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# 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., 1999; Kopan and Ilagan, 2009; Cau and Blader, 2009). In humans, N-signaling abnormalities cause diseases that include leukemia, other cancers, and pulmonary arterial hypertension (Ellisen et al., 1991; Nicolas et al., 2003; Li et al., 2009). *Drosophila N* encodes a transmembrane receptor with 36 epidermal growth factor (EGF)-like repeats in its extracellular domain (Wharton et al., 1985). During maturation of N, its extracellular domain is cleaved by Furin protease (S1 cleavage) in the Golgi (Logeat et al., 1998; Kidd and Lieber, 2002; Lake et al., 2009). After reaching the cell surface, the binding of N to its transmembrane ligand, Delta or Serrate, leads to a second cleavage in the extracellular domain of N by Kuzbanian (Kuz)/ADAM10 or ADAM17 (S2 cleavage). This cleavage removes most of the N extracellular domain and produces a membrane-tethered form of the N intracellular domain (NEXT) (Kopan and Goate, 2000). Subsequently, NEXT is cleaved within its transmembrane domain by γ-secretase (S3 cleavage), which liberates the intracellular domain, termed NICD (Mumm and 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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*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*, *pecanex1*, 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: $pcx^3$, a loss-of-function mutant (Mohler, 1977; Mohler and Carroll, 1984); Df(1)ED6574 and Df(1)ED409, deletions uncovering the *pcx* locus (Yan et al., 2009); $N^{55E11}$, a null mutant (Kidd et al., 1983); and $Presenilin^{C1}$ ($Psn^{C1}$), a null mutant (Lukinova et al., 1999). The Gal4 lines used were: *wingless*-Gal4 (*wg*-Gal4) (Pfeiffer et al., 2000), *armadillo*-Gal4 (*arm*-Gal4) (Sanson et al., 1996), *Aloxg*-Gal4 (Taniguchi et al., 2011), matα-Gal4 (Bossing 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{wt}* (Elefant and Palter, 1999), UAS-*Hsc70-3K97S* (Elefant and Palter, 1999), UAS-*Xbp1-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 (Lecourtois and Schweisguth, 1995). *P{Crey}1b* overexpresses *cre recombinase* (*cre*) in the female germ line (Siegal and Hartl, 1996). *tubP-Gal80$^{ts}$* 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,I, Fig. 3C,E,G-M, Fig. 5B,D,G,H and Fig. 6A-H,K-M′,O-O″ were obtained by the following genetic crosses. Fig. 1B: $pcx^3/pcx^3 \times pcx^3$/Y; Fig. 1F: $pcx^3/pcx^3$; *arm*-Gal4/*arm*-Gal4 × $pcx^3$/Y; UAS-*pcxGFP*/UAS-*pcxGFP*; Fig. 2B: $pcx^3/pcx^3$; *E*(*spl*) *m8-lacZ*/*E*(*spl*) *m8-lacZ* × $pcx^3$/Y; *E*(*spl*) *m8-lacZ*/*E*(*spl*) *m8-lacZ*; Fig. 2D: $pcx^3/pcx^3 \times pcx$/Y; Fig. 2F: $pcx^3/pcx^3 \times pcx^3$/Y; Fig. 2H: $pcx^3/pcx^3 \times pcx^3$/Y; Fig. 2I: $pcx^3$, *cre*/$pcx^3$, *cre*; UAS-*pcxGFP*/UAS-*pcxGFP* × $pcx^3$/Y; *Aloxg*-Gal4/*Aloxg*-Gal4; Fig. 3C: $pcx^3/pcx^3$; hs-*ΔECN*/hs-*ΔECN* × $pcx^3$/Y; hs-*ΔECN*/hs-*ΔECN*; Fig. 3E: $pcx^3/pcx^3$; *matα*-Gal4/*matα*-Gal4 × $pcx^3$/Y; UAS-*NICD*/UAS-*NICD*; Fig. 3F,G: $pcx^3/pcx^3$; hs-*ΔECN*/hs-*ΔECN* × $pcx^3$/Y; hs-*ΔECN*/hs-*ΔECN*; Fig. 3H-J: $pcx^3/pcx^3$; *arm*-Gal4/*arm*-Gal4 × $pcx^3$/Y; UAS-*NICD*/UAS-*NICD*; Fig. 5B,D: $pcx^3/pcx^3 \times pcx^3$/Y; Fig. 5G: $pcx^3/pcx^3$; *arm*-Gal4/*arm*-Gal4 × $pcx^3$/Y; UAS-*pcxGFP*/UAS-*pcxGFP*; Fig. 5H: $pcx^3/pcx^3$; *arm*-Gal4/*arm*-Gal4 × $pcx^3$/Y; UAS-*NICD*/UAS-*NICD*; Fig. 6A-C: $pcx^3/pcx^3$; *arm*-Gal4/*arm*-Gal4 × $pcx^3$/Y; UAS-*Xbp1-RB*/UAS-*Xbp1-RB*; Fig. 6D,E: $pcx^3/pcx^3$; *arm*-Gal4/*arm*-Gal4 × $pcx^3$/Y; UAS-*Hsc70-3{wt}*/UAS-*Hsc70-3{wt}*; Fig. 6F-H: $pcx^3/pcx^3$; *arm*-Gal4/*arm*-Gal4 × $pcx^3$/Y; UAS-*Hsc70-3K97S*/UAS-*Hsc70-3K97S*; Fig. 6K: $pcx^3/pcx^3$; *matα*-Gal4/*matα*-Gal4 x $pcx^3$/Y; UAS-*Hsc70-3K97S*/UAS-*Hsc70-3K97S*; Fig. 6L,L′: $pcx^3/pcx^3$; *arm*-Gal4/*arm*-Gal4 × $pcx^3$/Y; UAS-*Xbp1-RB*/UAS-*Xbp1-RB*; Fig. 6M,M′: $pcx^3/pcx^3$; *arm*-Gal4/*arm*-Gal4 × $pcx^3$/Y; UAS-*Hsc70-3K97S*/UAS-*Hsc70-3K97S*; Fig. 6O-O″: $pcx^3/pcx^3$; *arm*-Gal4/*arm*-Gal4 × $pcx^3$/Y; UAS-*O-fut1*/UAS-*O-fut1*. $pcx^3$ and other $pcx^3$ derivatives carrying transgenes in other chromosomes were balanced with *FM6*, *Bar* (*B*). Thus, $pcx^3/pcx^3$ females and $pcx^3$/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 $pcx^3/pcx^3$ × +/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 $pcx^3$ *P{Crey}1b*/$pcx^3$; *Aloxg*-Gal4/ UAS-*pcxGFP* or $pcx^3$ *P{Crey}1b*/Y; *Aloxg*-Gal4/ UAS-*pcxGFP* (Taniguchi et al., 2011; Siegal and Hartl, 1996). To obtain these embryos, we crossed $pcx^3$ *P{Crey}1b*/$pcx^3$ *P{Crey}1b*; UAS-*pcxGFP* females with $pcx^3$/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). *EGFP* cDNA (Clontech) was combined in-frame with the 5′ end of the full-length *pcx* cDNA using PCR. The resulting fragment was inserted into the *Not*I 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 (Matsuno 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-lethal (M18, 1:20) (Bopp et al., 1991), mouse anti-β-galactosidase (Z378B, 1:100; Promega), mouse anti-Engrailed (1:25) (Braid et al., 2010), rabbit anti-active MAPK (1:200, Promega), mouse anti-RFP (1:500, MBL), mouse anti-Protein disulfide isomerase (Pdi) (1:100, Stressgen) (Vaux et al., 1990), rabbit anti-GM130 (1:50, Abcam), rabbit anti-COPII (1:100, ABR), guinea pig anti-Hrs (1:400) (Lloyd et al., 2002), rabbit anti-Rab7 (1:5000) (Tanaka and Nakamura, 2008), rabbit anti-Rab11 (1:10,000) (Tanaka and Nakamura, 2008), mouse anti-Notch intracellular domain (C17. 9C6, 1:200) (Fehon et al., 1990) and rat anti-*Drosophila* E-Cadherin (*D*E-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 *pcx* encodes an N-signaling component, based on its mutant phenotypes (Perrimon et al., 1984; LaBonne et al., 1989). However, although *pcx* homo/hemizygotes lacking maternal *pcx* show the neurogenic phenotype, the contribution of *pcx* 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 (Fig. 1C) (Poulson, 1937; Perrimon et al., 1984). Consistent with Perrimon's report, we confirmed that embryos homo/hemizygous for $pcx^3$ and lacking a maternal contribution of *pcx* showed the neurogenic phenotype in all cases examined ($n$>50) (Fig. 1B) (Perrimon et al., 1984). In the rest of this paper, these embryos are referred to as, '$pcx^{m/z}$ embryos'. As reported previously, the maternal neurogenic phenotype of *pcx* 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 *pcx* locus (Yan et al., 2009). We also found that Df(1)ED6574/$pcx^3$ or Df(1)ED409/$pcx^3$ females mated with $pcx^3$/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 $pcx^{m/z}$ embryos, suggesting that $pcx^3$ is a null allele.

Next, we determined the molecular lesion of the $pcx^3$ mutant by sequencing its *pcx* locus. A nonsense mutation was found in the genomic DNA sequence of the *pcx* locus corresponding to the 2030th amino acid of the Pcx protein, which resulted in the production of a truncated Pcx 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 *pcx* functions is fully responsible for the neurogenic phenotype, we examined whether *pcx* overexpression in $pcx^{m/z}$ embryos could rescue this phenotype. We overexpressed GFP-tagged Pcx protein (PcxGFP), and detected this protein in the extracts of UAS-*pcxGFP*/*arm*-Gal4, but not wild-type embryos, by western blotting (Fig. 1E). The neuronal hyperplasia in $pcx^{m/z}$ embryos was effectively suppressed by the overexpression of *pcxGFP*, at 73% frequency ($n$=26) (Fig. 1F). This result demonstrated that the maternal neurogenic phenotype of $pcx^3$ was caused by the mutation of the *pcx* gene. These results also showed that *pcxGFP* retains *pcx*'s wild-type function.

We then examined the expression pattern of *pcx* in embryos and imaginal discs by in situ hybridization. *pcx* 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 *pcx* is a maternal neurogenic gene (Perrimon et al., 1984). By contrast, no *pcx* expression was detected in imaginal discs (data not shown). This might explain why adult *pcx* homozygotes do not show obvious defects besides female sterility (Perrimon et al., 1984), although other explanations are also possible.

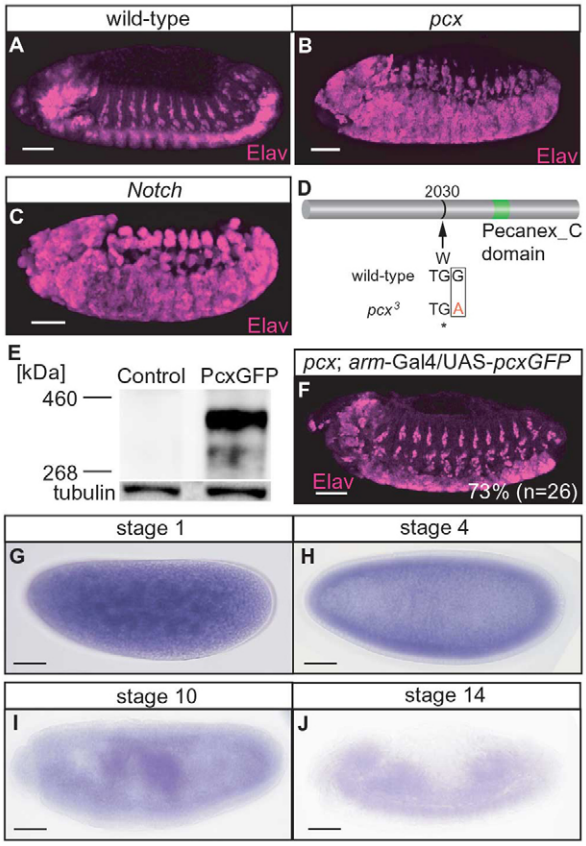


**Fig. 1. *pcx* is a neurogenic gene.** (**A-C**) Lateral views of a wild-type *Drosophila* embryo at stage 14 (A), a $pcx^{m/z}$ embryo (B) and an embryo homozygous for *N* (C) in which the neuronal cells were stained with an anti-Elav antibody (magenta). (**D**) Diagram of the mutant Pcx protein derived from the nonsense mutation found in $pcx^3$. This mutation changes tryptophan (2030th amino acid) to a stop codon. The conserved Pecanex C domain is shown in green. (**E**) Western blot analysis to detect the PcxGFP protein in embryonic extracts. Control, wild-type embryos; PcxGFP, UAS-*pcxGFP*/*arm*-Gal4 embryos. The positions of molecular weight markers (kDa) are shown on the left. Endogenous Tubulin was used as a loading control (bottom). (**F**) $pcx^{m/z}$ embryo, in which *pcxGFP* was ubiquitously overexpressed (*pcx*; *arm*-Gal4/UAS-*pcxGFP*). Neuronal cells were stained with the anti-Elav antibody (magenta). (**G-J**) *pcx* expression in wild-type embryos at stage 1 (G), 4 (H), 10 (I) and 14 (J) detected by in situ hybridization. Scale bars: 50 μm.

### *pcx* encodes an essential component of N signaling

The maternal neurogenic phenotype associated with the *pcx* mutant supported the idea that *pcx* 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 *pcx* in N signaling, we examined the expression of two N-signal target genes, *m8* and *single-minded* (*sim*), in $pcx^{m/z}$ embryos.

*m8* is a member of the *Enhancer of split* [*E*(*spl*)] complex and encodes a basic helix-loop-helix protein (Delidakis 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 $pcx^{m/z}$ 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 $pcx^{m/z}$ 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 *engrailed* (*en*), a target gene of Wnt signaling, by anti-En antibody staining (White et al., 1998). The En expression in $pcx^{m/z}$ 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 $pcx^{m/z}$ 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 *Aloxg*-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 $pcx^{m/z}$ embryos. The cells comprising the mosaic clones expressing *pcxGFP* did not assume a neuronal fate, whereas the $pcx^{m/z}$ 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 $pcx^{m/z}$ 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* (*matα*-Gal4/UAS-*ΔECN*) (82%, $n$=17) (Fig. 3B) or *NICD* (*matα*-Gal4/UAS-*NICD*) (91%, $n$=22) (Fig. 3D) resulted in the ectopic

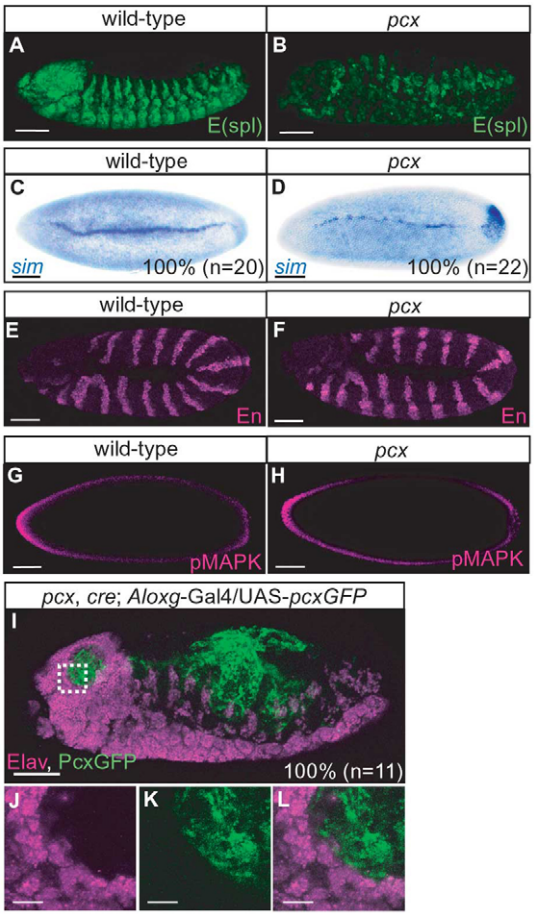


**Fig. 2. *pcx* cell-autonomously functions in Notch signaling.** (**A**,**B**) Expression of *E(spl) m8-lacZ* detected by anti-β-galactosidase antibody staining in an *E(spl) m8-lacZ Drosophila* embryo (otherwise wild type) at stage 13 (A) and an *E(spl) m8-lacZ* embryo lacking zygotic and maternal *pcx* (B). (**C**,**D**) *sim* expression detected by in situ hybridization at stage 6 in a wild-type embryo (C) and an embryo lacking zygotic and maternal *pcx* (D). (**E**,**F**) Lateral views of a wild-type embryo at stage 9-10 (E) and an embryo lacking zygotic and maternal *pcx* (F) stained with an anti-En antibody. (**G**,**H**) Lateral views of a wild-type embryo at stage 5 (G) and an embryo lacking zygotic and maternal *pcx* (H) stained with an anti-phosphorylated MAPK antibody. (**I-L**) $pcx^{m/z}$ embryos containing somatic mosaic cells overexpressing *pcxGFP* (green). Neuronal cells were stained with an anti-Elav antibody (magenta). J, K, and L (merged image of J and K) are high-magnification images of the region indicated by a dashed box in I. Scale bars: 50 μm in A-I; 5 μm in J-L.

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 $pcx^{m/z}$ 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 $pcx^{m/z}$ 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 $pcx^{m/z}$ 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),

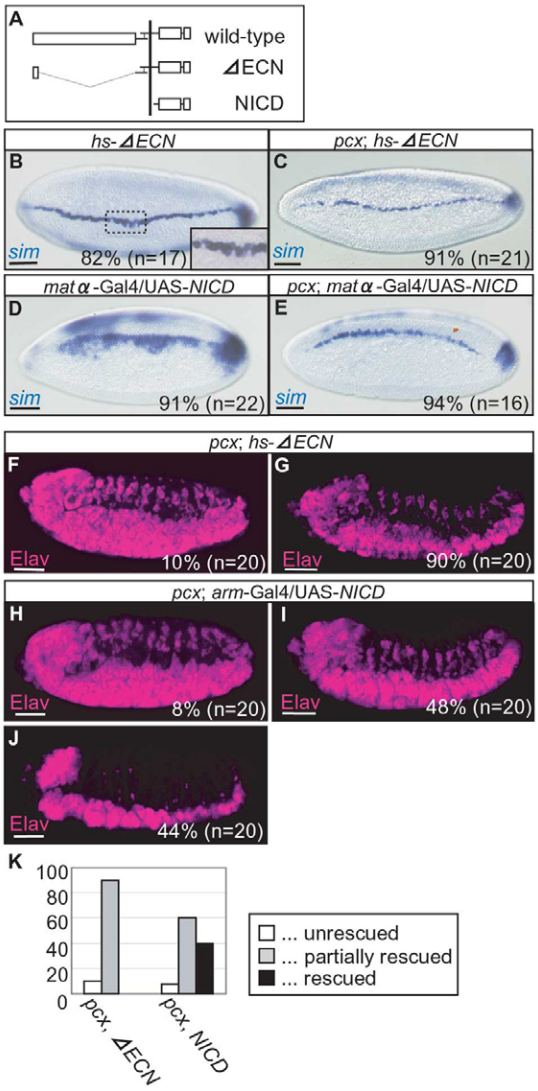


**Fig. 3. *pcx* functions upstream of activated N derivatives.** (**A**) Structures of the wild-type and activated forms of N, ΔECN and NICD. (**B-E**) Expression of *sim* detected by in situ hybridization at stage 6. Inset in B is a higher-magnification image of the area indicated by the dashed box. (B) Wild-type *Drosophila* embryos overexpressing *ΔECN* (*hs-ΔECN*/UAS-*ΔECN*). (C) $pcx^{m/z}$ embryos overexpressing *ΔECN* (*pcx; hs-ΔECN/UAS-ΔECN*). (D) Wild-type embryos overexpressing *NICD* (*matα*-Gal4/UAS-*NICD*). (E) Embryo lacking zygotic and maternal *pcx*, overexpressing *NICD* (*pcx*; *matα*-Gal4/UAS-*NICD*). The numbers at the bottom are the percentage of embryos showing the presented phenotype for *sim* expression. The number of embryos (*n*) examined in each experiment is indicted in parentheses. Genotypes of embryos are indicated at the top. (**F-J**) Lateral views of $pcx^{m/z}$ embryos, in which the neuronal cells were stained with an anti-Elav antibody (magenta). (F,G) Embryos overexpressing *ΔECN* (*pcx; hs-ΔECN*). The neurogenic phenotype was not rescued in 10% of the embryos (F) but was partially rescued in 90% of them (G). (H-J) Embryos overexpressing *NICD* (*pcx*; *arm*Gal4/UAS-*NICD*). The neurogenic phenotype was not rescued in 8% of the embryos (H), was partially rescued in 48% (I) and was rescued in 44% (J). The anti-neurogenic activity of *NICD* led to a smaller number of peripheral neurons in these embryos. The number of embryos (*n*) examined in each experiment is indicted in parentheses. Genotypes of embryos are indicated at the top. (**K**) The percentage of embryos showing an unrescued (white bar), partially rescued (gray bar) or rescued (black bar) neurogenic phenotype associated with the absence of *pcx* function, by the overexpression of *ΔECN* (*pcx*, *ΔECN*) or *NICD* (*pcx*, *NICD*). Scale bars: 50 μm.

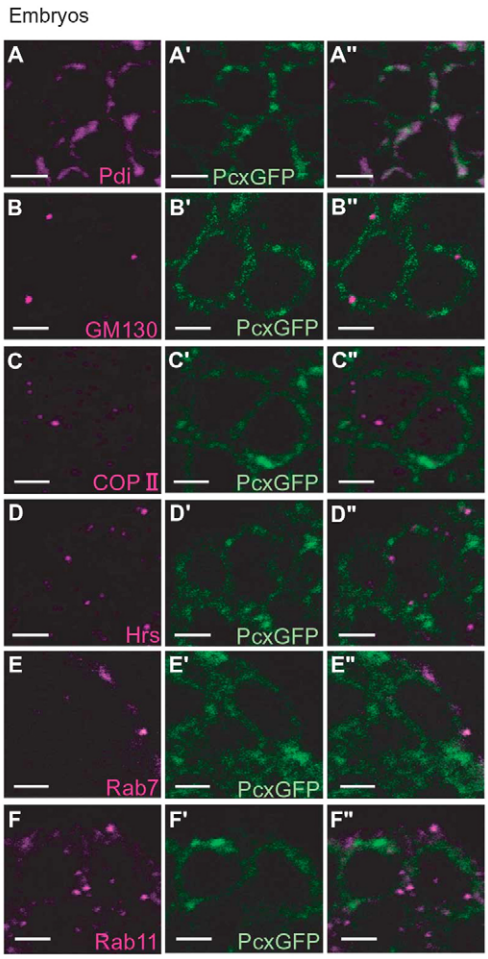


**Fig. 4. Pcx is an ER-resident protein in vivo.** (**A-F″**) Subcellular localization of PcxGFP (green) detected by anti-GFP antibody staining in the neuroectoderm of *Drosophila* embryos at stage 14 (middle and right panels). Various intracellular compartments (magenta), including the ER (Pdi in A), Golgi (GM130 in B), ERGIC (COPII in C), early endosomes (Hrs in D), late endosomes (Rab7 in E) and recycling endosomes (Rab11 in F) were also detected (left and right panels). The right panels (A″-F″) are merged images of the left (A-F) and middle (A′-F′) panels. Scale bars: 2 μm.

suggesting that this reduction might not be a specific effect in $pcx^{m/z}$ embryos. Based on these results, we speculated that Pcx functions upstream of ΔECN and NICD, although we could not exclude the possibility that Pcx also functions downstream of NICD.

To confirm these results, we also examined whether *ΔECN* 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 *ΔECN* (90%, *n*=20) (Fig. 3F,G) or *NICD* (92%, *n*=20) (Fig. 3H-J) suppressed the neurogenic phenotype of $pcx^{m/z}$ embryos. This result was compatible with the idea presented above that Pcx functions upstream of ΔECN and NICD. However, these analyses did not clarify whether N, Dl or both were affected in the $pcx^{m/z}$ embryos.

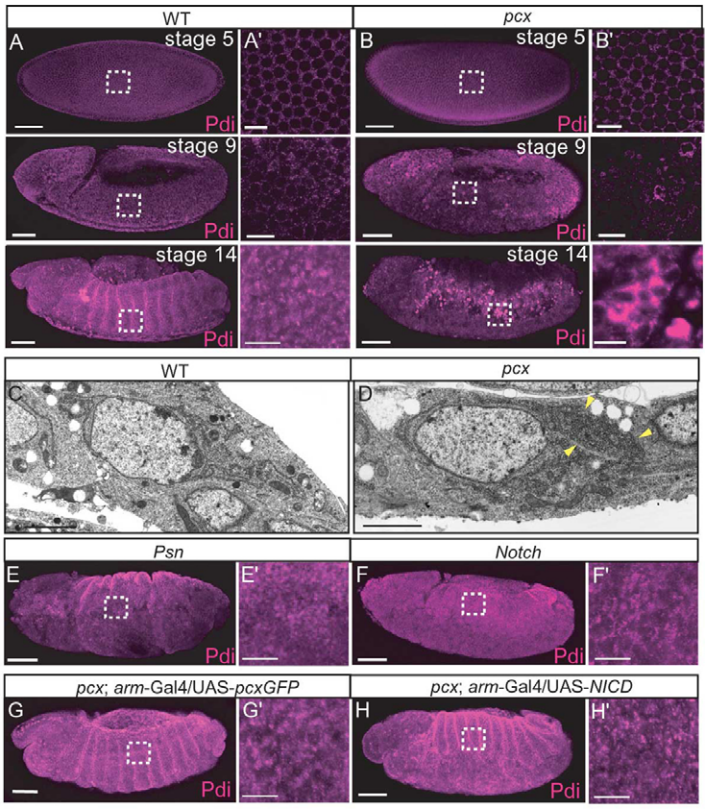


**Fig. 5. Pcx is involved in controlling the ER architecture.** (**A-B′**) Lateral views of a wild-type *Drosophila* embryo (A,A′) and an embryo lacking zygotic and maternal *pcx* (B,B′), in which the ER was visualized by anti-Pdi antibody staining. (**C**,**D**) Electron micrographs of embryonic cells in the region corresponding to the dorsal epidermis at stage 14 in a wild-type embryo (C) and a $pcx^{m/z}$ embryo (D). Yellow arrowheads indicate the ER. (**E-H′**) Lateral views of embryos in which the ER was visualized by anti-Pdi antibody staining. (E) Embryo lacking zygotic and maternal *Psn*. (F) Embryo homozygous for *N*. (G) Embryo lacking zygotic and maternal *pcx* and overexpressing *pcxGFP* (*pcx*; *arm*-Gal4/UAS-*pcxGFP*). (H) Embryo lacking zygotic and maternal *pcx* 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′.

## 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 $pcx^3$, as described above (Fig. 1E,F).

We expressed *pcxGFP* driven by *arm*-Gal4 in the neuroectoderm of embryos at stage 14, because $pcx^{m/z}$ 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, $Gal80^{ts}$, in the wing imaginal discs of third instar larvae (Hewes et al., 2006). Under these conditions, we could control the expression level of *pcxGFP* so that it was just above the detection limit of the product by immunostaining (data not shown). Consistent with the results obtained in embryos, PcxGFP was mostly colocalized with an ER marker (Pdi), but not with other markers (supplementary material Fig. S2A-F) (McKay et al., 1995; Bobinnec et al., 2003). These results suggest that Pcx might be an ER-resident protein, although the localization of the endogenous protein still needs to be determined.

## 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 $pcx^{m/z}$ embryos were stained with an anti-Pdi antibody. The ER appeared to be normal in $pcx^{m/z}$ 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 $pcx^{m/z}$ 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 $pcx^{m/z}$ 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 (Rab5), 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*, an N-signaling component (Fig. 5E-F′), although these embryos showed the neurogenic phenotype (Simpson, 1990; Ye et al., 1999). These results indicate that the enlargement of the ER was not due to the disruption of N signaling. We also found that this ER defect was rescued by the overexpression of *pcx-GFP* in $pcx^{m/z}$ embryos, indicating that this phenotype was induced by the lack of *pcx* function (Fig. 5G,G′). Therefore, we speculated that the enlargement of the ER might be the reason for the severe reduction in N signaling in these embryos. However, the overexpression of

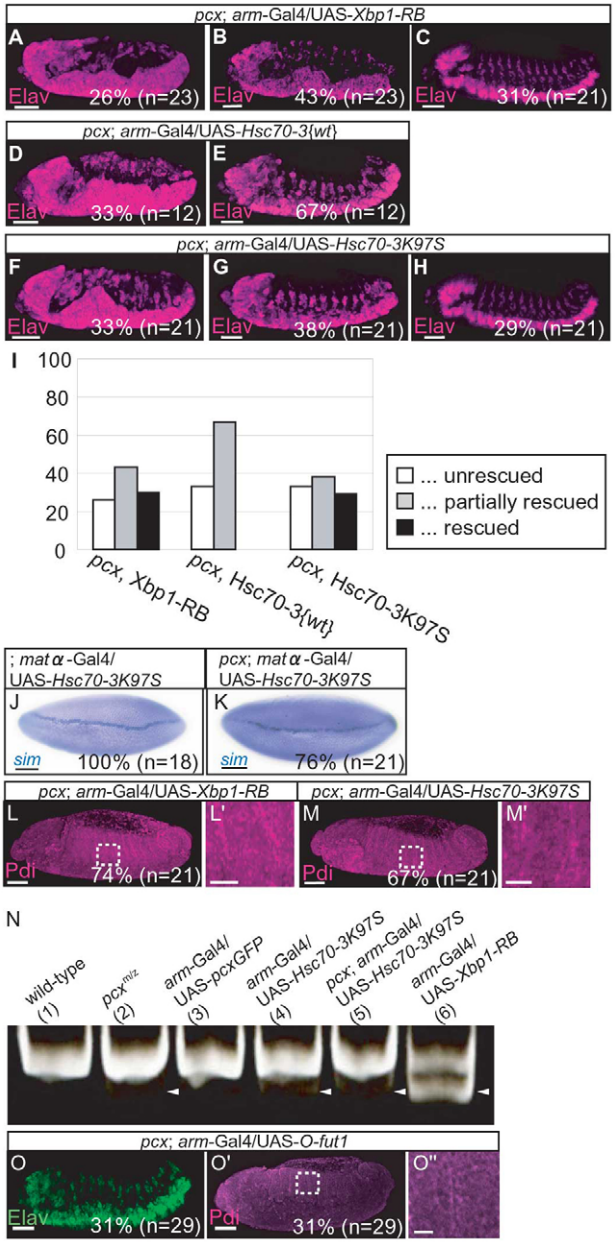


**Fig. 6. *pcx* function is rescued by upregulation of UPR.** (**A-H**) Lateral views of *pcx*$^{m/z}$ embryos, in which the neuronal cells were stained with an anti-Elav antibody (magenta). (A-C) Embryos overexpressing *Xbp1-RB*. The neurogenic phenotype was not rescued in 26% of the embryos (A), but was partially rescued in 43% (B) and was fully rescued in 31% (C). (D,E) Embryos overexpressing wild-type *Hsc70-3*, *Hsc70-3{wt}*. The neurogenic phenotype was not rescued in 33% of the embryos (D) but was partially rescued in 67% (E). (F-H) Embryos overexpressing a dominant-negative form of *Hsc70-3*, *Hsp70-3K97S*. The neurogenic phenotype was not rescued in 33% of the embryos (F), but was partially rescued in 38% (G) and was fully rescued in 29% (H). The number of embryos (*n*) examined in each experiment is indicated in parentheses. Genotypes of the embryos are indicated at the top. (**I**) Percentage of embryos showing an unrescued (white bar), partially rescued (gray bar) or fully rescued (black bar) neurogenic phenotype associated with the absence of *pcx* function, by the overexpression of *Xbp1-RB* (*pcx*, *Xbp1-RB*), *Hsc70-3{wt}* (*pcx*, *Hsc70-3{wt}*) or *Hsp70-3K97S* (*pcx*, *Hsp70-3K97S*). (**J**,**K**) Expression of *sim* detected by in situ hybridization at stage 6 in a wild-type embryo overexpressing *Hsc70-3K97S* (J) and a *pcx*$^{m/z}$ embryo overexpressing *Hsc70-3K97S* (K). (**L-M′**) Lateral views of *pcx*$^{m/z}$ embryos , in which the ER was visualized by anti-Pdi antibody staining (magenta). (L) Embryo overexpressing *Xbp1-RB*. (M) Embryo overexpressing *Hsc70-3K97S*. L′ and M′ are high-magnification views of the region indicated by a dashed box in L and M, respectively. (**N**) Spliced form of *Xbp1* mRNA (Xbp1-RB) was detected by RT-PCR (lower band indicated by arrowheads). (**O-O″**) Lateral views of *pcx*$^{m/z}$ embryos overexpressing *O-fut1*, in which the neuronal cells were stained with an anti-Elav antibody (green in O) and the ER was visualized by anti-Pdi antibody staining (magenta in O′ and O″). O″ is a high-magnification view of the region indicated by a dashed square in O′. Scale bars: 50 μm in A-H,J-M,O,O′; 10 μm in L′,M′,O″.

*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 *pcx* 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 *pcx* function

Based on the fact that Pcx was mostly detected in the ER and that defects in the ER structure were found in *pcx*$^{m/z}$ embryos, we next examined whether Pcx participates in some biological process that takes place in the ER. For example, the proper folding of N is known to be essential for its activation. The formation of disulfide bonds in the extracellular domain of N in *Drosophila* is known to require Ero1L, which has a broad role in protein folding in yeast (Tien et al., 2008).

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* (*Xbp1-RB*) mRNA results in the transcriptional induction of these genes (Back et al., 2006). We produced *Xbp1-RB* mRNA in *pcx*$^{m/z}$ 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 *pcx*$^{m/z}$ embryos (67%, *n*=12) (Fig. 6D,E,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 *pcx*$^{m/z}$ embryos as efficiently as *Xbp1-RB* (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 *sim* (Hong et al., 2008). The expression of *sim* in mesectoderm cells was restored by the overexpression of *Hsc70-3K97S* in $pcx^{m/z}$ embryos, at 76% frequency ($n$=21) (Fig. 6J,K). These results suggested that the attenuation of N signaling associated with the absence of *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 $pcx^{m/z}$ embryos was rescued by inducing the UPR. The ER enlargement was efficiently suppressed by the overexpression of *Xbp1-RB* (74%, $n$=21) or *Hsc70-3K97S* (67%, $n$=21) in these embryos (Fig. 6L-M′). Therefore, the condition causing the enlargement of the ER in the absence of *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 *pcx* in the UPR, we detected the spliced form of endogenous *Xbp-1* (*Xbp1-RB*) mRNA, which reflects the activation of the UPR in vivo, by RT-PCR (Haecker et al., 2008). As reported previously (Elephant and Palter, 1999), overexpression of UAS-*Hsc70-3K97S*, which encodes a dominant-negative form of Hsc70 driven by *arm*-Gal4, induced the production of *Xbp1-RB* mRNA (Fig. 6N, lane 4). In $pcx^{m/z}$ embryos, the overexpression of UAS-*Hsc70-3K97S* also induced *Xbp1-RB* mRNA as efficiently as in the wild-type background (Fig. 6N, lane 5). These results suggest that *pcx* is not essential for induction of the UPR. We also found that overexpressing *pcx* did not induce an ectopic UPR in wild-type embryos (Fig. 6N, lane 3). However, we detected a weak but reproducible induction of *Xbp1-RB* mRNA production in $pcx^{m/z}$ embryos (Fig. 6N, lane 2). Thus, an ectopic UPR might be induced in $pcx^{m/z}$ 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 $pcx^{m/z}$ embryos. To test this possibility, we overexpressed *O-fut1*, which has an N-specific chaperone activity, in $pcx^{m/z}$ embryos. We found that the neurogenic phenotype and the ER enlargement were suppressed in 31% ($n$=29) of the $pcx^{m/z}$ embryos overexpressing *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 $pcx^{m/z}$ embryos. It is conceivable that the transportation of N, and possibly Dl, was disrupted in $pcx^{m/z}$ embryos. However, N and Dl were properly localized to the apical region of the epithelial cells where *Drosophila* E-cadherin was accumulated at stage 5, when the expression of *sim* had already started (supplementary material Fig. S4). These results suggest that the transportation of N was not severely disrupted in $pcx^{m/z}$ 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 *pcx* was previously suggested to be involved in N signaling, based on the neurogenic phenotype associated with its mutant in *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 $pcx^{m/z}$ embryos, the ER was abnormally enlarged. Various factors regulating the architecture of the ER have been identified. In *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 $pcx^{m/z}$ 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 *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 $pcx^{m/z}$ embryos, the ectopic activation of N signaling by overexpression of *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 $pcx^{m/z}$ 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 $pcx^{m/z}$ embryos might be attributable to the enlargement of the ER. However, we cannot exclude the possibility that *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 *pcx* function. We found that overexpression of *O*-fut1, an N-specific chaperone, partially compensated for the loss of *pcx* function (Okajima et al., 2005). Thus, a disruption of N signaling in the absence of *pcx* function might be partly due to the mis-folding of N, which is consistent with our hypothesis that *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 *pcx* function.

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**Competing interests statement**
The authors declare no competing financial interests.

**Supplementary material**
Supplementary material available online at
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