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

An-Chi Tien,1 Akhila Rajan,2 Karen L. Schulze,3 Hyung Don Ryoo,5,6 Melih Acar,1 Hermann Steller,6 and Hugo J. Bellen1,2,3,4

1Program in Developmental Biology; 2Department of Molecular and Human Genetics; 3Howard Hughes Medical Institute, and 4Department of Neuroscience, Baylor College of Medicine, Houston, TX 77030
2Department of Cell Biology, New York University School of Medicine, New York, NY 10016
3Howard Hughes Medical Institute, The Rockefeller University, New York, NY 10065

© 2008 Tien et al. This article is distributed under the terms of an Attribution–Noncommercial–Share Alike–No Mirror Sites license for the first six months after the publication date (see http://www.jcb.org/misc/terms.shtml). After six months it is available under a Creative Commons License (Attribution–Noncommercial–Share Alike 3.0 Unported license, as described at http://creativecommons.org/licenses/by-nc-sa/3.0/).

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

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.

A.-C. Tien and A. Rajan contributed equally to this paper.

Correspondence to Hugo J. Bellen: hbellen@bcm.tmc.edu

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; 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.
Published September 22, 2008

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).

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 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. 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* mutations. 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. Interestingly, these cells are clearly 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').

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 notchig 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...
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 germ line 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.

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 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 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 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, 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;
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 altered. 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 altered.
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 (Hooper 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 (Hooper 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 nubbins (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. 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 affects 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.
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 EGF double-stranded RNA (dsRNA) was used (Fig. 7 C, lane 2). Although 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).

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 peptide Y, Kpm-bla, and Gaslp are severely compromised, leading to their accumulation in the ER (Frands 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 (Frands 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-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 -β 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 (papalysin-1) and pregnancy-associated plasma protein A2 (papalysin-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 auto-inhibitory 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 auto-inhibitory 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+ FRT80B/TM3, Sb, (2) y w; FRT80B (isogenized), (3) y w/UbxFp FRT19A, (4) y w UbxFp; Rp517w Ubi-GFP FRT80B/TM3, Ser, (5) y w; UAS-FIP, C684-GAL4 P[w g] FRT80B/TM6B, (6) y w/Necri FRT19A/ FM7, (7) y w hs-FIP, UAS-N164(3P)GAL4/CY0; MKRS/TM2 [Wang and Struhl, 2004], (8) y w hs-FIP, UAS-N/CY0; MKRS/TM2 [Wang and Struhl, 2004], (9) y w hs-FIP, UAS-DI/CY0; MKRS/TM2, (10) y w; UAS-Ero1L, (11) y w; UAS-HERO1-Lα, (12) y w; UASHERO1-Lα C394A, (13) y w hs-FIP tubx 1-G4AL4 UAS-GFP, nls; tub-CALBO80 P w, (14) y w hs-FIP, tubx 1-G4AL4 FRT20B, (15) y w hs-FIP, tubx 1-G4AL4 UAS-GFP, nls; tub-CALBO80 FRT20A females were crossed with y w; Y; UAS+/+, Ero1L23T FRT2A/+ males (where X = N, N164(3P)GAL4 or Dl). The rescue experiments were also performed using the MARCM technique. Female flies of y hs-FIP tubx 1-G4AL4 UAS-GFP; tub-CALBO80 P w, FRT20A, Tb [Jafar-Nejad et al., 2006], (14) UAS-Hsc3. D2315 [Elefant and Patler, 1999], (15) UAS-Ero1L/RNAi [Dietzl et al., 2007], (16) UAS-CG4670/RNAi, and (17) tub-GAL4 [Collara et al., 1996]. Mosaic analysis with a repressible cell marker [MARC1 experiments were performed as described previously [Wang and Struhl, 2004]. In brief, y w hs-FIP tubx 1-G4AL4 UAS-GFP; tub-CALBO80 P w, UAS+/+, Ero1L23T FRT2A/+ where X = Ero1L, HERO1-Lα, or HERO1-Lα C394A. The homozygous mutant bristles with longer and thicker appearance were differentiated from the short and thin Rp517w [minute phenotype] bristles. The homozygous mutant clones in follicle cells were obtained by dissecting the ovaries of females of the genotype y hs-FIP+/+, Ero1L23T FRT80B/Rp517w Ubi-GFP FRT80B. To obtain double mutant clones of Notch and Ero1L, the larvae with the genotype y w hs-FIP FRT19A/ N164(3P)FRT19A, Ero1L23T FRT80B/ Ero1L23T FRT80B were dissected.

Immunohistochemistry and Western blotting
For conventional immunostaining, ovaries, wing discs from third instar lar- vae, or pupal nota 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 3% 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 a-Cut (1:500; Developmental Studies Hybridoma Bank [DSHB]; Blobligner et al., 1990); rat a-Elav (1:200; DSHB; Robinow and White, 1991), guinea pig a-Vg (1:100; 4D4; DSHB; Brook and Cohen, 1996), mouse a-Vg (1:1,000; Williams et al., 1991), rabbit a-spalt (1:200; Kuhnlein et al., 1994), rabbit a-P-Mad (1:500; Nakao et al., 1997), rat a-Ci (1:100; 2A1; Motzny and Holmgren, 1995), mouse a-N164(3P) (1:1,000; C17.96C; DSHB; Feohon et al., 1990), mouse a-N164(3P) (1:1,000; C458.2H; DSHB; Diederich et al., 1994), rabbit a-NO (1:1,000; Kidd and Lieber, 2002), mouse a-DICER (1:1,000; C594.9B; DSHB; Feohon et al., 1990), guinea pig a-Hsc3 (1:200; Ryoo et al., 2007),
rabbit α-Drosophila EGFP receptor (1:500; Lesokhin et al., 1999), mouse α-Fas1 (1:500; DSHB, Schuster et al., 1996), mouse α-crumbs (1:500, C4; DSHB, Tepass and Knust, 1993), and phallolidin 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 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 α-Nun (1:5,000; C17;9C6; DSHB, Fehon 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 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 twotailed t test.

Molecular biology and generation of antibodies

Sequencing of the Ero1 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-1T-1 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 equilibrated 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 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 was cloned into pUAST between KpnI and Xhol sites and into pAC 5.1 between KpnI and Xhol sites, and into pGEX-4T-1 between BglII–AvrII sites, and into pGEX-4T-1 between BglII–AvrII sites, and into pGEX-4T-1. Two additional CXXC motifs were introduced by site-directed mutagenesis into the CXXCXXC motif of Ero1L. Mouse Ero1L was cloned into pUAST as a XhoI–BamHI fragment and into pGEX-4T-1 between BglII–AvrII sites, and into pGEX-4T-1 between EcoRI and BglII sites with a C-terminal His tag. The CXXCXXC motif determines the folding, structure and stability of human Ero1L (EMBO J. 19:4493–4502).

Acknowledgments

We thank members of the Bellen laboratory for sharing reagents and discussions, Tien Stock Center for fly stocks and the DSHB for monoclonal antibodies. We are thankful to the Bloomington Stock Center for fly stocks and the DSHB for monoclonal antibodies. We thank members of the Bellen laboratory for sharing reagents and discussions, especially H. Jafar-Nejad. We thank Y. He for transgenic injections and S. Yamanoto, H. Andrews, H. Tsuda, N. Giajtzoglou, and H.F. Gilbert for critical reading of the manuscript.

Submitted: 1 May 2008
Accepted: 20 August 2008

References


Downloaded by on June 5, 2017
Published September 22, 2008


JCB • VOLUME 182 • NUMBER 6 • 2008 1124


