure 3). Statistical analysis indicates no significant difference in the degree of enhancement in the rrf-1(+) vs. rrf-1(pk1417) backgrounds; therefore we conclude that UBR-5 acts in the germ line to limit GLP-1 signaling and germ cell proliferation.

**UBR-5 negatively regulates LIN-12 activity**

As a result of LIN-12-mediated lateral signaling between cells in the L2 stage gonad primordium, Z1.ppp and Z4.aaa, one cell becomes the AC and the other cell becomes a VU precursor cell. In the absence of LIN-12 signaling activity, both Z1.ppp and Z4.aaa take on an AC fate (Greenwald et al. 1993; Seydoux and Greenwald 1989). To determine if UBR-5 activity limits signaling via LIN-12/Notch, we evaluated the ability of ubr-5(RNAi) and ubr-5(q345) to suppress the 2-AC phenotype in the late L2 (ar170ts) mutant at 25°. We included a cdh-3::gfp transgene in our strains to aid in identification of ACs (Karp and Greenwald 2003). CDH-3 is a cadherin protein expressed by the AC but not by other nearby cells during late L2/early L3 stage (Petitt et al. 1996). At 25°, we observed two ACs in ~64–68% of lin-12(ar170) controls scored at late L2 stage (Table 3). In contrast, we observed two ACs in ~33% of ubr-5(om2) and ubr-5(q345) to suppress the 2-AC phenotype in the lin-12(ar170ts) mutants at 25°. We included a cdh-3::gfp transgene in our experiments, where all four treatments were run in parallel. In the rrf-1(+) background, ubr-5 RNAi is active in all tissues; in the rrf-1(0) background, ubr-5 RNAi is active in the germline, but not in the somatic gonad (Kumsta and Hansen 2012). The Glp-1 gf phenotype is significantly enhanced in both rrf-1(+) and rrf-1(0) backgrounds relative to the respective non-RNAi control assay (animals cultured on OP50 bacteria). A paired t-test indicates no difference between enhancement of glp-1(ar202gf) in rrf-1(+) and rrf-1(0) samples. In contrast to the 4- to 5.5-fold increase in % tumorous animals upon ubr-5(RNAi), negative controls performed with “empty vector” (L4440 plasmid without an insert) increased the % tumorous animals by 0.4- to 0.5-fold relative to controls grown in parallel [n = 449 glp-1(ar202), n = 78 rrf-1;glp-1(ar202), n, total number of animals scored in replicate treatments. (B) Examples of the glp-1(ar202gf) Tumorous and non-Tumorous phenotypes observed under our assay conditions.

![Figure 3](image-url)
Table 3 Loss of ubr-5 function suppresses the lin-12 2-AC defect

| Strain | % 2 AC | n |
|--------|--------|---|
| unc-32(e189) lin-12(ar170); cdh-3::gfp | 67.6 | 108 |
| ubr-5(om2); unc-32(e189) lin-12(ar170); cdh-3::gfp | 33.3 | 96 |
| unc-32(e189) lin-12(ar170); cdh-3::gfp | 63.6 | 110 |
| ubr-5(q345); unc-32(e189) lin-12(ar170); cdh-3::gfp | 33.0 | 100 |

Assays were conducted at 25°C. Control unc-32(e189) lin-12(ar170); cdh-3::gfp animals were assayed in parallel with each ubr-5; unc-32(e189) lin-12(ar170); cdh-3::gfp strain. In each case, the value for “% 2 AC” was significantly different in control vs. experimental strains, P < 0.03 (Z-test): n, number of larvae evaluated; AC, anchor cell.

Table 4 Loss of ubr-5 function does not suppress lag-1(om13ts) phenotypic defects

| Genotype | Avg No. Embryos Produced (± SE) | % Dead Embryos | % Dead Larvae | % Viable Progeny | n |
|----------|-------------------------------|----------------|--------------|------------------|---|
| lag-1(om13ts) | 188 ± 18                     | 39.6           | 57.2         | 3.2              | 6 |
| ubr-5(ok1108);lag-1(om13ts) | 225 ± 11                     | 48.6           | 47.7         | 3.7              | 10 |

Tests were performed at 20°C. Most nonviable larvae died at early L1 stage, as is characteristic of the Lag phenotype (Lambie and Kimble 1991). n, number of full broods counted.
Extensive genetic and molecular analysis has revealed that the outcome of Notch-type signaling is extremely sensitive to the level (“strength”) of signaling activity in a wide variety of contexts (Louvi and Artavanis-Tsakonas 2012; Yamamoto et al. 2014). Our data are consistent with these previous observations. Eliminating just one negative regulator of GLP-1 and LIN-12 activity, UBR-5, increased signaling activity sufficiently to rescue moderately severe loss-of-function phenotypes and strongly enhance a weak gain-of-function phenotype.

### The relationship between UBR-5 and SEL-10

Our data suggest a complex relationship between UBR-5 and SEL-10 with respect to Notch signaling. These findings may reflect, in part, the equivalent receptor activity of GLP-1 and LIN-12 (Fitzgerald et al. 1993) and the fact that both GLP-1 and LIN-12 are active in some tissues, e.g., in the embryo, whereas only GLP-1 or LIN-12 is active in other tissues, e.g., GLP-1 in the gonad. Whereas UBR-5 has a major role in limiting Notch signaling in the embryo and gonad, SEL-10 appears to have a larger role in limiting Notch signaling in the embryo than in the gonad. Our observation of a synergistic genetic interaction between *ubr-5* and *sel-10* with respect to suppression of the *glp-1(q231ts)* embryonic lethality indicates that UBR-5 and SEL-10 do not function in a simple linear pathway, at least in embryonic tissues. One model for the relationship between these factors in the embryo is that UBR-5 restricts GLP-1 activity broadly during embryogenesis, whereas SEL-10 restricts GLP-1 primarily in the late embryo – where it also restricts LIN-12 (Figure 4B). Only rarely would development of the *glp-1(q231ts)* embryo be rescued by *sel-10(ok1632)* alone. An alternative hypothesis is that SEL-10 primarily restricts LIN-12 signaling activity and has little impact on GLP-1 signaling activity. This would explain the very minor role of SEL-10 in the gonad, where LIN-12 signaling is not active. One way to think about these data is that UBR-5 may primarily limit GLP-1 signaling, SEL-10 may primarily limit LIN-12 signaling, and reduced UBR-5 or SEL-10 activity may better suppress reduced *glp-1* or *lin-12* activity in those cells or tissues where increased activity of one receptor can compensate for reduced activity of the other, e.g., in the embryo (Figure 4A).

### UBR5 activity in development and disease

UBR5 proteins in mammals and *Drosophila* have been implicated in numerous developmental processes (reviewed by Shearer et al. 2015). Vertebrate UBR5 regulates cell cycle progression, and misregulation of UBR5 activity is linked to cancer in many tissues (Schefchner and Kumar 2014; Shearer et al. 2015). UBR5 activity appears to promote cell proliferation in some tissues and limit it in others, as the loss of UBR5 activity promotes cancer in some tissues whereas UBR5 overexpression promotes cancer in other tissues. Presumably these differences reflect the multitude of UBR5 targets that may contribute to oncogenesis. The *Drosophila* ortholog, Hyperplastic discs (Hyd), regulates cell proliferation in developing imaginal discs and promotes development of other tissues, as well (Mansfield et al. 1994).

UBR5 appears to contribute to cell proliferation control in a number of ways. One role for UBR5 is in modulating activity of the mitotic spindle assembly checkpoint (SAC) mechanism, thereby impacting the ability of cells to enter anaphase. SAC activity ensures cells remain in metaphase until all chromosomes have attached to the mitotic spindle; once chromosomes have done so, then SAC activity must be reduced in order to allow anaphase entry. Evidence suggests that UBR5 functions both to promote SAC activity when needed, e.g., if microtubules are disrupted (Scialpi et al. 2015), and to reduce SAC activity once chromosomes have attached to the mitotic spindle (Jiang et al. 2015). UBR5 targets different SAC components in these different situations, and its subcellular localization changes during the cell cycle (Scialpi et al. 2015; Jiang et al. 2015).

---

**Table 5 Tests for redundancy between UBR-5 and SEL-10**

| Genotype                  | Avg No. Embryos Produced (± SE) | N  | % Viable Progeny | n  |
|---------------------------|---------------------------------|----|------------------|----|
| *ubr-5(om2); unc-32(e189) glp-1(q231ts); sel-10(ok1632)* | 124 ± 5                          | 10 | 74.8             | 1243|
| *ubr-5(om2); unc-32(e189) glp-1(q231ts)*            | 174 ± 10                         | 10 | 40.0             | 1740|
| *unc-32(e189) glp-1(q231ts); sel-10(ok1632)*        | 119 ± 15                         | 12 | 0.6              | 1428|
| *unc-32(e189) glp-1(q231ts)*                       | 129 ± 8                          | 16 | 0.0              | 2065|
| *ubr-5(om2); sel-10(ok1632)*                       | 248 ± 6                          | 12 | 97.2             | 2972|
| *ubr-5(om2)*                                        | 276 ± 17                         | 7  | 98.4             | 1901|
| *sel-10(ok1632)* original RB1432*                  | 157 ± 17                         | 8  | 32.0             | 1441|
| *sel-10(ok1632)* reisolated from *ubr-5(om2); sel-10(ok1632)* | 225 ± 7                          | 5  | 98.6             | 1125|

Assays were conducted at 20°C. N, number of full broods counted; n, number of individuals counted.

*The reported broods were produced by animals with a functional vulva. Some animals of these genotypes have a defective vulva and consequently fail to lay eggs and/or die prematurely, in each case producing a limited number of offspring that does not reflect the degree of germline proliferation. Hence, the effective brood size of this strain is smaller than the value listed here. These data also are listed in Table 1.

*The embryonic lethality and reduced brood size of strain RB1432 do not appear to be caused by *sel-10(ok1632)*. See text.*
Another function of UBR5 is in modulating stability of nuclear myosin 1 (NM1), a factor required for RNA polymerase I transcriptional activity. NM1 is stabilized by GSK3β-mediated phosphorylation, which prevents UBR5-mediated ubiquitination and subsequent degradation (Sarshad et al. 2014). Progression beyond G1 stage requires NM1 activity, and hence modulation of NM1 is one means by which UBR5 limits cell cycle progression. Antagonistic activity of GSK3β vs. Hyd/UBR5 appears to be a general regulatory mechanism as it also modulates Hedgehog signaling in Drosophila (Lee et al. 2002; Moncrieff et al. 2015). As we observed for Notch signaling, Hedgehog signaling is limited by Hyd/UBR5 activity. Interestingly, mammalian FBW7/SEL10 is recruited to certain targets upon GSK3β-mediated phosphorylation (Flugel et al. 2012).

Other studies have implicated UBR5 in modulating the DNA damage response (DDR), where it participates at several steps, and in regulation of certain transcription factors (Shearer et al. 2015). One aspect of UBR5 activity is that it limits activity of the DDR machinery to sites of double-strand breaks and prevents inappropriate/unnecessary activity elsewhere on the chromosome. In regulating transcription factor activity, UBR5 functions in many cell types, positively regulating certain transcription factors and negatively regulating others. We present the first evidence, to our knowledge, of a UBR5 family E3 ligase modulating Notch signaling activity. In the future, it will be informative to identify the targets of UBR-5 activity.

ACKNOWLEDGMENTS

We thank Jim Lissемore and Mark Edgley for invaluable assistance in the WGS analysis, Yiаng Guo, Bing Yang, and Yini Li for technical advice, and two reviewers for insightful comments on the manuscript. This study was supported by funds from Syracuse University, including the Coronat Scholars Program and the Korczynski-Lundgren Fund. Work in the laboratory of D.G.M. was supported by a grant from the Canadian Institute for Health Research. D.G.M. is a Senior Fellow of the Canadian Institute for Advanced Research. Some strains used in this study were obtained from the Caenorhabditis Genetics Center, which is funded by the National Institutes of Health (NIH) Office of Research Infrastructure Programs (P40 OD010440).

LITERATURE CITED

Baron, M., 2012 Endocytic routes to Notch activation. Semin. Cell Dev. Biol. 23: 437–442.
Barth, J. M., and K. Kohler, 2014 How to take autophagy and endocytosis up a notch. BioMed Res. Int. 10.1155/2014/960803.
Berry, L. W., B. Westlund, and T. Schedl, 1997 Germ-line tumor formation caused by activation of glp-1, a Caenorhabditis elegans member of the Notch family of receptors. Development 124: 925–936.
Callaghan, M. J., A. J. Russell, E. Woollatt, G. R. Sutherland, R. L. Sutherland et al., 1998 Identification of a human HECT family protein with homology to the Drosophila tumour suppressor gene hyperplastic discs. Oncogene 17: 3479–3491.
Epstein, H. F., and D. C. Shakes, 1995 Caenorhabditis elegans: Biological Analysis of an Organism, in Methods in Cell Biology, Vol. 48. Academic Press, San Diego.
Fitzgerald, K., H. A. Wilkinson, and I. Greenwald, 1993 glp-1 can substitute for lin-12 in specifying cell fate decisions in Caenorhabditis elegans. Development 119: 1019–1027.
Flibotte, S., M. L. Edgley, I. Chaudhry, J. Taylor, S. E. Neil et al., 2010 Whole-genome profiling of mutagenesis in Caenorhabditis elegans. Genetics 185: 431–441.
Flugel, D., A. Gorlach, and T. Kietzmann, 2012 GSK-3β regulates cell growth, migration, and angiogenesis via Fbw7 and USP28-dependent degradation of HIG-1α. Blood 119: 1292–1301.
Ghai, V., and J. Gaudet, 2008 The CSL transcription factor LAG-1 directly represses htl-1 expression in C. elegans. Dev. Biol. 322: 334–344.
Greenwald, I., and R. Kovall, 2013 Notch signaling: genetics and structure (January 17, 2013), WormBook, ed. The C. elegans Research Community Wormbook, doi/10.1895/wormbook.1.10.2, http://www.wormbook.org.
Greenwald, I. S., P. W. Sternberg, and H. R. Horvitz, 1983 The lin-12 locus specific cell fates in Caenorhabditis elegans. Cell 34: 435–444.
Hansen, D., and T. Schedl, 2013 Stem cell proliferation versus meiotic fate decision in Caenorhabditis elegans. Adv. Exp. Med. Biol. 757: 77–91.
Hershko, A., and A. Ciechanover, 1998 The ubiquitin system. Annu. Rev. Biochem. 67: 425–479.
Hubbard, E. J., G. Wu, J. Kitajewski, and I. Greenwald, 1997 sel-10, a negative regulator of lin-12 activity in Caenorhabditis elegans, encodes a member of the CDC4 family of proteins. Genes Dev. 11: 3182–3193.
Hubbard, E. J., D. Z. Korta, and D. Dalfó, 2013 Physiological control of germline development. Adv. Exp. Med. Biol. 757: 101–131.
Inoue, T., D. R. Sherwood, G. Aspöck, J. A. Butler, B. P. Gupta et al., 2002 Gene expression marks for Caenorhabditis elegans vulval cells. Mech. Dev. Suppl. 1: S203–S209.
Jiang, H., X. He, D. Feng, X. Zhu, and Y. Zheng, 2015 RanGTP aids anaphase entry through UBR5-mediated protein turnover. J. Cell Biol. 211: 7–18.
Johnson, J. E., and R. J. MacDonald, 2011 Notch-independent functions of Curr. Curr. Top. Dev. Biol. 97: 55–74.
Kandchar, V., and F. Roegers, 2012 Endocytosis and control of Notch signaling. Curr. Opin. Cell Biol. 24: 534–540.
Karp, X., and I. Greenwald, 2003 Post-transcriptional regulation of the E/Daughterless ortholog HLH-2, negative feedback, and birth order bias during the AC/VU decision in C. elegans. Genes Dev. 17: 3100–3111.
Killian, D. J., and E. J. Hubbard, 2005 Caenorhabditis elegans germline patterning requires coordinated development of the somatic gonadal sheath and the germ line. Dev. Biol. 279: 322–335.
Killian, D. J., E. Harvey, P. Johnson, M. Otori, S. Mitani et al., 2008 SKR-1, a homolog of Skp1 and a member of the SCF Eyl–1 complex, regulates sex-determination and LIN-12/Notch signaling in C. elegans. Dev. Biol. 322: 322–331.
Kimble, J., and H. Seidel, 2013 C. elegans germ line stem cells and their niche (November 15, 2013). StemBook, ed. The Stem Cell Research Community StemBook, doi/10.3824/stembook.1.95.1, http://www.stembook.org.
Kipreos, E. T., 2005 Ubiquitin-mediated pathways in C. elegans (December 1, 2005). WormBook, ed. The C. elegans Research Community WormBook, doi/10.1895/wormbook.1.361.1, http://www.wormbook.org.
Koch, U., R. Lelah, and F. Radlke, 2013 Stem cells living with a Notch. Development 140: 689–704.
Kumsta, C., and M. Hansen, 2012 C. elegans rfl-1 mutations maintain RNAi efficiency in the soma in addition to the germline. PLoS One 7: e35428.
Lai, E. C., 2002 Protein degradation: four E3s for the Notch pathway. Curr. Biol. 12: R74–R78.
Lambie, E., and J. Kimble, 1991 Two homologous regulator genes, lin-12 and glp-1, have overlapping functions. Development 112: 231–240.
Le Bras, S., N. Loyer, and R. Le Borgne, 2011 The multiple facets of ubiquitination in the regulation of notch signaling pathway. Traffic 12: 149–161.
Lee, J. D., K. Amanai, A. Shearn, and J. E. Treisman, 2002 The ubiquitin ligase Hyperplastic discs negatively regulates hedgehog and decapentaplegic expression by independent mechanisms. Development 129: 5697–5706.
Loui, A., and S. Artavanis-Tsakonas, 2012 Notch and disease: a growing field. Semin. Cell Dev. Biol. 23: 473–480.
Maine, E. M., and J. Kimble, 1989 Identification of genes that interact with glp-1, a gene required for inductive cell interactions in Caenorhabditis elegans. Development 105: 133–143.
Maine, E. M., and J. Kimble, 1993 Suppressors of glp-1, a gene required for cell communication during development in Caenorhabditis elegans, define a set of interacting genes. Genetics 135: 1011–1022.
Mansfield, E., E. Hersperger, J. Biggs, and A. Shearn, 1994 Genetic and molecular analysis of hyperplastic discs, a gene whose product is required for regulation of cell proliferation in Drosophila melanogaster imaginal discs and germ cells. Dev. Biol. 165: 507–526.

Moncrieff, S., M. Moncan, F. Scialpi, and M. Ditzel, 2015 Regulation of hedgehog ligand expression by the N-end rule ubiquitin protein ligase Hyperplastic Discs and the Drosophila GSK3β homolog, Shaggy. PLoS One 10: e0136760.

Ntziachristos, P., J. S. Lim, J. Sage, and I. Afantis, 2014 From fly wings to targeted cancer therapies: a centennial for notch signaling. Cancer Cell 25: 318–334.

Penton, A. L., L. D. Leonard, and N. B. Spinner, 2012 Notch signaling in human development and disease. Semin. Cell Dev. Biol. 23: 450–457.

Pepper, A. S., D. J. Killian, and E. J. Hubbard, 2003 Genetic analysis of Caenorhabditis elegans glp-1 mutants suggests receptor interaction or competition. Genetics 163: 115–132.

Pettitt, J., W. B. Wood, and R. H. Plasterk, 1996 cdh-3, a gene encoding a member of the cadherin superfamily, functions in epithelial cell morphogenesis in Caenorhabditis elegans. Development 122: 4149–4157.

Priess, J. R., 2005 Notch signaling in the C. elegans embryo (June 25, 2005). WormBook, ed. The C. elegans Research Community Wormbook, doi/10.1895/wormbook.1.4.1, http://www.wormbook.org.

Qiao, L., J. L. Lissimore, P. Shu, A. Smardon, M. B. Gelber et al., 1995 Enhancers of glp-1, a gene required for cell-signaling in Caenorhabditis elegans, define a set of genes required for germline development. Genetics 141: 551–569.

Rodriguez, J. A., 2014 Interplay between nuclear transport and ubiquitin/SUMO modifications in the regulation of cancer-related proteins. Semin. Cancer Biol. 27: 11–19.

Sarshad, A. A., M. Corcoran, B. Al-Muzzaini, L. Borgonovo-Brandter, A. Von Euler et al., 2014 Glycogen synthase kinase (GSK) 3β phosphorylates and protects nuclear myosin 1c from proteasome-mediated degradation to activate rDNA transcription in early G1 cells. PLoS Genet. 10: e1004390.

Scheffner, M., and S. Kumar, 2014 Mammalian HECT ubiquitin-protein ligases: biological and pathophysiological aspects. Biochim. Biophys. Acta. 843: 61–74.

Scialpi, F., D. Mellis, and M. Ditzel, 2015 EDD, a ubiquitin-protein ligase of the N-end rule pathway, associates with spindle assembly checkpoint components and regulates the mitotic response to nocodazole. J. Biol. Chem. 290: 12585–12594.

Seydoux, G., and I. Greenwald, 1989 Cell autonomy of lin-12 function in a cell fate decision in C. elegans. Cell 57: 1237–1245.

Shearer, R. F., M. Iconomou, C. K. Watts, and D. N. Saunders, 2015 Functional roles of the E3 ubiquitin ligase UBR5 in cancer. Mol. Cancer Res. 13: 1523–1532.

Sijen, T., J. Fleenor, F. Simmer, K. L. Thijssen, S. Parrish et al., 2001 On the role of RNA amplification in dsRNA-triggered gene silencing. Cell 107: 465–476.

Starich, T. A., D. H. Hall, and D. Greenstein, 2014 Two classes of gap junction channels mediate soma-germline interactions essential for germline proliferation and gametogenesis in Caenorhabditis elegans. Genetics 198: 1127–1153.

Sternberg, P., 2005 Vulval development (June 25, 2005). WormBook, ed. The C. elegans Research Community WormBook, doi/10.1895/wormbook.1.6.1, http://www.wormbook.org.

Sundaram, M., and I. Greenwald, 1993 Suppressors of a lin-12 hypomorph define genes that interact with both lin-12 and glp-1 in Caenorhabditis elegans. Genetics 135: 765–783.

Suresh, S., and A. E. Irvine, 2015 The NOTCH signaling pathway in normal and malignant blood cell production. J. Cell Commun. Signal. 9: 5–13.

Tasaki, T., L. C. Mulder, A. Iwamatsu, M. J. Lee, I. V. Davydov et al., 2005 A family of mammalian E3 ubiquitin ligases that contain the UBR box motif and recognize N-degrons. Mol. Cell. Biol. 25: 7120–7136.

Thompson, O., M. Edgley, P. Strasbourger, S. Filipotse, B. Ewing et al., 2013 The million mutation project: a new approach to genetics in Caenorhabditis elegans. Genome Res. 23: 1749–1762.

Timmons, L., D. L. Court, and A. Fire, 2001 Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in Caenorhabditis elegans. Gene 263: 103–112.

Wang, Z., H. Inuzuka, H. Fukushima, L. Wan, D. Gao et al., 2011 Emerging roles of the FBW7 tumour suppressor in stem cell differentiation. EMBO Rep. 13: 36–43.

Weinmaster, G., and J. A. Fischer, 2011 Notch ligand ubiquitylation: what is it good for? Dev. Cell 21: 124–144.

Yamamoto, S., K. L. Schulze, and H. J. Bellen, 2014 Introduction to Notch signaling, pp. 1–14 in Notch Signaling: Methods and Protocols, in Methods in Molecular Biology, Vol. 1187, edited by H. Bolen and S. Yamamoto. Springer Science + Business Media, New York.

Communicating editor: S. Lee