

# Ero1L, a thiol oxidase, is required for Notch signaling through cysteine bridge formation of the Lin12-Notch repeats in *Drosophila melanogaster*

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**N**otch-mediated cell–cell communication regulates numerous developmental processes and cell fate decisions. Through a mosaic genetic screen in *Drosophila melanogaster*, we identified a role in Notch signaling for a conserved thiol oxidase, endoplasmic reticulum (ER) oxidoreductin 1–like (Ero1L). Although Ero1L is reported to play a widespread role in protein folding in yeast, in flies *Ero1L* mutant clones show specific defects in lateral inhibition and inductive signaling, two characteristic processes regulated by Notch signaling. *Ero1L* mutant

cells accumulate high levels of Notch protein in the ER and induce the unfolded protein response, suggesting that Notch is misfolded and fails to be exported from the ER. Biochemical assays demonstrate that Ero1L is required for formation of disulfide bonds of three Lin12-Notch repeats (LNRs) present in the extracellular domain of Notch. These LNRs are unique to the Notch family of proteins. Therefore, we have uncovered an unexpected requirement for Ero1L in the maturation of the Notch receptor.

## Introduction

Cell–cell signaling mediated by receptors of the Notch family has been implicated in a wide variety of developmental processes in organisms ranging from nematode to man (Artavanis-Tsakonas et al., 1999; Schweisguth, 2004; Bray, 2006). In addition, aberrant Notch signaling has been associated with several diseases and cancers. The core components of Notch signaling are cell surface single-pass type I transmembrane proteins: the receptor, Notch, and the ligands Delta (Dl) and Serrate (Jagged in vertebrates). Ligand binding to the extracellular domain of Notch results in a series of proteolytic cleavages mediated by kuzbanian, a disintegrin and metalloprotease (Lieber et al., 2002), and by the  $\gamma$ -secretase activity of the presenilin complex (De Strooper et al., 1999; Struhl and

Greenwald, 1999; Okochi et al., 2002). As a result, the intracellular domain of Notch is released from the cell membrane and translocates into the nucleus to activate the downstream target genes.

Notch signaling is iteratively involved in numerous developmental processes of various organs and affects lateral inhibition, binary cell fate decision, and inductive signaling (Bray, 1998; Lai, 2004). A model system to study Notch signaling is the development of adult external sensory organs (ESOs) of *Drosophila melanogaster* (Modolell, 1997; Artavanis-Tsakonas et al., 1999; Lai, 2004). Each ESO develops from a field of equivalent cells that express similar levels of proneural proteins (Jan and Jan, 1993). Each cell in this group has the potential to develop into a sensory organ precursor (SOP), the mother cell of an ESO. However, because of lateral inhibition mediated by Notch signaling, only one or few cells are selected to acquire the SOP fate (Hartenstein and Posakony, 1990; Artavanis-Tsakonas et al., 1999). The SOP then undergoes a series of asymmetrical

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Abbreviations used in this paper: AMS, 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid; AP, anterior posterior; APF, after puparium formation; Ci, cubitus interruptus; Dl, Delta; Dpp, decapentaplegic; dsRNA, double-stranded RNA; DV, dorsal ventral; Ero1L, ER oxidoreductin 1–like; ESO, external sensory organ; *ey-FLP*, eyeless-flipase; Hh, hedgehog; LNR, Lin12-Notch repeat; MARCM, mosaic analysis with a repressible cell marker; NECD, Notch extracellular domain; NEXT, Notch extracellular truncation; PBST, PBS-Tween; PDI, protein disulfide isomerase; QSOX, quiescin/Sox; SOP, sensory organ precursor; UPR, unfolded protein response.

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divisions to generate four daughter cells that form the mature ESO, during which Notch signaling plays a crucial role in binary cell fate decisions (Hartenstein and Posakony, 1990; Gho et al., 1999; Bardin et al., 2004). Defects in Notch signaling lead to bristle aberrations that can easily be identified under a dissection microscope. Thus, screens to identify novel components of the Notch signaling pathway can be based on assaying mitotic clones on the adult *Drosophila* thorax for bristle abnormalities (Berdnik et al., 2002; Jafar-Nejad et al., 2005; Gallagher and Knoblich, 2006).

The activity of Notch signaling is highly regulated through posttranslational modifications. For instance, the activity of the Notch receptor in certain contexts is modulated by extracellular posttranslational glycosylation mediated by ER enzymes, including rumi (Acar et al., 2008) and *O*-fucosyl transferase-1, and the Golgi enzymes Fringe (glycosaminyl transferase) and Fringe connection (UDP sugar transporter; Haines and Irvine, 2003). In addition, *O*-fucosyl transferase-1 has been shown to act as a chaperone for Notch (Okajima et al., 2005). Whether maturation and folding of Notch within the secretory pathway require additional enzymes within the ER or Golgi has yet to be determined.

By performing a mosaic genetic screen for bristle aberrations, we show that a disulfide bond forming oxidase, ER oxidoreductin 1-like (*Ero1L*), is required for Notch-dependent developmental processes. We find that Notch is a key target in *Ero1L* mutant cells. These cells exhibit a much-reduced ability of Notch to reach the cell membrane, thereby accumulating a vast amount of the protein in the ER. Our biochemical assays indicate that *Ero1L* plays a role in disulfide bond formation of the three Lin12-Notch repeats (LNRs). The specificity of this phenotype is quite surprising, as *Ero1L* is proposed to be a global regulator of disulfide bond formation in yeast.

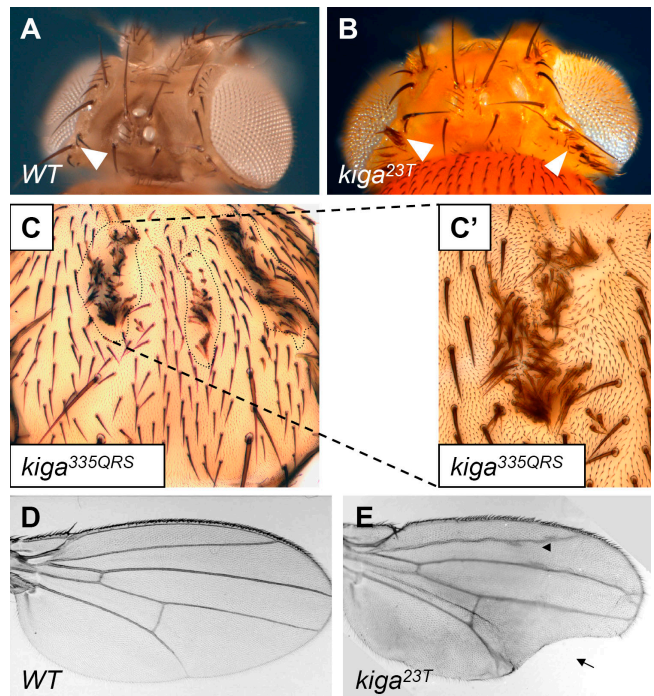
## Results

### Mutations in *kiga* cause bristle tufting and wing defects

To identify novel genes required for ESO development, we performed a mosaic genetic screen on chromosome 3L using the *eyeless-flipase* (*ey-FLP*) system (Stowers and Schwarz, 1999; Newsome et al., 2000). We generated ethyl methanesulfonate-induced mutant clones during larval development and screened for bristle defects in adult heads (Fig. 1, A and B). Mutations in a single complementation group (two alleles, *23T* and *335QRS*) cause a bristle-tufting phenotype in homozygous mutant clones on the head epidermis (Fig. 1 B) and thorax (Fig. 1, C and C'). In addition, mutations in this complementation group cause notching of the wing margin and thickening of the wing veins (Fig. 1, D and E). As these phenotypes are reminiscent of the loss of Notch function, we named this group *kiga*, which is phonetic for "notched" in Taiwanese.

### *kiga* is required for lateral inhibition and inductive signaling, Notch-dependent developmental processes

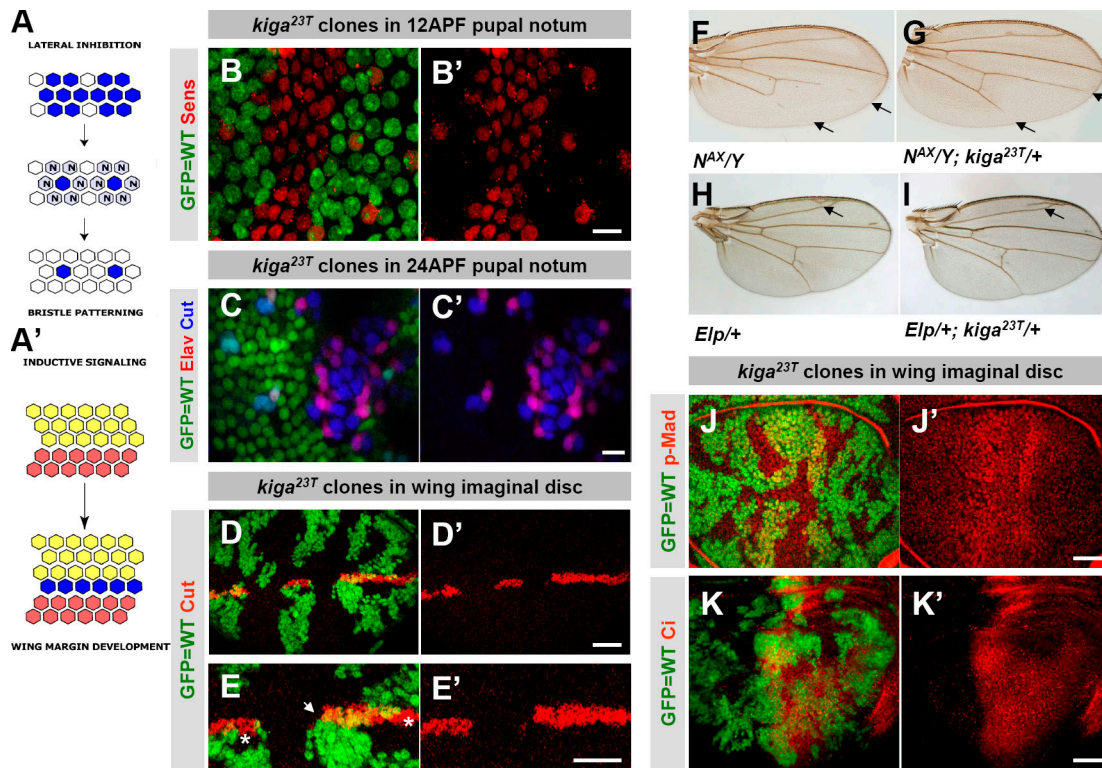
The tufting phenotype associated with *kiga* mutant clones suggests a defect in lateral inhibition (Fig. 2 A; Ghysen et al., 1993).



**Figure 1. Alleles of *kiga* cause bristle tufting and wing defects.** (A) The bristle and socket cells are external structures of a mechanosensory organ (arrowhead). They are present on the head epidermis of a wild-type (WT) fly at stereotypic positions. (B) Homozygous *kiga* mutant clones show a bristle-tufting phenotype indicating a defect in lateral inhibition. Compared with the regularly spaced bristle patterning on the wild-type head epidermis (A), clear expansion of bristles are observed in *kiga* clones induced by *ey-FLP* on the head epidermis (arrowheads). (C) Bristle patterning on the thorax is also affected in homozygous *kiga* mutant clones. Homozygous mutant *kiga* clones induced by *Ubx-FLP* are identified by the trichome marker multiple wing hairs and marked by dashed lines. A higher magnification of a mutant clone in C is shown in C'. (D and E) Wing formation is affected in homozygous *kiga* mutant clones. Compared with wild-type wing (D), loss of wing tissue around the wing margin (arrow) and wing vein-thickening phenotypes (arrowhead) are associated with homozygous *kiga* mutant clones (E).

Loss of Notch signaling in this process causes transformation of most or all epithelial cells within a proneural cluster into SOPs, resulting in a bristle-tufting phenotype (Hartenstein and Posakony, 1990). To probe whether lateral inhibition is affected in *kiga* mutant clones, we performed immunostaining using anti-Sens (Nolo et al., 2000) to label the SOPs in pupal notum at 12 h after puparium formation (APF) when the primary SOPs of the bristles are specified. As shown in Fig. 2 (B and B'), all cells within this clone are labeled with Sens, whereas only a few regularly spaced cells outside of the mutant clone are labeled with Sens. This indicates that lateral inhibition is indeed affected in the absence of *kiga*.

At 24 h APF, the ESO consists of four Cut-positive cells and one neuron expressing Elav. Consistent with the role of *kiga* in lateral inhibition, Cut expression in *kiga* mutant clones is expanded (Fig. 2, C and C'). However, fate specification occurs normally, as the neuronal marker Elav is expressed in the *kiga* clones at 24 h APF (Fig. 2, C and C'), and the adult phenotype indicates that the shaft and socket cells of the ESO can differentiate properly. These data indicate that *kiga* is specifically required for lateral inhibition but not for cell fate decisions and differentiation.



**Figure 2. *kiga* is specifically required for Notch-dependent processes and interacts genetically with *Notch* mutants.** (A) Schematic illustration of the lateral inhibition process mediated by Notch signaling. (A') Schematic illustration of inductive signaling. The "N's" in the cells represent high Notch activity. The wing margin cell fate (blue) is induced by Notch signaling between dorsal and ventral compartments (yellow and orange). In B–E and J–K, homozygous mutant regions lack GFP expression (green). (B) Lateral inhibition is impaired in homozygous *kiga* mutant clones. The SOPs of the pupal notum at 12 h APF are labeled for Sens (red). (B and B') Note that SOPs in the wild-type region are spaced regularly between epithelial cells, whereas no epithelial cells are present between mutant SOPs. (C and C') Binary fate decision at 24 h APF in *kiga* mutant clones. Cut (blue) marks all of the cells of the SOP progeny. Elav (red) stains the neuronal cells. In the wild-type domain, there are well-spaced clusters of four Cut-positive cells and one Elav-positive cell. In *kiga* mutant clones, Cut expression is expanded, and it is difficult to identify single clusters, indicating a lateral inhibition defect. However, the presence of neuronal cells suggests that cell fate specification and differentiation are likely normal. (D and D') Defective wing margin formation is associated with *kiga* mutants. Wing imaginal disc from a third instar larva stained for Cut (red). Cells in the large homozygous mutant clones lack expression of Cut at the DV boundary. A higher magnification of a small portion of D is shown in E and E'. Note that in large clones, mutant cells at the boundary of the clone are not Cut positive (arrow), whereas in some smaller mutant clones and near the edge of wild-type cells, some margin cells are Cut positive (\*). (F and G) Removal of one copy of *kiga* suppresses a gain of function phenotype of *Notch*. A male adult wing from an  $N^{AxE-2}/Y$  fly is shown (F) with a reduced length of veins (F, arrows). The loss of wing veins is suppressed (G, arrows) when one copy of *kiga* is removed in  $N^{AxE-2}/Y; kiga/+$  male wings (G). (H and I) Removal of one copy of *kiga* does not suppress a gain of function phenotype of *Egfr*. A male adult wing from an  $Elp/+$  fly is shown (H) with an ectopic wing vein (H, arrow). The gain of wing vein (I, arrow) is not suppressed when one copy of *kiga* is removed. (J–K') Dpp and Hh signaling are unaffected in *kiga* mutant clones. Wing imaginal disc with *kiga* mutant clones stained for phospho-Mad (J and J', red) and Ci (K and K', red). Bars: (B–C') 10  $\mu$ m; (D–E' and J–K') 40  $\mu$ m.

During adult wing development, the formation of the dorsal–ventral (DV) boundary is crucial for compartmentalization and is dependent on Notch signaling (Fig. 2 A'; de Celis et al., 1996; de Celis and Bray, 1997). In agreement with the notching phenotype (Fig. 1 E), we observed a loss of Cut, a downstream target of Notch signaling at the DV boundary in large *kiga* mutant clones (Fig. 2, D and D'), indicating that *kiga* plays a role in inductive Notch signaling. Note that *kiga* mutant cells located in the vicinity of wild-type cells (Fig. 2, D and D'; green) do express Cut at the boundary. Interestingly, these cells are clearly less affected by the loss of *kiga* (see Fig. 5, B and B').

We then sought to examine whether *kiga* can genetically modify Notch-related phenotypes. For example, a hypermorphic allele of Notch ( $N^{AxE-2}$ ; de Celis and Garcia-Bellido, 1994) exhibits a loss of the fourth and fifth wing veins in male flies (Fig. 2 F) caused by a gain of function of Notch. Because the wing vein-thickening phenotype associated with *kiga* mutants might be caused by a loss of Notch signaling activity (Fig. 1 E),

we examined whether loss of *kiga* can suppress the severity of the wing phenotype in  $N^{AxE-2}$  mutant flies. As shown in Fig. 2 G, removal of one copy of *kiga* in  $N^{AxE-2}$  mutant flies leads to a suppression of the wing vein loss phenotype. In addition, we examined whether *kiga* can genetically modify the ectopic wing vein phenotype of a gain of function allele of EGF receptor, *Elp* (Lesokhin et al., 1999). We found that removal of one copy of *kiga* does not affect the ectopic wing vein formation of  $Elp/+$  (Fig. 2, compare the arrow in H with the arrow in I). In summary, these observations indicate that *kiga* selectively affects Notch signaling.

To determine whether *kiga* affects other signaling pathways, we examined the readouts for the decapentaplegic (Dpp) and hedgehog (Hh) pathways. Dpp and Hh signaling are required for anterior–posterior (AP) boundary formation during wing development. We found that the levels of phospho-Mad, a downstream effector of Dpp (Tanimoto et al., 2000), in mutant clones are comparable with wild-type cells near the AP boundary, indicating that



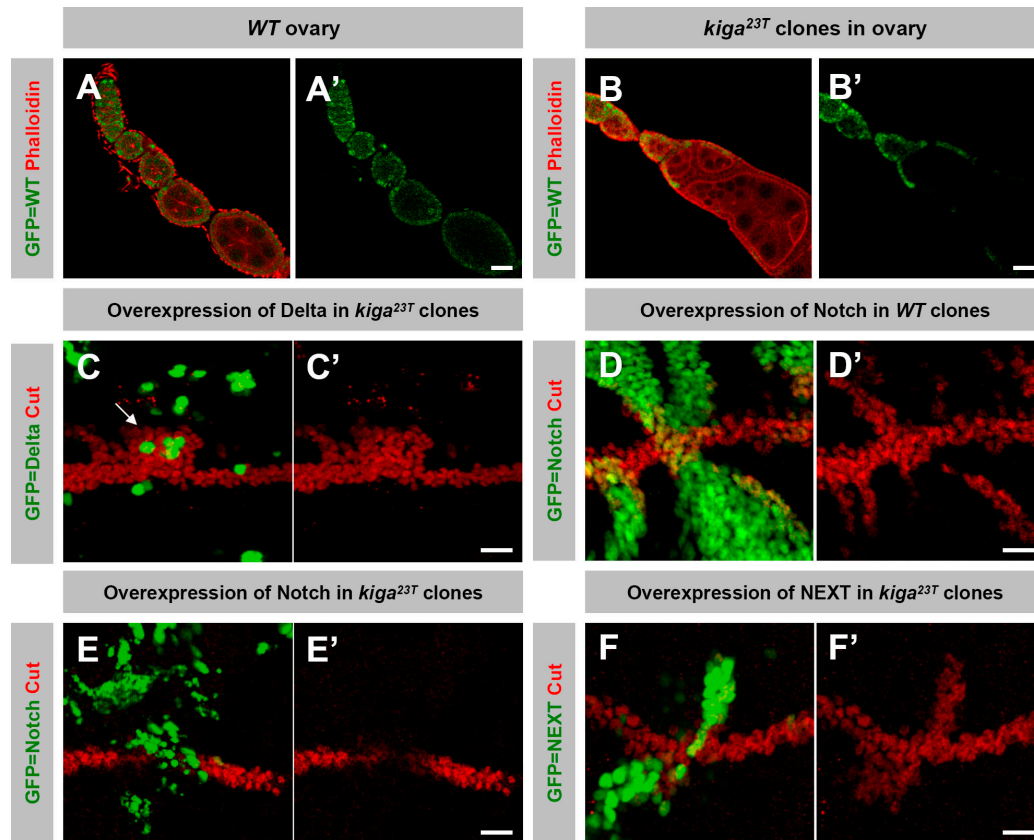


Figure 3. ***kiga* is required in signal-receiving cells for Notch signaling.** (A–B') Wild-type (WT) ovary (A and A') and ovary with *kiga* mutant clones (B and B') were stained with phalloidin (red) to outline the cell boundary. (B and B') A large *kiga* mutant clone marked by loss of GFP expression (green) results in a fused egg chamber phenotype. (C–F') Notch signaling activity reported by Cut expression at the DV boundary using the MARCM technique. Part of the wing discs stained for Cut (red) is shown, and the mutant region is marked positively with GFP expression (green). (C and C') Overexpression of DI in *kiga* mutant cells can induce Cut expression in the adjacent cells near the DV boundary at the dorsal compartment. (D and D') Overexpression of Notch in wild-type cells can induce Cut expression cell autonomously near the DV boundary. (E and E') Overexpression of Notch in *kiga* mutant cells fails to induce Cut expression near the DV boundary. Note that when the clones are crossing the boundary, Cut expression is lost in the mutant cells. (F and F') Overexpression of NEXT in *kiga* mutant cells can induce Cut expression cell autonomously. Bars: (A–B') 20  $\mu$ m; (C–F') 10  $\mu$ m.

the Dpp pathway is unaffected (Fig. 2, J and J'). Similarly, the expression of cubitus interruptus (Ci), the target of Hh signaling (Motzny and Holmgren, 1995; Wang and Holmgren, 1999), is unaffected in the posterior compartment in *kiga* mutant clones (Fig. 2, K and K'). These observations are in agreement with the adult wing phenotypes that indicate that *kiga* is not required for AP patterning of the wing and suggest that loss of *kiga* results in specific defects in Notch signaling.

#### ***kiga* is required for Notch signaling in the signal-receiving cells**

To determine whether *kiga* is required for the Notch signaling in the signal-sending or signal-receiving cell, we analyzed the role of *kiga* in ovary development. In ovaries, there is a distinct requirement for the ligand (DI) and the receptor (Notch); DI is expressed in the germ cells to activate Notch in the surrounding somatic follicle cells (Lopez-Schier and St Johnston, 2001). Loss of *Notch* in the follicle cells results in failure of follicle cell differentiation and leads to formation of giant compound egg chambers in which multiple germline cysts are surrounded by a single follicular epithelium (Xu et al., 1992). Loss of *DI* in the follicle cells does not affect encapsulation (Lopez-Schier and

St Johnston, 2001). We found that clones of *kiga* in follicle cells result in formation of giant compound egg chambers (Fig. 3, A and B), indicating that loss of *kiga* phenocopies loss of *Notch* in this developmental context.

To probe whether the role of *kiga* is also required for the signal-receiving cell during wing formation, we performed an assay (de Celis and Bray, 1997; Lee and Luo, 2001; Wang and Struhl, 2004) in which DI or Notch were overexpressed in mutant clones under the control of GAL4 (Brand and Perrimon, 1993). In wild-type clones, it has been shown that ectopic expression of DI in proximity to the DV boundary can induce ectopic Notch signaling in surrounding cells (de Celis and Bray, 1997), leading to the expression of the Notch downstream target gene *cut*.

When DI is overexpressed in *kiga* mutant clones, these cells are capable of inducing Cut expression in adjacent cells in proximity to the DV boundary in the dorsal compartment (Fig. 3, C and C'), suggesting that *kiga* is not required for proper function of DI in the signal-sending cells. Next, full-length Notch was overexpressed in either wild-type clones or *kiga* mutant clones. Overexpression of Notch results in a cell-autonomous expression of Cut in clones of wild-type cells (Fig. 3, D and D'), but it fails to induce Cut expression in *kiga* mutant cells (Fig. 3, E and E').

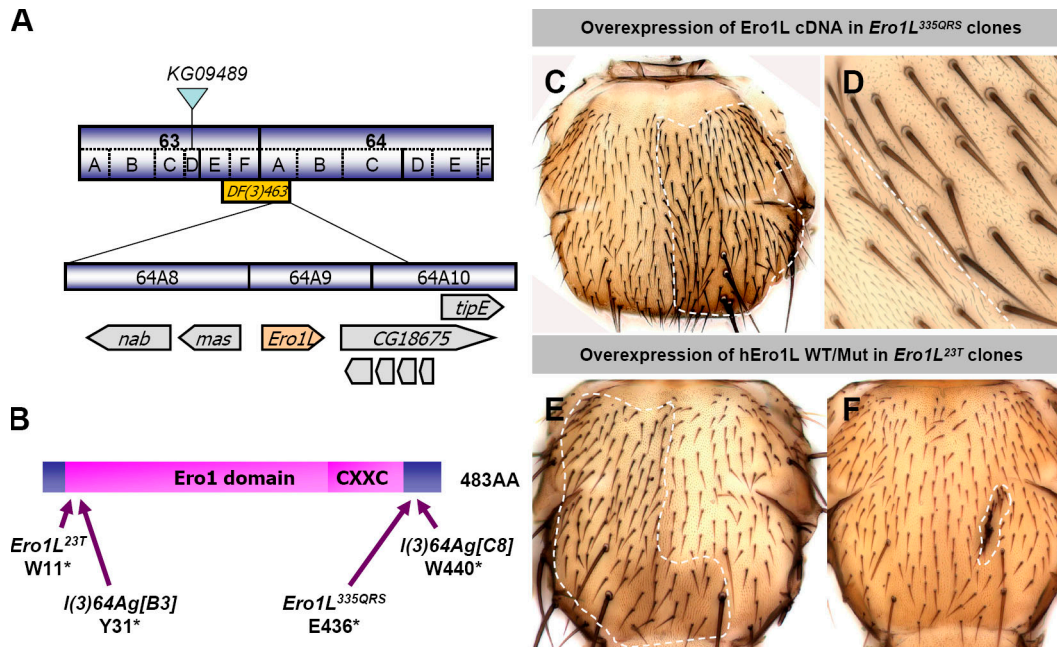


Figure 4. **Mutations in *Ero1L* cause the *kiga* mutant phenotypes.** (A) Schematic illustration of *Ero1L* and neighboring genes in chromosomal region 64A9. (B) Position of the stop codons (indicated by asterisks) in four *Ero1L* alleles; 237 and 335QRS were generated in the screen in this study, whereas others were isolated previously in a screen for essential complementation groups in the 63E-64A region (Harrison et al., 1995). Complementation tests and sequencing revealed that they are alleles of *kiga*. (C) Overexpression of *Ero1L* with *tub-GAL4* rescues the tufting phenotype associated with *kiga* mutant clones. A thorax of an adult fly harboring *kiga* mutant clones (marked by dashed lines) expressing the *Ero1L* cDNA is shown. (D) The rescued region is shown at higher magnification. (E and F) Only the enzymatically active form of the human homologue of *Ero1L* can rescue the tufting phenotype in *Ero1L* mutant clones. A thorax of an adult fly harboring *Ero1L* clones (marked by dashed line) expressing a wild-type human *ERO1-L $\alpha$*  (E) or C394A mutant (F) cDNA is shown. Only the enzymatically active (wild type) form of human *ERO1-L $\alpha$*  restores normal bristle patterning (E). Conversely, *Ero1L* mutant clones expressing enzymatically inactive human *ERO1-L $\alpha$*  (C394A) exhibit tufting phenotypes (F).

These data strongly suggest that *kiga* is required in the signal-receiving cells.

We then examined whether the function of *kiga* is required at a step after or before ligand activation of the Notch receptor using a constitutively active form of Notch, Notch extracellular truncation (NEXT; Struhl and Greenwald, 1999). NEXT is an activated membrane-tethered form of the Notch intracellular domain, which is ligand independent but is dependent on presenilin cleavage. When NEXT is overexpressed in *kiga* mutant clones, Cut expression is activated cell autonomously within the clones (Fig. 3, F and F'), suggesting that the role of *kiga* is in the signal-receiving cells and that it is required at a step that is likely to be upstream of the S3 cleavage of Notch.

#### ***kiga* encodes an *Ero1L* protein**

We mapped the lethality associated with *kiga* to chromosomal region 64A1-A9 (Fig. 4 A) using meiotic recombination with *P* elements (Zhai et al., 2003). Sequencing of several genes in the region showed molecular lesions in the *Ero1L* gene (Fig. 4 B). In addition, two lethal mutations (*l(3)64AgB3* and *l(3)64AgC8*) isolated in a previous screen failed to complement our *kiga* alleles (Harrison et al., 1995). The four identified mutations (Fig. 4 B) correspond to stop codons (W11\*, Y31\*, E436\*, and W440\*). A cDNA rescue experiment showed that the tufting phenotype associated with *kiga* mutant clones was rescued by *Ero1L* overexpression (Fig. 4, C and D). Thus, *kiga* corresponds to *Ero1L*.

The protein encoded by *Ero1L* has a signal peptide at its N terminus followed by an Ero1 domain. In yeast and mammalian cell culture, *Ero1L* is an oxidase involved in disulfide bond formation (Frandsen and Kaiser, 1998, 1999; Pollard et al., 1998; Cabibbo et al., 2000) by oxidizing several protein disulfide isomerase (PDI) proteins. These PDIs then oxidize substrate proteins in the ER. The enzyme activity of *Ero1L* depends on a cofactor flavin adenine dinucleotide and the active site, the Cys-X-X-Cys-X-X-Cys (CXXCXXC) motif in the Ero1 domain (Benham et al., 2000; Gross et al., 2004). Two mammalian homologues of *Ero1L* have been identified: *ERO1-L $\alpha$*  and *ERO1-L $\beta$*  (Cabibbo et al., 2000; Pagani et al., 2000). To assess whether *Ero1L* is functionally conserved, we performed rescue experiments with overexpression of the human cDNA of *ERO1-L $\alpha$*  in *Ero1L* mutant clones and found that the lateral inhibition phenotype is rescued (Fig. 4 E). However, overexpression of an enzymatically inactive form (C394A) of human *ERO1-L $\alpha$* , which has a mutation in the CXXCXXC motif (Cabibbo et al., 2000), was unable to rescue the lateral inhibition phenotype (Fig. 4 F). These data provide strong evidence that the function of *Ero1L* is evolutionarily conserved and that the enzymatic function of *Ero1L* is required for its role in Notch signaling.

#### **Loss of *Ero1L* induces the unfolded protein response (UPR) and results in the accumulation of Notch in the ER**

When unfolded or misfolded proteins accumulate in the ER, they cause stress in this organelle (Frandsen and Kaiser, 1998;

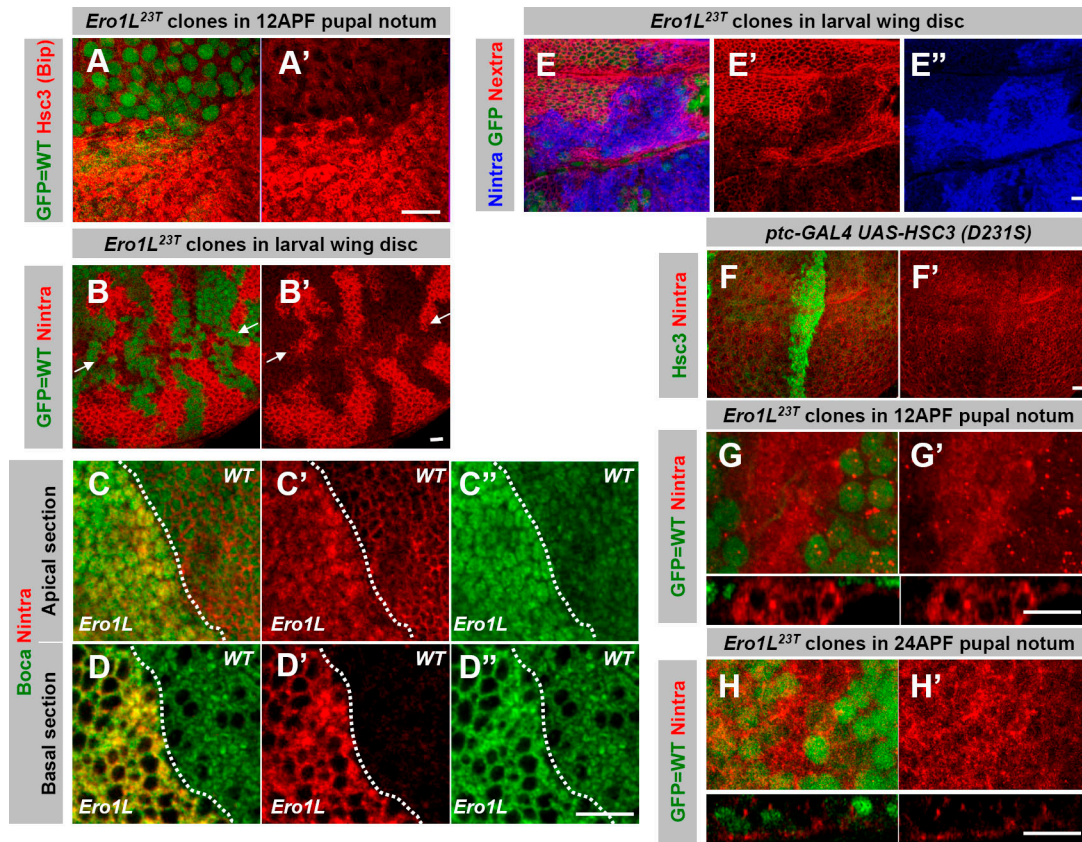


Figure 5. ***Ero1L* mutant cells exhibit a UPR and accumulate Notch in the ER.** (A–H) Immunostainings of the pupal nota (A and A' and G–H') or wing imaginal discs (B–F) that harbor *Ubx-FLP*-induced *Ero1L* homozygous clones (A–E'' and G–H', marked by lack of GFP expression). (A) *Ero1L* mutant cells exhibit a UPR. Pupal nota (12 h APF) labeled with Hsc3 (red). Significantly higher levels of Hsc3 expression are observed in mutant tissue. (B and B') Cell-autonomous up-regulation of Notch in *Ero1L* mutant clones. Wing imaginal disc stained with an antibody raised against the intracellular portion of Notch (red). Much higher levels of Notch are observed in *Ero1L* mutant cells when compared with wild-type cells. Note that accumulation of Notch near the DV boundary (arrows) is less pronounced. (C–D'') Accumulation of Notch colocalizes with ER markers in *Ero1L* mutant cells. Wing imaginal disc stained for Notch (red) and Boca (green), an ER chaperone. Apical honeycomb-like localization of Notch is lost in the *Ero1L* mutant clones (C–C''), whereas the basal section shows that Notch is accumulated intracellularly and colocalized with Boca (D–D''). The dotted lines mark the clone boundary. (E–E'') Membrane-bound Notch is severely decreased in *Ero1L* mutant cells. Wing imaginal disc stained with an antibody raised against the NECD (E', red; no permeabilization). Anti-Notch staining performed after permeabilization shows a very strong intracellular accumulation of Notch (E'', blue). (F) Up-regulation of a mutant Hsc3 known to induce a UPR does not affect the levels or localization of Notch. Wing imaginal disc overexpressing mutant Hsc3 (D231S) in the AP boundary driven by *patched-GAL4* were stained for Notch (red) and Hsc3 (green). No accumulation or mislocalization of Notch in the patched expression domain is observed. (G–H') Different levels of Notch accumulation are observed at different developmental stages in mutant cells. Wing imaginal disc at the third instar larval stage (B and B') or pupal nota at 12 h APF (G and G') and 24 h APF (H and H') were stained for Notch (red). The bottom panels of G–H' show the z-section slide. Note that significantly less accumulated Notch is present at 24 h APF (when four SOP progeny initiate differentiation) in mutant cells relative to wild-type cells when compared with 12 h APF, the time point when lateral inhibition is terminating. Bars, 10  $\mu$ m.

Pollard et al., 1998) and often result in an activation of the UPR (Schroder and Kaufman, 2005). In yeast, loss of *ERO1* leads to retention of misfolded disulfide-containing proteins in the ER, and a potent UPR is induced (Frand and Kaiser, 1998; Pollard et al., 1998). To test whether loss of *Ero1L* causes ER stress, we examined the expression of Bip/Hsc3, a well-established transcriptional target and marker for the UPR (Schroder and Kaufman, 2005; Ryoo et al., 2007), in *Ero1L* mutant clones. The expression levels of Bip/Hsc3 are strongly up-regulated in most of the *Ero1L* mutant clones (Fig. 5 A and see Fig. 6 A), indicating that the UPR is activated when *Ero1L* is nonfunctional.

Based on our *in vivo* data, the target of *Ero1L* should correspond to the Notch extracellular domain (NECD), which contains two major segments with multiple disulfide bonds: the 36 EGF repeats and the three LNRs. Each EGF repeat and LNR has three

disulfide bonds. As unpaired cysteine residues induce a prominent UPR (Frand and Kaiser, 1998), it is likely that some or many disulfide bonds of the NECD require *Ero1L* to fold properly. Furthermore, the unpaired cysteines of Notch should result in the misfolding and accumulation of Notch in the ER. Indeed, we noticed strikingly high levels of Notch in *Ero1L* mutant cells (Fig. 5, B and B') except in the vicinity of the DV boundary (Fig. 5 B', arrows), where Notch accumulation is less obvious (Fig. 2, D–E). In addition, intracellularly accumulated Notch colocalizes with another ER marker, Boca, a chaperone protein of the ER (Fig. 5, C–D''); Culi and Mann, 2003). Expression of Boca is also up-regulated in *Ero1L* mutant clones (Fig. 5, C'' and D''), indicating that the ER is likely expanded and that the levels of multiple chaperones are up-regulated during a UPR.

In the apical section in Fig. 5 (C–C''), the normal cortical localization of Notch in the mutant region is dramatically



decreased, suggesting that Notch cannot be inserted into cell membrane in *Ero1L* mutant cells. To examine this, we immunostained unpermeabilized cells in wing imaginal discs using an antibody recognizing the NECD. We found that membrane-associated Notch is greatly reduced in *Ero1L* mutant clones (Fig. 5, E and E'), whereas total Notch staining is greatly enhanced (Fig. 5 E''). These data indicate that the vast majority of Notch is presumably misfolded in the ER and unable to reach the cell membrane in the absence of *Ero1L*.

To rule out the possibility that higher levels of Notch are secondary to induction of the UPR, we overexpressed a mutant form of Hsc3 to ectopically induce ER stress (Elefant and Palter, 1999) under the control of the *patched-GAL4* driver. Immunostaining of Notch indicates that neither the localization nor the expression levels of Notch are affected in the *Hsc3* expression domain (Fig. 5, F and F'), indicating that the accumulation of Notch in the ER is caused by the loss of *Ero1L*.

It is important to emphasize that the accumulation of Notch in *Ero1L* mutant tissue gradually decreases with time. When the SOPs are selected from the proneural cluster at 12 h APF, the accumulation of Notch in mutant tissue is most extreme (Fig. 5, G and G'). However, by 24 h APF, the accumulation of Notch in *Ero1L* mutant tissue has decreased to near wild-type levels (Fig. 5, H and H'). This correlates with the adult phenotype, in which only inductive signaling and lateral inhibition are affected. As a significant decrease in Notch protein levels between 12 and 24 h APF, it is reasonable to hypothesize that the UPR may help fold Notch and/or promote degradation of misfolded proteins through a mechanism known as ER-associated protein degradation, whose components are induced by the UPR (Travers et al., 2000). This process may thereby allow Notch to function properly during the later cell fate specification events.

### Notch is a major target of Ero1L

Our phenotypic analyses suggest that loss of Ero1L causes a rather specific defect in Notch signaling. If Notch is one of a few key affected proteins in *Ero1L* mutants, the UPR should be significantly diminished when *Notch* is removed in *Ero1L* mutant clones. To test this, we generated mutant clones that lack *Notch* and *Ero1L* as well as *Ero1L* alone in the same wing imaginal disc (Fig. 6 A) to compare the different levels of the UPR associated with different genotypes. Using Hsc3 as a readout for the UPR (Ryoo et al., 2007), we observed very low levels of Hsc3 in wild-type tissue (Fig. 6 A'', middle) and high levels of Hsc3 in *Ero1L* mutant clones (Fig. 6 A', left). In clones mutant for *Notch* and *Ero1L* (Fig. 6 A''', right), Hsc3 is present at much lower levels than in *Ero1L* mutant cells (Fig. 6, compare A' with A'''; quantified in Fig. 6 B). Although the residual up-regulation of Hsc3 suggests that there are unidentified targets of Ero1L, the result strongly suggests that Notch is a major target of Ero1L and that the accumulation of misfolded Notch in the ER activates a strong and transient UPR in the absence of Ero1L.

As previously described, the NECD contains 36 EGF repeats, each containing three pairs of disulfide bonds. However, EGF repeats are also found in DI. As our in vivo analysis shows

that DI can signal properly, we found that neither the levels nor the localization of DI are affected in *Ero1L* mutant clones (Fig. 6 C), suggesting that EGF repeats may not be affected by loss of Ero1L. Furthermore, other important membrane proteins that contain EGF repeats, including EGF receptors crumbs and *Drosophila* epithelial-cadherin, are unaffected in their localization in the absence of Ero1L (Fig. 6, D and D'; and not depicted). Finally, proteins that contain Ig-like repeats with disulfide bonds such as FasII are also unaffected in their subcellular distribution (unpublished data). Together, these data indicate that Ero1L is quite specifically required for Notch to exit the ER and reach the cell membrane.

In the contexts that we have examined so far, it seems that disulfide bond formation of many proteins does not depend on Ero1L function. It has been shown that a quiescin/Sox (QSOX) protein family (Hoover et al., 1999b; Thorpe and Coppock, 2007) can carry out thiol oxidation in vitro, and overexpression of QSOX in yeast can rescue the lethality of *ero1*. Because there are three uncharacterized homologues of this QSOX family in the *Drosophila* genome (Hoover et al., 1999a), we surmise that these proteins may have a previously unidentified role in oxidative folding in the ER. Two of the QSOX genes, *CG6690* and *CG17843*, are less likely to be involved in general protein folding in the ER because a microarray study has shown that these proteins have a specific expression pattern in male accessory gonads of *Drosophila* (Chintapalli et al., 2007). In contrast, another QSOX gene, *CG4670* (*QSOX1*), exhibits widespread expression in different developmental stages (Chintapalli et al., 2007), suggesting a role for this gene in general ER protein folding. To characterize the function of *CG4670*, we knocked down *CG4670* using RNAi (Dietzl et al., 2007) with the ubiquitous drivers *da-GAL4* and *act-GAL4* at 29°C. Although the mRNA of *CG4670* is significantly knocked down (Fig. 6 E), this did not result in any lethality or developmental defects. In addition, knocking down *CG4670* with tissue-specific drivers, including *patched-GAL4*, *neuralized-GAL4*, and *nubbin (nub)-Gal4*, did not result in any observable phenotypes in the wing or notum (Fig. 6 F; and not depicted), suggesting that *CG4670* is not specifically required for Notch signaling.

To examine whether *CG4670* can contribute to disulfide bond formation when Ero1L function is compromised, we performed genetic interaction experiments between these two genes. Reducing the levels of *Ero1L* using the *nub-GAL4/UAS-RNAi Ero1L* results in a mild wing vein-thickening phenotype (Fig. 6 G), providing us with a sensitized background to examine the genetic interaction. Although knocking down *CG4670* alone does not show any significant phenotype in the wing (Fig. 6 F), knocking down both *Ero1L* and *CG4670* results in a relatively strong wing vein-thickening and reduced wing size phenotype (Fig. 6 H), suggesting that *CG4670* can contribute to cell growth and Notch-related phenotypes in *Ero1L* knockdown cells. Because the knockdown of *CG4670* only shows phenotypes when *Ero1L* is compromised, we propose that *CG4670* plays a redundant role, probably as an alternative mechanism to deal with misfolded proteins in the absence of *Ero1L*. Together, these data suggest that in the context of Notch signaling, *Ero1L* plays a primary role in disulfide bond formation.

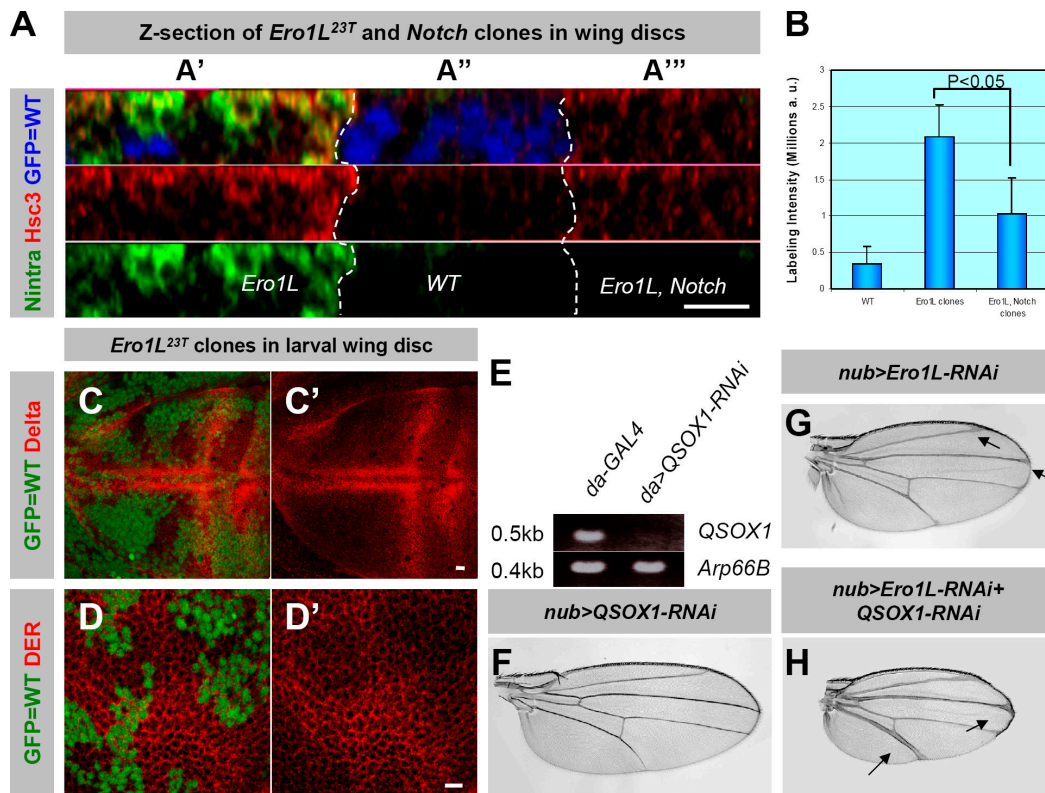


Figure 6. **Notch is a major target in *Ero1L* mutant cells.** (A–D') Confocal sections through wing imaginal discs harboring *Ubx-FLP*-induced mutant clones (lack of GFP expression). (A–A'') Cells without *Notch* and *Ero1L* have lower levels of Hsc3 expression compared with cells that lack only *Ero1L*. A z section through a wing imaginal disc harboring *Ero1L* and/or *Notch* mutant clones (marked by dashed lines) is shown. It was stained for Notch (green) and Hsc3 (red). In A', wild-type cells expressing GFP (blue) show low levels of expression for both Hsc3 and Notch. In A'', high levels of Hsc3 and Notch are observed in cells that are only mutant for *Ero1L*. In A''', a group of *Notch* and *Ero1L* double mutant cells, indicated by the loss of GFP (blue) and Notch (green) expression, show lower levels of Hsc3 when compared with *Ero1L* mutant cells in the left panel (compare A''' with A'). (B) Quantification of Hsc3 expression in the cells of different genotypes is shown in the bar graph (a.u., arbitrary unit). Error bars indicate SEM. (C–D') Normal expression levels and localization of various membrane proteins are observed in *Ero1L* mutant clones. DI (C–C', red) and *Drosophila* EGF receptor (D and D', red) are localized normally and are not up-regulated in *Ero1L* mutant cells. (E) Knockdown of CG4670 revealed by semiquantitative RT-PCR. In larva with ubiquitous expression of CG4670 RNAi (left), the mRNA of CG4670 is significantly lower than control larva (right). (F–H) Genetic interaction between QSOX protein and *Ero1L*. Wings from adult female flies incubated at 25°C. The genotype of each fly is indicated near the figure, and the arrows indicate the wing vein-thickening phenotypes. Bars, 10  $\mu$ m.

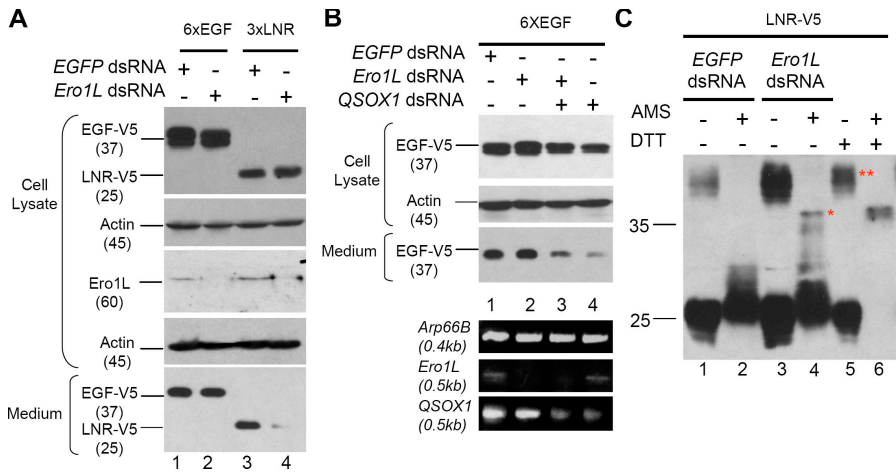
### ***Ero1L* is required for cysteine bridge formation of the LNR domain of Notch**

As our *in vivo* analysis suggests that the folding of EGF domains might not depend on *Ero1L*, we hypothesized that *Ero1L* may act on the LNRs. LNRs have special disulfide bonds that are unique to Notch proteins (Vardar et al., 2003). Most misfolded proteins, especially when disulfide bonds are not properly formed (Frand and Kaiser, 1998; Pollard et al., 1998), are expected to be trapped in the ER. We examined the secretion efficiency of LNR domains (3XLNR) and EGF domains (6XEGF) tagged with a signal peptide for secretion in S2 cells when *Ero1L* is reduced. The constructs were transfected in S2 cells, and similar levels of secreted proteins in the medium were observed for both proteins (Fig. 7 A, bottom, lanes 1 and 3). Although secretion of 6XEGF is unaffected in *Ero1L* knockdown cells (Fig. 7 A, bottom, lanes 1 and 2), the amount of 3XLNR in the medium of cells in which *Ero1L* is reduced through RNAi is much diminished (Fig. 7 A, compare lane 3 with lane 4), indicating that *Ero1L* is required for proper 3XLNR secretion but not for 6XEGF. These data support the hypothesis that *Ero1L*

function is required specifically for folding of the LNRs. Although knockdown of *Ero1L* does not affect the EGF secretion, we further examined whether knockdown of CG4670 (*QSOX1*) in S2 cells affect the secretion of the EGF domains. To our surprise, whereas CG4670 seems not to be required for *in vivo* disulfide bond formation in our RNAi experiments, knockdown of CG4670 in S2 cells affects EGF (Fig. 7 B, lane 4). Together, the secretion assay suggests that *Ero1L* plays a rather specific role for folding the LNR domain, whereas CG4670 may be generally required for ER folding in S2 cells.

To directly test whether disulfide bond formation is perturbed in 3XLNR by knocking down *Ero1L*, we performed an *in vitro* oxidation experiment using the thiol-conjugating reagent 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS). This compound can covalently react with a free thiol on the cysteine residue and increase the molecular mass by 490 D per cysteine residue (Kobayashi et al., 1997). Therefore, if there is a defect in disulfide bond formation of the 3XLNR domain when *Ero1L* is knocked down, we expect a mobility shift of this domain by  $\sim$ 9 kD using Western blot analysis after incubation





**Figure 7. Ero1L is involved in secretion and disulfide bond formation of the LNR domain of Notch.** (A) Secretion of the LNR domain into the medium is affected when Ero1L is reduced (lane 4, bottom, Western blot). S2 cells treated with either dsRNA against EGFP (lanes 1 and 3) or Ero1L (lanes 2 and 4) were followed by transfection with EGF-V5 (lanes 1 and 2) and LNR-V5 (lanes 3 and 4). Western blots of the cell lysate (top) and the medium from these samples are shown (bottom). (B) Secretion of the EGF domain into the medium is affected when QSOX1 (CG4670) is knocked down. S2 cells treated with various dsRNA are indicated in the table above the Western blotting. The bottom panel shows the relative abundance of mRNA of Arp66B (control), Ero1L, and QSOX1, which was measured by RT-PCR. (C) Disulfide bonds in the LNR domain of Notch

fail to form properly in Ero1L knockdown S2 cells. An AMS thiol-modifying analysis was used to reveal disulfide bond formation under various conditions. As a positive control, lysate was treated with DTT followed by AMS incubation. The expected molecular weight shift of the LNR domain is observed in lane 6 (\*). A similar molecular weight shift is observed in Ero1L knockdown lysate without prior DTT treatment (lane 4), indicating a loss of disulfide bond formation in a portion of the LNR domain. Note that the LNR domain can form homodimers without AMS treatment (\*\*\*) that can be removed in a high concentration of urea (not depicted).

with AMS. As a positive control, the LNRs were reduced with DTT incubation before AMS incubation, and a clear mobility shift by ~10 kD was observed (Fig. 7 C, compare lane 6 with lane 5). When Ero1L knockdown was performed, a fraction of the LNR domain showed a similar mobility shift as in our positive control (Fig. 7 C, compare lane 4 with lane 6), indicating that a certain proportion of the cysteine residues of the LNRs are in the thiol form. This is in contrast with the result obtained from a negative control experiment in which an EGFP double-stranded RNA (dsRNA) was used (Fig. 7 C, lane 2). Although a considerable fraction of the LNRs does not exhibit a mobility shift in our Ero1L knockdown experiments (Fig. 7 C, lane 4), we propose that this is likely caused by an incomplete knockdown of Ero1L. Alternatively, the remaining capacity of disulfide bond formation in the S2 cell may be dependent on QSOX protein, allowing formation of the disulfide bonds in Notch.

In summary, the data indicate that disulfide bond formation of the LNR domain and the subsequent trafficking of this domain from the ER require Ero1L, supporting our hypothesis that Notch is a major target of Ero1L.

## Discussion

In this study, we have isolated mutations in *Ero1L* in a mosaic screen that cause *Notch*-related phenotypes. Ero1L is conserved from yeast to man at the sequence and functional level (Cabibbo et al., 2000). In yeast-conditional *ero1-1* mutants, the disulfide bond formation in carboxyl peptidase Y, Kpn-bla, and Gas1p are severely compromised, leading to their accumulation in the ER (Frand and Kaiser, 1998; Pollard et al., 1998). Biochemical and structural analyses have shown that Ero1p can directly transfer disulfide equivalents to PDI proteins, thereby oxidizing the PDIs (Frand and Kaiser, 1999; Tu et al., 2000) that then catalyze disulfide bond formation of substrate proteins in the ER (Freedman, 1989; Ellgaard and Ruddock, 2005; Maattanen et al., 2006; Sevier and Kaiser, 2006). Typically, most organisms contain one or two *Ero1L* genes but many different PDIs (5 in yeast and 13 in *Drosophila*).

Two human *ERO1* homologues, *ERO1- $\alpha$*  and *ERO1- $\beta$* , have been shown to be able to rescue the lethality of the *ero1* mutants in yeast, indicating that the enzymatic function of Ero1p might be conserved in multicellular organisms (Cabibbo et al., 2000; Pagani et al., 2000). *ERO1- $\alpha$*  and *- $\beta$*  are highly expressed in organs with a high demand for secretion, consistent with their roles in oxidative folding of ER proteins. In vitro studies indicate that Ero1L exhibits biochemical properties in mammalian cells that are similar to yeast (Cabibbo et al., 2000; Pagani et al., 2000). However, it is not clear whether Ero1L can affect specific subsets of disulfide-containing proteins in vivo in different physiological contexts.

### Ero1L is required for Notch signaling in *Drosophila*

Several datasets indicate that Ero1L function affects Notch signaling rather specifically during development. First, lateral inhibition, inductive signaling, and follicle cell differentiation, which are hallmarks of Notch signaling (Bray, 1998), are defective in *Ero1L* mutant clones. Second, downstream targets of the Dpp and Hh signaling pathways are properly expressed in *Ero1L* mutants. Third, several membrane proteins other than Notch are expressed and localized normally in *Ero1L* mutant clones. Fourth, the UPR induced in *Ero1L* mutant clones is considerably reduced in clones that are double mutant for *Notch* and *Ero1L*. Thus, the data strongly argue that Notch is a major substrate for Ero1L or that Notch is an extremely sensitive target when Ero1L function is compromised.

In yeast, Ero1p relies on PDIs to transfer oxidizing equivalents for folding substrates (Tu et al., 2000). Because certain members of the PDI family exhibit substrate specificity (Ellgaard and Ruddock, 2005; Jessop et al., 2007), it has been proposed that different disulfide bond structures may be oxidized by specific sets of PDI family members. Although it is quite surprising that *Drosophila* Ero1L has a quite specific role in Notch signaling, it is likely that one or several PDIs are required to interact with Ero1L to function in the Notch signaling. Furthermore, the

Notch loss of function phenotype associated with the loss of *Drosophila Ero1L* can be rescued by overexpressing human *Ero1L- $\alpha$* , suggesting that the role of Ero1L in Notch signaling is conserved in vertebrates.

### Ero1L affects the LNR domain of Notch

Notch, Dl, and Serrate have numerous EGF motifs in their extracellular domain, and each motif has three cysteine bridges (Downing et al., 1996; Hambleton et al., 2004), leading to the possibility that these proteins are likely candidate substrates for Ero1L. The in vivo overexpression experiments using Dl, full-length Notch, and NEXT in *Ero1L* mutant clones strongly suggest that Ero1L is involved in folding of the NECD. Furthermore, clonal analyses show that only Notch exhibits strong ER accumulation, whereas other EGF-containing membrane proteins are unaffected. This suggests that a unique portion in NECD depends critically on Ero1L function. In addition to the EGFs, the NECD also contains another type of domain with cysteine bridges, namely three LNRs. The LNRs are found in all Notch homologues, including Lin12, Notch, and Notch 1–4 (Sanchez-Irizarry et al., 2004). The only other LNR-containing proteins are mammalian proteins termed pregnancy-associated plasma protein A (pappalysin-1) and pregnancy-associated plasma protein A2 (pappalysin-2; Boldt et al., 2004), which have no homologues in *Drosophila*. This observation prompted us to test whether the LNRs are the target of Ero1L. Our biochemical data indicate that cysteine bridge formation in the LNRs is clearly impaired in *Ero1L* knockdown cells. Thus, Ero1L-dependent disulfide bond formation in the LNR domain but not the EGF domain may also underlie the specificity of the phenotype associated with the loss of Ero1L.

Before ligand activation, the interaction between the LNRs and the nearby heterodimer domain of Notch mediates an autoinhibitory function that renders Notch resistant to S2 cleavage and thus regulates ligand-dependent Notch activation (Sanchez-Irizarry et al., 2004; Gordon et al., 2007). Deletions in the autoinhibitory domain result in a gain of function activity of the Notch receptor (Greenwald and Seydoux, 1990; Lieber et al., 1993; Berry et al., 1997). It is therefore reasonable to surmise that if the LNRs of Notch are misfolded and Notch were able to reach the membrane, it may have detrimental effects, as the protein is likely to be constitutively active. However, all of the gain of function mutations identified in T cell acute lymphoblastic leukemia patients reside in the heterodimer domain of Notch1 (Weng et al., 2004). Quite possibly, a stringent quality control mechanism in the ER ensures proper folding of the LNRs. If mutations in the LNRs disrupt its proper folding, Notch will likely not exit the ER and reach the cell membrane. Furthermore, our genetic interaction data suggest that *Ero1L* loss of function can specifically suppress a *Notch* gain of function phenotype (Fig. 2, F and G). A plausible translational aspect is that partially knocking down Ero1L expression in Notch-dependent T cell leukemia could be considered as a therapeutic strategy.

### Different thiol oxidases in ER-protein folding of metazoans

It is surprising that Ero1L, a thiol oxidase proposed to be involved in global disulfide bond formation in yeast, does not cause

cell lethality in *Drosophila*. How do disulfide bonds form in the absence of Ero1L? We explored the possibility that other thiol oxidases (QSOX) are involved in ER disulfide bond formation. Although compromising QSOX does not cause a visible phenotype, genetic interaction between *Ero1L* and *QSOX1* (*CG4670*) suggests that a QSOX protein can indeed contribute to disulfide bond formation when Ero1L function is decreased. Together with the strong UPR observed in *Ero1L* mutant cells, we propose that the UPR can either enhance the expression of QSOX or potentiate similar proteins at a later developmental phase. This also suggests that regulation of thiol oxidases in multicellular organisms is more complex than in yeast.

In summary, through characterization of the loss of function phenotypes of *Ero1L* in *Drosophila*, we find that Ero1L is involved in Notch signaling. Our study not only contributes to a previously unknown requirement for the maturation of the Notch LNR domain in the ER but also suggests that the thiol oxidase Ero1L plays a surprisingly specific role during development.

## Materials and methods

### *Drosophila* genetics

Stocks used in this study are as follows: (1) *y w ey-FLP GMR-lacZ; P{w<sup>+</sup>}70C RpS17<sup>4</sup> FRT80B/TM3, Sb*, (2) *y w; FRT80B* (isogenized), (3) *y w Ubx-FLP FRT19A*, (4) *y w Ubx-FLP; RpS17<sup>4</sup> Ubi-GFP FRT80B/TM3, Ser*, (5) *y w; UAS-FLP; C684-GAL4 P{y<sup>+</sup> w<sup>+</sup>} FRT80B/TM6B*, (6) *y w N<sup>5419</sup> FRT19A/FM7*, (7) *y w hs-FLP; UAS-NECN<sup>(NEXT)</sup>/CyO; MKRS/TM2* (Wang and Struhl, 2004), (8) *y w hs-FLP; UAS-N/CyO; MKRS/TM2* (Wang and Struhl, 2004), (9) *y w hs-FLP; UAS-Dl/CyO; MKRS/TM2*, (10) *y w; UAS-Ero1L*, (11) *y w; UAS-hERO1- $\alpha$* , (12) *y w; UAS-hERO1- $\alpha$ .C394A*, (13) *y hs-FLP tub $\alpha$ 1-GAL4 UAS-GFP.nls; tub-GAL80 RpS17<sup>4</sup> FRT80B/TM6B*, *Tb* (Jafar-Nejad et al., 2006), (14) *UAS-Hsc3. D231S* (Elefant and Palter, 1999), (15) *UAS-Ero1L-RNAi* (Dietzl et al., 2007), (16) *UAS-CG4670-RNAi*, and (17) *nub-GAL4* (Calleja et al., 1996). Mosaic analysis with a repressible cell marker (MARCM) experiments were performed as described previously (Wang and Struhl, 2004). In brief, *y w Ubx-FLP tub-GAL4 UAS-GFP.nls; tub-GAL80 FRT2A* females were crossed with *y w/Y; UAS-X/+; Ero1L<sup>23T</sup> FRT2A/+* males (where X = N, NECN<sup>(NEXT)</sup>, or Dl). The rescue experiments were also performed using the MARCM technique. Female flies of *y hs-FLP tub $\alpha$ 1-GAL4 UAS-GFP.nls; tub-GAL80 RpS17<sup>4</sup> FRT80B/TM6B*, *Tb* were crossed to male flies of *y w/Y; UAS-X/+; Ero1L<sup>23T</sup> FRT2A/+* (where X = *Ero1L*, *hERO1- $\alpha$* , or *hERO1- $\alpha$ .C394A*). The homozygous mutant bristles with longer and thicker appearance were differentiated from the short and thin *RpS17<sup>4</sup>* (*minute* phenotype) bristles. The homozygous mutant clones in follicle cells were obtained by dissecting the ovaries of females of the genotype *y w hs-FLP/+; Ero1L<sup>23T</sup> FRT80B/ RpS17<sup>4</sup> Ubi-GFP FRT80B*. To obtain double mutant clones of *Notch* and *Ero1L*, the larvae with the genotype *y w Ubx-FLP FRT19A/ N<sup>5419</sup> FRT19A; Ero1L<sup>23T</sup> FRT80B/ RpS17<sup>4</sup> Ubi-GFP FRT80B* were dissected.

### Immunohistochemistry and Western blotting

For conventional immunostaining, ovaries, wing discs from third instar larvae, or pupal notum were dissected in PBS and fixed with 4% formaldehyde for 20 min. The samples were permeabilized in PBS + 0.2% Triton X-100 (PBS-Tween [PBST]) for 20 min and blocked with 5% normal donkey serum in PBST for 1 h. The samples were incubated with primary antibodies at 4°C overnight. The following primary antibodies were used: mouse  $\alpha$ -Cut (1:500; Developmental Studies Hybridoma Bank [DSHB]; Blochlinger et al., 1990), rat  $\alpha$ -Elav (1:200; DSHB; Robinow and White, 1991), guinea pig  $\alpha$ -Sens (1:1,000; Nolo et al., 2000), mouse  $\alpha$ -Wg (1:200; 4D4; DSHB; Brook and Cohen, 1996), mouse  $\alpha$ -Vg (1:1,000; Williams et al., 1991), rabbit  $\alpha$ -spalt (1:200; Kuhnlein et al., 1994), rabbit  $\alpha$ -p-Mad (1:500; Nakao et al., 1997), rat  $\alpha$ -Ci (1:100; 2A1; Motzny and Holmgren, 1995), mouse  $\alpha$ -N<sup>mra</sup> (1:1,000; C17.9C6; DSHB; Fehon et al., 1990), mouse  $\alpha$ -NECD (1:100; C458.2H; DSHB; Diederich et al., 1994), rabbit  $\alpha$ -NO (1:1,000; Kidd and Lieber, 2002), mouse  $\alpha$ -D<sup>EC</sup> (1:1,000; C594.9B; DSHB; Fehon et al., 1990), guinea pig  $\alpha$ -Hsc3 (1:200; Ryoo et al., 2007),

rabbit  $\alpha$ -*Drosophila* EGF receptor (1:500; Lesokhin et al., 1999), mouse  $\alpha$ -FasII (1:500; 1D4; DSHB; Schuster et al., 1996), mouse  $\alpha$ -crumbs (1:500; Cq4; DSHB; Tepass and Knust, 1993), and phalloidin Alexa Fluor 647 (1 U for every reaction; Invitrogen). The samples were incubated with Cy3- and Cy5-conjugated secondary antibodies (1:500; Jackson ImmunoResearch Laboratories). Images were captured at RT using a confocal microscope (LSM510; Carl Zeiss, Inc.) with the LSM5 software (Carl Zeiss, Inc.). In Fig. 2 (B–E' and J–K'), Fig. 3 (C–F'), Fig. 5 (A–H'), and Fig. 6 (A and C–D'), a 40 $\times$  1.0 NA differential interference contrast plan-Neofluor oil-immersion lens (Carl Zeiss, Inc.) was used. In Fig. 3 (A–B'), a 20 $\times$  0.75 NA lens without oil (Carl Zeiss, Inc.) was used. The images were processed with Amira (3.1; Mercury Computer Systems) and PhotoShop (version 7.0; Adobe). Non-permeabilized immunostaining was performed as previously described (Wang and Struhl, 2004). In brief, the wing discs from third instar larvae were fixed and incubated with primary antibody without detergent at 4°C overnight. The sample was washed three times with PBS and permeabilized with PBST followed by normal staining procedures. Western blot analysis was performed according to standard procedures with the primary antibodies of mouse  $\alpha$ -N<sup>intra</sup> (1:5,000; C17.9C6; DSHB; Fehon et al., 1990), mouse  $\alpha$ -V5 (1:5,000) and mouse  $\alpha$ -Flag (1:5,000; M2; Sigma-Aldrich), and rat  $\alpha$ -Ero1L (1:3,000). Coimmunoprecipitations were performed as described previously (Acar et al., 2006).

#### Quantification

To determine the immunolabeling intensity of Hsc3 in cells with different genotypes, the number of pixels in a fixed area of a certain genotype was measured using the LabelVoxel and TissueStatistics functions of Amira (Indeed-Visual Concepts GmbH). For each genotype, the mean and standard deviation were calculated. Significance was based on the two-tailed *t* test.

#### Molecular biology and generation of antibodies

Sequencing of the *Ero1L* alleles was performed using homozygous mutant embryos with standard techniques. The *Ero1L* cDNA was obtained from the *Drosophila* Gene Collection (Rubin et al., 2000). It was subcloned into pUAST, pAC, and pGEX-4T-1 vectors. To generate antibody against Ero1L, the GST fusion protein was produced in bacteria and purified using glutathione–Sephadex 4B beads (GE Healthcare). The beads were boiled in sample buffer, the eluate was separated by SDS-PAGE, and a gel slice with the corresponding band was sent to Cocalico Biologicals for use as an antigen in rats for antibody production. Human *ERO1L* $\alpha$  and C394A mutant cDNAs were provided by A. Benham (University of Durham, Durham, England, UK; Cabibbo et al., 2000) and were subcloned into pUAST for generating transgenic flies. Human *ERO1L* $\alpha$  was cloned into pUAST between the KpnI and XhoI sites. *Drosophila Ero1L* was cloned into pUAST between KpnI and XhoI sites, into pAC 5.1 between KpnI and XhoI sites, and into pGEX-4T-1 between BglII–BamHI and XhoI sites. The three LNRs and six EGF (28–33 EGF) repeats from Notch were cloned into pMT/V5-HisB within the EcoRI site with additional signal peptide at its 5' end to allow secretion.

#### RT-PCR, RNAi, and AMS oxidation reaction

10 larvae were lysed to obtain the total RNA using an RNA Miniprep kit (Stratagene). The semiquantitative abundance of the mRNA of CG4670 and Arp66B (control) was determined using one-step RT-PCR (Ambion). Knockdown experiments using dsRNA against either *Ero1L* or *EGFP* were performed according to standard procedures described previously (Clemens et al., 2000). In brief, dsRNA was generated using the MEGAscript RNAi kit (Ambion). S2 cells incubated with 30  $\mu$ g dsRNA for 4 d were transfected with either LNR-V5 or EGF-Flag using the FuGene transfection reagent (Roche) and an additional 10  $\mu$ g of dsRNA. The cells were harvested and incubated with or without 10 mM DTT (Sigma-Aldrich) in PBS for 20 min at RT. After lysing, cells were precipitated with 20% TCA, and the protein pellet was washed with cold acetone twice and air dried for 3 min. The pellet was dissolved and incubated in lysis buffer of 2.5% SDS, 100 mM Tris, pH 8, and bromophenyl blue with or without 20 mM AMS for 20 min at RT. After adding the reducing agent  $\beta$ -mercaptoethanol to 10% of the final concentration, the cell lysates were boiled for 2 min and subjected to SDS-PAGE analysis.

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