hecd-1 modulates C. elegans Notch activity in *C. elegans*

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Abstract

Notch is a receptor that mediates cell-cell interactions that specify binary cell fate decisions in development and tissue homeostasis. Inappropriate Notch signaling is associated with cancer, and mutations in Notch pathway components have been associated with developmental diseases and syndromes. In *C. elegans*, suppressors of phenotypes associated with constitutively active LIN-12/Notch have identified many conserved core components and direct or indirect modulators. Here, we molecularly identify *sel(ar584)*, originally isolated as a suppressor of a constitutively active allele of *lin-12*. We show that *sel(ar584)* is an allele of *hecd-1*, the ortholog of human HECDT1, a ubiquitin ligase that has been implicated in several different mammalian developmental events. We studied interactions of *hecd-1* with *lin-12* in the somatic gonad and with the other *C. elegans* Notch gene, *glp-1*, in the germ line. We found that *hecd-1* acts as a positive modulator of *lin-12*/Notch activity in a somatic gonad context--the original basis for its isolation--but acts autonomously as a negative modulator of *glp-1*/Notch activity in the germ line. As the yeast ortholog of HECD-1, Ufd4p, has been shown to function in quality control, and *C. elegans* HECD-1 has been shown to affect mitochondrial maintenance, we propose that the different genetic interactions between *hecd-1* and *Notch* genes we observed in different cell contexts may reflect differences in quality control regulatory mechanisms or in cellular metabolism.

INTRODUCTION

Notch is a receptor that mediates cell-cell interactions during animal development. Virtually all of the core components and many modulators of Notch signaling were first identified through genetic analysis in *C. elegans* and Drosophila [reviewed in (Greenwald 2012; Greenwald and
Mutant forms of Notch, as well as of other core components and modulators of the signal transduction system, have been associated with congenital human disease syndromes, cancer, or Alzheimer's disease.

Notch is a type 1 transmembrane receptor protein that is essentially a membrane-tethered transcription factor (reviewed in Greenwald and Kovall 2013). Ligand-binding leads to proteolytic cleavages that release the intracellular domain, which translocates to the nucleus and promotes the transcription of target genes. Core components of the signaling system include the proteases that mediate the cleavage events and a sequence-specific DNA binding protein generically called "CSL" that is part of the nuclear transcription activation complex.

Missense mutations in the ectodomain can mimic ligand-binding, leading to ligand-independent cleavage and release of the intracellular domain and therefore to constitutive activity. In *C. elegans*, such mutant forms cause cell fate transformations (Greenwald and Seydoux 1990; Greenwald et al. 1983). Similar missense forms of *NOTCH1* have been found in many patients with T-cell Acute Lymphoblastic Leukemia (T-ALL), where they result in aberrant cell fate decisions and drive growth to contribute to oncogenesis (Weng et al. 2004); reviewed in (Ferrando 2009). Reducing the activity of components involved in the cleavage events or in the nuclear complex is an effective way to reduce constitutive signaling, and has been the basis for genetic screens in *C. elegans* (Dunn et al. 2010; Katie et al. 2005; Levitan and Greenwald 1995; Tax et al. 1997).

In *C. elegans*, genetic analysis of potential components and modulators of Notch involves the two Notch orthologs, *lin-12* and *glp-1* (Greenwald 2012; Greenwald and Kovall 2013). These genes have unique roles in some cell fate decisions and are functionally redundant for others (Lambie and Kimble 1991). In this study, we include genetic analysis of a cell fate
decision in early gonadogenesis uniquely mediated by \textit{lin-12}, and a role in germ-line proliferation uniquely mediated by \textit{glp-1}.

The \textit{C. elegans} somatic gonad has a single anchor cell (AC), which induces and organizes the vulva. Two cells of the developing somatic gonad have the potential to be the AC; interactions between them, mediated by LIN-12, result in one becoming the AC and the other a ventral uterine precursor cell (VU). The process by which the cells resolve their fates is called the "AC/VU decision," and involves differential transcriptional regulation of \textit{lin-12} and other feedback elements (Seydoux and Greenwald 1989; Wilkinson et al. 1994). In a \textit{lin-12} null mutant, both cells become ACs; in "\textit{lin-12(d)}" constitutively active missense mutants, both cells become VUs (Greenwald et al. 1983). Since the AC is required for vulval induction, \textit{lin-12(d)} mutants lack a vulva and are egg-laying defective.

The germ line has a distal-to-proximal axis, with a mitotic zone in a distal region, and a proximal zone in which the germline nuclei undergo meiosis and, further proximally, gametogenesis. Mitosis in the distal region is promoted by a ligand produced by the Distal Tip Cell, which activates GLP-1 in the underlying germ line. Mutations that cause strong constitutive \textit{glp-1} activity result in a "Tumorous" (Tum) phenotype, in which germ cells always remain in the mitotic cycle (Berry et al. 1997). In contrast, in the absence of \textit{glp-1}, germline stem cells do not proliferate (Austin and Kimble 1987; Priess et al. 1987).

The allele \textit{sel(ar584)} was originally identified as a suppressor of the Vulvaless phenotype of a \textit{lin-12(d)} mutant (Katic et al. 2005). We molecularly identify \textit{sel(ar584)} as an allele of \textit{hecd-1}, the ortholog of the human HECDT1 ubiquitin ligase. We show that \textit{hecd-1} behaves as a positive regulator of \textit{lin-12}/Notch in the AC/VU decision and as a cell-autonomous negative
hecd-1 modulates C. elegans Notch regulator of glp-1/Notch in germline proliferation. We propose that the different genetic interactions reflect a difference in cell context between the somatic gonad and germ line.

RESULTS

sel(ar584) is an allele of hecd-1

We performed whole genome sequencing of strain GS3347 to identify sel(ar584). On LG IV, where sel(ar584) had been mapped (Katic et al. 2005), we identified a single predicted premature stop mutation in the hecd-1 gene (Fig. 1). C. elegans HECD-1 is the ortholog of yeast Ufd4p and of human HECTD1 (Shaye and Greenwald 2011). There are eight predicted isoforms, ranging in length from 2607-2650 amino acids (Harris et al. 2014). All isoforms contain a carboxy-terminal HECT ubiquitin ligase domain, and the stop codon associated with sel(ar584) is predicted to truncate all eight isoforms before the HECT domain (Fig. 1). The domain structure of isoform a (2648 amino acids) is shown in Fig. 1, as is the domain structure of a comparable isoform of its human ortholog.

We performed further genetic analysis using an available deletion allele, hecd-1(ok1437), which is predicted to cause a more severe truncation in all eight HECD-1 isoforms (see Materials & Methods and Fig. 1). The results support the conclusion that sel(ar584) is an allele of hecd-1. First, hecd-1(ok1437), like sel(ar584), is a recessive suppressor of the 0 AC-Egl defect of lin-12(n302), a mutation that results in elevated lin-12 activity. Second, the two mutations fail to complement for suppression (Fig. 2A). Third, as described below, hecd-1(ok1437), sel(ar584), and hecd-1(RNAi) behave similarly in several genetic assays in combination with alleles of lin-12 and/or glp-1. We now call this allele hecd-1(ar584).
**hecd-1 is a positive modulator of lin-12/Notch activity in the somatic gonad**

Both alleles of *hecd-1*, the original *hecd-1(ar584)* suppressor mutation and the independent deletion allele *hecd-1(ok1437)* are likely to be strong loss-of-function or null alleles. Genetic analysis using *hecd-1(ok1437)* indicates that *hecd-1* is a positive modulator of *lin-12* activity in the somatic gonad. In the AC/VU decision, *hecd-1(ok1437)* suppresses the 0 AC-Egl defect of *lin-12(n302)* (Fig. 2A) and also enhances the 2 AC defect of a hypomorphic allele, *lin-12(n676n930ts)* (Fig. 2B). *hecd-1(ok1437)* also enhances a different egg-laying problem associated with reduced *lin-12* activity, the "late defect" (Sundaram and Greenwald 1993). The late defect has a complex basis including aberrations in uterine and sex muscle development (Eimer et al. 2002; Newman et al. 1995) (Fig. 2B); we did not characterize the cellular basis of this enhancement further.

**hecd-1 is a negative modulator of glp-1/Notch activity in the germ line**

To examine the effect of *hecd-1* on *Notch* activity in the germ line, we used *glp-1* alleles that increase or decrease activity. Mitotic proliferation in the distal region of the germ line is driven by *glp-1* activity, and strong constitutive *glp-1* activity results in a "Tumorous" (Tum) phenotype (Berry et al. 1997). The *glp-1(ar202ts)* allele is a milder variant constitutive allele, which at 25°C causes a "Pro" phenotype: a zone of ectopic proximal proliferation due to elevated *glp-1* activity in cells that would otherwise be meiotic (Pepper et al. 2003).

Since *hecd-1* behaves as a positive regulator in the somatic gonad, we were surprised to see that loss of *hecd-1* enhances the sterility of *glp-1(ar202)* at 25°C (Fig. 3A-C), consistent with increased activity of *glp-1(ar202)*. To corroborate this inference, we examined the cellular basis of this phenotype in greater detail. We observed a range of phenotypes when either *hecd-1*
hecd-1 modulates C. elegans Notch

Chen and Greenwald

1(ok1437) or hecd-1(ar584) is combined with glp-1(ar202) at 25°C, including a strong Tumorous phenotype associated with strong elevation of glp-1 activity (Berry et al. 1997) and enhancement of ectopic proximal proliferation, the "Pro" phenotype, associated with milder elevations of glp-1 activity (Pepper et al. 2003).

To see if this unexpected interaction was a property of glp-1(ar202), we also combined hecd-1(ok1437) with glp-1(bn18) at 23°C, a condition in which glp-1 activity is partially reduced (Fig. 3D) (Kodoyianni et al. 1992). We observed that loss of hecd-1 suppresses the sterility associated with loss of glp-1, indicating that the interaction is not allele-specific. Thus, the results with both gain- and loss-of-function alleles indicates that hecd-1 acts as a negative regulator of glp-1 activity in the germ line, in contrast to the positive role it plays for lin-12 for somatic cell fate decisions.

Maternally-provided glp-1 mediates many different decisions in the early embryo (Priess 2005), and loss of maternal glp-1 activity results in embryonic lethality (Austin and Kimble 1987; Priess et al. 1987). We allowed hermaphrodites to reach fertility at the permissive temperature and lay eggs at the restrictive temperature: a higher proportion of glp-1(bn18); hecd-1(ok1437) eggs than glp-1(bn18) eggs hatched [40/151(26%), vs. 7/83 (8%), p < 0.01)]. This observation is consistent with hecd-1 acting as a negative regulator of maternal glp-1 activity. However, the hatched eggs arrested as L1 larvae, so embryonic development is still abnormal; we do not know the cellular basis of the improved rate of hatching observed.

**hecd-1 acts autonomously in the germ line to modulate glp-1 activity**

In the AC/VU decision, both interacting cells within the somatic gonad express ligand and receptor. However, for germline proliferation, the ligand-expressing distal tip cell of the
somatic gonad and the receptor-expressing germline stem cells are distinct, making it more straightforward to determine the cellular focus of *hecd-1* activity for influencing *Notch* activity in this context. We asked whether *glp-1(ar202)* activity was increased by loss of *hecd-1* activity in the soma (signaling cell) or germ line (receiving cell) by comparing the effect of *hecd-1(RNAi)* in the background of *rrf-1(+) or rrf-1(pk1417)*, a mutation that preferentially eliminates RNAi in many somatic tissues, including the somatic gonad, without compromising RNAi in the germ line (Kumsta and Hansen 2012; Sijen et al. 2001). We found that *hecd-1(RNAi)* enhanced *glp-1(ar202)* regardless of the *rrf-1* genotype (Fig. 4), suggesting that *hecd-1* acts autonomously in the germ line to modulate *glp-1* activity.

**DISCUSSION**

HECD-1 is the ortholog of human HECTD1 and yeast Ufd4p. We have identified *hecd-1* as a new modulator of Notch signaling in *C. elegans* with unusual genetic properties: loss of *hecd-1* leads to reduced *lin-12/Notch* activity in the AC/VU decision but increased *glp-1/Notch* activity in the germ line. We are unaware of any other modulator that has this distinctive genetic behavior. It is possible that the different genetic interactions reflect an intrinsic difference between LIN-12 and GLP-1. However the functional redundancy of LIN-12 and GLP-1 in several somatic cell fate decisions (Lambie and Kimble 1991) and the ability of GLP-1 to substitute for LIN-12 the AC/VU decision and other decisions uniquely mediated by *lin-12* (Fitzgerald et al. 1993) lead us to propose instead that the different genetic interactions we observed reflects differences in cell context that are not directly related to the Notch paralogs per se.
The yeast ortholog of HECD-1, Ufd4p, has been shown to be a quality control ubiquitin ligase (Hwang et al. 2010; Johnson et al. 1995; Ju et al. 2007; Ju and Xie 2006), and there appears to be substantial feedback regulation in the clearance of misfolded, aggregated proteins by quality control ubiquitin ligases including Ufd4p (Theodoraki et al. 2012). As quality control is fundamental to eukaryotic cells, the conservation between HECD-1 and Ufd4p may reflect a conserved function in quality control for HECD-1 and at least some of the mechanisms that regulate it. C. elegans hecd-1 was also recently identified in a screen for mutations that result in reduced ubiquitin-proteasome activity, and further implicated in mitochondrial maintenance (Segref et al. 2014). The role in mitochondrial maintenance suggests a possible effect of loss of hecd-1 on energy production or metabolism.

Different cell contexts may affect the way proteins fold or aggregate when misfolded, the dynamics or regulation of quality control mechanisms, or energetics. Thus, hecd-1 may influence Notch activity indirectly through regulating one or more of these cellular properties. However, it is possible that HECD-1 acts directly, although at this level of genetic analysis, we cannot know the target: Notch signaling involves many components, both membrane-associated and cytosolic, and many modulators, some of which are cell type-specific (Greenwald and Kovall 2013).

Many genes identified through genetic analysis in C. elegans have proven to play similar roles in mammals. In mice, the ortholog HECTD1 has been shown to be a functional ubiquitin ligase required for normal craniofacial development (Sarkar and Zohn 2012; Zohn et al. 2007). As aberrations in Notch signaling can also cause craniofacial abnormalities, we speculate that craniofacial abnormalities resulting from loss of HECTD1 may, at least in part, reflect effects on Notch signaling.
MATERIALS AND METHODS

Strains and genetic analysis

*Caenorhabditis elegans* var. Bristol strain N2 was the wild-type parent strain of all mutants and markers used. All strains were grown using standard procedures at 20°C, except for strains containing *glp-1(ar202)* and *glp-1(bn18)* background, which were maintained at 15°C. For strains that were scored at 23°C or 15°C, animals were maintained and handled at the temperature of interest prior to scoring. Key strains used are listed in Table 1.

Whole genome sequencing and data analysis

The strain GS3347 containing *sel(ar584)* mutation was backcrossed 4x with N2 before deep sequencing. Genomic DNA library of GS3347 was prepared following Illumina’s WGS sample preparation manual. Paired-end library preparation, sequencing and base calling were done according to the manufacturers recommendations through Illumina’s FastTrack Sequencing Services Laboratory. Initial sequence data was mapped to the sequence of wild-type N2 reference genomic sequence using Illumina Genome Analyzer. Further data analysis was performed with MAQGene using general parameters previously described (Bigelow et al. 2009).

The lesion associated with *hecd-1(ar584)* was verified by performing PCR followed by Sanger sequencing. The sequence of *hecd-1(ok1437)* was determined by Sanger sequencing using primers flanking the predicted deletion region.

Design of RNAi constructs for *C. elegans*
hecd-1 modulates C. elegans Notch

RNAi constructs targeting the HECT domain encoding region of C34D4.14 was designed using a web tool E-RNAi version 3.0 (http://www.dkfz.de/signaling/e-rnai3//). Primer pairs for HECT domain were yc-334 (AAAAACCGGTAGTTCAAGAATTGGCCTGGA) and yc-335 (AAAAGGTACCTTCTTGGTTGCTTCACATTCC). Target regions were amplified and cloned into vector pL4440 (Addgene). Each construct was confirmed by Sanger sequencing and thereafter transformed into E. coli strain HT115(DE3).

**RNAi experiments**

Feeding RNAi experiments were performed at 20°C as described (Timmons and Fire 1998). Briefly, gravid adults were bleached and the eggs were placed on plates seeded with HT115 cells expressing the dsRNA targeting the region of hecd-1 encoding the HECT domain. A clone corresponding to the HECT domain, which should target all predicted isoforms, was used for the experiments shown in Figs. 3 and 4. T7 polymerase expression in the HT115 cells had been induced with 6 mM IPTG for at least four hours at room temperature before plating the eggs. To score the Pro or Tum phenotype at the adult stage, animals were DAPI-stained and scored three days after eggs were placed on plates.

**Imaging**

All microscopy done on live animals was performed on a Zeiss Axioplan2 microscope, with a consistent exposure time used for each marker assayed.

**ACKNOWLEDGEMENTS**

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sequencing and analysis, Caroline Goutte for advice and a preliminary assessment of another genotype for embryonic effects, all members of the Greenwald laboratory for suggestions during the course of this work, and Dan Shaye and Michelle Attner for comments on this manuscript. Some of the strains used in this study were provided by the Caenorhabditis Genetics Center, which is supported by the NIH-Office of Research Infrastructure Programs (P40 OD010440). This work was supported in part by a grant to I.G. from the National Institutes of Health (R01 CA095389). Y.C. was a Postdoctoral Associate and I.G. is an Investigator with the Howard Hughes Medical Institute.
FIGURE LEGENDS

Figure 1. *C. elegans* HECD-1, its human ortholog, and mutations.

(A) Genomic structure of *C. elegans* hecd-1 (*C34D4.14*) isoform a. The deletion identified in *ok1437* causes a frameshift and results in a stop codon in all predicted isoforms.

(B) Domain structure of *C. elegans* HECD-1, its human ortholog, and mutations. Using the sequence analysis protein SMART (Letunic et al. 2014), the human Hectd1 protein is predicted to contain three Ankyrin repeats, a SAD1/UNC domain, MIB domain and HECT domain. The *C. elegans* HECD-1 isoform a is predicted by SMART to have similar domain architecture as human Hectd1, except the third Ankyrin repeat (grey) is slightly below threshold. The predicted stops associated with the *hecd-1*(ar584) point mutation and the *hecd-1*(ok1437) deletion mutation are indicated. Prior to the stop codon in *hecd-1*(ok1437) is a 78 amino acid-frame shift caused by the deletion mutation.

Figure 2. Genetic interactions between *hecd-1* and *lin-12*. Here and in all other figures, * indicates significant P-value from Fisher’s test is < 0.05 versus control, and ** indicates P is <0.01 versus control.

(A) The egg-laying defect of *lin-12*(n302), a constitutively active allele (Greenwald and Seydoux 1990; Greenwald et al. 1983), is mildly suppressed by *hecd-1*(ar584) and *hecd-1*(ok1437) at 20°C. Trans-heterozygotes for the two alleles fail to complement: mild suppression of *lin-12*(n302) egg-laying defect by *hecd-1*(ar584)/*hecd-1*(ok1437) is observed. For the first set of three strains, the full genotype on chromosome III is *unc-36(e251) lin-12*(n302)/ + *lin-12*(n302).
(B) *lin-12(n676n930)* behaves like a partial loss-of-function allele at 25°C and causes a "2 AC defect" (left) and a "late Egl defect" (right) (Sundaram and Greenwald 1993). *hecd-1(ok1437)* enhances both of these defects, indicating that loss of *hecd-1* further reduces *lin-12* activity. The full genotype on chromosome III of all the strains is *unc-32(e189) arIs131[lag-2p::2nls-yfp::unc-54 3'UTR] lin-12(n676n930)*. *arIs131* marks the anchor cell.

**Figure 3.** *hecd-1(ar584) and hecd-1(ok1437) increase glp-1 activity in the germ line.* All experiments were performed at 20°C.

(A) *glp-1(ar202ts)* has elevated activity in the germ line, increasing mitotic proliferation and causing incompletely-penetrant sterility (Ste) at 20°C (Pepper et al. 2003). Both *hecd-1(ar584)* and *hecd-1(ok1437)* enhance the penetrance of this defect, indicating that *glp-1* activity is increased.

(B) *glp-1(bn18ts)* is a partial loss of function allele of *glp-1* (Kodoyianni et al. 1992). At 23°C, *glp-1(bn18ts)* is ~ 50% sterile, and its sterility is suppressed by *hecd-1(ar584)* or *hecd-1(ok1437)*.

(C) DAPI-staining reveals defects in germline anatomy. At 25°C, *glp-1(ar202ts)* displays a "weak Pro" phenotype associated with mild elevation of *glp-1* activity (Pepper et al. 2003). Under our growth conditions at 23°C, *glp-1(ar202ts)* display a mixture of weak Pro and wild-type. When *glp-1(ar202ts)* animals also carry *hecd-1(ok1437)* or *hecd-1(ar584)* and grown at 23°C, a "strong Pro" and tumorous (Tum) phenotype is also evident, further supporting the inference that loss of *hecd-1* increases *glp-1* activity.

(D) Images of *glp-1(ar202); hecd-1(ok1437)* animals scored in (C), with cartoons depicting the interpretation of the phenotype. DTC = Distal Tip Cell.
Figure 4. Evidence that loss of hecd-1 acts autonomously in the germ line to increase glp-1 activity. Bar graph shows the expression of mean ± standard error from three trials.

(A) Cartoon depicting the cell-cell interactions: the somatic Distal Tip Cell (DTC) is the origin of the LAG-2 signal that activates GLP-1/Notch in the germ line.

(B) hecd-1(RNAi) enhances glp-1(ar202) sterility. Enhancement is still seen in the presence of rrf-1(pk1417), a mutation that prevents RNAi in some somatic cells, including cells of the somatic gonad (Kumsta and Hansen 2012), indicating that hecd-1 is likely to act to increase glp-1(ar202) in the germ line. There is no statistically significant difference between the values obtained in the rrf-1(+) and rrf-1(pk1417) background.
LITERATURE CITED


Greenwald, I., 2012 Notch and the awesome power of genetics. *Genetics*.


hecd-1 modulates C. elegans Notch

Chen and Greenwald 17


**Table 1. Strains analyzed in this study.**

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"Scoring control" indicates that a *hecd-1(+)* strain was segregated from the same heterozygous genetic background used to generate the comparison with a *glp-1* mutant strain indicated.
**Fig. 1**
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Fig. 2
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Fig. 3D
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**Fig. 4**

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