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# Windbeutel is required for function and correct subcellular localization of the *Drosophila* patterning protein Pipe

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## SUMMARY

***Drosophila* embryonic dorsal-ventral polarity originates in the ovarian follicle through the restriction of *pipe* gene expression to a ventral subpopulation of follicle cells. Pipe, a homolog of vertebrate glycosaminoglycan-modifying enzymes, directs the ventral activation of an extracellular serine proteolytic cascade which defines the ventral side of the embryo. When *pipe* is expressed uniformly in the follicle cell layer, a strong ventralization of the resulting embryos is observed. Here, we show that this ventralization is dependent on the other members of the dorsal group of genes controlling dorsal-ventral polarity, but not on the state of the Epidermal Growth Factor Receptor signal transduction pathway which defines egg chamber polarity. Pipe protein expressed in vertebrate tissue culture cells localizes to the endoplasmic reticulum. Strikingly, coexpression of the dorsal group gene *windbeutel* in those cells directs Pipe to the Golgi. Similarly, Pipe protein exhibits an altered subcellular localization in the follicle cells of females mutant for *windbeutel*. Thus, Windbeutel protein enables the correct subcellular distribution of Pipe to facilitate its pattern-forming activity.**

Key words: *Drosophila*, *pipe*, *windbeutel*, Follicle cell, Oogenesis, Dorsal-ventral axis, Golgi, Chaperone, Quality control

## INTRODUCTION

Pattern formation along the dorsal-ventral axis of the developing *Drosophila* embryo depends upon two sequentially acting signal transduction cascades functioning in the ovarian egg chamber and in the egg, respectively (Ray and Schüpbach, 1996; Morisato and Anderson, 1995). In the egg chamber, spatially restricted activation of the *Drosophila* EGF receptor homolog, Torpedo, leads to the establishment of dorsal cell fate in the follicle cells in which Torpedo is activated. The spatially restricted activation of Torpedo (Top; Egrf – FlyBase) is mediated by the TGFα homolog, Gurken (Grk), whose mRNA is associated with the oocyte nucleus which is asymmetrically positioned along the DV circumference of the oocyte (Neumann-Silberberg and Schüpbach, 1993). Thus, the position of the oocyte nucleus defines the dorsal side of the egg chamber and only the follicle cells overlying the nucleus receive the signal to assume a dorsal fate. In females carrying loss of function mutations in *torpedo* (*top*), *gurken* (*grk*), or other members of the receptor signal transduction pathway acting downstream of Top, the egg chambers, eggs and embryos are ventralized, owing to the failure of any follicle cells to assume a dorsal fate (Schüpbach, 1987; Ray and Schüpbach, 1996).

The establishment of dorsal-ventral polarity in the embryo depends upon the prior establishment of polarity in the egg chamber. A key event in the establishment of embryonic polarity is the ventrally restricted activation of the uniformly distributed Toll receptor protein (Toll) whose putative ligand-binding domain extends into the perivitelline space lying between the egg shell and the embryonic membrane (Hashimoto et al., 1988). Spatial restriction of Toll activation is thought to result from ventrally restricted processing of a precursor form of the putative Toll ligand, encoded by the *spätzle* locus (Morisato and Anderson, 1994). Spatially restricted processing of Spätzle occurs via the action of a proteolytic cascade comprising the products of the genes *gastrulation-defective* (*gd*), *snake* and *easter*, with the activation of the cascade taking place only ventrally in the perivitelline space, thereby restricting the formation of active Toll ligand to the ventral side of the embryo (Morisato and Anderson, 1995).

We recently identified the *pipe* (*pip* – FlyBase) gene as providing the link between ovarian and embryonic dorsal ventral polarity (Sen et al., 1998). *pipe* is expressed in the ventral follicle cells under the transcriptional control of the Grk/Top signal transduction pathway. Moreover, this spatial restriction of *pipe* expression determines the ultimate polarity of the progeny embryo. *pipe* encodes an enzyme with structural similarity to the vertebrate glycosaminoglycan-modifying enzyme, heparan sulfate 2-O-sulfotransferase (Kobayashi et al., 1997), which led us to propose that spatially restricted

modification of carbohydrate chains arising during oogenesis plays a pivotal role in the spatial restriction of activation of the protease cascade leading to the formation of the Toll ligand.

When *pipe* is expressed uniformly in the follicle cell layer a strong ventralization of the resulting embryos is observed (Sen et al., 1998). Here, we show that this ventralization is dependent on the activity of other genes of the dorsal group that control embryonic dorsal-ventral patterning, but not on the state of the EGF receptor signal transduction pathway, which defines egg chamber polarity. Consistent with its putative identification as an oligosaccharide-modifying enzyme, we have obtained evidence indicating that Pipe is normally a resident of the Golgi apparatus but that this subcellular localization depends on the presence of activity encoded by the *windbeutel* (*wind*; *wbl* – FlyBase) (Konsolaki and Schüpbach, 1998) locus. Pipe expressed together with Windbeutel in vertebrate COS7 cells exhibits a characteristic perinuclear distribution consistent with Golgi localization, co-localizes with a known Golgi marker protein and exhibits an alteration in its distribution in cells treated with brefeldin A, a drug known to alter Golgi morphology. Pipe expressed in the absence of Windbeutel assumes an endoplasmic reticulum distribution. Similarly, the subcellular targeting of the Pipe protein was found to be dependent on the action of *windbeutel* in ovarian follicle cells. These findings demonstrate that Windbeutel plays a specific role in facilitating the function and subcellular targeting of Pipe.

## MATERIALS AND METHODS

### Fly stocks

All stocks were maintained employing standard conditions and procedures. The wild-type stock was OregonR. Mutant alleles of *gd*, *fs(1)K10*, *nudel* (*ndl*) and *wind* used in this study are described at FlyBase (http://flybase.bio.indiana.edu). The pUAST-*pipe* transgenic stock (Sen et al., 1998) and the λtop-expressing stock (Queenan et al., 1997) have been described previously. The Gal4-3 enhancer trap stock expressing throughout the follicle cell layer was a gift of L. Stevens. Larval cuticles were prepared according to van der Meer, 1977.

### Plasmid constructs

A full length cDNA encoding the PipeST2 isoform (Sen et al., 1998) was subcloned into pHSCaSpeR (Bang and Posakony, 1992) and introduced into flies. In some epistasis experiments, a ubiquitously expressed transgenic insert of this transgene was used. Otherwise the Gal4-3 enhancer trap line was used to direct expression of a transgenic pUAST-*pipe* insert (Sen et al., 1998).

For EM studies of FLAG-Pipe, a PCR fragment corresponding to the PipeST2 isoform was subcloned into *Bgl*II-cut pCMV-Tag1 (Strategene) downstream of and in frame with the FLAG epitope (pCMV-FLAG-Pipe). Next, a *Not*I/*Xho*I fragment containing the FLAG-Pipe cassette was introduced into similarly digested pUAST (Brand and Perrimon, 1993).

For analysis of the distribution of a GFP-Pipe fusion protein in follicle cells, a *Bam*HI/*Bgl*II fragment encoding the mGFP6 variant form of GFP (Schuldt et al., 1998) (a gift of Andrea Brand) was subcloned into similarly digested pCMV-FLAG-Pipe, placing mGFP6 upstream of and in frame with Pipe. Next, a *Not*I/*Xho*I fragment corresponding to GFP-Pipe was excised and introduced into similarly digested pUAST.

For the observation of N-acetylglucosaminyltransferase-GFP in follicle cells, an NAGT-GFP (Shima et al., 1997) -containing *Bam*HI insert (a kind gift from David Shima) was cloned into *Bgl*II-digested pUAST.

All P-element-based transgenic plasmids were introduced into the genome of a *w*/*w* stock by microinjection (Rubin and Spradling, 1982).

For expression in COS7 tissue culture cells, a Pipe-GFP construct was generated. An *Xho*I/*Bam*HI fragment corresponding to the Pipe-ST2 was cloned into a similarly digested pEFGP-N1(Clontech), placing a GFP tag at the C-terminus of Pipe-ST2. For studies of the effect of Windbeutel on Pipe localization, a PCR generated *Bgl*II/*Not*I fragment, corresponding to *wind* (Konsolaki and Schüpbach, 1998), was cloned into similarly digested pEGFP-N1. Δ81Wind-pEGFP-N1 was generated by digesting the above Wind-pEGFPN1 construct with *Pst*I, end-filling with Klenow, followed by ligation of a stop-linker(NEB). This generated a construct that expresses a truncated form of Windbeutel missing 81 amino acids at the C terminus. To generate the KEEL deleted form of Windbeutel, PCR amplification was used to generate a *Pst*I/*Not*I fragment in which lysine 254 had been converted to a stop codon. The *Pst*I/*Not*I fragment was subcloned into similarly cut Wind-pEGFPN1 in which the corresponding wild-type fragment had been excised. The SMH-4 construct expressing Myc-tagged Sialyltransferase was a kind gift from Dr S. Munro (Chapman and Munro, 1994).

### Preparation of monoclonal antibodies directed against Windbeutel

A 358 bp fragment (*Eco*RV-*Hin*dIII) from the 5′ end of the *wind* gene (Konsolaki and Schüpbach, 1998), excluding the signal peptide area, was fused in frame to the C terminus of the glutathione-S-transferase gene contained in the pGEX-3X vector (AMRAD). Purified fusion protein was used to generate monoclonal antibody producing cell lines (Princeton University Antibody Facility), using standard procedures. Supernatants were screened using ELISA assays for specificity against the Windbeutel part of the fusion protein and positive cells were further subcloned. Supernatant from a subclone designated 4GF was used in COS7 cell staining.

### COS7 cell transfection and immunofluorescence microscopy

COS7 cells were transfected with the 10 μg of the Pipe-GFP with or without 7 μg of the *wind* construct plus 10 μg SMH-4, or with 7 μg delta-Wind plus 10 μg SMH-4 (expressing Myc-tagged Sialyltransferase) as described (Wilson et al., 1994). Transfected cells were then processed for indirect immunofluorescence (Church and Wilson, 1997). Primary antibodies used were as follows: (1) 1:100 dilution of anti-Calnexin, raised in rabbit (Stress Gen Biotech Corp); (2) 1:1000 dilution of anti-Myc antibody, raised in rabbit (Santa Cruz Biotech Inc); and (3) 1:10 dilution of 4G7, anti-Wind monoclonal antibody. Secondary antibodies used were 1:100 anti-mouse IgG conjugated to Texas red and 1:100 anti-mouse IgG conjugated to FITC (Southern Biotech Associates). Following immunofluorescence cells were examined with a Biorad MRC 600 scanning laser confocal microscope.

For confocal microscopic analysis of GFP fusion proteins in follicle cells, ovaries were dissected in PBS, fixed in 4% paraformaldehyde in PBS for 10 minutes, washed in PBS and mounted in 60% glycerol in PBS. Egg chambers were examined by confocal microscopy as described for the COS7 cells.

### Brefeldin A treatment of COS7 cells

COS7 cells were co-transfected with the constructs that express Pipe-GFP, Wind and Myc-tagged Sialyltransferase (as above). Cells were plated on coverslips 24 hours after transfection. After a further 24 hours they were treated with DMEM culture medium alone or with medium containing 5 μg/ml of brefeldin A (Sigma) for 1 hour. Following treatment they were fixed in 2% paraformaldehyde in PBS and processed for indirect immunofluorescence and confocal microscopy as above.

### Electron microscopy

For immuno-electron microscopy, ovaries were dissected into PBS (pH 7.4) and fixed 30 minutes in fresh 4% paraformaldehyde + 0.1% glutaraldehyde. Fixed ovaries were washed with cacodylate buffer, then with cacodylate buffer + 0.5% BSA and 0.05% Tween-20 (CBT). Ovaries were stained 1:500 with anti-FLAG(M5) (Sigma) overnight at 4ºC, washed six times, for 30 minutes in CBT, then incubated with 5 nm gold-conjugated anti-mouse mAb (Polysciences; 1:500) overnight at 4ºC, then washed again six times, for 30 minutes in CBT. Dissected stages 8-11 follicles were post-fixed 1 hour with 1% $OsO_4$, dehydrated through ethanol and infiltrated overnight in 1:1 ethanol/PolyBed 812 (Polysciences). Samples were transferred to neat PolyBed 812 and cured for 48 hours at 60ºC. Lead citrate-stained silver/gold sections were visualized on a Jeol 100CX TEM at 60 kV.

For morphological examination, ovaries were dissected in PBS (pH 7.4). Stage 8-11 egg chambers were retained and fixed for 30 minutes in 4% paraformaldehyde, 2% glutaraldehyde in PBS. PBS washed egg chambers were post fixed with 1% $OsO_4$, dehydrated through an ethanol series and propylene oxide and embedded as above. Silver/gold sections were stained with uranyl acetate and lead citrate, and visualized on a Jeol 100CX TEM at 60 kV.

### RNA in situ hybridization

0-24 hour old OregonR embryos were collected and fixed, and whole-mount RNA in situ hybridization was carried out according to Tautz and Pfeifle, 1989, using digoxigenin-labeled *ndl* (Hong and Hashimoto, 1995) and *wind* (Konsolaki and Schüpbach, 1998) cDNA probes.

## RESULTS

### Pipe function requires *windbeutel*, *nudel* and *gastrulation-defective*

Directed expression of *pipe* in all follicle cells of wild-type or *pipe* mutant females results in ventralization of progeny embryos (Sen et al., 1998). In order to assess the requirement for other dorsal group gene products in facilitating Pipe function, we expressed *pipe* under Gal4 control throughout the follicle cell layer in egg chambers of females carrying mutations in dorsal group genes. When *pipe* was expressed uniformly in the background of females mutant for either *wind*, *ndl* or *gd* (Konrad et al., 1998), the resulting embryos were dorsalized (Fig. 1B,D,F), indicating that these genes are required for Pipe function.

In contrast to the dependence of Pipe activity on dorsal group gene function, mutations in genes affecting the EGF receptor signal transduction signaling cascade did not affect the function of ectopically expressed Pipe protein (Fig. 1H,J). *gurken* encodes a *Drosophila* homolog of TGF-α (Neumann-Silberberg and Schüpbach, 1993), whose activation of the *Drosophila* EGF receptor, Top, on the dorsal side of the follicle cell layer is required for normal dorsal-ventral polarity in the ovary (Ray and Schüpbach, 1996). Females mutant for the gene *fs(1)K10* produced outwardly dorsalized embryos and egg shells (Fig. 1G), owing to a mislocalization of the *grk* RNA to the anterior margin of the developing oocyte (Neumann-Silberberg and Schüpbach, 1993). When *pipe* was uniformly

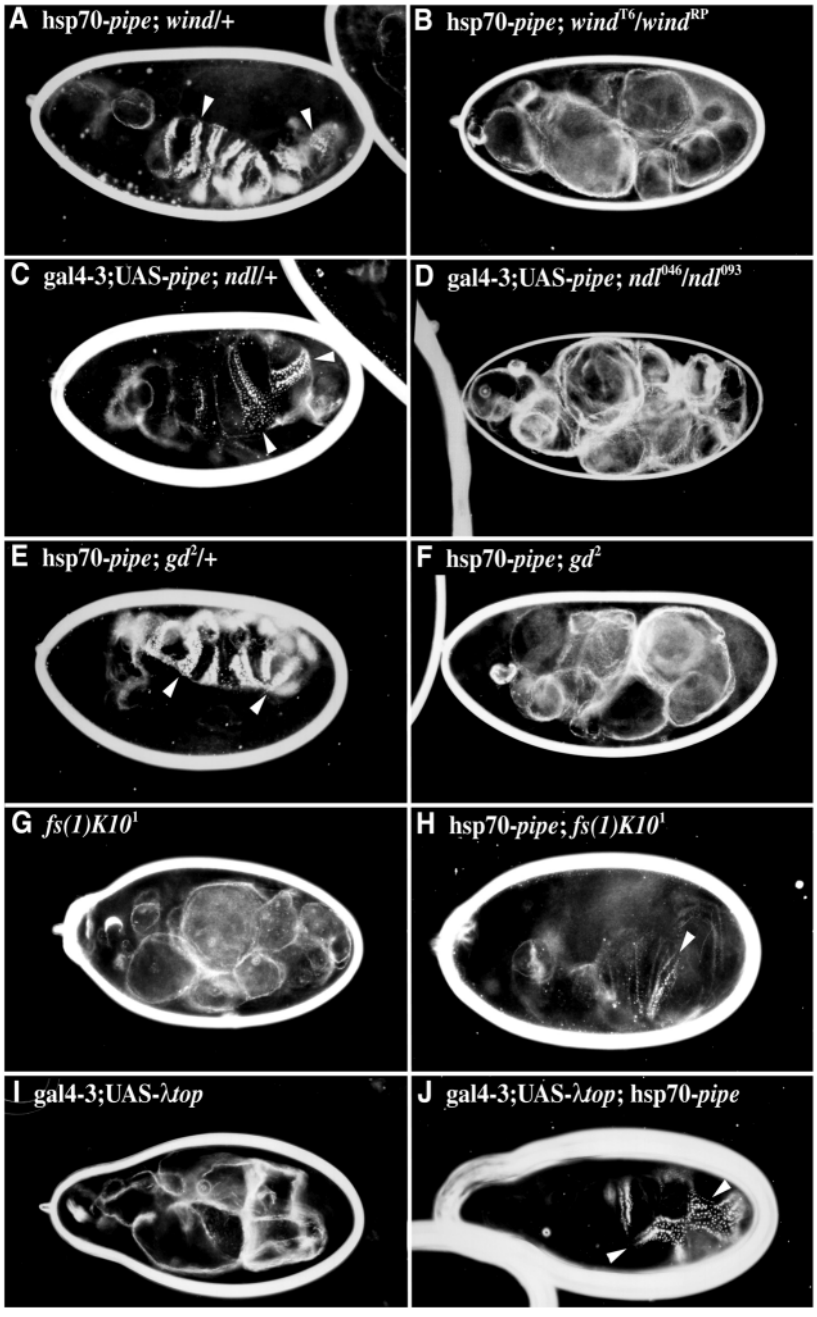


**Fig. 1.** Ventralization of embryos by uniform follicle cell expression of *pipe* is dependent upon dorsal group gene function. *pipe* was expressed uniformly in the follicle cell layers of females heterozygous (A,C,E) or homozygous (B,D,F) for mutations in the dorsal group genes *wind* (A,B), *ndl* (C,D) and *gd* (E,F), in egg chambers homozygous for *fs(1)K10* (H) or expressing constitutively active Torpedo (λtop) (J). Embryos from *fs(1)K10* mutant and λtop expressing females that do not uniformly express *pipe* are shown for comparison in G and I, respectively. Shown are dark-field images of embryonic cuticles within their vitelline membranes. Anterior is towards the left and dorsal is upwards. Arrowheads denote the position of ventral denticle material. Maternal genotypes are indicated.

expressed in the follicle cell layer of females mutant for *fs(1)K10*, ventralized embryos carrying ventral denticle material were produced within dorsalized egg shells (Fig. 1H). Ventralized embryos developing in dorsalized eggs also resulted when *pipe* was expressed uniformly in the background of females expressing an activated, ligand-independent form of Torpedo (Fig. 1J; Queenan et al., 1997). We have previously shown that the normal activation of Top on the dorsal side of the wild-type egg chamber results in an inhibition of transcription of the endogenous *pipe* gene in the dorsal cells of the follicle cell layer (Sen et al., 1998). The ability of uniformly expressed Pipe to exert its ventralizing function in the presence of ectopically active Top indicates that EGF receptor pathway-mediated regulation of endogenous Pipe is manifested solely at the level of transcriptional inhibition, and that Top activation has little or no effect on the activity of the Pipe protein itself.

### Pipe localization is altered in *wind* mutant ovaries

In order to assess the subcellular distribution of Pipe in *Drosophila* ovarian follicle cells, a GFP-tagged form of Pipe was introduced into the *Drosophila* genome under the transcriptional control of the *Saccharomyces cerevisiae Gal4* upstream activation sequences. Expression of the GFP-Pipe protein was directed in the ovary using Gal4-3, an enhancer trap line that is expressed throughout the follicle cell layer. When expressed in *pipe* mutant females, expression of the GFP-Pipe protein was seen to restore the production of ventral and lateral embryonic pattern elements to what would otherwise have been completely dorsalized embryos (data not shown). This demonstrated that the GFP-Pipe protein was functional and that the subcellular distribution observed using this fusion protein was likely to represent the bona fide localization of Pipe protein.

At the light microscopic level, GFP-Pipe fusion protein was observed in a particulate distribution throughout the follicle cell layer (Fig. 2A). GFP-tagged N-acetyl-glucosaminyltransferase, a known vertebrate Golgi resident protein (Shima et al., 1997) that has previously been demonstrated to localize to the Golgi in *Drosophila* tissue culture cells (Rabouille et al., 1999) exhibited a similar distribution (Fig. 2E), providing support for the notion that the subcellular distribution seen represented localization to the Golgi apparatus. We note that the observed distribution of Pipe in follicle cells bore little resemblance to the perinuclear, pericentriolar distribution of the Golgi apparatus observed in mammalian cells. However, the Golgi of *Drosophila* has previously been described as adopting a dispersed or punctate distribution (Ripoche et al., 1994), in comparison to the perinuclear distribution that is observed in vertebrate cells.

The localization of Pipe protein in vivo was also assessed by immuno-electron microscopic analysis of a functional N-terminal FLAG-tagged version of Pipe expressed throughout the follicle cell layer. In stained sections, we identified a multivesicular tubular structure as the Golgi apparatus (Fig. 3A). A structure with this morphology has previously been identified as the Golgi in *Drosophila* nurse cells (Wilsch-Brauninger et al., 1997). In immuno-gold stained sections, gold-particles identifying the FLAG-Pipe protein were observed to be associated with this same structure (Fig. 3B,C), suggesting that the subcellular organelle in which Pipe is localized in follicle cells is indeed the Golgi apparatus. No other structures within these cells were associated with high concentrations of gold particles.

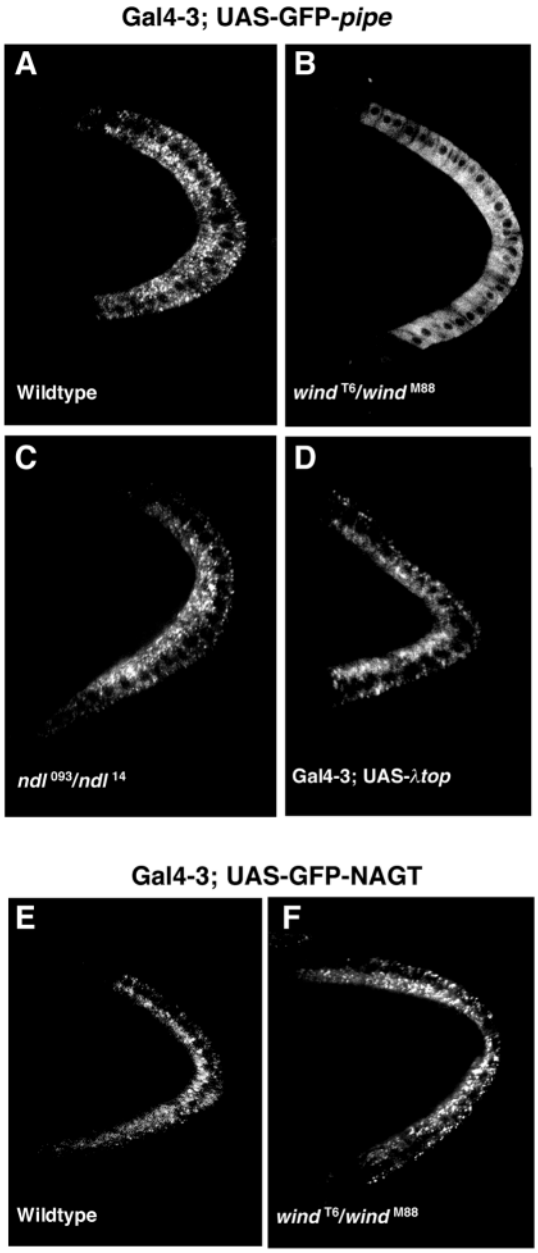


**Fig. 2.** The correct subcellular distribution of GFP-Pipe in follicle cells requires *wind* function. GFP-Pipe (A-D) or N-acetylglucosaminyltransferase-GFP (NAGT)(E,F) was expressed uniformly in the follicle cells of wild-type (A,E), *wind* (B,F), *ndl* mutant females (C) or in females expressing λtop (D). Confocal images of stage 10 follicle cell layers are shown.

As described above, both *wind* and *ndl* are, like *pipe*, required to be expressed in the somatic follicle cells of the ovary in order to exert their dorsal group function. In the epistasis studies described above, we have shown that the products of both genes are required to facilitate the ventralization of progeny embryos generated by uniform expression of Pipe in the follicle cell layer and are therefore required for Pipe function. This requirement could arise if Windbeutel and/or Nudel act downstream of Pipe in the transmission of the ventralizing signal generated by Pipe.

Alternatively, Windbeutel and/or Nudel could be required to generate functional Pipe activity, for example, by facilitating its correct folding or subcellular localization. Analysis of follicles mosaic for mutant alleles of *ndl*, *pipe* and *wind* has demonstrated a specific requirement for *pipe* and *wind* in the ventral follicle cells of the egg chamber (Nilson and Schüpbach, 1998). In contrast, *ndl* activity can be provided nonautonomously, even when expressed solely in follicle cells on the dorsal side of the egg chamber (Nilson and Schüpbach, 1998). This result is inconsistent with a cell-autonomous requirement for Nudel in generating functional Pipe in the cells in which Pipe is expressed. In contrast, the requirement for Windbeutel in the same cells in which Pipe activity is required, together with the identification of Windbeutel as a homolog of the vertebrate endoplasmic reticulum protein ERp29 (Konsolaki and Schüpbach, 1998; Demmer et al., 1997), suggest that Windbeutel might function in generating active Pipe or in properly localizing it. Indeed, it has been speculated that Windbeutel has a chaperone-like function because of the presence of a protein disulfide isomerase-like region (Konsolaki and Schüpbach, 1998; Noiva and Lennarz, 1992). Taken together, these observations suggested that Windbeutel might be involved in directing Pipe protein to its appropriate subcellular compartment. To assess the requirement for Windbeutel function in the correct localization of Pipe, the subcellular localization of GFP-Pipe was assayed in the background of females mutant for *wind*. In such females, the particulate pattern of GFP-Pipe observed in a wild-type background was converted to a uniform cytoplasmic distribution (Fig. 2B), providing strong evidence that Windbeutel protein is required to facilitate the correct Golgi localization of Pipe. Loss of Windbeutel expression did not affect the localization of the GFP-N-acetylglucosaminyltransferase (Shima et al., 1997; Fig. 2F). Similarly, Windbeutel was not required for the normal localization of a GFP fusion to a second *Drosophila* homolog of heparan sulfate 2-O-sulfotransferase (Powers and Ganetzky, 1991) distinct from *pipe* (data not shown). These observations indicate that Windbeutel function is specific for Pipe. In stark contrast to the results seen for *wind*, in females mutant for *ndl*, the distribution of GFP-Pipe was indistinguishable from that seen in a wild-type background (Fig. 2C). Similarly, GFP-Pipe expressed in females expressing a constitutively active form of the Top receptor (Queenan et al., 1997) exhibited a distribution that was indistinguishable from wild type (Fig. 2D), demonstrating that the Top signaling pathway does not influence Pipe subcellular localization (for comparison, see Fig. 2A).

## Windbeutel directs Pipe to the Golgi apparatus of COS7 cells

The predicted *pipe* translation product exhibits significant amino acid sequence similarity to heparan sulfate 2-O-sulfotransferase

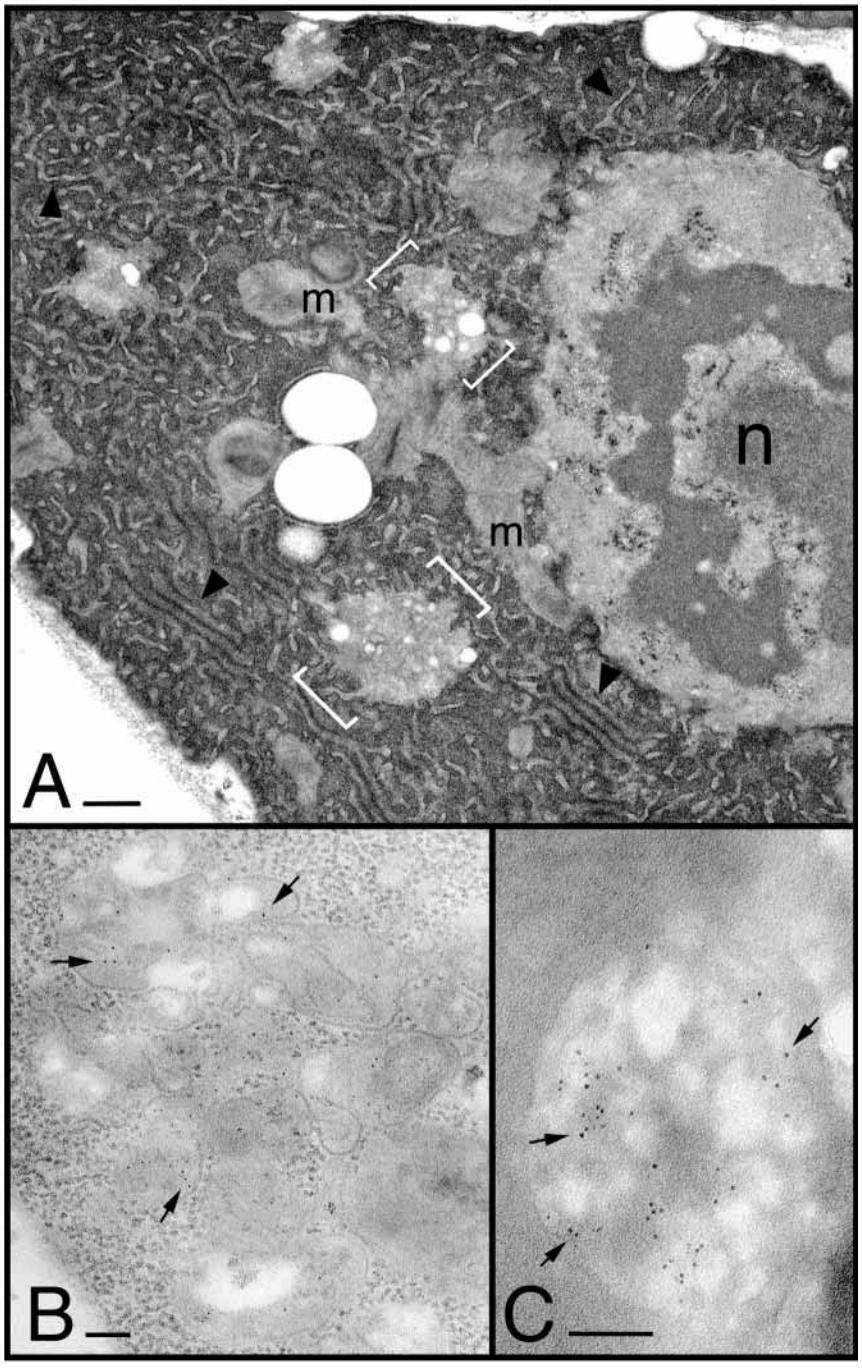


**Fig. 3.** Pipe localizes to the Golgi of ovarian follicle cells. (A) Uranyl acetate/lead citrate-stained follicle cell section; arrowheads indicate rough endoplasmic reticulum. n, nucleus, m, mitochondria. Golgi apparatus are enclosed within white brackets. (B,C) FLAG-tagged Pipe exhibits a Golgi distribution detected by electron microscopy of antibody-coupled immuno-gold particles. The arrows indicate the position of gold particles (note that they are localized to the vesicular structure morphologically similar to the one identified in A as Golgi). Scale bars: 400nm in A; 100 nm in B,C.

(HS2ST) from Chinese hamster ovary (CHO) cells (Sen et al., 1998; Kobayashi et al., 1997). A putative function in oligosaccharide modification together with a predicted type II transmembrane topology suggest that Pipe is a Golgi resident protein. While our observations of the distribution of GFP-Pipe in follicle cells are consistent with Golgi localization, the lack of useful gratuitous markers for *Drosophila* Golgi make an unambigous assignment of Pipe to the Golgi difficult. To confirm that Pipe is a Golgi resident protein, a GFP-tagged form of Pipe was expressed in COS7 monkey cells. These cells were used because of their large size, conspicuous organelle morphology and the availability of markers for a variety of subcellular compartments. Surprisingly, in spite of its predicted function in the Golgi apparatus and the presence of a putative Golgi-targeting type II transmembrane stretch, transfected Pipe-GFP fusion protein expressed in vertebrate COS7 tissue culture cells appeared to exhibit an endoplasmic reticulum (ER) distribution (Fig. 4A,C) as indicated by virtue of its co-localization with Calnexin (Hammond and Helenius, 1994; Fig. 4B,C) a known ER marker. We speculated that the failure of Pipe-GFP to localize to the Golgi could have resulted from our use of the heterospecific COS7 cells. The failure of Pipe-GFP to localize to the Golgi of these cells might arise from a lack of Windbeutel activity which, as we have shown above, is required for normal localization of Pipe in ovarian follicle cells. In a compelling demonstration of the ability of Windbeutel to influence Pipe localization, Pipe-GFP co-expressed with Windbeutel in COS7 cells was redirected to the Golgi (Fig. 4D,F), as demonstrated by its co-localization with Sialyltransferase (Chapman and Munro, 1994) (Fig. 5E,F) a resident Golgi protein.

When expressed in COS7 cells, Windbeutel itself appeared to be present in the ER (Fig. 5B,D,F) as indicated by its co-localization with Calnexin (Fig. 4E,F). This pattern of localization did not appear to depend on the presence of GFP-Pipe (compare Fig. 5B with 5D). Strikingly, a mutant form of Windbeutel, lacking the C terminal 81 amino acids which include the KEEL ER-retention signal (Pelham, 1990) exhibited primarily a Golgi localization pattern although some protein also appeared to be present in the endoplasmic reticulum (Fig. 5G,I). This deleted

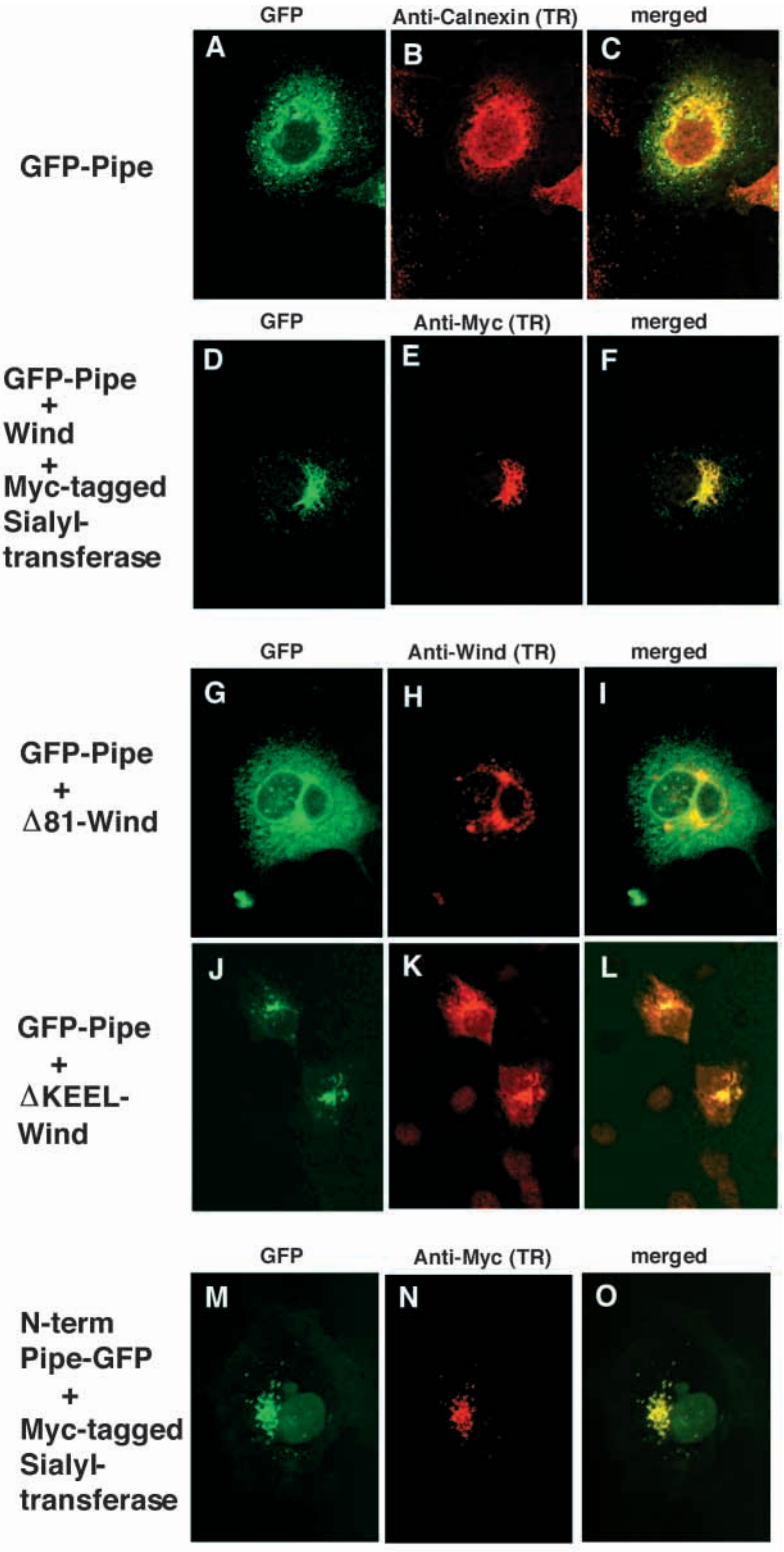


**Fig. 4.** Windbeutel directs Pipe-GFP to the Golgi of COS7 cells. Pipe-GFP expressed alone (A-C) or together with *wind* (D-F) was detected by confocal microscopy. Antibodies against Calnexin (B,C) and Myc-tagged Sialyltransferase (E,F) mark the ER and Golgi, respectively. In addition to ER, we have observed that the antibody against Calnexin also stains the nucleus of COS7 cells. For each transfection, the subcellular distribution of Pipe-GFP in 100 cells was counted. In the absence of Wind, Pipe-GFP exhibited an ER localization in 100% of cells. In the presence of Wind, Pipe-GFP exhibited a Golgi localization in 99% of the cells. (G,I) A mutant form of Wind lacking the C-terminal 81 amino acids was deficient in the ability to direct Pipe-GFP to the Golgi (in 97% of cells, Pipe-GFP was in the Golgi, in 3% of cells Pipe-GFP was in both the Golgi and ER), but was itself detected primarily in the Golgi (H, see also Fig. 5G,I). A mutant form of Wind, lacking only the C-terminal KEEL ER retention signal, was capable of directing Pipe-GFP to the Golgi (J,L) in 99% of cells but was itself localized to the Golgi and ER (K, see also Fig. 5J,L). The N-terminal 95 amino acids of Pipe constitute a Golgi localization signal (M,O). In all cases, transfected constructs are shown on the left, and markers used for imaging are shown above the panels. (TR) indicates Texas Red, and (FITC) fluorescein isothiocyanate.

form of Windbeutel was deficient in the ability to direct Pipe-GFP to the Golgi (Fig. 4G,I). A second mutant Windbeutel protein, lacking only the four amino acids comprising the C-terminal KEEL ER retention signal, assumed a mixed Golgi/ER distribution (Fig. 5J,L). In spite of this, the KEEL-deleted form was nevertheless capable of directing localization of Pipe-GFP to the Golgi (Fig. 4J,L).

When the first 95 amino acids of Pipe, containing the Type II transmembrane region, were fused directly to GFP and expressed in COS7 cells, the fusion protein assumed a putative Golgi distribution, even in the absence of Windbeutel (Fig. 4M,O). This observation suggests that the N-terminal transmembrane domain of Pipe contains the Golgi-specific targeting determinants for Pipe, similar to observations made for other Golgi-resident enzymes with a Type II transmembrane topology (Munro, 1998). Furthermore, this result indicates that the presumed catalytic C-terminal part of the Pipe protein restricts the ability of the N-terminal transmembrane domain to direct the protein to the Golgi unless Windbeutel is present, perhaps because the Pipe protein requires Windbeutel function for appropriate folding and ER export.

As a final confirmation that Pipe-GFP expressed together with Windbeutel was indeed localized to the Golgi, we treated those cells with brefeldin A, a drug that causes the Golgi to disassemble and directs Golgi proteins to be transported back to the ER (Lippincott-Schwartz et al., 1989). After 1 hour's treatment of the cells expressing Pipe-GFP and Windbeutel with 5 μg/ml brefeldin A, a large proportion of the Pipe-GFP appeared to be localized to the ER (Fig. 6D,F) while in cells treated with media alone, virtually all Pipe-GFP was present in the Golgi (6A,C). A similar mislocalization of Myc-tagged Sialyltransferase was also observed in response to brefeldin A (Fig. 6E,F).

### *wind* is expressed in tissues that express *pipe*

Our observation that Windbeutel protein is required for the Golgi localization and subsequent function of Pipe indicates that Windbeutel should be expressed in other tissues in which Pipe functions. BLAST analysis of the genomic DNA of the Pipe region indicates the presence of multiple sets of alternative exons homologous to HS2ST from CHO cells. Including PipeST2, as many as 10 independent isoforms of HS2ST appear to be encoded by the Pipe locus (data not shown). In addition to being expressed in the ventral follicle cells, we have previously shown that the ovary-specific

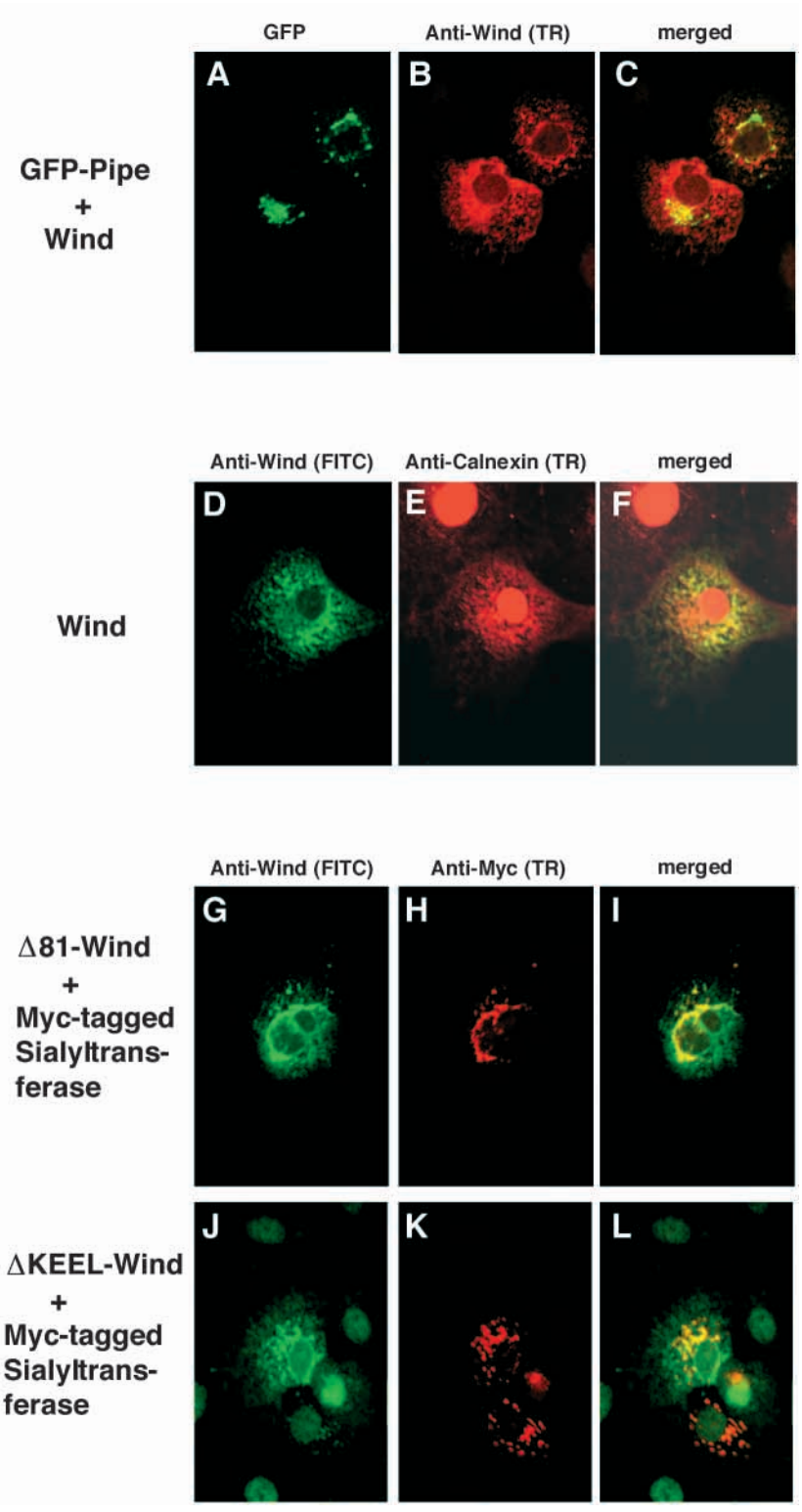


**Fig. 5.** The localization of Wind is altered in mutants, affecting the C terminus. COS7 cells expressing Wind in either the presence (A-C), or absence (D-F) of Pipe-GFP exhibit a diffuse reticular pattern consistent with ER. This is confirmed by co-staining with an antibody directed against Calnexin (E,F), which stains the ER. Although we also observe that the Calnexin antibody also stains the nucleus of COS7 cells (E,F), Wind staining is not seen in the nucleus (D,F). Deletion of the C-terminal 81 amino acids of Wind results in a protein that localizes primarily to the Golgi (G,I), although weak endoplasmic reticulum staining is also detected. When the C-terminal four amino acids of Wind are deleted, the resultant protein localizes in a mixed Golgi/ER pattern (J,L). In all cases, transfected constructs are shown on the left, and markers used for imaging are shown above the panels. (TR) indicates Texas Red, and (FITC) fluorescein isothiocyanate.

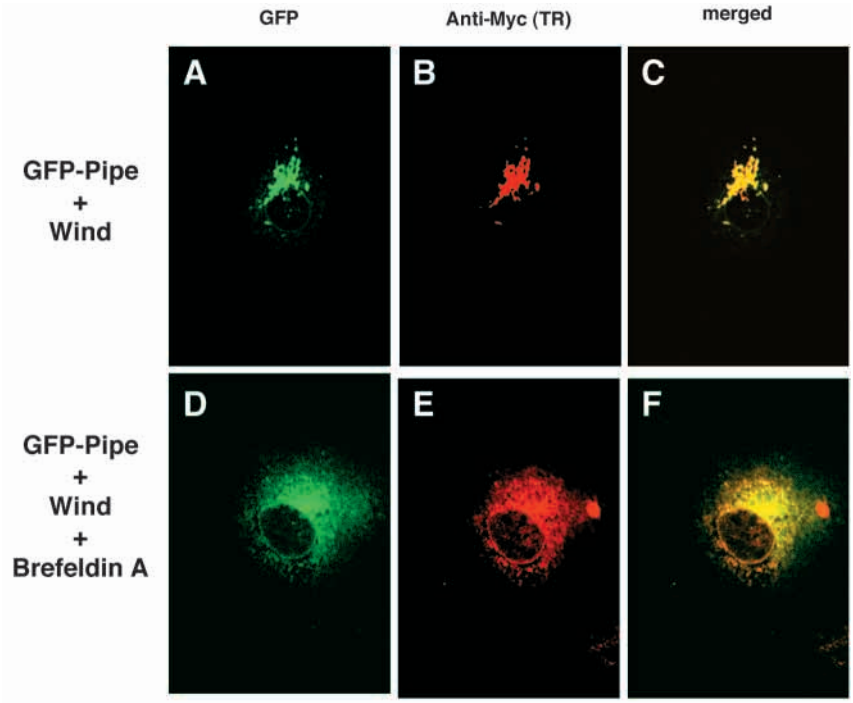


**Fig. 6.** Brefeldin A treatment of COS7 cells results in a redistribution of Pipe-GFP from the Golgi to the endoplasmic reticulum. Pipe-GFP coexpressed with Wind exhibited a tight perinuclear staining pattern representative of Golgi in COS7 cells that were mock treated with DMEM medium alone (A,C), co-localizing with the Golgi marker Sialyltransferase (B,C). In similar cells treated with 5 μg/ml brefeldin A, both the Pipe-GFP (D,F) and Sialyltransferase (E,F) exhibited, in addition to residual Golgi staining, a diffuse staining throughout the cell that is consistent with redistribution to the ER. In all cases, transfected constructs and drug treatment are shown at left, and markers used for imaging are shown above the panels. (TR) indicates Texas Red.

isoform of Pipe (PipeST2) is expressed strongly in the developing salivary gland during embryogenesis (Sen et al., 1998; Fig. 7A), as are at least two other splicing isoforms of the Pipe protein (data not shown). The role of Pipe in the salivary gland is currently unclear. However, we have observed that two *pipe* alleles that result in a loss of *pipe* mRNA expression, and presumably affect all Pipe isoforms through an effect on transcriptional regulatory signals (data not shown), lead to a zygotic semi-lethal phenotype, suggesting that Pipe has functions in addition to its follicle cell role in embryonic patterning. A similar semi-lethal phenotype has been observed for *wind* mutants (Konsolaki and Schüpbach, 1998). We carried out RNA in situ hybridization (Tautz and Pfeifle, 1989) using the *wind* cDNA as well as the *ndl* cDNA as probes of RNA expressed in embryos collected over the first 24 hours of embryogenesis. While we did not detect any consistent pattern of *ndl* expression during embryogenesis (data not shown) we were able to detect *wind* expression in embryonic salivary gland (Fig. 7B). Coexpression of *wind* in both tissues in which we have thus far detected *pipe* expression is consistent with the notion that Windbeutel is required for Pipe function and correct localization.

## DISCUSSION

The experiments described herein provide evidence that, consistent with its predicted function in the sulfation of glycoprotein-associated oligosaccharides and its type II transmembrane structure, Pipe functions as a resident of the Golgi apparatus. Our most compelling evidence of Golgi localization comes from several observations of Pipe expressed (together with Windbeutel) in COS7 cells. First, Pipe is present in a perinuclear distribution characteristic of Golgi. Second, Pipe co-localizes with Sialyltransferase, a well-characterized resident of the Golgi. Finally, the distribution of Pipe is altered dramatically in cells incubated with brefeldin A, an agent known to result in the depolymerization of the Golgi apparatus. Our studies of the expression of Pipe in *Drosophila* follicle cells are similarly consistent with the notion that Pipe acts in the Golgi. Here, Pipe protein exhibits a punctate distribution similar to that seen for another known Golgi enzyme, N-acetyl-glucosaminyltransferase I. Flag-tagged Pipe assessed by immuno-EM was detected in a structure morphologically similar to one previously identified as Golgi (Wilsch-

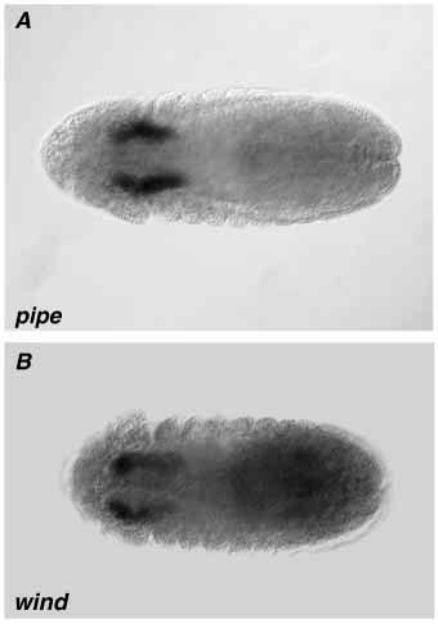


**Fig. 7.** *pipe* and *wind* are co-expressed in the embryonic salivary gland. In situ hybridization with digoxigenin-labelled probes specific for *pipe* (A) and *wind* (B) cDNAs indicate that both are expressed in the salivary gland. Anterior is towards left.

Brauninger et al., 1997). However, an unambiguous assignment of Pipe to the Golgi of follicle cells is associated with the following caveats. First, the structure that we have identified as Golgi in follicle cells does not exhibit the stacked cisternal structure that is characteristic of observations of Golgi in vertebrate cells. Second, we have thus far not been able to demonstrate co-localization of Pipe with a gratuitous marker of Golgi in *Drosophila*, owing to the paucity of such markers specific to *Drosophila*. That being the case, although our data are consistent with the view that Pipe acts in the Golgi of follicle cells, an unambiguous demonstration of that fact awaits the generation of robust markers for the identification of Golgi in follicle cells that could be used in co-localization studies with Pipe.

Although the precise function and catalytic target(s) of Pipe remain elusive, our demonstration that Pipe expressed in COS7 cells (with Windbeutel) resides in the Golgi strongly supports our previous suggestion that Pipe is involved in the modification of glycosaminoglycans or other oligosaccharides (Sen et al., 1998), thereby facilitating the activation of the serine proteolytic cascade activated on the ventral side of the egg that gives rise to active Toll ligand. Unexpectedly, we have found a rather specific role for the Windbeutel protein in directing the correct subcellular targeting of Pipe. This is consistent with our observation that the role of Pipe in dorsal-ventral patterning is dependent on Windbeutel activity. *wind* is expressed in both tissues in which we have detected Pipe expression. Pipe expressed in follicle cells lacking Windbeutel appears to be mislocalized in the ER, presumably rendering Pipe unable to modify its oligosaccharide target which is likely to be synthesized in the Golgi. Strikingly, in a heterospecific expression system, COS7 vertebrate tissue culture cells, Windbeutel expression appears to be sufficient to direct Pipe to the Golgi.

The mechanisms operating in the ER to ensure the fidelity of proteins traversing the secretory pathway have been collectively termed ‘quality control’ (Ellgard et al., 1999; Herrmann et al., 1999). These events comprise a stringent selection process in which only proteins that undergo proper folding and maturation are transported to their target compartments. Those quality control mechanisms which apply to all proteins expressed in the ER have been termed ‘primary quality control’. ‘Secondary quality control’ comprises a rapidly growing list of protein-specific factors influencing folding, maturation, and assembly of proteins in the ER as well as factors which act to facilitate or inhibit forward transport of mature proteins in the secretory pathway. Accessory factors which facilitate movement of proteins out of the ER have been categorized as ‘outfitters’, which establish or maintain a secretion-competent conformation of the transported protein; ‘escorts’, which additionally accompany their cognate transported proteins to the Golgi; and ‘guides’, which mediate the selective uptake of transported proteins into transport vesicles.

Our observations are most consistent with the notion that Windbeutel functions as either an ‘outfitter’ or an ‘escort’ for Pipe transport to the Golgi. The reported structural similarity between Windbeutel/ERp29 and PDI may point towards a role for Windbeutel as a folding catalyst or chaperone for Pipe folding, a likely prerequisite for the migration of Pipe from the ER to Golgi. PDI and its homologs have been demonstrated to possess chaperone-like activities (Puig and Gilbert, 1994).

In some respects, the relationship between Windbeutel and Pipe is reminiscent of that between receptor associated protein (RAP) and low-density lipoprotein (LDL) receptor (Bu et al., 1995). A physical interaction between RAP and LDL receptor prevents aggregation and premature ligand binding in the ER, with RAP escorting LDL receptor to the Golgi. Here, the complex dissociates and RAP is retrieved to the ER by the KDEL receptor (Pelham, 1990). We consider it likely that in the highly synthetic environment of the follicle cell it is important that inappropriate interaction between Pipe and its target(s) be prevented, allowing only spatially and temporally appropriate oligosaccharide modification by Pipe. Ongoing studies will address the precise mechanism of Pipe regulation by Windbeutel and identify the specific targets of Pipe action during *Drosophila* dorsal-ventral pattern formation.

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