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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Notch-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 γ-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

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.
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 oxidoeductin 1–like (Ero1L), is required for Notch-dependent developmental processes. We find that Notch is a key target in Ero1L mutant clones. 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). 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 nota 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 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].
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 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 smaller mutant clones and near the edge of wild-type cells, some mutant cells are Cut positive (*). (F and G) Removal of one copy of kiga does not suppress a gain of function phenotype of Notch. A male adult wing from an N^{A1E-2}/Y fly is shown (F) with a reduced length of veins (F, arrows). The loss of wing veins is suppressed (G) when one copy of kiga is removed in N^{A1E-2}/Y; kiga/+ male wings (G). (H and I) Removal of one copy of kiga does not suppress a gain of function phenotype of Egr. 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 μm; (D–E' and J–K') 40 μm.

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 notch 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^{A1E-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^{A1E-2} mutant flies. As shown in Fig. 2 G, removal of one copy of kiga in N^{A1E-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...
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 Dl 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 Dl 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 Dl is overexpressed in kiga mutant cells, these cells are capable of inducing Cut expression in adjacent cells near the DV boundary in the dorsal compartment (Fig. 3, C and C’), suggesting that kiga is not required for proper function of Dl 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’).

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 (Dl) and the receptor (Notch); Dl 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 Dl 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.

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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 (Frand 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 Ero1p 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/H9251 and ERO1-L/H9252 (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/H9251 in Ero1L mutant clones and found that the lateral inhibition phenotype is rescued (Fig. 4E). However, overexpression of an enzymatically inactive form (C394A) of human ERO1-L/H9251, which has a mutation in the CXXCXXC motif (Cabibbo et al., 2000), was unable to rescue the lateral inhibition phenotype (Fig. 4F). 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 (Frand and Kaiser, 1998;
disulfi de bonds. As unpaired cysteine residues induce a prominent UPR (Frand and Kaiser, 1998), it is likely that some or many disulfi de 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.

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 disulfi de-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 disulfi de bonds: the 36 EGF repeats and the three LNRs. Each EGF repeat and LNR has three disulfi de bonds. As unpaired cysteine residues induce a prominent UPR (Frand and Kaiser, 1998), it is likely that some or many disulfi de 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).

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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 by Hsc3 (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 lasstil 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 (Hoober 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 (Hoober et al., 1999a), we surmise that these proteins may have a previously unidentifiable 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. The reducing 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.
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 ~9 kD using Western blot analysis after incubation...
with AMS. As a positive control, the LNRs were reduced with DTT incubation before AMS incubation, and a clear mobility shift by $\sim 10$ 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 (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-Lα* and *ERO1-Lβ*, 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-Lα* and *ERO1-Lβ* 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 ERO1-Lα, 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 (CGG4670) 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) w υ y-FRT080, UAS-GFP, FRT80B/TM3, Ser, (2) y w; FRT80B [isoegenized], (3) y w UbxFP FRT19A, (4) y w UbxFp, Rps17m Ubi-GFP FRT80B/TM3, Ser, (5) y υ w; UAS-βgal, FRT80B, TM6B, (6) y υ w NAD1 FRT19A/FM7, (7) y υ hs-FSP, UAS-NAD1/Cyo; MKRS/TM2 [Wang and Struhl, 2004], (8) y υ hs-FSP, UAS-NAD1/Cyo; MKRS/TM2 [Wang and Struhl, 2004], (9) y υ hs-FSP, UAS-D/Cyo; MKRS/TM2, (10) y υ w, UAS-Ero1L, (11) y υ w; UAS-Erho1-Lα, (12) y υ UAS-Erho1-Lα, C394A, (13) y υ hs-FSP tubx-1-GAL4 UAS-GFP, nls; tub-GAL80 Rps17m FRT80B/TM6B, Tb [Jafar-Nejad et al., 2006], (14) UAS-Hsc3. D2315 [Elefant and Pauler, 1999], (15) UAS-Ero1LRNAi [Dietzl et al., 2007], (16) UAS-CG4670RNAi, and (17) nub-GAL4 [Callia et al., 1996]. Mosaic analysis with a repressible cell marker (MARCM) experiments were performed as described previously [Wang and Struhl, 2004]. In brief, y υ UbxFp tub-GAL4 UAS-GFP, nls; tub-GAL80 FRT2A females were crossed with y w; UAS+/+, Ero1L-ΔTub FRT2A/+ [where X = Ero1L, Erho1-Lα, or Erho1-Lα, C394A]. The homozygous mutant bristles with longer and thicker appearance were differentiated from the short and thin Rs17m [minute phenotype] bristles. The homozygous mutant clones in follicle cells were obtained by dissecting the ovaries of females of the genotype y υ hs-FSP/+; Ero1L-ΔTub FRT80B/Rps17m Ubi-GFP FRT80B. To obtain double mutant clones of Notch and Ero1L, the larvae with the genotype y υ UbxFp FRT19A/ NAD1 FRT19A; Ero1L-ΔTub FRT80B/Rps17m Ubi-GFP FRT80B were dissected.

Immunohistochemistry and Western blotting
For conventional immunostaining, ovaries, wing discs from third instar larve, or prepup noto 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 α-Cut (1:500; Developmental Studies Hybridoma Bank [DSHB]; Blochlinger et al., 1990), rat α-EIav (1:200; DSHB; Robinow and White, 1991), guinea pig α-Cut (1:1000; Nolo et al., 2000), mouse α-Vg (1:200; Diederich et al., DSHB; Brook and Cohen, 1996), mouse α-Vg (1:1000; Williams et al., 1991), rabbit α-spalt (1:200; Kuhnlein et al., 1994), rabbit α-εMAD (1:500; Nakao et al., 1997), rat α-Ci (1:100; 2A1; Motzny and Holmgren, 1995), mouse α-Nf (1:1000; C17.9C6; DSHB; Feohan et al., 1990), mouse α-Nfn (1:100; C458.2H; DSHB; Diederich et al., 1994), rabbit α-NO (1:1000; Kidd and Lieber, 2002), mouse α-ΔFIC (1:1000; C592.9B; DSHB; Feohan et al., 1990), guinea pig α-Hsc3 (1:200; Ryoo et al., 2007),...
rabbit α-Drosophila EGF receptor (1:500; Lesokhin et al., 1999), mouse α-Fas (1:500; ID4; DSHB; Schuster et al., 1996), mouse α-crumbs (1:500, C4; DSHB; Tepass and Knust, 1993), and phospho-Alxera 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 LSMS software (Carl Zeiss, Inc., Inc.). In Fig. 2B–E and Fig. 3C–F, Fig. 5A–H, and Fig. 6A (A and C–D), a 40×/1.0 NA differential interference contrast plan-neofluor oil-immersion lens (Carl Zeiss, Inc.) was used. In Fig. 3 (A–B), a 20× 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 Stuhl, 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 PBS followed by normal staining procedures. Western blot analysis was performed according to standard procedures with the primary antibodies of mouse α-N-cad (1:5,000; C17:9C6; DSHB; Fellan et al., 1990), mouse α-V5 (1:5,000) and mouse α-Flag (1:5,000; M2, Sigma-Aldrich), and rat α-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 LabelVox 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-AT1 vectors. To generate antibody against Ero1L, the GST fusion protein was produced in bacteria and purified using glutathione-Sepharose 4B beads (GE Healthcare). The beads were washed and in some cases 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 ERO1-Lα 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 ERO1-Lα was cloned into pUAST between the KpnI and XhoI sites.

**References.**

Acar, M., H. Jafari-Nejad, N. Giagtzoglou, S. Yallampalli, G. David, Y. He, C. Delidakis, and H.J. Bellen. 2006. Senseless physically interacts with proneural proteins and functions as a transcriptional co-activator. Development. 133:1979–1989.

Acar, M., H. Jafari-Nejad, H. Takeuchi, A. Rajan, D. Ibrani, N.A. Rana, H. Pan, R.S. Haltiwanger, and H.J. Bellen. 2005. Rumi is a CAP10 domain glycosyltransferase that modifies Notch and is required for Notch signaling. Proc. Natl. Acad. Sci. USA 102:13322–13331.

Artavanis-Tsakonas, S., M.D. Rand, and R.J. Lake. 1999. Notch signaling: cell fate control and signal integration in development. Science. 284:770–776.

Bard, A.J., R. Le Borgne, and F. Schweiguth. 2004. Asymmetric localization and function of cell-fate determinants: a fly’s view. Curr. Opin. Neurobiol. 14:6–14.

Benham, A.M., A. Cabibbo, A. Fassio, N. Bulleid, R. Sitia, and I. Braakman. 2000. The CXXCXXC motif determines the folding, structure and stability of human Ero1-Lα. EMBO J. 19:4493–4502.

Berdnik, D., T. Torok, M. Gonzalez-Gaitan, and J.A. Knoblich. 2002. The endocytic protein alpha-Adaptin is required for numb-mediated asymmetric cell division in Drosophila. Dev. Cell. 3:221–231.

Berry, L.W., B. Westlund, and T. Sch sidelines. 1997. Germ-line tumor formation caused by activation of gpl-1, a Caenorhabditis elegans member of the Notch family of receptors. Development. 124:925–936.

Blochinger, K., R. Bodmer, L.Y. Jan, and YN. Jan. 1990. Patterns of expression of cut, a protein required for external sensory organ development in wild-type and cut mutant Drosophila embryos. Genes Dev. 4:1322–1331.

Boldt, H.B., K. Kjaer-Sorensen, M.T. Overgaard, K. Weyer, C.B. Poulsen, L. Slottrup-Jensen, C.A. Conover, L.C. Giudice, and C. Oxtvig. 2004. The Lin12-notch repeats of pregnancy-associated plasma protein-A bind calcium and determine its proteolytic specificity. J. Biol. Chem. 279:38525–38532.

Brand, A.H., and N. Perrimon. 1993. Targeted gene expression as a means of altering cell fate and generating dominant phenotypes. Development. 118:401–415.

Bray, S. 1998. Notch signalling in Drosophila: three ways to use a pathway. Semin. Cell Dev. Biol. 9:591–597.

Bray, S.J. 2006. Notch signalling: a simple pathway becomes complex. Nat. Rev. Mol. Cell Biol. 7:578–613.

Brook, W.J., and S.M. Cohen. 1996. Antagonistic interactions between wingless and decapentaplegic responsible for dorsal-ventral pattern in the Drosophila Leg. Development. 273:1373–1377.

Cabibbo, A., M. Pagani, M. Fabbricci, M. Rocchi, M.R. Farmery, N.J. Bulleid, and R. Sita. 2000. ERO1-L, a human protein that favors disulfide bond formation in the endoplasmic reticulum. J. Biol. Chem. 275:4827–4833.

Calleja, M., E. Moreno, S. Pelaz, and G. Morata. 1996. Visualization of gene expression in living adult Drosophila. Science. 274:252–255.

Chintapalli, V.R., J. Wang, and J.A. Dow. 2007. Using FlyAtlas to identify better Drosophila melanogaster models of human disease. Nat. Genet. 39:715–720.

Clemens, J.C., C.A. Worby, N. Simonson-Leff, M. Muda, T. Maehama, B.A. Hemmings, and J.E. Dixon. 2000. Use of double-stranded RNA interference in Drosophila cell lines to dissect signal transduction pathways. Proc. Natl. Acad. Sci. USA. 97:6499–6503.

Culi, J., and R.S. Mann. 2003. Boca, an endoplasmic reticulum protein required for wingless signaling and trafficking of LDL receptor family members in Drosophila. Cell. 112:343–354.

de Celis, J.F., and S. Bray. 1997. Feed-back mechanisms affecting Notch activation at the dorsoventral boundary in the Drosophila wing. Development. 124:3241–3251.


de Celis, J.F., and A. Garcia-Bellido. 1994. Modifications of the notch function by Abreptax mutations in Drosophila melanogaster. Genetics. 136:183–194.
De Celis, J.F., A. Garcia-Bellido, and S.J. Bray. 1993. HLH proteins, fly neurogenesis, and vertebrate myogenesis. Cell. 75:827–830.

Jassar, H.K., Andrews, M., Acar, B., Bayat, F., Witzel-Peitz, S.Q., Mehta, J.K., and Bellen, H.J. 2005. A Genome-wide transgene RNAi library for conditional gene inactivation in Drosophila. Nature. 448:151–156.

Downing, A.K., W. Szymański, J.D. Campbell, and P.A. Handford. 1996. Solution structure of a pair of calcium-binding epidermal growth factor-like domains: implications for the Marfan syndrome and other genetic disorders. Cell. 85:597–605.

Elefant, F., and K.B. Palter. 1999. Tissue-specific expression of dominant negative mutant Drosophila HSC70 causes developmental defects and lethality. Mol. Biol. Cell. 10:2101–2117.

Ellgaard, L., and L.W. Ruddock. 2005. The human protein disulfide isomerase family: substrate interactions and functional properties. EMBO Rep. 6:28–32.

Fehon, R.G., P.J. Kioh, J. Rebay, C.L. Regan, T. Xu, M.A. Muskavitch, and S. Artavanis-Tsakonas. 1990. Molecular interactions between the protein products of the neurogenic loci Notch and Delta, two EGF-homologous genes in Drosophila. Cell. 61:523–534.

Freedman, R.B. 1989. Protein disulfide isomerase: multiple roles in the modification of nascent secretory proteins. Cell. 57:1069–1072.

Gallagher, C.M., and J.A. Knoblich. 2006. The conserved c2 domain protein lethal (2) giant discs regulates protein trafficking in Drosophila. Dev. Cell. 11:641–653.

Ghio, M., Y. Bellaiche, and F. Schweiguth. 1999. Revisiting the Drosophila microchaete lineage: a novel intrinsically asymmetric cell division generates a glial cell. Development. 126:3573–3584.

Glysson, A., C. Dambly-Chaudière, L.Y. Jan, and Y.N. Jan. 1993. Cell interactions and gene interactions in peripheral neurogenesis. Genes Dev. 7:723–733.

Gordon, W.R., D. Vardar-Ulu, G. Histen, C. Sanchez-Irizarry, J.C. Aster, and S.C. Blacklow. 2007. Structural basis for autoinhibition of Notch. Nat. Struct. Mol. Biol. 14:295–300.

Greenwald, I., and G. Seydoux. 1990. Analysis of gain-of-function mutations of the lin-12 gene of Caenorhabditis elegans. Genes Dev. 4:469–477.

Freedman, R.B. 1989. Protein disulfide isomerase: multiple roles in the modification of nascent secretory proteins. Cell. 57:1069–1072.

Gallagher, C.M., and J.A. Knoblich. 2006. The conserved c2 domain protein lethal (2) giant discs regulates protein trafficking in Drosophila. Dev. Cell. 11:641–653.

Ghio, M., Y. Bellaiche, and F. Schweiguth. 1999. Revisiting the Drosophila microchaete lineage: a novel intrinsically asymmetric cell division generates a glial cell. Development. 126:3573–3584.

Glysson, A., C. Dambly-Chaudière, L.Y. Jan, and Y.N. Jan. 1993. Cell interactions and gene interactions in peripheral neurogenesis. Genes Dev. 7:723–733.

Gordon, W.R., D. Vardar-Ulu, G. Histen, C. Sanchez-Irizarry, J.C. Aster, and S.C. Blacklow. 2007. Structural basis for autoinhibition of Notch. Nat. Struct. Mol. Biol. 14:295–300.

Greenwald, I., and G. Seydoux. 1990. Analysis of gain-of-function mutations of the lin-12 gene of Caenorhabditis elegans. Genes Dev. 4:469–477.

Gross, E., D.B. Kastner, C.A. Kaiser, and D. Fass. 2004. Structure of Ero1p, source of disulfide bonds for oxidative protein folding in the cell. Cell. 117:601–610.

Haines, N., and K.D. Irvine. 2003. Glycosylation regulates Notch signalling. Nat. Rev. Mol. Cell Biol. 4:786–797.

Hambleton, S., N.V. Valeev, A. Muranyi, V. Knott, J.M. Werner, A.J. McMichael, P.A. Handford, and A.K. Downing. 2004. Structural and functional properties of the human notch-1 ligand binding region. Structure. 12:2173–2183.

Harrison, S.D., N. Solomon, and G.M. Rubin. 1995. A genetic analysis of the 63E-64A genomic region of Drosophila melanogaster: identification of mutations in a replication factor C subunit. Genetics. 139:1701–1709.

Hartenstein, V., and J.W. Posakony. 1990. A dual function of the Notch gene in Drosophila sensillum development. Dev. Biol. 142:13–30.

Hober, K.L., N.M. Glynn, J. Burnside, D.L. Coppock, and C. Thorpe. 1999a. Homology between egg white sulfhydryl oxidase and quiescin Q6 defines a new class of flavin-linked sulfhydryl oxidas es. J. Biol. Chem. 274:31759–31762.

Hober, K.L., S.L. Sheasley, H.F. Gilbert, and C. Thorpe. 1999b. Sulfhydryl oxidase from egg white. A facile catalyst for disulfide bond formation in proteins and peptides. J. Biol. Chem. 274:22147–22150.

Jafar-Nejad, H., H.K. Andrews, M. Acar, B. Bayat, F. Witzel-Peitz, S.Q. Mehta, J.K. Knoblich, and J.H. Bellen. 2005. Sec15, a component of the exocyst, promotes notch signaling during the asymmetric division of Drosophila sensory organ precursors. Dev. Cell. 9:351–363.

Jafar-Nejad, H., A.C. Tien, M. Acar, and H.J. Bellen. 2006. Senseless and Daughterless confer neuronal identity to epithelial cells in the Drosophila wing margin. Development. 133:1683–1692.

Jan, Y.N., and L.Y. Jan. 1993. HLH proteins, fly neurogenesis, and vertebrate myogenesis. Cell. 75:827–830.

Jassar, H.K., Andrews, M., Acar, B., Bayat, F., Witzel-Peitz, S.Q., Mehta, J.K., and Bellen, H.J. 2005. A Genome-wide transgene RNAi library for conditional gene inactivation in Drosophila. Nature. 448:151–156.

Downing, A.K., W. Szymański, J.D. Campbell, and P.A. Handford. 1996. Solution structure of a pair of calcium-binding epidermal growth factor-like domains: implications for the Marfan syndrome and other genetic disorders. Cell. 85:597–605.

Elefant, F., and K.B. Palter. 1999. Tissue-specific expression of dominant negative mutant Drosophila HSC70 causes developmental defects and lethality. Mol. Biol. Cell. 10:2101–2117.

Ellgaard, L., and L.W. Ruddock. 2005. The human protein disulfide isomerase family: substrate interactions and functional properties. EMBO Rep. 6:28–32.

Fehon, R.G., P.J. Kioh, J. Rebay, C.L. Regan, T. Xu, M.A. Muskavitch, and S. Artavanis-Tsakonas. 1990. Molecular interactions between the protein products of the neurogenic loci Notch and Delta, two EGF-homologous genes in Drosophila. Cell. 61:523–534.

Freedman, R.B. 1989. Protein disulfide isomerase: multiple roles in the modification of nascent secretory proteins. Cell. 57:1069–1072.

Gallagher, C.M., and J.A. Knoblich. 2006. The conserved c2 domain protein lethal (2) giant discs regulates protein trafficking in Drosophila. Dev. Cell. 11:641–653.

Ghio, M., Y. Bellaiche, and F. Schweiguth. 1999. Revisiting the Drosophila microchaete lineage: a novel intrinsically asymmetric cell division generates a glial cell. Development. 126:3573–3584.

Glysson, A., C. Dambly-Chaudière, L.Y. Jan, and Y.N. Jan. 1993. Cell interactions and gene interactions in peripheral neurogenesis. Genes Dev. 7:723–733.

Gordon, W.R., D. Vardar-Ulu, G. Histen, C. Sanchez-Irizarry, J.C. Aster, and S.C. Blacklow. 2007. Structural basis for autoinhibition of Notch. Nat. Struct. Mol. Biol. 14:295–300.

Greenwald, I., and G. Seydoux. 1990. Analysis of gain-of-function mutations of the lin-12 gene of Caenorhabditis elegans. Genes Dev. 4:469–477.

Gross, E., D.B. Kastner, C.A. Kaiser, and D. Fass. 2004. Structure of Ero1p, source of disulfide bonds for oxidative protein folding in the cell. Cell. 117:601–610.

Haines, N., and K.D. Irvine. 2003. Glycosylation regulates Notch signalling. Nat. Rev. Mol. Cell Biol. 4:786–797.
of ligand-independent proteolytic activation depend, respectively, on a novel domain and the LNR repeats. Mol. Cell. Biol. 24:9265–9273.

Schroder, M., and R.J. Kaufman. 2005. ER stress and the unfolded protein response. Mutat. Res. 569:29–63.

Schuster, C.M., G.W. Davis, R.D. Fetter, and C.S. Goodman. 1996. Genetic dissection of structural and functional components of synaptic plasticity. I. Fasciclin II controls synaptic stabilization and growth. Neuron. 17:641–654.

Schweisguth, F. 2004. Regulation of notch signaling activity. Curr. Biol. 14:R129–R138.

Sevier, C.S., and C.A. Kaiser. 2006. Conservation and diversity of the cellular disulfide bond formation pathways. Antioxid. Redox Signal. 8:797–811.

Stowers, R.S., and T.L. Schwarz. 1999. A genetic method for generating Drosophila eyes composed exclusively of mitotic clones of a single genotype. Genetics. 152:1631–1639.

Struhl, G., and I. Greenwald. 1999. Presenilin is required for activity and nuclear access of Notch in Drosophila. Nature. 398:522–525.

Tanimoto, H., S. Itoh, P. ten Dijke, and T. Tabata. 2000. Hedgehog creates a gradient of DPP activity in Drosophila wing imaginal discs. Mol. Cell. 5:59–71.

Tu, B.P., S.C. Ho-Schleyer, K.J. Travers, and J.S. Weissman. 2000. Biochemical basis of oxidative protein folding in the endoplasmic reticulum. Science. 290:1571–1574.

Vardar, D., C.L. North, C. Sanchez-Irizarry, J.C. Aster, and S.C. Blacklow. 2003. Nuclear magnetic resonance structure of a prototype Lin12-Notch repeat module from human Notch1. Biochemistry. 42:7061–7067.

Wang, Q.T., and R.A. Holmgren. 1999. The subcellular localization and activity of Drosophila cubitus interruptus are regulated at multiple levels. Development. 126:5097–5106.

Wang, W., and G. Struhl. 2004. Drosophila Epsin mediates a select endocytic pathway that DSL ligands must enter to activate Notch. Development. 131:5367–5380.

Weng, A.P., A.A. Ferrando, W. Lee, J.P. Morris IV, L.B. Silverman, C. Sanchez-Irizarry, S.C. Blacklow, A.T. Look, and J.C. Aster. 2004. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science. 306:269–271.

Williams, J.A., J.B. Bell, and S.B. Carroll. 1991. Control of Drosophila wing and haltere development by the nuclear vestigial gene product. Genes Dev. 5:2481–2495.

Xu, T., L.A. Caron, R.G. Fehon, and S. Artavanis-Tsakonas. 1992. The involvement of the Notch locus in Drosophila oogenesis. Development. 115:913–922.

Zhai, R.G., P.R. Hiesinger, T.W. Koh, P. Verstreken, K.L. Schulze, Y. Cao, H. Jafar-Nejad, K.K. Norga, H. Pan, V. Bayat, et al. 2003. Mapping Drosophila mutations with molecularly defined P element insertions. Proc. Natl. Acad. Sci. USA. 100:10860–10865.