LIN-12/Notch trafficking and regulation of DSL ligand activity during vulval induction in *Caenorhabditis elegans*

Daniel D. Shaye[1,2,*] and Iva Greenwald[1,3,†]

[1]Howard Hughes Medical Institute, Columbia University, New York, NY 10032, USA
[2]Department of Genetics and Development, Columbia University, New York, NY 10032, USA
[3]Department of Biochemistry and Molecular Biophysics, Columbia University, New York, NY 10032, USA
*Present address: Institut de Biología Molecular de Barcelona-CSIC, Parc Científic de Barcelona, Lab PBB31, C/ Josep Samitier, 1-5, 08028 Barcelona, Spain
†Author for correspondence (e-mail: greenwald@cancercenter.columbia.edu)

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Summary

A novel mode of crosstalk between the EGFR-Ras-MAPK and LIN-12/Notch pathways occurs during the patterning of a row of vulval precursor cells (VPCs) in *Caenorhabditis elegans*: activation of the EGFR-Ras-MAPK pathway in the central VPC promotes endocytosis and degradation of LIN-12 protein. LIN-12 downregulation in the central VPC is a prerequisite for the activity of the lateral signal, which activates LIN-12 in neighboring VPCs. Here we characterize *cis*-acting targeting sequences in the LIN-12 intracellular domain and find that in addition to a di-leucine motif, serine/threonine residues are important for internalization and lysine residues are important for post-internalization trafficking and degradation. We also identify two *trans*-acting factors that are required for post-internalization trafficking and degradation: ALX-1, a homolog of yeast Bro1p and mammalian *Alix* and the WWP-1/Su(dx)/Itch ubiquitin ligase. By examining the effects of mutated forms of LIN-12 and reduced *wwp-1* or *alx-1* activity on subcellular localization and activity of LIN-12, we provide evidence that the lateral signal-inhibiting activity of LIN-12 resides in the extracellular domain and occurs at the apical surface of the VPCs.

Key words: Notch, Ras, Endocytosis, *C. elegans*

Introduction

In *Caenorhabditis elegans*, six vulval precursor cells (VPCs), consecutively numbered P3.p-P8.p, have the potential to adopt one of three fates, termed 1°, 2° or 3° (reviewed by Sternberg, 2005). In wild-type hermaphrodites, cell-cell interactions result in an invariant pattern of fates termed 3°-3°-2°-1°-2°-3° (Fig. 1A). Descendants of the 1° and 2° cells will form the vulva, and descendants of the 3° cells will adopt a non-vulval fate. Two of the signaling events that specify the pattern of VPC fates are 'inductive' and 'lateral' signaling (Fig. 1A). An EGF-like inductive signal produced by the anchor cell of the gonad activates an EGF receptor-Ras-MAP kinase cascade in the underlying VPCs. Among other outputs, this cascade activates transcription via SUR-2/MDT-23, the MED23 subunit of the 'Mediator' transcription activation complex (Singh and Han, 1995; Boyer et al., 1999; Stevens et al., 2002; Bourbon et al., 2004). The centralmost VPC, P6.p, has the highest level of EGFR-Ras-MAPK activation; it becomes the presumptive 1° VPC and produces the lateral signal. The lateral signal is composed of three proteins of the Delta/Serrate/LAG-2 (DSL) family (Chen and Greenwald, 2004), which activate LIN-12 in the neighboring VPCs, P5.p and P7.p, to promote the 2° fate.

Crosstalk between the EGFR-Ras-MAPK and LIN-12/Notch pathways is important for proper VPC patterning. In P5.p and P7.p, the presumptive 2° VPCs, activation of LIN-12 results in the expression of multiple negative regulators of the EGFR-Ras-MAPK pathway, restricting the effects of the inductive signal to P6.p (Berset et al., 2001; Yoo et al., 2004). In P6.p, the presumptive 1° VPC, the EGFR-Ras-MAPK pathway leads to the transcription of lateral signal genes (Chen and Greenwald, 2004), but expression of these genes in P6.p is not sufficient to activate LIN-12 in neighboring VPCs. Instead, LIN-12 must be downregulated in P6.p in response to EGFR-Ras-MAPK activation in order for lateral signaling to occur: the LIN-12 intracellular domain has a 'downregulation targeting sequence' (DTS; see Fig. 1B), and if LIN-12 is stabilized in P6.p, by removing the DTS, lateral signaling is compromised (Shaye and Greenwald, 2002).

The DTS contains a di-leucine sorting motif, and we have proposed that endocytic trafficking of LIN-12 is altered upon EGFR-Ras-MAPK activation, leading to degradation. Indeed, we found that if the DTS is removed, LIN-12 is not internalized efficiently and instead accumulates on the apical surface of the VPCs. Furthermore, modified LIN-12 trafficking in response to EGFR-Ras-MAPK activation is likely to depend on the activity of at least one *trans*-acting factor transcribed in response to activation of Ras, as degradation of LIN-12 does not occur when the function of the SUR-2/Mediator transcription activator complex is removed (Shaye and Greenwald, 2002).

Endocytic downregulation has been well studied as a mechanism for attenuating the activity of activated receptors (reviewed by Sorkin and Von Zastrow, 2002). Downregulation of LIN-12 is a novel variation on this theme, as LIN-12 is downregulated as a consequence of activating a different signaling pathway. LIN-12 is likely to undergo a generic

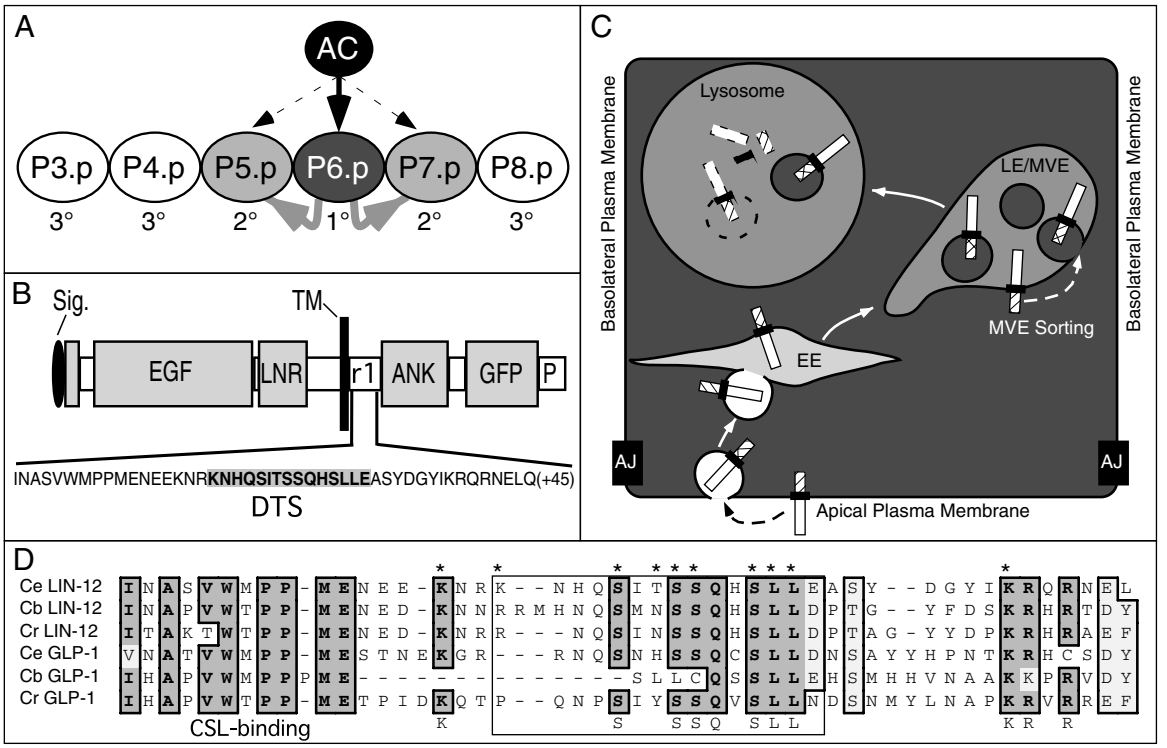

Fig. 1. Relevant background information. (A) In the third larval stage, an inductive signal (black arrow) from the gonadal anchor cell (AC) and a lateral signal (gray arrows) from P6.p impose a 3°-3°-2°-1°-2°-3° pattern of cell fates on six equivalent VPCs, P3.p-P8.p. Cell fates can be distinguished by appropriate markers (see text and legends of Figs 5, 6). (B) Full-length LIN-12, with hallmark regions of EGF-like (EGF), LIN-12/Notch repeat (LNR), cdc10/ankyrin (ANK) repeat and PEST sequence (P). The position of the green fluorescent protein (GFP) tag is also shown. 'Region 1' (r1) was shown to be sufficient to promote downregulation of membrane tethered GFP in P6.p (Shaye and Greenwald, 2002). (C) Canonical endocytic downregulation. Sub-apical adherens junctions (AJ), marked by the protein AJM-1 (Koppen et al., 2001), separate the apical surface from the basolateral surface. A receptor (white bar: extracellular domain; hatched bar: intracellular domain) marked for downregulation is internalized and trafficked to early endosomes (EE). From EEs, receptors that are tagged for downregulation are trafficked to late endosomes (LE)/multi-vesicular endosomes (MVE). An MVE-sorting step, by which the receptor is sorted into invaginating lumenal vesicles, removes the intracellular domain from the cytosol. MVE lumenal vesicles are delivered to the lysosome. (D) The first 45 amino acids of region 1 from LIN-12 and GLP-1 in *C. elegans (Ce)*, *C. briggsae (Cb)* and *C. remanei (Cr)* (Rudel and Kimble, 2001; Rudel and Kimble, 2002) have regions of conservation. The DTS is boxed, and conserved residues analyzed in this study are marked by asterisks. The conserved CSL-binding region (Kovall and Hendrickson, 2004) is also indicated. Alignments were obtained with the ClustalW feature of MacVector (Accelrys).

trafficking process, involving a series of protein sorting decisions that occur at different points in the endocytic pathway (Fig. 1C) (reviewed by Katzmann et al., 2002; Sorkin and Von Zastrow, 2002; Bonifacino and Traub, 2003). At the plasma membrane, receptors can be retained at the cell surface or be sorted into invaginating endocytic vesicles for internalization. In early endosomes, receptors destined to be recycled to the plasma membrane are sorted from those marked for downregulation, which are trafficked to late endosomes. En route to, or at, late endosomes, receptors marked for downregulation undergo a crucial sorting step known as multivesicular endosome (MVE) sorting (reviewed by Katzmann et al., 2002; Gruenberg and Stenmark, 2004). From late endosomes, downregulated receptors are trafficked to the lysosome, where they are degraded.

MVEs have lumenal vesicles that are formed by inward invagination and pinching of the outer endosomal membrane into the lumenal space (reviewed by Gruenberg and Stenmark, 2004). Transmembrane receptors targeted for degradation are typically transferred from the outer endosomal membrane into invaginating lumenal vesicles (Fig. 1C). This sorting step is usually dependent on ubiquitination of the receptor, and is achieved by three multi-protein ESCRT complexes, which recognize ubiquitinated receptors and de-ubiquitinate them as they are transferred into lumenal vesicles (reviewed by Katzmann et al., 2002). The MVE sorting step is crucial to degrade both the extracytosolic and cytosolic domains of transmembrane receptors; in the absence of this step, the cytosolic domain of receptors would remain exposed to the cytosol, potentially resulting in prolonged signaling function.

Here we address how and why LIN-12 is downregulated, through the analysis of mutations within and near the DTS and other engineered forms of LIN-12, and the identification and analysis of *trans*-acting factors that mediate downregulation. Our results suggest that LIN-12 is degraded via the MVE sorting pathway, that the extracellular domain of LIN-12 is the agent that inhibits lateral signaling, and that internalization without degradation is sufficient to relieve this inhibition.

Table 1. Plasmids and transgenes

Plasmid (concentration injected)	Protein expressed	Lines analyzed	Figure	*lin-12(0)* rescue¶ (number of lines/total)
pLIN12GFP* (5 μg/ml)	LIN-12(+)::GFP (wild type) ^{952}EEKNRKNHQSITSSQHSLLEASYDGYIKRQ^{981}	*arEx541* *arEx542* *arEx543*	2	6/7
p410† (5 μg/ml)	LIN-12(LLtoA)::GFP ^{952}EEKNRKNHQSITSSQHS***AA***EASYDGYIKRQ^{981}	*arEx495* *arEx496* *arEx497*	2	4/5
p411† (5 μg/ml)	LIN-12(S/TtoA)::GFP ^{952}EEKNRKNHQ***A***I***A***A***A***QH***A***LLEASYDGYIKRQ^{981}	*arEx498* *arEx499* *arEx500*	2	5/6
p413† (5 μg/ml)	LIN-12(KtoA)::GFP ^{952}EE***A***NR***A***NHQSITSSQHSLLEASYDGYI***A***RQ^{981}	*arEx514* *arEx515* *arEx516*	2	7/8**
p415* (5 μg/ml)	LIN-12(S/TtoD)::GFP ^{952}EEKNRKNHQ***D***I***DDD***QH***D***LLEASYDGYIKRQ^{981}	*arEx524* *arEx525* *arEx526*	3	7/11
p405‡ (20 μg/ml)	LIN-12(ΔEΔDTS)::GFP††	*arEx409* *arEx410*	6	
p406‡ (20 μg/ml)	LIN-12(extra)::TM::GFP	*arEx411* *arEx412*	6	
p408§ (20 μg/ml)	LIN-12(extra)::TM::DTS::GFP	*arEx482* *arEx483* *arEx485*	6	
p409§ (20 μg/ml)	LIN-12(extra)::TM::region1::GFP	*arEx492* *arEx493*	6	

Transgenes containing *unc-4(+)* were analyzed in an *unc-4(e120)* background. Transgenes containing *pha-1(+)* were analyzed in a *pha-1(e2123ts)* background. Constructs driven by *lin-12* genomic sequences (Figs 2, 3) were analyzed at 20°C. Constructs driven by the *egl-17* promoter (Fig. 6) were analyzed at 25°C.

DTS is underlined.

*Mixed with *unc-4(+)* (30 μg/ml pNC4.21) (see Miller and Niemeyer, 1995), *ttx-3*p::*gfp* (60 μg/ml pTTX-3GFP) (see Hobert et al., 1997) and *egl-17*p::*lacZ* (20 μg/ml pNH291) (see Burdine et al., 1998).

†Mixed with *unc-4(+)* (30 μg/ml), *ttx-3*p::*gfp* (60 μg/ml) and pBluescript (Stratagene, USA) carrier (20 μg/ml).

‡Mixed with *unc-4(+)* (30 μg/ml), *egl-17*p::*lacZ* (20 μg/ml) and pBluescript carrier (30 μg/ml).

§Mixed with *pha-1(+)* (50 μg/ml pBX) (see Granato et al., 1994), *ttx-3*p::*gfp* (60 μg/ml) and *egl-17*p::*lacZ* (20 μg/ml).

¶Rescue was assessed in a *lin-12(n941)* background, and the effects on downregulation and lateral signaling were assessed in a *lin-12(+)* background (see Shaye and Greenwald, 2002).

**Essentially all hermaphrodites carrying this transgene display a Multivulva (Muv) phenotype, which suggests elevated *lin-12* activity (see text).

††The DTS, ^{957}K through ^{971}E, was deleted and replaced by four amino acids, MAAG. LIN-12(ΔEΔDTS)::GFP is highly toxic but causes a Muv phenotype when expressed using regulatory sequences from the *sel-12* gene, which drives expression in all VPCs and many other cells (data not shown).

Materials and methods

Strains, genetics and transgenes

Information about all mutations can be found in WormBase (http://www.wormbase.org). The *arIs82* transgene, which expresses LIN-12(+)::GFP, is described in Shaye and Greenwald (Shaye and Greenwald, 2002). Plasmids and transgenes generated in this study are shown in Table 1. Transgenes were generated by germline injection using standard methods (Mello and Fire, 1995).

Yeast two-hybrid screen

Yeast strains, plasmids and methods are described in the ProQuest two-hybrid system manual (Invitrogen). The starting strain contained the bait plasmid, LIN-12(region 1) fused in frame to the Gal4 DNA binding domain in plasmid pDBLeu, in strain MaV203. Approximately 3.84×10^6 clones of a commercially available *C. elegans* cDNA library (Invitrogen) were screened, and five clones containing cDNAs from four different genes were not self-activating and interacted with LIN-12(region1) upon retransformation. These clones were tested by RNAi for an effect on LIN-12 downregulation (see below). Only clone pB8-1 showed an effect. This clone carried an insert corresponding to most of the gene R10E12.1B (coding sequences for the first 37 residues are missing from pB8-1), which is now named *alx-1* based on sequence homology (see Results).

Feeding RNAi

For *alx-1(RNAi)*, we cloned the cDNA insert from clone pB8-1 (see above) into the double-T7 promoter plasmid pPD129.36 (Timmons et al., 2001) to generate plasmid p402. For *wwp-1(RNAi)*, we cloned the cDNA insert from clone yk1104a10 (kindly provided by Y. Kohara) into pPD129.36 to generate plasmid p416. For *lacZ(RNAi)* we used plasmid pXK10 (Karp and Greenwald, 2003). RNAi plasmids were transformed into HT115 bacteria and used for RNAi as described by Timmons and Fire (Timmons and Fire, 2001), except that IPTG was omitted from the overnight seed culture and the plates contained 60 μg/ml AMP and 6 mmol/l IPTG. Synchronized starved L1-stage *arIs82* hermaphrodites were placed on bacterial lawns and grown at 25° until the mid-L3 stage (about 18 hours). Larvae were then fixed and stained as described below.

Immunofluorescence and image analysis

For lines expressing mutant proteins from *lin-12* genomic constructs (Figs 2, 3), eggs were collected and grown for 42 hours at 20°C. For lines carrying *egl-17*p-driven constructs (Fig. 6), eggs were collected

and grown for 32 hours at 25°C. Fixation, staining and image acquisition were essentially as described (Shaye and Greenwald, 2002), except that the monoclonal antibody MH27 (Developmental Studies Hybridoma Bank, USA), which recognizes AJM-1, an apical component of the adherens junction (Priess and Hirsh, 1986; Koppen et al., 2001), was used at a dilution of 1:600. For measurements of endocytic puncta in LIN-12(+)::GFP and LIN-12(KtoA)::GFP images, we analyzed single confocal sections from five individual animals. Measurements were done with ImageJ software (National Institutes of Health, USA).

Results

In a previous study (Shaye and Greenwald, 2002), we showed that a 90-amino-acid juxtamembrane fragment of the LIN-12 intracellular domain, 'region 1', is sufficient to promote downregulation in P6.p. When we deleted a 15-amino-acid sequence within this region, we blocked internalization and downregulation of LIN-12. We termed this 15-amino-acid sequence the 'downregulation targeting sequence' (DTS). In this study, we will show that in addition to a di-leucine motif and serine/threonine residues within the DTS, lysine residues that flank the DTS are also crucial for downregulation. Region 1 and the DTS are indicated in Fig. 1B,D.

A di-leucine motif and nearby serine/threonine and lysine residues play distinct roles in LIN-12 trafficking

The DTS contains two adjacent leucine residues (Fig. 1B,D). Many di-leucine-based endocytic sorting signals that mediate internalization and post-internalization trafficking to endosomes/lysosomes have been characterized (reviewed by Bonifacino and Traub, 2003). Both leucines are required for all activities of the signal, although in some cases one leucine can be substituted by a valine, isoleucine or methionine. When these residues are mutated to alanine in the context of the full-length, GFP-tagged LIN-12 protein LIN-12(+)::GFP, the

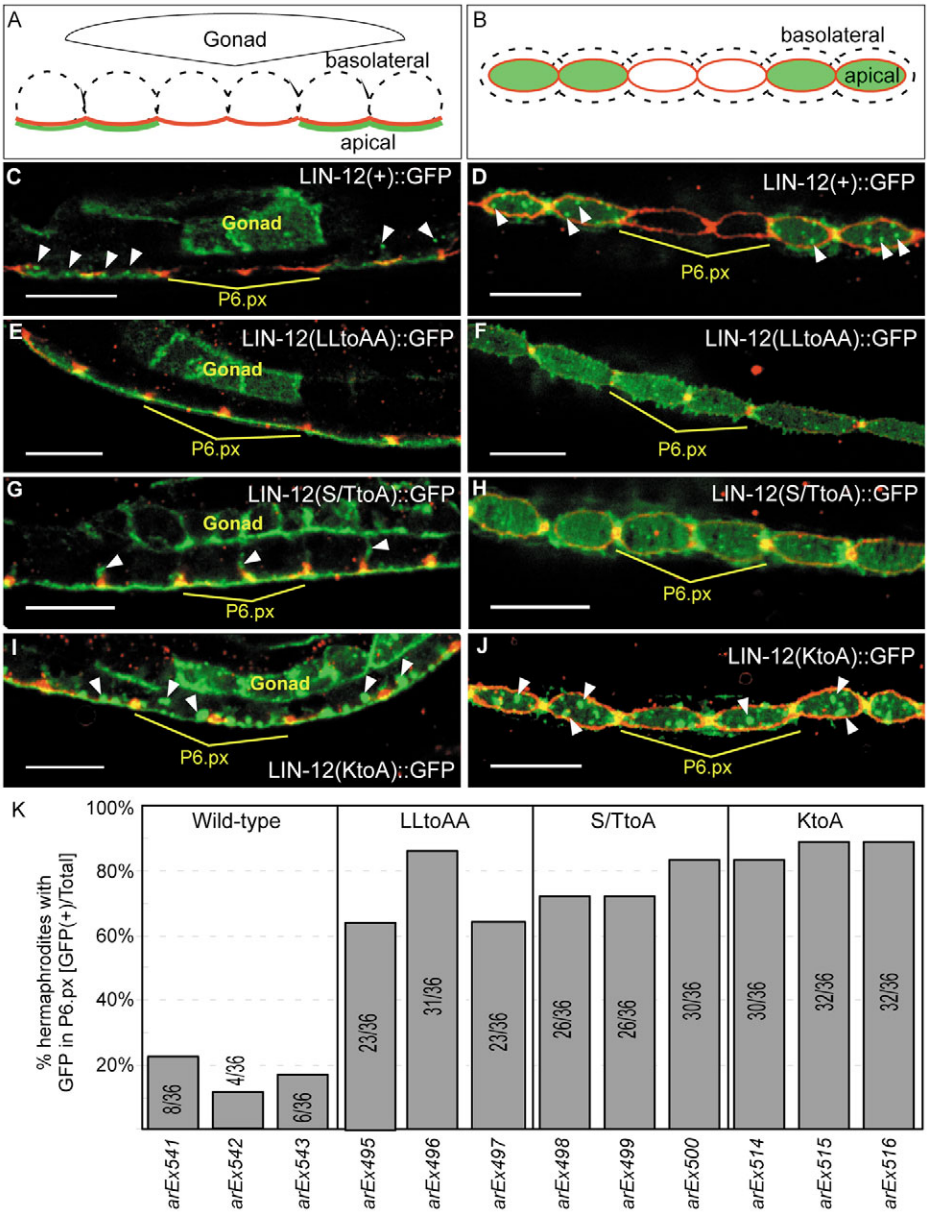

Fig. 2. *Cis*-determinants required for LIN-12 downregulation. GFP (green), and AJM-1 (red) were visualized by immunofluorescence (see Materials and methods). Left panels show lateral views, with the apical surface toward the bottom. Right panels show ventral views, with the apical surface surrounded by AJM-1 staining. Throughout this study, the effect on downregulation was assessed in the daughters of the VPCs – the Pn.px stage – to ensure that P6.p has been induced and that there has been sufficient time for downregulation. (A,B) Schematic representation of VPCs at the Pn.px stage; green indicates LIN-12 accumulation in the apical domain, dashed lines the basolateral domain. Note that LIN-12 remains apical wherever it is detected in VPCs and their descendants. (C,D) LIN-12(+)::GFP is downregulated in the 1° lineage (P6.px), and can be seen at the apical membrane and in endocytic puncta (arrowheads) in the 2° lineage (P5.px and P7.px). (E,F) The DTS di-leucine is required for internalization and degradation. (G,H) The DTS serine/threonine residues are required for internalization and degradation. We note that these residues may regulate other aspects of LIN-12 trafficking or localization, as we sometimes see some basolateral accumulation of LIN-12(S/TtoA)::GFP (arrowheads in G). (I,J) LIN-12(KtoA)::GFP is internalized but not degraded in the 1° lineage; it accumulates in large and pleiomorphic endocytic puncta (arrowheads), suggestive of late endosomes (see also Fig. 4C). (K) Quantification of downregulation of different LIN-12 proteins. Scale bar: 10 μm.

mutant protein [LIN-12(LLtoAA)::GFP] effectively rescues the sterility and lethality of *lin-12(0)* mutants (Table 1). However, LIN-12(LLtoAA)::GFP appears to accumulate mostly at the apical membrane with little accumulation in intracellular puncta (Fig. 2E,F), indicating that the di-leucine is crucial for LIN-12 internalization. It is possible that the di-leucine is also involved in other aspects of trafficking, but the strong internalization defect in the mutant precludes assessing these other potential roles.

Other amino acids contribute to the activity of characterized di-leucine motifs, including upstream negatively charged amino acids and serine/threonine residues that can be phosphorylated to modulate the activity of these signals (Bonifacino and Traub, 2003). We compared the sequence of region 1 from *C. elegans* LIN-12 with the corresponding sequence of GLP-1, the other *C. elegans* Notch protein, which can functionally substitute for LIN-12 (Fitzgerald et al., 1993), and with LIN-12 and GLP-1 homologs for *C. briggsae* and *C. remanei* (Fig. 1D). In addition to the conserved leucine residues, we identified conserved serines and a non-conserved threonine as potential sites of phosphorylation, and conserved lysine residues as potential sites of ubiquitination. LIN-12(S/TtoA)::GFP and LIN-12(KtoA)::GFP mutants effectively rescued the sterility and lethality of *lin-12(0)* mutants (Table 1), but had different effects on downregulation.

LIN-12(S/TtoA)::GFP, like LIN-12(LLtoAA)::GFP, appears to accumulate mostly at the apical membrane (Fig. 2G,H), suggesting that these residues are also important in regulating internalization. Some LIN-12(S/TtoA)::GFP hermaphrodites display mislocalization to the basolateral plasma membrane (Fig. 2G, arrowheads), suggesting that the Ser/Thr residues within the DTS may also modulate post-internalization trafficking. Internalization is not affected by mutation of the lysine residues; instead, LIN-12(KtoA)::GFP showed a striking accumulation in large and irregularly shaped intracellular vesicles (Fig. 2I,J arrowheads). We measured the area of these vesicles (see Materials and methods), and found that LIN-12(+)::GFP accumulated in vesicles that measured on average 0.250 μm^2 (±0.078, n=66). By contrast, LIN-12(KtoA)::GFP accumulated in vesicles that measured on average 0.442 μm^2 (±0.227, n=122). This difference in size was highly significant ($P=2.8\times10^{-10}$, two-tailed, unpaired Student's t-test). Therefore, we believe that LIN-12(KtoA)::GFP accumulates in endocytic compartments distinct from those where internalized LIN-12(+)::GFP is seen. These large vesicles may be late endosomes and/or lysosomes, as these compartments tend to be significantly larger and more pleiomorphic than earlier compartments in the endocytic system (Gruenberg and Stenmark, 2004; Patton et al., 2005). Such accumulation of LIN-12(KtoA)::GFP would be consistent with a block in MVE sorting, which depends on ubiquitination and occurs at late steps in the endocytic pathway.

Mutations that may mimic phosphorylated Ser/Thr enhance internalization from the apical membrane and do not affect downregulation of LIN-12 in P6.p

LIN-12(S/TtoA)::GFP was inefficiently internalized and was not downregulated (Fig. 2G,H), suggesting that phosphorylation of some, or all, of these serine/threonine residues is required for internalization, and perhaps for post-internalization trafficking of LIN-12. If these residues serve as a substrate for a protein kinase, then mutation of these residues to aspartate might mimic phosphorylated forms. LIN-12(S/TtoD)::GFP rescues *lin-12(0)* defects (Table 1) and is efficiently downregulated in the 1° lineage (Fig. 3A,B,E), suggesting that the S/TtoD mutations are not detrimental to LIN-12 function, internalization and downregulation.

If these residues were phosphorylated during the process of internalization and downregulation, then we would expect to see an effect of these mutations on subcellular localization or the promotion of downregulation in cells where we usually do not see it. Indeed, the phospho-mimicking mutations appeared to enhance internalization of LIN-12 from the apical plasma membrane, as LIN-12(S/TtoD)::GFP appeared to accumulate mostly in internal vesicles (Fig. 3A,B, white arrowheads). Additionally, even though these mutations did not promote downregulation in cells other than P6.p, we observed conspicuous GFP accumulation at what appeared to be the basolateral, instead of the apical, plasma membrane in the daughters of P5.p/P7.p (Fig. 3A,B, yellow arrowheads; compare with Fig. 2A,B).

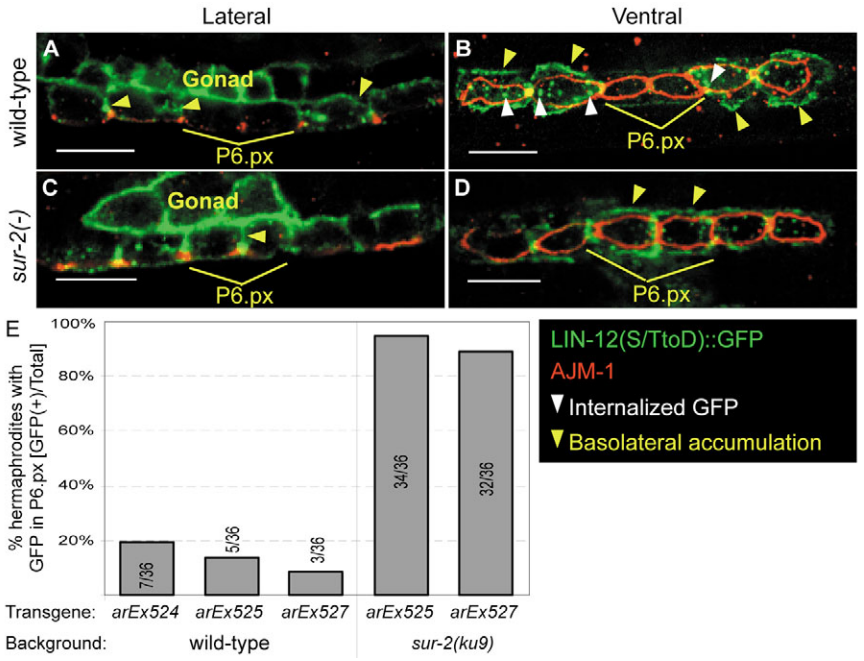

Fig. 3. The phosphomimicking mutant LIN-12(S/TtoD)::GFP. (A,B) LIN-12(S/TtoD)::GFP is downregulated normally in the 1° lineage. Enhanced internalization and increased accumulation in endocytic puncta (white arrowheads) and the basolateral plasma membrane (yellow arrowheads) is seen in the 2° lineage. (C,D) Loss of *sur-2* activity prevents LIN-12(S/TtoD)::GFP downregulation in the 1° lineage and causes accumulation in the basolateral plasma membrane (yellow arrowheads). (E) Quantification of LIN-12(S/TtoD)::GFP downregulation. Scale bars: 10 μm.

However, phospho-mimicking mutations are not sufficient to promote downregulation in VPCs where the EGFR-Ras-MAPK pathway and SUR-2/Mediator are not active. This observation could indicate that a Ser/Thr kinase that phosphorylates the LIN-12 DTS is not a target, or at least not the only target, of *sur-2*. Alternatively, the phospho-mimicking mutations may functionally bypass the requirement for *sur-2*, but the basolateral localization of this mutant protein in P5.p and P7.p precludes effective downregulation. We address these questions below.

LIN-12(S/TtoD)::GFP downregulation is dependent on *sur-2*

If phosphorylation of serine/threonine residues were the only step regulated by a target of the EGFR-Ras-MAPK pathway, then we would expect that the phospho-mimicking mutations could bypass the requirement for *sur-2*. However, LIN-12(S/TtoD)::GFP was not downregulated in *sur-2(–)* hermaphrodites (Fig. 3C,D,E). Thus, the EGFR-Ras-MAPK pathway does not solely regulate a kinase that modifies these residues to mediate LIN-12 downregulation. There may be at least one other factor regulated by *sur-2* required for downregulation. Alternatively, the putative kinase that phosphorylates the LIN-12 DTS may also phosphorylate another *trans*-acing factor required for downregulation. We note that the LIN-12(S/TtoD)::GFP that persisted in the 1° lineage of *sur-2(–)* hermaphrodites accumulated mostly at the basolateral membrane (Fig. 3C,D, yellow arrowheads), as in P5.p and P7.p daughters in a wild-type background (Fig. 3A,B). This suggests that the failure to downregulate LIN-12(S/TtoD)::GFP in P5.p and P7.p in wild-type hermaphrodites is not due to the basolateral localization of this protein, but instead that this re-localization is a consequences of reduced or absent *sur-2* activity in these cells.

ALX-1 is required for LIN-12 degradation, but not internalization

To identify potential *trans*-acting factors, we performed a yeast two-hybrid screen for proteins that interact with the 90-amino-acid juxtamembrane fragment of the LIN-12 intracellular domain (region 1) that is sufficient to promote downregulation of a membrane-tethered GFP reporter. We obtained four candidate interactors (Fig. 4A and data not shown), and analyzed all using RNAi to reduce their activity in hermaphrodites that carry a LIN-12(+)::GFP transgene, followed by immunofluorescence to assess downregulation (see Materials and methods). Only *alx-1(RNAi)* affected downregulation: significantly more *alx-1(RNAi)* hermaphrodites than *lacZ(RNAi)* hermaphrodites failed to downregulate LIN-12(+)::GFP (Fig. 5A,C,I; $P=3.5 \times 10^{-3}$, two-tailed, unpaired Student's *t*-test). Additionally, LIN-12(+)::GFP was able to accumulate in intracellular puncta in *alx-1(RNAi)* hermaphrodites (Fig. 5C, arrowheads). These

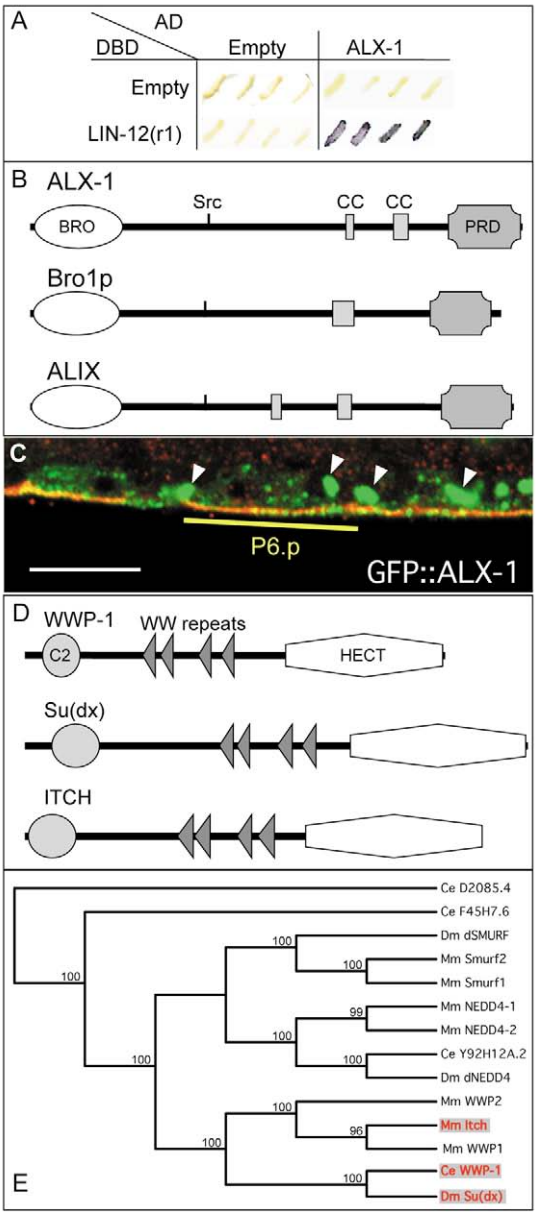

Fig. 4. *Trans*-acting factors required for LIN-12 downregulation. (A) β-Galactosidase filter lift assay showing that ALX-1 interacts with LIN-12(region 1) in the yeast two-hybrid system. Four independent transformants carrying the activation domain (AD) and DNA-binding domain (DBD) fusions indicated are shown. (B) Domain structure of *Ce* ALX-1, *Saccharomyces cerevisiae (Sc)* Bro1p and *H. sapiens (Hs)* Alix, showing the N-terminal BRO1 domain, which mediates localization to late endosomes (Kim et al., 2005), a highly conserved Src kinase phosphorylation consensus site, one or two coiled-coils and a C-terminal proline-rich domain (PRD). (C) A GFP::ALX-1 translational reporter is highly and widely expressed (data not shown), and is seen in all the VPCs. This is a lateral view, with AJM-1 (red) detected by immunofluorescence, while native fluorescence of fixed GFP::ALX-1 is visualized without the need for anti-GFP staining. Note that GFP::ALX-1 appears to accumulate in vesicular structures (arrowheads) that are probably LE/MVEs. (D) Domain structure of *Ce* WWP-1, *D. melanogaster* (*Dm)* Su(dx) and *Mus musculus* (*Mm)* Itch, showing the C2 phospholipid-binding motif, WW motifs, and the ubiquitin-conjugating HECT domain (reviewed by Ingham et al., 2004). (E) Phylogenetic relationship among Nedd4-family members from *C. elegans*, *Drosophila* and mouse. Neighbor-joining analysis of the full-length proteins was done with MacVector (Accelrys), using an uncorrected 'p' setting for distance calculation and 1000 bootstrap repetitions to calculate the percent branch support, shown above each branch. *Ce*D2085.4 (HECT domain only) and *Ce*F45H7.6 (WW repeats and HECT domain) were used to anchor the tree. Scale bar: 10 μm.

results implicate *alx-1* in LIN-12 downregulation at a post-internalization step.

ALX-1 is the *C. elegans* ortholog of yeast Bro1p and mammalian *Alix* (Fig. 4B). Bro1p plays a role in MVE sorting at a late step, probably after the function of the ESCRT-III complex (Springael et al., 2002; Nikko et al., 2003; Odorizzi et al., 2003; Luhtala and Odorizzi, 2004). *Alix* promotes MVE formation and interacts with various endocytic proteins, including components of the ESCRT-I and ESCRT-III complexes (Martin-Serrano et al., 2003; Strack et al., 2003; Matsuo et al., 2004). Our data are consistent with a role for *alx-1* in MVE sorting, and imply that downregulation of LIN-12 is achieved via this endocytic pathway.

We note that *alx-1(RNAi)* hermaphrodites resemble *sur-2* mutant hermaphrodites, in that LIN-12 is internalized but not degraded (Shaye and Greenwald, 2002), raising the possibility that *alx-1* is a target of *sur-2*. However, a GFP::ALX-1 translational reporter (kindly provided by B. Grant) appeared to be highly and ubiquitously expressed, including in all the VPCs and their descendants (data not shown), suggesting that *alx-1* is not a transcriptional target of the EGFR-Ras-MAPK pathway. GFP::ALX-1 appeared to accumulate in very large and pleiomorphic vesicular structures in the VPCs, which seem likely to be *C. elegans* MVEs (Fig. 4C).

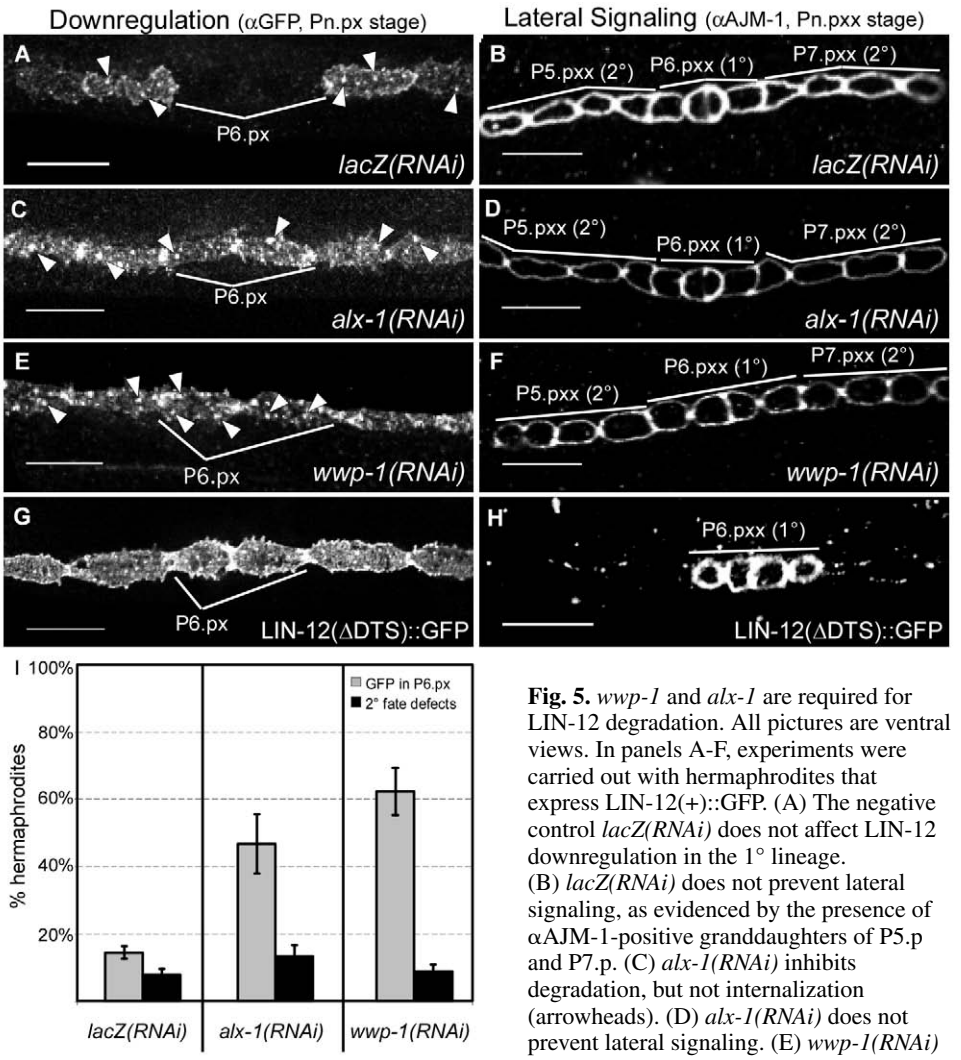

Fig. 5. *wwp-1* and *alx-1* are required for LIN-12 degradation. All pictures are ventral views. In panels A-F, experiments were carried out with hermaphrodites that express LIN-12(+)::GFP. (A) The negative control *lacZ(RNAi)* does not affect LIN-12 downregulation in the 1° lineage. (B) *lacZ(RNAi)* does not prevent lateral signaling, as evidenced by the presence of αAJM-1-positive granddaughters of P5.p and P7.p. (C) *alx-1(RNAi)* inhibits degradation, but not internalization (arrowheads). (D) *alx-1(RNAi)* does not prevent lateral signaling. (E) *wwp-1(RNAi)* inhibits degradation but not internalization, as LIN-12(+)::GFP accumulates in endocytic puncta (arrowheads). (F) *wwp-1(RNAi)* does not prevent lateral signaling. (G,H) For comparison, LIN-12(ΔDTS)::GFP is not downregulated, and inhibits lateral signaling. (I) Quantification of downregulation and lateral signaling in RNAi experiments. Each set was performed in parallel three different times. Each time, 30 hermaphrodites at the Pn.px and Pn.pxx stages were scored for presence of GFP (gray bars) and 2° fate defects (black bars). Scale bars: 10 μm.

WWP-1 is required for LIN-12 degradation, but not internalization

Our analysis of the DTS suggests the existence of a kinase that can phosphorylate the serine/threonine residues of the DTS and a ubiquitin ligase that targets the DTS-flanking lysines. The yeast two-hybrid screen did not yield predicted serine/threonine kinases or ubiquitin ligases, so we considered a candidate gene approach.

Unfortunately, there are approximately 230 predicted serine/threonine kinases in *C. elegans* (WormBase release WS144, searched for Interpro motif IPR002290), and it would be prohibitively difficult to test them all for failure of downregulation. Although there are also many potential ubiquitin ligases, we focused on the Nedd4 family of E3 ubiquitin ligases (Ingham et al., 2004), because Suppressor of Deltex [Su(dx)], a *Drosophila* Nedd4-like protein, was known to be a negative regulator of Notch (Cornell et al., 1999), and the mouse Nedd4-like protein Itch was also known to ubiquitinate mouse Notch1 (Qiu et al., 2000; McGill and McGlade, 2003). Analysis of the *C. elegans* genome for proteins with the domain structure of Nedd4-family proteins (Fig. 4D) identified Y65B4BR.4 (WWP-1) (see also Huang et al., 2000) as the apparent ortholog of Su(dx) and Itch (Fig. 4E).

We depleted *wwp-1* activity by RNAi and examined the effect on LIN-12(+)::GFP accumulation (Materials and methods). About 62% of *wwp-1(RNAi)* hermaphrodites failed to downregulate LIN-12(+)::GFP (Fig. 5E,I), as opposed to 14% of *lacZ(RNAi)* hermaphrodites (Fig. 5A,I). This difference was highly statistically significant ($P=3.3\times10^{-4}$, two-tailed, unpaired Student's t-test), indicating that *wwp-1* is required for LIN-12 downregulation. Additionally, LIN-12(+)::GFP was still able to accumulate in intracellular puncta in *wwp-1(RNAi)* hermaphrodites (Fig. 5E, arrowheads). These results lead us to conclude that *wwp-1* is required for LIN-12 downregulation at

a post-internalization step. A GFP::WWP-1 translational reporter, in which GFP is fused in frame to the amino terminus of WWP-1 in a genomic context, was widely expressed and appeared to accumulate in the cytoplasm of all VPCs and their descendants (data not shown), suggesting that *wwp-1* is unlikely to be a transcriptional target of the EGFR-Ras-MAPK pathway.

Lateral signaling is normal in *wwp-1(RNAi)* and *alx-1(RNAi)* hermaphrodites: correlation between subcellular localization and lateral signal inhibition

If lateral signaling occurs, P5.p and P7.p undergo three rounds of division, and their descendants stain with the adherens junction marker αAJM-1 (see Fig. 5B). If lateral signaling fails, then P5.p and P7.p undergo one round of division and their daughters join the hypodermal syncytium, so they do not stain with αAJM-1 (see Fig. 5H). Deletion of the LIN-12 DTS results in a concomitant failure of downregulation and lateral signaling (Fig. 5G,H) (Shaye and Greenwald, 2002). By contrast, *wwp-1(RNAi)* and *alx-1(RNAi)* hermaphrodites displayed significant persistence of LIN-12 without compromised lateral signaling (Fig. 5C-F,I). Internalization appeared to be normal, but post-internalization trafficking and degradation appeared to be affected, suggesting that the crucial event that allows lateral signaling to occur is clearance of LIN-12 from the surface. The experiments presented in subsequent sections address why persistent LIN-12 may inhibit lateral signaling at the cell surface, but not after internalization.

LIN-12 activity is not necessary, and the extracellular domain is sufficient, for lateral signal inhibition

We first considered the possibility that persistent LIN-12 at the cell surface of P6.p is activated for signal transduction, perhaps in an autocrine fashion by ligands produced by P6.p (Chen and Greenwald, 2004). Ectopic activation of LIN-12 in P6.p might extinguish the production of the lateral signal through reducing

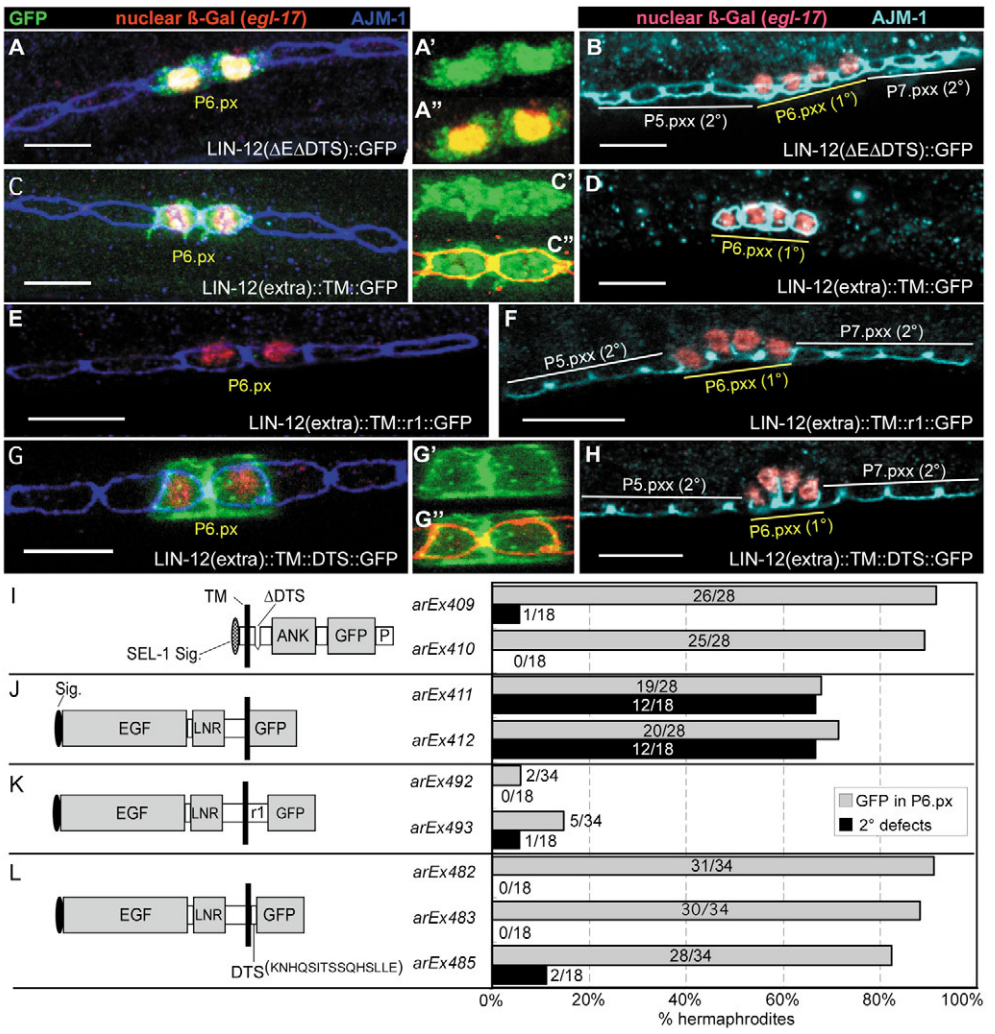

Fig. 6. Mechanism of lateral signaling inhibition by persistent LIN-12 in the 1° lineage. All pictures are ventral views. In left panels, GFP (green), AJM-1(blue) and nuclear β-galactosidase expressed from *egl-17*p::lacZ (red) are visualized in Pn.px stage hermaphrodites. In right panels, AJM-1 (teal) and *egl-17*p::LacZ (red) are visualized in Pn.pxx stage hermaphrodites. (A) LIN-12(ΔEΔDTS)::GFP is an activated form of LIN-12 that is not downregulated. Most of the GFP is nuclear localized. A' shows magnification of GFP staining, and A" shows overlapping nuclear β-galactosidase and GFP. (B) LIN-12(ΔEΔDTS)::GFP does not affect VPC fates. The 1° fate is assessed by the presence of P6.pxx cells marked with *egl-17*p::LacZ and AJM-1. The 2° fate is scored as described in Fig. 5B. (C) LIN-12(extra)::TM::GFP is not downregulated, and accumulates at the apical plasma membrane. C' shows magnification of GFP staining, while C" shows that most of the GFP is enclosed inside the AJM-1 boundary (pseudocolored red). (D) LIN-12(extra)::TM::GFP does not affect the 1° fate, but inhibits lateral signaling as evidenced by loss of AJM-1-marked granddaughters of P5.p/P7.p. (E,F) Adding region 1 promotes downregulation of LIN-12(extra)::TM::GFP and allows for normal lateral signaling. (G) Adding the DTS to LIN-12(extra)::TM::GFP does not promote downregulation. G' shows magnification of GFP staining, while G" shows that most of the GFP is outside the AJM-1 boundary (pseudocolored red), consistent with basolateral accumulation. (H) LIN-12(extra)::TM::DTS::GFP does not inhibit lateral signaling. (I-L) Structure of GFP-tagged proteins expressed above, and quantification of downregulation (gray bars) and 2° fate defects (black bars).

the activity of the EGFR-Ras-MAPK pathway or by another mechanism.

To test this model, we expressed a constitutively active form of LIN-12 that cannot be downregulated and assessed whether lateral signaling is compromised. We activated LIN-12 by truncating its ectodomain (see Struhl and Adachi, 2000) and stabilized it in P6.p by removing the DTS. We expressed this form, LIN-12(ΔEΔDTS)::GFP, under the control of the *egl-17* promoter, which is continuously expressed in P6.p after inductive signaling (Burdine et al., 1998; Shaye and Greenwald, 2002; Yoo et al., 2004). We found that LIN-12(ΔEΔDTS)::GFP was not downregulated (Fig. 6A,I), and did not cause a lateral signaling defect (Fig. 6B,I), indicating that inhibition of lateral signaling is not caused by activation of LIN-12 in P6.p. GFP staining was found mostly in the nucleus (Fig. 6A',A''), consistent with the expected constitutive transmembrane cleavage and signal transduction of this form. In addition, we found that LIN-12(ΔEΔDTS)::GFP did not appear to affect the 1° fate of P6.p (Fig. 6B), suggesting that a presumptive 1° cell is refractory to activated LIN-12.

As activated LIN-12 did not cause lateral signal inhibition, we considered the possibility that the extracellular domain of LIN-12, which was deleted from the activated form, inhibits the function of the DSL proteins expressed in P6.p. We made LIN-12(extra)::TM::GFP, which consists of the extracellular and transmembrane domains of LIN-12 and a cytosolic GFP tag (Fig. 6J). When this protein was expressed in P6.p, it was not downregulated (Fig. 6C,J), and appeared to accumulate mostly at the apical plasma membrane, as it was mostly restrained to the apical domain demarcated by AJM-1 (Fig. 6C',C''). LIN-12(extra)::TM::GFP caused a highly penetrant lateral signaling defect (Fig. 6D,J). Therefore, persistence of the LIN-12 extracellular domain is sufficient to cause lateral signal inhibition. The addition of region 1 to LIN-12(extra)::TM::GFP restored downregulation and relieved lateral signal inhibition (Fig. 6E,F,K), indicating that the mechanism by which LIN-12(extra)::TM::GFP inhibits lateral signaling is related to the inhibition caused by persistence of full-length LIN-12.

Apical localization of the LIN-12 extracellular domain is necessary for lateral signal inhibition

We assessed whether the DTS is sufficient to cause downregulation by adding it to LIN-12(extra)::TM::GFP, and found that it was not (Fig. 6G,L). However, we noticed that this protein was mostly found outside the apical domain, and instead appeared to accumulate on the basolateral membrane (Fig. 6G',G''). Thus, the DTS is not sufficient to promote downregulation, but appears to promote basolateral re-localization or to inhibit apical localization. The basolaterally localized LIN-12(extra)::TM::DTS::GFP protein is not degraded, nor does it inhibit lateral signaling (Fig. 6H,L). These observations suggest that apical localization of the LIN-12 extracellular domain is crucial for lateral signal inhibition.

We note that the subcellular localization of LIN-12(extra)::TM::DTS::GFP was similar to that of LIN-12(S/TtoD)::GFP, the phospho-mimicking mutant described above, in cells where *sur-2* was not active (Fig. 3). This similarity leads us to speculate that the DTS is phosphorylated in LIN-12(extra)::TM::DTS::GFP, but this protein lacks other determinants required for degradation, such as the conserved lysines that may mediate ubiquitination.

Discussion

In a previous study (Shaye and Greenwald, 2002), we showed that ectopic stabilization of LIN-12 in P6.p abrogates lateral signaling, which is necessary to activate LIN-12/Notch in neighboring VPCs and specify their correct fates. This established LIN-12 downregulation in response to EGFR-Ras-MAPK activation as a crucial event during VPC patterning. We also showed that deletion of a downregulation targeting sequence (DTS) in the juxtamembrane region of the LIN-12 intracellular domain blocks both internalization and degradation, suggesting that degradation requires endocytic trafficking. Here we have characterized *cis*-acting targeting sequences and *trans*-acting factors that mediate internalization and degradation, and the relationship between endocytic trafficking and the ability of LIN-12 to inhibit the paracrine action of ligands for LIN-12/Notch proteins. We discuss the implications of our results for the regulation of internalization and degradation of LIN-12, the differences in the apparent mechanisms for internalization of LIN-12 and *Drosophila* Notch, and the mechanism of lateral signal inhibition by surface LIN-12.

Regulation of LIN-12 internalization

We have found that a di-leucine motif and serine/threonine residues in the DTS are required for internalization, and that lysine residues near the DTS are required for degradation (see below). A mutant form in which the DTS serine/threonine residues have been mutated to alanine shows diminished internalization of LIN-12 in all VPCs, suggesting that at least some of these serine/threonine residues are likely to be constitutively phosphorylated to promote basal internalization.

A mutant form in which the serine/threonine residues have been mutated to aspartate displayed enhanced internalization of LIN-12 from the apical plasma membrane in all VPCs and basolateral accumulation of LIN-12 in VPCs with reduced *sur-2* activity, i.e. P5.p and P7.p in a wild-type background, or P5.p, P6.p and P7.p in a *sur-2(–)* background. We also found that the wild-type DTS was sufficient to cause basolateral accumulation when flanking sequences that mediate degradation were not present. This observation leads us to speculate that basolateral localization indicates a phosphorylated state of LIN-12 that would usually not be detected because such a protein is normally marked for degradation. Basolateral localization may represent an intermediate step in the normal downregulation process. If so, *sur-2* may regulate the activity of a basolaterally located factor required for degradation of phosphorylated LIN-12, and thus this factor would not be found in P5.p and P7p. Alternatively, basolateral accumulation may be an aberrant consequence of the inability to degrade a phosphorylated LIN-12 in cells where *sur-2* is not active. At this point, we are unable to distinguish between these possibilities.

The mutant form of LIN-12 in which the DTS serine/threonine residues had been mutated to aspartate was downregulated in P6.p, but was not ectopically downregulated in other VPCs. Furthermore, this form did not bypass the requirement for *sur-2*, as this protein accumulated in the

basolateral membrane of P6.p in a *sur-2(–)* background. These observations suggest that phosphorylation of LIN-12 is not the limiting step in downregulation, and thus that the EGFR-Ras-MAPK pathway does not simply lead to transcription of a kinase that promotes LIN-12 phosphorylation. However, the nature of the link between EGFR-Ras-MAPK activation remains to be determined.

Regulation of LIN-12 post-internalization trafficking: MVE sorting and degradation

LIN-12 appears to be downregulated via MVEs. Although mutation of the conserved lysines near the DTS does not affect internalization of LIN-12, degradation in P6.p is blocked, and in all VPCs this mutant form accumulates in large pleiomorphic internal vesicles. Furthermore, the ubiquitin ligase WWP-1 and the MVE-associated factor ALX-1 are required for LIN-12 degradation after internalization. As *sur-2* mutants display a similar phenotype, transcriptional targets of the EGFR-Ras-MAPK pathway may be involved in directing LIN-12 to MVEs.

Mutating the conserved lysines near the DTS caused the 'Multivulva' phenotype associated with constitutive LIN-12 activation (Table 1). This phenotype is consistent with an MVE sorting defect. If a transmembrane protein does not go through the MVE sorting step, then upon delivery to the lysosome its extracellular domain will be degraded whereas its intracellular domain will remain exposed to the cytosol. For LIN-12/Notch, the mechanism of signal transduction involves cleavage and release of the intracellular domain. Thus, if MVE sorting is disrupted, degradation of the extracellular domain in the lysosome could mimic ectodomain shedding, creating a substrate for Presenilin-dependent release of the intracellular domain of LIN-12, or perhaps would release the intracellular domain by an alternative mechanism.

Recent reports have described 'ligand-independent' activation of *Drosophila* Notch in late endosomes. In these studies, overexpression of the protein Deltex was shown to promote internalization and accumulation of Notch in late endosomes, correlated with activation of Notch signaling. It was suggested that such endosomal activation of Notch might represent a novel and relevant mode of activating this pathway (Hori et al., 2004). However, our finding that an apparent block in MVE sorting can lead to LIN-12 activation suggests an alternative explanation for the effect of Deltex overexpression: the enhanced internalization and endosomal accumulation of Notch may saturate the MVE sorting machinery, so that some Notch is not correctly internalized into MVE lumenal vesicles, leading to degradation of the extracellular domain without concomitant degradation of the intracellular domain.

Different mechanisms for regulating trafficking of LIN-12 and *Drosophila* Notch, and possible evolutionary conservation in vertebrates

We have provided evidence that internalization of LIN-12 is mediated by the di-leucine motif and basal phosphorylation of flanking serine/threonine residues. By contrast, for *Drosophila* Notch, recent evidence suggests that ubiquitination by the dNedd4 ubiquitin ligase is required for Notch internalization (Sakata et al., 2004). *Drosophila* Notch does not have a di-leucine-based motif similar to the one we have described for LIN-12 (Fig. 7). Conversely, LIN-12 does not have a C-terminal PPXY signal (data not shown), which in *Drosophila* Notch promotes interaction with dNedd4 (Sakata et al., 2004). We suggest that *C. elegans* and *Drosophila* may utilize different mechanisms for targeting LIN-12/Notch for internalization.

Both of these mechanisms may be utilized in vertebrate Notch proteins. Sequence analysis of vertebrate Notch proteins shows an intriguing inverse correlation between the presence of a di-leucine based motif and a PPXY signal. The corresponding juxtamembrane regions of vertebrate Notch1 and Notch2 proteins have a segment that is strikingly similar to the LIN-12 DTS, including conserved flanking lysines (Fig. 7), but these proteins do not have a conserved PPXY signal at their C-termini (data not shown). By contrast, most vertebrate Notch3 proteins appear more divergent in this region (Fig. 7), but possess a PPXY signal at their C-termini (data not shown); it is curious that zebrafish Notch3 lacks the PPXY motif (data not shown), but has instead a canonical di-leucine motif (Fig. 7). These observations raise the possibility that the two modes of internalizing Notch proteins (di-leucine based versus

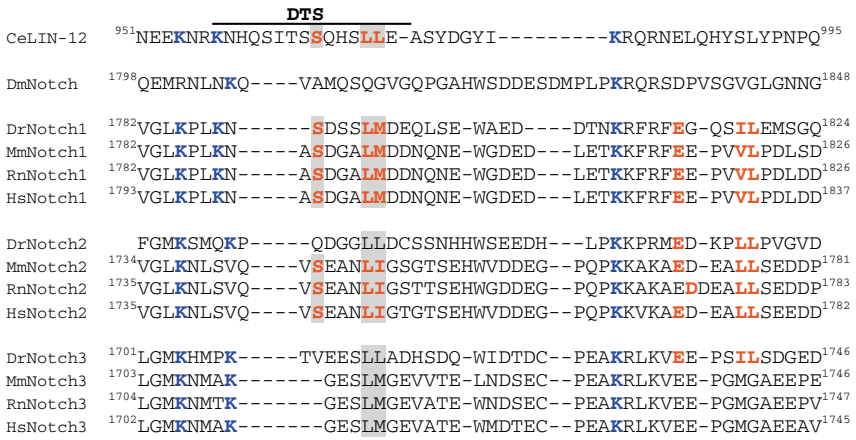

Fig. 7. Conservation of *cis*-acting determinants of LIN-12 trafficking in vertebrate Notch proteins. The relevant portion of LIN-12 region 1 was aligned with the equivalent region from *Dm* Notch, *Danio rerio (Dr)* Notch1-3, *Mm* Notch 1-3, *Rattus norvegicus (Rn)* Notch1-3 and *Hs* Notch 1-3. The requirement for the leucines and upstream serine/threonines in internalization has been verified experimentally for the LIN-12 DTS. We have marked the conserved di-leucine and serine residues in red, with shading. The conserved di-leucine in *Dr* Notch2 and *Dr* Notch3 and the di-leucine-like 'LM' in other Notch3 proteins are shaded, but not marked in red to emphasize the lack of upstream negative or phospho-accepting amino acids that may be important for function as an internalization signal. Most vertebrate Notch proteins also have a canonical (D/E)XXXLL consensus motif (Bonifacino and Traub, 2003); the key residues are marked in red without shading. Conserved lysines that have been verified experimentally as being involved in degradation of LIN-12 are marked in blue.

ubiquitination via a PPXY motif) have been conserved in different vertebrate Notch proteins through evolution. Perhaps other modes exist as well, as vertebrate Notch4 does not seem to have either of these conserved motifs (data not shown). Mutational analysis of these potential internalization sequences in vertebrate Notch proteins will be necessary to test their roles.

Regulation of DSL ligand activity by LIN-12/Notch in the signaling cell

We previously proposed that the lateral signaling defect of *sur-2* was caused by the failure to downregulate LIN-12 (Shaye and Greenwald, 2002). Subsequently, it was found that *dsl* gene transcription is regulated by *sur-2* (Chen and Greenwald, 2004), and here, we have found that internalized LIN-12, as is seen in *sur-2* mutants (Shaye and Greenwald, 2002), did not appear to inhibit lateral signaling. Thus, persistence of LIN-12 does not appear to be the basis of the *sur-2* lateral signaling defect; rather, loss of lateral signaling in *sur-2* mutants is likely to result simply from the failure to transcribe the lateral signal.

We observed that expression of a constitutively active LIN-12 that could not be downregulated in P6.p did not affect the fate of this cell or its ability to signal laterally, implying that the principal role of LIN-12 downregulation is to permit DSL ligands to activate LIN-12 in P5.p and P7.p in a paracrine mode, rather than to prevent autocrine LIN-12 activation in P6.p. However, this result also indicates that activation of the EGFR-Ras-MAPK pathway in P6.p causes it to become refractory to activated LIN-12, suggesting another potentially novel mode of crosstalk between EGFR-Ras-MAPK and LIN-12/Notch signaling in P6.p.

In certain gain-of-function mutants or ectopic overexpression situations in *Drosophila*, Notch also appears to be able to inhibit the ability of Delta to signal laterally (Heitzler and Simpson, 1993; Jacobsen et al., 1998). These results were interpreted as suggesting the formation of a DSL-Notch inhibitory complex, but whether such interactions occur during normal *Drosophila* development is not clear. Our results establish that inhibition of ligand activity occurs at the surface of the signaling cell, consistent with a DSL-Notch inhibitory complex. Furthermore, our results suggests that relief of this inhibition by internalization of LIN-12 appears to be part of the normal mechanism for coordinating EGFR-Ras-MAPK-mediated inductive signal and LIN-12-mediated lateral signaling. Given that the ability of LIN-12/Notch proteins to inhibit DSL signaling activity appears to be conserved, and the presence of conserved endocytic sorting motifs in all Notch proteins, there may be other natural situations in which inhibition of DSL ligands by endogenous LIN-12/Notch proteins, and regulated relief of such inhibition, may be relevant to patterning cell fates.

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