Deficient Notch signaling associated with neurogenic pecanex is compensated for by the unfolded protein response in Drosophila

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SUMMARY
The Notch (N) signaling machinery is evolutionarily conserved and regulates a broad spectrum of cell-specification events, through local cell-cell communication. pecanex (pcx) encodes a multi-pass transmembrane protein of unknown function, widely found from Drosophila to humans. The zygotic and maternal loss of pcx in Drosophila causes a neurogenic phenotype (hyperplasia of the embryonic nervous system), suggesting that pcx might be involved in N signaling. Here, we established that Pcx is a component of the N-signaling pathway. Pcx was required upstream of the membrane-tethered and the nuclear forms of activated N, probably in N signal-receiving cells, suggesting that pcx is required prior to or during the activation of N. pcx overexpression revealed that Pcx resides in the endoplasmic reticulum (ER). Disruption of pcx function resulted in enlargement of the ER that was not attributable to the reduced N signaling activity. In addition, hyper-induction of the unfolded protein response (UPR) by the expression of activated Xbp1 or dominant-negative Heat shock protein cognate 3 suppressed the neurogenic phenotype and ER enlargement caused by the absence of pcx. A similar suppression of these phenotypes was induced by overexpression of O-fucosyltransferase 1, an N-specific chaperone. Taking these results together, we speculate that the reduction in N signaling in embryos lacking pcx function might be attributable to defective ER functions, which are compensated for by upregulation of the UPR and possibly by enhancement of N folding. Our results indicate that the ER plays a previously unrecognized role in N signaling and that this ER function depends on pcx activity.

KEY WORDS: Drosophila, Notch signaling, Unfolded protein response

INTRODUCTION
Cell-cell signaling mediated by the Notch (N) receptor is implicated in a wide variety of developmental processes in multicellular organisms, across phyla (Artavanis-Tsakonas et al., 1991; Nicolas et al., 2003; Li et al., 2009). In humans, N-signaling abnormalities cause diseases that include leukemia, other cancers, and pulmonary arterial hypertension (Ellisen et al., 1991; Kopan, 2000). NICD then translocates to the nucleus and regulates the transcription of downstream genes (Struhl et al., 1993; Lecourtois and Schweisguth, 1995). N requires various post-translational modifications to its extracellular domain to be activated. For example, O-glycosylation of the N extracellular domain by O-fucosyltransferase 1 (O-fut1) and Fringe regulates the binding between N and its ligands (Bruckner et al., 2000). O-fut1 is also known to act as an N-specific chaperone in Drosophila (Okajima et al., 2005). In addition, analysis of a Drosophila thiol oxidase, endoplasmic reticulum (ER) oxidoreductin 1-like (Ero1L), showed that disulfide-bond formation in the extracellular domain of N is indispensable for the activation of the N signal (Tien et al., 2008).

Many roles played by N signaling in Drosophila development are crucial and have been studied extensively. Its best-known role during the early development of the central nervous system, is to prevent cells that neighbor a neuroblast from choosing the neuroblast fate, a phenomenon called ‘lateral inhibition’ (Simpson, 1990). This is achieved when the neuroblast-fated cell activates N signaling in its neighbors; these cells become epidermoblasts. Thus, disruption of N signaling in Drosophila embryos results in the failure of lateral inhibition and the consequent hyperplasia of neuroblasts at the expense of epidermoblasts (Cau and Blader, 2009), which is referred to as the ‘neurogenic’ phenotype (Simpson, 1990). Because most of the genes that encode N-signaling components are essential for lateral inhibition, these genes were first identified by the neurogenic phenotype resulting from their disruption (Lehmann et al., 1983).

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Accepted 24 November 2011
pecanex (pcx) was originally identified as a mutant showing recessive female sterility (Perrimon et al., 1984). Thus, pcx homozygous or hemizygous embryos obtained from pcx heterozygous females survive until adulthood. However, embryos obtained from pcx homozygous females mated with pcx hemizygous males, which are fertile, show neuronal hyperplasia, i.e., the neurogenic phenotype, suggesting that the maternally supplied pcx function rescues this phenotype (LaBonne and Mahowald, 1985). Therefore, pcx is considered to be a maternal neurogenic gene. pcx encodes a multi-pass transmembrane protein consisting of 3433 amino acids that is highly conserved from Drosophila to humans (LaBonne et al., 1989). A rat homolog of pcx, pecanex, is expressed in spermatocytes and probably functions in the testes (Geisinger et al., 2005). However, no molecular function of the Pcx protein has been identified in any species. Here, we established that pcx is an N-signaling component in Drosophila. We also provide evidence that Pcx might be involved in ER functioning.

MATERIALS AND METHODS
Drosophila stocks
All experiments were performed at 25°C on standard Drosophila culture medium. Canton-S was used as wild type. The mutants used were: pcx1, a loss-of-function mutant (Mohler, 1977; Mohler and Carroll, 1984); Df(1)ED6574 and Df(1)ED409, deletions uncovering the psc locus (Yan et al., 2009); X(EF1), a null mutant (Kidd et al., 1983); and Presentin1 (Pst1), a null mutant (Lukinova et al., 1999). The Gal4 lines used were: wingless-Gal4 (Pfeiffer et al., 2000), armadillo-Gal4 (arm-Gal4) (Sanson et al., 1996),Aloxg-Gal4 (Taniguchi et al., 2011), matrix-Gal4 (Bosson et al., 2002) and MS1096 (Capdevila and Guerrero, 1994). The UAS lines used were: UAS-NICD (Go et al., 1998), UAS-pcxGFP (see below), UAS-Hsc70-3/4 (Elefant and Palter, 1999), UAS-Hsc70-3K97S and UAS-Hsc70-3K97S (Elefant and Palter, 1999), UAS-Xpl-RB (Ryoo et al., 2007), UAS-endoplasmic reticulum-Cyan fluorescent protein (UAS-ER-CFP), a GFP variant with an ER-retention signal (KDEL) (BD Biosciences), and UAS-O-fut1 (Sasamura et al., 2003). hs–ΔECN expresses ΔECN under the control of a heat-shock promoter (Rebay et al., 1993). The heat-shock conditions were as described in Rebay et al. (Rebay et al., 1993). The Enhancer of split [E(spl)] m8-lacZ line carries a lacZ reporter controlled by the E(spl) m8 enhancer (Lecourtoux and Schweisguth, 1995). P{Crey}1b overexpresses cre recombinase (cre) in the female germ line (Siegal and Hartl, 1996). tubP-Gal80b overexpresses temperature-sensitive Gal80 (Hewes et al., 2006).

Genetic crosses to obtain pcx homo/hemizygous embryos lacking its maternal contribution
The pcx homo/hemizygous embryos shown in Fig. 1B,F, Fig. 2B,D,F,H, Fig. 3C,E, Fig. 5B,D,G,H and Fig. 6A-H,K-M,O-O were obtained by the following genetic crosses. Fig. 1B: ppc1/ppc3 × ppc1/ppc3; Fig. 1F: ppc2/ppc2; arm-Gal4/arm-Gal4 × ppc3/Y; UAS-pcxGFP/UAS-pcxGFP; Fig. 2B: ppc1/ppc1; E(spl) m8-lacZ/E(spl) m8-lacZ × ppc3/Y; E(spl) m8-lacZ/E(spl) m8-lacZ; Fig. 2D: ppc2/ppc2 × ppc2/ppc2; Fig. 2F: ppc2/ppc2 × ppc2/ppc2; Fig. 2H: ppc3/ppc3 × ppc3/ppc3; Fig. 2I: ppc4, cre/ppc4, cre; UAS-pcxGFP/UAS-pcxGFP × ppc3/Y; Aloxg-Aloxg GAL4; Fig. 3C: ppc2/ppc2; hs–ΔECN/hs–ΔECN × ppc3/Y; hs–ΔECN/hs–ΔECN; Fig. 3E: ppc3/ppc3; mata-Gal4/mata-Gal4 × ppc3/Y; UAS-NICD/UAS-NICD; Fig. 3F,G: ppc2/ppc2; hs–ΔECN/hs–ΔECN × ppc3/Y; hs–ΔECN/hs–ΔECN; Fig. 3H-J: ppc3/ppc3; arm-Gal4/arm-Gal4 × ppc3/Y; UAS-NICD/UAS-NICD; Fig. 5B,D: ppc2/ppc2 × ppc2/ppc2; Fig. 5G: ppc2/ppc2; arm-Gal4/arm-Gal4 × ppc3/Y; UAS-pcxGFP/UAS-pcxGFP; Fig. 5H: ppc3/ppc3; arm-Gal4/arm-Gal4 × ppc3/Y; UAS-NICD/UAS-NICD; Fig. 6A-D: ppc1/ppc1; arm-Gal4/arm-Gal4 × ppc3/Y; UAS-Xpl-RB/UAS-Xpl-RB; Fig. 6E: ppc3/ppc3; arm-Gal4/arm-Gal4 × ppc3/Y; UAS-Hsc70-3K97S; Fig. 6F-H: ppc2/ppc2; arm-Gal4/arm-Gal4 × ppc3/Y; UAS-Hsc70-3K97S; Fig. 6L-L′: ppc3/ppc3; arm-Gal4/arm-Gal4 × ppc3/Y; UAS-Xpl-RB/UAS-Xpl-RB; Fig. 6M: ppc1/ppc1; arm-Gal4/arm-Gal4 × ppc3/Y; UAS-Hsc70-3K97S/UAS-Hsc70-3K97S; Fig. 6O-O′: ppc3/ppc3; arm-Gal4/arm-Gal4 × ppc3/Y; UAS-O-fut1/UAS-O-fut1; ppc2 and other ppc3 derivatives carrying transgenes in other chromosomes were balanced with FMr, Bar (B). Thus, ppc2/ppc2 females and ppc3/Y males were identified based on the absence of the B phenotype.

The embryo shown in supplementary material Fig. S1A was obtained by the genetic cross ppc2/ppc2 × ppc3/Y. Female embryos were selected by immunostaining with an anti-Sex lethal antibody (Bopp et al., 1991).

Mosaic analysis in embryos
Mosaics were generated in embryos of ppc3 P{Crey}1b/ppc3; Aloxg-Gal4/UAS-pcxGFP or ppc3 P{Crey}1b/Y; Aloxg-Gal4/UAS-pcxGFP (Tanguchi et al., 2011; Siegal and Hartl, 1996). To obtain these embryos, we crossed ppc3 P{Crey}1b/ppc3 P{Crey}1b/Y; UAS-pcxGFP females with ppc3/Y; Aloxg-Gal4 males.

Construction of UAS-pcxGFP
To obtain full-length pcx cDNA, we performed rapid amplification of cDNA ends (RACE) with an already-known partial pcx cDNA sequence (SD01552). EGF F DNA (Clontech) was combined in-frame with the 5′ end of the full-length pcx cDNA using PCR. The resulting fragment was inserted into the NotI site of the pUAST vector (Brand and Perrimon, 1993). pUAS-pcxGFP was introduced into the Drosophila genome using P-element-mediated transformation (Brand and Perrimon, 1993).

Overexpression of UAS-pcxGFP
To produce MS1096/UAS-pcxGFP, Gal80ts, we crossed MS1096 females with UAS-pcxGFP, Gal80ts males at 25°C. The second instar larvae of MS1096/UAS-pcxGFP, Gal80ts were displaced to 30°C and raised to the third instar stage. These third instar larvae were dissected and immunostained.

Immunostaining
The antibody staining of embryos (Rhyu et al., 1994), wing imaginal discs (Matsumo et al., 2002) and S2 cells (Trammell et al., 2008) was performed as previously described. Confocal microscopy images were collected on an LSM 510 META (Zeiss) and analyzed on an LSM Image Browser. The following primary antibodies were used: rat anti-Elav (7E8A10, 1:20) (O’Neill et al., 1994), mouse anti-Elav (9F8A9, 1:20) (O’Neill et al., 1994), rat anti-GFP (GF090R, 1:200; Nacalai Tesque), mouse anti-Sex- lethargic (M18, 1:20) (Bopp et al., 1991), mouse anti-b-galactosidase (Z378B, 1:100; Promega), mouse anti-Engrailed (1:25) (Braud et al., 2010), rabbit anti-active MAPK (1:200, Promega), mouse anti-RFP (1:500, MBL), mouse anti-Protein disulide isomerase (Pdi) (1:100, Stressgen) (Vaux et al., 1990), rabbit anti-β-galactosidase (β-gal) (1:500) (Tanaka and Nakamura, 2008), rabbit anti-β-galactosidase (β-gal) (1:500) (Tanaka and Nakamura, 2008), mouse anti-Notch intracellular domain (C17: 9C6, 1:200) (Fehon et al., 1990) and rat anti-Drosophila E-Cadherin (DE-Cad) (DCAD2, 1:10) (Oda et al., 1994). The fluorescent secondary antibodies, Cy3-conjugated goat anti-mouse (Jackson ImmunoResearch), Alexa488-conjugated goat anti-rat (Molecular Probes), Cy3-conjugated goat anti-rabbit (Jackson ImmunoResearch) and Cy3-conjugated donkey anti-guinea pig (Jackson ImmunoResearch) were used at 1:500.

Western blotting analysis of PcxGFP
Each protein sample was prepared from five embryos of wild-type or UAS-pcxGFP arm-Gal4, incubated at 68°C for 10 minutes, and subjected to 5% SDS-PAGE and western blotting as described previously (Crevel et al., 2001). To detect PcxGFP and β-tubulin, anti-GFP (1:1000) and anti-β-tubulin antibodies (E7, 1:2000) (Wong et al., 2010) were used, respectively.

In situ hybridization
The pcx and single-minded (sim) RNA probes were prepared, and the in situ hybridization of embryos was performed as described previously (Takashima and Murakami, 2001).
Electron microscopy
For electron microscopy, specimens were prepared as described previously (Tepass and Hartenstein, 1994). These specimens were observed by electron microscopy using standard techniques, as described previously (Suzuki and Hirosawa, 1994).

Detection of spliced Xbp1 mRNA by RT-PCR
Primers and total RNA of embryos were prepared as described previously (Haecker et al., 2008).

RESULTS
pcx is a maternal neurogenic gene
Previous studies proposed that pcd encodes an N-signaling component, based on its mutant phenotypes (Perrimon et al., 1984; LaBonne et al., 1989). However, although pcd homo/hemizygotes lacking maternal pcd show the neurogenic phenotype, the contribution of pcd to N signaling has not been examined directly (Perrimon et al., 1984). A similar neurogenic phenotype is observed in embryos homozygous for N, as described previously (LaBonne et al., 1989). Df(1)ED6574 and Df(1)ED409 are deletions lacking the pcd locus (LaBonne et al., 1989). Df(1)ED6574 and Df(1)ED409 are deletions lacking the pcd locus (Yan et al., 2009). We also found that Df(1)ED6574/pcdx or Df(1)ED409/pcdx females mated with pcd/Y males produced embryos with the neurogenic phenotype in all cases examined (n=57) (supplementary material Fig. S1A) (LaBonne et al., 1989). In the rest of this paper, these embryos are referred to as, 'pcdx m8 embryos'. As reported previously, the maternal neurogenic phenotype of pcd was paternally rescued in 80% of the embryos (n=57) (supplementary material Fig. S1A) (LaBonne et al., 1989). Df(1)ED6574 and Df(1)ED409 are deletions lacking the pcd locus (Yan et al., 2009). We also found that Df(1)ED6574/pcdx or Df(1)ED409/pcdx females mated with pcd/Y males produced embryos with the neurogenic phenotype in all cases examined (n=22, supplementary material Fig. S1B; n=24, supplementary material Fig. S1C). The extent of the neurogenic phenotype in these embryos was equivalent to that of pcd m8 embryos, suggesting that pcd x is a null allele.

Next, we determined the molecular lesion of the pcd x mutant by sequencing its pcd locus. A nonsense mutation was found in the genomic DNA sequence of the pcd locus corresponding to the 2030th amino acid of the Pcd protein, which resulted in the production of a truncated Pcd protein (Fig. 1D). This mutant protein lacks the C-terminal half, which contains an evolutionarily conserved Pecanex C domain (Fig. 1D) (Gilbert et al., 1992). To confirm that the disruption of pcd functions is fully responsible for the neurogenic phenotype, we examined whether pcd overexpression in pcd m8 embryos could rescue this phenotype. We overexpressed GFP-tagged Pcd protein (PcdGFP), and detected this protein in the extracts of UAS-pcdGFP/arm-Gal4, but not wild-type embryos, by western blotting (Fig. 1E). The neuronal hyperplasia in pcd m8 embryos was effectively suppressed by the overexpression of pcdGFP, at 73% frequency (n=26) (Fig. 1F). This result demonstrated that the maternal neurogenic phenotype of pcd x was caused by the mutation of the pcd gene. These results also showed that pcdGFP retains pcd’s wild-type function.

We then examined the expression pattern of pattern of pcd in embryos and imaginal discs by in situ hybridization. pcd expression was strong in the early embryos, from stage 1 to 4, and then diminished from stage 5 (Fig. 1G-J). These results are consistent with the previous finding that pcd is a maternal neurogenic gene (Perrimon et al., 1984). By contrast, no pcd expression was detected in imaginal discs (data not shown). This might explain why adult pcd homozygotes do not show obvious defects besides female sterility (Perrimon et al., 1984), although other explanations are also possible.

pcd encodes an essential component of N signaling
The maternal neurogenic phenotype associated with the pcd mutant supported the idea that pcd encodes an essential component of N signaling. However, neuronal hyperplasia can also be induced by the mutation of genes that do not contribute to N signaling directly, such as shaggy (Simpson et al., 1988). Therefore, to confirm the involvement of pcd in N signaling, we examined the expression of two N-signaling target genes, m8 and single-minded (sim), in pcd m8 embryos.

m8 is a member of the Enhancer of split [E(spl)] complex and encodes a basic helix-loop-helix protein (Deshikkar and Artavanis-Tsakonas, 1992; Knust et al., 1992). The transcription of m8 is a direct target of N signaling (Bailey and Posakony, 1995). We detected m8 expression using E(spl)m8-lacZ, which carries a lacZ reporter controlled by the E(spl)m8 enhancer (Lecourtois and...
Schweisguth, 1995). As shown in Fig. 2A, m8 expression was detected in the central and peripheral nervous systems of wild-type embryos. By contrast, the m8 expression was drastically reduced in pcx<sup>m/z</sup> embryos, in all cases examined (Fig. 2B).

sim is expressed in mesectoderm cells and is required for the specification of the midline cells that arise from them (Crews et al., 1988). Furthermore, sim expression depends on the activation of N signaling in these cells (Hong et al., 2008). In wild-type cells, sim expression was detected by in situ hybridization in a single row of mesectoderm cells in the lateral half of each embryo, as reported previously (Fig. 2C) (Morel and Schweisguth, 2000). We found that the row of cells expressing sim was severely interrupted in pcx<sup>m/z</sup> embryos, in all cases examined (n=22), indicating that sim expression was reduced in these embryos (Fig. 2D). These results indicate that pcx has an essential role in N signaling.

We also investigated whether other signaling pathways were affected by the absence of pcx function. To examine the activity of Wnt signaling, we detected the expression of en<sup>graled</sup> (en), a target gene of Wnt signaling, by anti-En antibody staining (White et al., 1998). The En expression in pcx<sup>m/z</sup> embryos was not significantly different from that observed in wild type (Fig. 2E,F). We also examined the activity of receptor tyrosine kinase signaling pathways, which can be detected by anti-phosphorylated MAPK antibody staining (Peri et al., 1999). The intensity of the anti-phosphorylated MAPK antibody staining was almost the same in the wild-type embryos and the pcx<sup>m/z</sup> embryos (Fig. 2G,H). The results above indicate that the function of pcx might be specifically required for the activation of N signaling.

**pcx is required in the signal-receiving cells**

To understand the role of Pcx in N signaling, we examined whether N activation depended on Pcx activity in the signal-receiving or the signal-sending cells, by determining whether the pcx function was cell-autonomous. We recently developed a modified Cre/loxP system to efficiently induce somatic mosaic clones in Drosophila embryos (Taniguchi et al., 2011). The Aloxx-Gal4 line drives Gal4 expression as a consequence of Cre-mediated cis-recombination between its two loxP sites, leading to the overexpression of UAS-pcxGFP in clonal cells in pcx<sup>m/z</sup> embryos. The cells comprising the mosaic clones expressing pcxGFP did not assume a neuronal fate, whereas the pcx<sup>m/z</sup> embryonic cells surrounding them cells became neurons (100%, n=11) (Fig. 2I-L). This result suggests that the pcxGFP-expressing cells, in which the level of N signaling was higher than in the surrounding pcx<sup>m/z</sup> cells, preferentially differentiated into epithelial cells. We also noted that the clones overexpressing pcxGFP frequently formed circular clusters, which indicated a global change in tissue architecture, probably because the cell-adhesion property was different between the neurons and epithelial cells. Thus, although these results need to be interpreted cautiously, this potential cell-autonomous behavior of the pcx gene might support the hypothesis that pcx is required in the signal-receiving cells.

**pcx functions upstream of the activated forms of N**

To elucidate how Pcx contributes to N signaling, we examined whether various forms of N, including the membrane-tethered form of activated N (ΔECN) and the nuclear form of activated N (NICD) (Fig. 3A), could activate N signaling in embryos lacking pcx function. In wild-type embryos, ubiquitous expression of ΔECN (mato-Gal4/UAS-ΔECN) (82%, n=17) (Fig. 3B) or NICD (mato-Gal4/UAS-NICD) (91%, n=22) (Fig. 3D) resulted in the ectopic expression of sim in a few cells neighboring the row of mesectoderm cells that expressed sim endogenously, as reported previously (Morel and Schweisguth, 2000).

Although sim expression was severely reduced in pcx<sup>m/z</sup> embryos (Fig. 2D), its expression was increased by the overexpression of ΔECN (91%, n=21) (Fig. 3C) or NICD (94%, n=16) (Fig. 3E) in pcx<sup>m/z</sup> embryos. Thus, we speculated that NICD and ΔECN are epistatic to pcx. However, we also noted that NICD’s ability to induce ectopic sim expression was reduced in pcx<sup>m/z</sup> embryos, compared with wild type (Fig. 3D,E). Therefore, it is possible that Pcx plays some role(s) downstream of NICD. However, a similar reduction in NICD’s ability to induce ectopic sim expression was observed in N homozygotes (supplementary material Fig. S1D).
suggesting that this reduction might not be a specific effect in pcXm/z embryos. Based on these results, we speculated that Pcx functions upstream of \( \Delta ECN \) and NICD, although we could not exclude the possibility that Pcx also functions downstream of NICD.

To confirm these results, we also examined whether \( \Delta ECN \) and NICD could rescue the neurogenic phenotype associated with the absence of pcX function. As reported previously, in wild-type embryos, the overexpression of NICD (93%, \( n=14 \)) suppressed neuronal differentiation, which is called the ‘anti-neurogenic phenotype’ (supplementary material Fig. S1E) (Lieber et al., 1993). Overexpression of \( \Delta ECN \) (90%, \( n=20 \)) (Fig. 3F,G) or NICD (92%, \( n=20 \)) (Fig. 3H-J) suppressed the neurogenic phenotype of pcXm/z embryos. This result was compatible with the idea presented above that Pcx functions upstream of \( \Delta ECN \) and NICD.

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Pcx localizes mainly to the ER
To gain insight into the biochemical roles of Pcx, we examined its subcellular localization. Because it has been difficult to obtain a specific antibody against Pcx, we decided to study the subcellular localization of PcxGFP, which rescued the maternal neurogenic phenotype of pcx3, as described above (Fig. 1E,F).

We expressed pcxGFP driven by arm-Gal4 in the neuroectoderm of embryos at stage 14, because pcx3 embryos exhibit their phenotype in this tissue and at this stage. Under these conditions, PcxGFP mostly colocalized with the Pdi-positive ER (Fig. 4A). By contrast, we did not detect the colocalization of PcxGFP with markers of the Golgi (GM130), ER-Golgi intermediate compartment termed ERGIC (COPII), or endocytic compartments, including the early endosomes (Hrs), late endosomes (Rab7) and recycling endosomes (Rab11) (Fig. 4B-F).

To reduce the possibility that the overexpression of pcxGFP led to the mislocalization of its product, we drove the pcxGFP expression weakly, using the Gal4-UAS system in combination with a temperature-sensitive Gal 80, Gal80ts, in the wing imaginal discs (supplementary material Fig. S3A-B). To confirm the surplus of ER in the cells of pcx3 embryos, we also observed these embryos by electron microscopy. Consistent with the results above, an overabundance of ER was observed in the electron microscopy images (Fig. 5C,D). By contrast, the structures of the ER-Golgi intermediate compartment (ERGIC), the Golgi, and the endocytic compartments, including the early endosomes (Rab8), late endosomes (Rab7) and recycling endosomes (Rab11) were normal in these embryos (supplementary material Fig. S3C-L).

The ER is enlarged in embryos lacking pcx function
The specific localization of Pcx-GFP to the ER suggested that its function might be ER-related. Protein disulfide isomerase (Pdi) is located specifically in the ER (Roth and Pierce, 1987; Koivu and Myllyla, 1987). To detect possible defects in the ER, wild-type embryos and pcx3 embryos were stained with an anti-Pdi antibody. The ER appeared to be normal in pcx3 embryos at stage 5 (Fig. 5A-B, top). However, an abnormality of the ER was observable in these embryos at stage 9, when neuroblast segregation is just starting (Fig. 5A-B, middle) (Hartenstein and Campos-Ortega, 1984). Enlarged ER was observed predominantly in the region corresponding to the dorsal epidermis of the wild-type embryos at stage 14 (Fig. 5A-B, bottom) (Bokor and DiNardo, 1996). These observations suggest that the sensitivity to the absence of pcx function might vary among cells. A similar enlargement was detected in pcx3 embryos expressing KDEL-CFP, which encodes CFP with an ER retention signal (supplementary material Fig. S3A-B’). To confirm the surplus of ER in the cells of pcx3 embryos, we also observed these embryos by electron microscopy. Consistent with the results above, an overabundance of ER was observed in the electron microscopy images (Fig. 5C,D). By contrast, the structures of the ER-Golgi intermediate compartment (ERGIC), the Golgi, and the endocytic compartments, including the early endosomes (Rab8), late endosomes (Rab7) and recycling endosomes (Rab11) were normal in these embryos (supplementary material Fig. S3C-L’).

The abnormal ER phenotype was not observed in embryos homozygous for N or in embryos lacking zygotic and maternal Psn. (F) Embryo homozygous for N. (G) Embryo lacking zygotic and maternal pcn and overexpressing psn-GFP (pcx; arm-Gal4/UAS-PCNF). (H) Embryo lacking zygotic and maternal pcn and overexpressing NICD (pcx; arm-Gal4/UAS-NICD). A’, B’, E’, F’, G’ and H’ are higher magnifications of the regions indicated by dashed squares in A, B, E, F, G and H, respectively. Scale bars: 50 μm in A,B,E-H; 10 μm in A’,B’,E’-H’; 2 μm in C’,D’.
NICD also rescued the ER enlargement in these embryos (Fig. 5H, H'), even though a disruption of N signaling did not cause the ER enlargement and pce is required upstream of NICD. Therefore, we speculate that the ectopic activation of N signaling by the overexpressed NICD also influences the structure of the ER.

Induction of the UPR restores the N signal in the absence of pce function

Based on the fact that Pcx was mostly detected in the ER and that defects in the ER structure were found in pce

When unfolded or misfolded proteins appear in the ER, the organelle suffers a type of stress (ER stress) that can induce apoptosis (Lee, 1987; Gething and Sambrook, 1992; Li et al., 2006). Cells suffering from ER stress can reduce the stress level through a response known as the unfolded protein response (UPR) (Kaufman, 1999). In stressed cells, Ire-1, which is an ER-tethered endonuclease that acts as a sensor of ER stress, splices the Xbp1 mRNA, which encodes a basic helix-loop-helix (bHLH) transcription factor; this factor then activates the transcription of genes that promote protein folding (Lee et al., 2003; Yoshida et al., 2003). Therefore, the ectopic production of spliced Xbp1 mRNA results in the transcriptional induction of these genes (Back et al., 2006). We produced Xbp1 mRNA in pce embryos and observed that the neurogenic phenotype was effectively suppressed (Fig. 6B, C, I). In 31% (n=21) of these embryos, the metameric structures of the central nervous system were restored (Fig. 6C, I).

Heat-shock cognate 70-3 (Hsc70-3) encodes Drosophila binding protein (Bip), a major chaperone that recognizes misfolded proteins in the lumen of the ER (Rubin et al., 1993). We found that the overexpression of wild-type Hsc70-3, Hsc70-3(wt), weakly rescued the neurogenic phenotype of pce embryos (67%, n=12) (Fig. 6E, I). A dominant-negative form of Hsc70-3, Hsc70-3K97S, induces the UPR through the activation of Xbp1 (Elefant and Palter, 1999). Overexpression of Hsc70-3K97S suppressed the neurogenic phenotype of pce embryos as efficiently as Xbp1-RR (Fig. 6F-I). In 29% of these embryos (n=21), an almost normal
central nervous system was observed (Fig. 6H,I). To confirm that the rescue of the neurogenic phenotype by the upregulation of the UPR was due to the recovery of N signaling, we examined the expression of \textit{sim} (Hong et al., 2008). The expression of \textit{sim} in mesectoderm cells was restored by the overexpression of \textit{Hsc70-3K97S} in \textit{pcx}\textsuperscript{m/z} embryos, at 76% frequency (\(n=21\)) (Fig. 6J,K). These results suggested that the attenuation of N signaling associated with the absence of \textit{pcx} function could be restored by the upregulation of some ER functions that are under control of the UPR.

Next, we examined whether the enlargement of the ER in \textit{pcx}\textsuperscript{m/z} embryos was rescued by inducing the UPR. The ER enlargement was efficiently suppressed by the overexpression of \textit{Xbp1-RB} (74%, \(n=21\)) or \textit{Hsc70-3K97S} (67%, \(n=21\)) in these embryos (Fig. 6L-M'). Therefore, the condition causing the enlargement of the ER in the absence of \textit{pcx} function, which could be responsible for the disruption of N signaling, is restored by the upregulation of the UPR.

To understand the possible roles of \textit{pcx} in the UPR, we detected the spliced form of endogenous \textit{Xbp1} (\textit{Xbp1-RB}) mRNA, which reflects the activation of the UPR in vivo, by RT-PCR (Haecker et al., 2008). As reported previously (Elefant and Palter, 1999), overexpression of \textit{UAS-Hsc70-3K97S}, which encodes a dominant-negative form of Hsc70 driven by \textit{arm-Gal4}, induced the production of \textit{Xbp1-RB} mRNA (Fig. 6N, lane 4). In \textit{pcx}\textsuperscript{m/z} embryos, the overexpression of \textit{UAS-Hsc70-3K97S} also induced \textit{Xbp1-RB} mRNA as efficiently as in the wild-type background (Fig. 6N, lane 5). These results suggest that \textit{pcx} is not essential for induction of the UPR. We also found that overexpressing \textit{pcx} did not induce an ectopic UPR in wild-type embryos (Fig. 6N, lane 3). However, we detected a weak but reproducible induction of \textit{Xbp1-RB} mRNA production in \textit{pcx}\textsuperscript{m/z} embryos (Fig. 6N, lane 2). Thus, an ectopic UPR might be induced in \textit{pcx}\textsuperscript{m/z} embryos, although ectopic apoptosis was not detected in these embryos (data not shown).

The UPR increases chaperone activities in the ER (Lee et al., 2003). Thus, it is possible that upregulation of the UPR could restore the defective folding of N in \textit{pcx}\textsuperscript{m/z} embryos. To test this possibility, we overexpressed \textit{O-fut1}, which has an N-specific chaperone activity, in \textit{pcx}\textsuperscript{m/z} embryos. We found that the neurogenic phenotype and the ER enlargement were suppressed in 31% (\(n=29\)) of the \textit{pcx}\textsuperscript{m/z} embryos overexpressing \textit{O-fut1} (Fig. 6O-O'), although the suppression was less efficient than that seen with the ectopic induction of the UPR. Although further analysis is required, this result is consistent with the idea that the disruption of N’s folding might account, in part, for the attenuation of N signaling in \textit{pcx}\textsuperscript{m/z} embryos. It is conceivable that the transportation of N, and possibly DI, was disrupted in \textit{pcx}\textsuperscript{m/z} embryos. However, N and DI were properly localized to the apical region of the epithelial cells where \textit{Drosophila} E-cadherin was accumulated at stage 5, when the expression of \textit{sim} had already started (supplementary material Fig. S4). These results suggest that the transportation of N was not severely disrupted in \textit{pcx}\textsuperscript{m/z} embryos, even if the folding of N was disrupted.

**DISCUSSION**

**Pcx is a component of N signaling**

The Pcx family proteins are evolutionarily conserved, large transmembrane proteins with multi-pass transmembrane domains (LaBonne et al., 1989). However, no motifs that might suggest Pcx’s biochemical function have been found in its amino acid sequence. Although \textit{pcx} was previously suggested to be involved in N signaling, based on the neurogenic phenotype associated with its mutant in \textit{Drosophila}, this possibility had not been explored. In this study, we provide evidence that Pcx is a component of the N-signaling pathway.

**Pcx might play a role in controlling the ER architecture**

In \textit{pcx}\textsuperscript{m/z} embryos, the ER was abnormally enlarged. Various factors regulating the architecture of the ER have been identified. In \textit{Drosophila}, Atlastin, a dynamin-like GTPase, is required for fusion of the ER membrane (Orso et al., 2009). Thus, the overexpression of Atlastin induces an enlarged ER (Orso et al., 2009). In addition, the peripheral ER shows two distinct structures: tubules and sheets (Puhka et al., 2007). Several factors organizing the shape of the ER membrane into tubules or sheets have been identified (English et al., 2009). Therefore, Pcx might contribute to the regulatory machinery that accomplishes the normal organization of the ER.

In \textit{pcx}\textsuperscript{m/z} embryos, the enlarged ER was observed predominantly in the region corresponding to the dorsal epidermis of wild-type embryos (Bokor and DiNardo, 1996). Therefore, sensitivity to the absence of \textit{pcx} function might differ among groups of cells. This distinct behavior could reflect differences in the cell-cycle phase or level of UPR activity.

Although our results showed that the reduction of N signaling was not responsible for the enlargement of the ER in \textit{pcx}\textsuperscript{m/z} embryos, the ectopic activation of N signaling by overexpression of \textit{NICD} also suppressed this ER defect. We speculate that the ectopic activation of N signaling might affect the progression of the cell-cycle or the level of UPR, which could in turn affect the regulation of the ER architecture. It has been shown that N signaling directly or indirectly affects the cell cycle (Johnston and Edgar, 1998; Simon et al., 2009). However, the biological significance and mechanisms of this phenomenon remain elusive.

**Possible role of Pcx in the activation of N signaling**

We found that induction of the UPR suppressed the ER enlargement in \textit{pcx}\textsuperscript{m/z} embryos. The suppression of the ER enlargement by the expression of genes that induce the UPR coincided with the rescue of N signaling activity in these embryos. Therefore, the reduced N signaling in \textit{pcx}\textsuperscript{m/z} embryos might be attributable to the enlargement of the ER. However, we cannot exclude the possibility that \textit{pcx} is independently involved in the activation of N signaling and the regulation of the ER architecture. Nevertheless, our results suggest that some downstream events induced by the UPR compensate for the defect of N signaling associated with the absence of \textit{pcx} function. We found that overexpression of \textit{O-fut1}, an N-specific chaperone, partially compensated for the loss of \textit{pcx} function (Okajima et al., 2005). Thus, a disruption of N signaling in the absence of \textit{pcx} function might be partly due to the mis-folding of N, which is consistent with our hypothesis that \textit{pcx} acts upstream of the activated forms of N and probably functions in signal-receiving cells.

The UPR induces various downstream events, including the attenuation of protein synthesis, the enhancement of misfolded ER protein degradation, and the induction of genes encoding various chaperones (Kaufman, 1999). Therefore, in future experiments, it will be important to determine the specific defects that are compensated for by the UPR in the absence of \textit{pcx} function.


