Development 134, 1347-1356 (2007) doi:10.1242/dev.02811

The *O*-fucosyltransferase O-fut1 is an extracellular component that is essential for the constitutive endocytic trafficking of Notch in *Drosophila*

Takeshi Sasamura[1,2], Hiroyuki O. Ishikawa[3], Nobuo Sasaki[2], Syunsuke Higashi[2], Maiko Kanai[1,2], Shiho Nakao[2], Tomonori Ayukawa[2], Toshiro Aigaki[4], Katsuhisa Noda[5], Eiji Miyoshi[5], Naoyuki Taniguchi[5] and Kenji Matsuno[1,2,3,*]

Notch is a transmembrane receptor that mediates the cell-cell interactions necessary for many cell-fate decisions. Endocytic trafficking of Notch plays important roles in the activation and downregulation of this receptor. A *Drosophila O*-FucT-1 homolog, encoded by *O-fut1*, catalyzes the *O*-fucosylation of Notch, a modification essential for Notch signaling and ligand binding. It was recently proposed that O-fut1 acts as a chaperon for Notch in the endoplasmic reticulum and is required for Notch to exit the endoplasmic reticulum. Here, we report that O-fut1 has additional functions in the endocytic transportation of Notch. O-fut1 was indispensable for the constitutive transportation of Notch from the plasma membrane to the early endosome, which we show was independent of the *O*-fucosyltransferase activity of O-fut1. We also found that O-fut1 promoted the turnover of Notch, which consequently downregulated Notch signaling. O-fut1 formed a stable complex with the extracellular domain of Notch. In addition, O-fut1 protein added to conditioned medium and endocytosed was sufficient to rescue normal Notch transportation to the early endosome in O-fut1 knockdown cells. Thus, an extracellular interaction between Notch and O-fut1 is essential for the normal endocytic transportation of Notch. We propose that O-fut1 is the first example, except for ligands, of a molecule that is required extracellularly for receptor transportation by endocytosis.

KEY WORDS: Notch, *O*-fucosyltransferase, O-fut1, Endocytosis, *Drosophila*

INTRODUCTION

Notch signaling is an evolutionarily conserved mechanism that regulates a broad spectrum of cell-specification events through local cell-cell communication (Artavanis-Tsakonas et al., 1999). *Notch* encodes a single-pass transmembrane receptor protein with 36 epidermal growth factor-like (EGF) repeats and three Notch/LIN-12 repeats in its extracellular domain and six CDC10/Ankyrin repeats and a PEST-like sequence in its intracellular domain (Kidd et al., 1983; Wharton et al., 1985). In *Drosophila*, two ligands for Notch, Delta and Serrate, which are also transmembrane proteins, are known (Fleming, 1998). The binding between Notch and its ligands leads to a proteolytic cleavage within the transmembrane domain that releases the intracellular domain Notch^{ICD} (Mumm and Kopan, 2000). Notch^{ICD} then translocates to the nucleus and acts as a coactivator for a sequence-specific DNA-binding protein, Suppressor of Hairless, and this complex activates various target genes of the Notch signal (Bray and Furriols, 2001).

This main Notch pathway is evolutionarily conserved from nematodes to mammals (Lai, 2004). However, several additional processes, such as the intracellular transportation of Notch and its ligands, tightly regulate Notch signaling (Le Borgne et al., 2005). For example, the activation of Notch requires its endocytic incorporation and/or that of its ligand (Seugnet et al., 1997), and Notch endocytosis is also involved in the downregulation of Notch activity (Jékely and Rørth, 2003). Several regulators of the endocytic trafficking of Notch, such as the Nedd4 family proteins, Arrestin, Numb and Deltex, have been identified (Sakata et al., 2004; Wilkin et al., 2004; Mukherjee et al., 2005; Berdnik et al., 2002; Hori et al., 2004). In addition, recent findings show that mutations of genes that are generally involved in endocytosis affect the amount and activity of Notch (Giebel and Wodarz, 2006). Although the amount of Notch increased in these mutants, the Notch signal was inactivated in some of them and hyperactivated in others (Lu and Bilder, 2005; Vaccari and Bilder, 2005; Thompson et al., 2005; Moberg et al., 2005; Maitra et al., 2006; Herz et al., 2006). However, it is largely unknown how the endocytic pathway influences the activity of Notch.

In addition to its trafficking, the signaling activity of Notch is also influenced by its glycosylation. Analyses of the Notch signal in a mutant of the UDP-GlcNAc transporter gene, which is probably required for protein *N*-glycosylation, suggested that this modification is essential for the normal functioning of Notch (Goto et al., 2001; Selva et al., 2001). Furthermore, Notch undergoes *O*-linked fucosylation, and the functions of this modification have been studied extensively (Haines and Irvine, 2003). The EGF-like repeats of the Notch extracellular domain, which contain a consensus sequence, are modified by the *O*-linked tetrasaccharide Sia-α2,3-Gal-β1,4-GlcNAc-β1,3-Fuc (Moloney et al., 2000). A GDP-fucose protein *O*-fucosyltransferase1 catalyzes this *O*-linked fucosylation in mammals and *Drosophila* (Wang et al., 2001; Okajima and Irvine, 2002). In *Drosophila*, this enzyme is encoded by *O-fut1* (Okajima and Irvine, 2002). This *O*-fucosylation of Notch is essential for Notch signaling and Notch-ligand interactions (Okajima and Irvine, 2002; Sasamura et al., 2003; Okajima et al.,

[1]Precursory Research for Embryonic Science and Technology (PRESTO), Japan Science and Technology Agency, 4-1-8 Honcho Kawaguchi, Saitama, Japan. [2]Department of Biological Science and Technology and [3]Genome and Drug Research Center, Tokyo University of Science, Chiba 278-8510, Japan. [4]Department of Biological Sciences, Tokyo Metropolitan University, Tokyo 192-0397, Japan. [5]Department of Biochemistry, Osaka University, Graduate School of Medicine, Osaka 565-0871, Japan.

*Author for correspondence (e-mail: matsuno@rs.noda.tus.ac.jp)

Accepted 17 January 2007

2003; Shi and Stanley, 2003). *N*-acetylglucosamine is subsequently added to this fucose moiety by a fucose-specific β1,3 *N*-acetylglucosaminyltransferase, Fringe (Moloney et al., 2000). This modification modulates Notch-ligand interactions (Panin et al., 1997; Brückner et al., 2000).

More recently, O-fut1 was shown to act as a chaperon for Notch, a function that does not require the *O*-fucosyltransferase enzymatic activity of O-fut1 (Okajima et al., 2005). Knocking down *O-fut1* by RNA interference (RNAi) in *Drosophila* cultured cells prevents the Notch extracellular domain polypeptide from being secreted into the medium (Okajima et al., 2005). When this Notch fragment and a mutant O-fut1 that lacks *O*-fucosyltransferase activity are co-expressed, the mutant O-fut1 still promotes the binding between the Notch polypeptide and its ligands (Okajima et al., 2005). These observations led to the proposal that O-fut1 is required for the proper folding of the Notch extracellular domain, and this function is independent of the enzymatic activity of O-fut1 (Okajima et al., 2005). Here, we demonstrated that O-fut1 has another, distinct, function in Notch signaling. Extracellular O-fut1 was required for the constitutive endocytic transportation of Notch to the early endosome, and this function was also independent of the *O*-fucosyltransferase activity of O-fut1.

MATERIALS AND METHODS

Fly stocks and genetics

We used *Canton-S* as the wild-type strain, *O-fut1*^{4R6} as the *O-fut1* mutant (Sasamura et al., 2003), *Delta*^{RevF10} *Serrate*^{RX106} as the double mutant of *Delta* and *Serrate* (Ligoxygakis et al., 1998) and *Notch*^{55e11} (Kidd et al., 1983) as the *Notch* mutant. *daughterless* (*da*)-*GAL4* (*GAL4*^{daG32}) (Wodarz et al., 1995), *engrailed* (*en*)-*GAL4* (Johnson et al., 1995) and *scalloped* (*sd*)-*GAL4* (Roy et al., 1997) were used as the GAL4 drivers. We used FLP/FRT (Xu and Rubin, 1993) and the MARCM (Lee and Luo, 1999) system to generate somatic mutant clones. To isolate the *Gmd* mutant, 171 independent *white* derivative strains were established from *GS13045* and screened using a genomic PCR method (Toba et al., 1999). To generate *O-fut1*$^{-}$ clones in the *Gmd*^{H78} mutant, *hs-flp*; *Gmd*^{H78} *FRT42D O-fut1*^{4R6}*/CyO*, *Kr-GFP* males were crossed to *Gmd*^{H78} *FRT42D P{πM}45F/CyO*, *Kr-GFP* females.

Constructs

For the O-fut1-ER^{-} construct, three tandem oligonucleotides encoding GSEEQKLISEEDLL were inserted into an artificially created *Bam*HI site before the stop codon of O-fut1 cDNA. *UAS-O-fut1*, *UAS-O-fut1-G3*, *UAS-O-fut1-ER*$^{-}$ and *UAS-ER-CFP* (unpublished, provided by A. Sato, Purde University, West Lafayette, IN) were made by inserting cDNAs encoding wild-type O-fut1, Nti-G3, a mutant O-fut1 lacking its *O*-fucosyltransferase activity (Sasamura et al., 2003), O-fut1-ER^{-}, a mutant O-fut1 lacking a functional ER-retention signal, and ECFP-ER (Clontech), an ECFP with an ER-retention signal, respectively, into pUAST.

Cell culture

S2 cells were cultured, transfected and stained as described previously (Fehon et al., 1990; Sasamura et al., 2003). To express O-fut1-ER^{-} in S2 cells, a cDNA encoding O-fut1-ER^{-} was cloned into pRmHa-3 and introduced into S2 cells. We also used pRmHa-3-Notch (Fehon et al., 1990), pRmHa-3-NotchΔEC (Rebay et al., 1991), pRmHa-3-O-fut1-myc and pRmHa-3-O-fut1-IR (Sasamura et al., 2003). To detect O-fut1 incorporated into Notch-expressing S2 cells, conditioned medium containing O-fut1-ER^{-} was collected from S2 cells transfected with pRmHa-3-O-fut1-ER^{-} and added to S2 cells transfected with pRmHa-3-Notch. After a 20-minute incubation, the cells were fixed and stained as described previously (Fehon et al., 1990). To detect the effect of the O-fut1 knockdown on Notch endocytosis, the transfected S2 cells were incubated in *Drosophila* M3 medium (Sigma) on glass slides coated with concanavalin A (Sigma) for 2 hours at 25°C, anti-Notch extracellular antibody (rat1, 1/500) was added, and the slides were incubated at 4°C for 1 hour. After being washed in M3 medium three times at 4°C, the cells were incubated in M3 medium for 15 minutes at 25°C. In some cases, conditioned medium containing O-fut1-ER^{-} was used in place of the M3 medium, beginning 20 minutes before the antibody was added and continuing until the end of the culture period. The cells were subsequently fixed and stained.

Immunohistochemistry

The primary antibodies used in this study were mouse anti-Notch C17.9C6 (1/500) (Fehon et al., 1990), rat anti-Notch rat1 (1/500, a gift from S. Artavanis-Tsakonas), rat anti-*D*E-cadherin DCAD2 (1/20), guinea pig anti-Hrs (1/1,000) (Lloyd et al., 2002), mouse anti-GAL4 RK5C1 (1/500, Santa Cruz), mouse anti-engrailed (1/1000) (Patel et al., 1989), mouse anti-Wg 4D4 (1/500) (Brook and Cohen, 1996), rat anti-GFP GF090R (1/1000, Nacalai) and mouse anti-Myc MC045 (1/500, Nacalai). An O-fut1 guinea pig antibody was raised against O-fut1 (amino acids 27 to 402) that had six histidines added to the C-terminus and was expressed in SF9 insect cells (used in 1/1000 dilution). Immunostaining of wing discs and Garland cells was performed as previously described (Matsuno et al., 2002). To detect cell-surface Notch, dissected wing discs were incubated in 1/100-diluted rat1 in M3 medium at 4°C for 2 hours. They were rinsed four times with M3 medium at 4°C, and then incubated for 20 minutes or 10 hours in M3 medium. At this point the M3 medium was supplemented with 1 μl/ml 20-OH ecdysone (Sigma) and 1% fetal calf serum (Gibco). The endocytic tracer uptake assay was performed as described (Entchev et al., 2000). Confocal images were taken with LSM5 PASCAL and LSM510 META. We used Auto Deblur (AutoQuant) as the deconvolution tool.

Measurement of cytosolic GDP-L-fucose concentration

GDP-L-fucose levels contained in whole-larva homogenates were measured using previously described procedures with minor modifications (Noda et al., 2002). Briefly, larvae were homogenized in a Dounce homogenizer under crushed ice in 250 μl of 0.25 mol/l sucrose buffer containing Protease Inhibitor Mix/DMSO diluted 1/1000 (Wako, Osaka, Japan), 5 mmol/l adenosine-5-monophosphate (AMP) (pH 7.4) (Wako, Osaka, Japan), 10 mmol/l Tris-HCl (pH 7.4), 10 mmol/l KCl and 10 mmol/l $MgCl_2$. Larva homogenates were spun and the supernatants were subjected to ultracentrifugation at 105,000×***g*** for 1 hour at 4°C to obtain the cytosolic fraction. The protein concentration in these fractions was quantified using a BCA kit (Pierce, IL, USA). In a typical experiment, 120 μg protein from the cytosolic fraction was adjusted to a volume of 20 μl with chilled autoclaved water and then boiled at 100°C for 20 seconds. Then, 8.5 μl ice-cold 200 mmol/l MES-NaOH (pH 7.0) was added, and the samples were spun, mixed with 1 μl 10% Triton X-100 and 0.5 μl (36.8 pmol) GnGn-bi-Asn-4-(2-pyridylamino) butylamine (PABA) (Sigma), subjected to a series of enzymatic digestions and coupled with PABA through a peptide bond and 5 μl purified α1-6 FucT (1050 nmol/l). The mixtures were incubated at 37°C for 2 hours and the reaction was terminated by boiling at 100°C for 1 minute. The samples were then spun at 15,000×***g*** for 10 minutes, and 10 μl of the 35 μl of supernatant was subjected to high performance liquid chromatography for the GDP-L-fucose assay as described (Noda et al., 2003).

Immunoprecipitation and western blotting

Whole-cell extract was prepared from S2 cells as previously described (Sasamura et al., 2003). Anti-Notch (C17.9C6) or anti-Myc (9E10) antibodies were added to cell lysates and immunoprecipitated with Protein G Sepharose 4 Fast Flow (Amersham). The beads were washed five times with an extraction buffer and subjected to western blotting as described (Sasamura et al., 2003). The primary antibodies used for blotting were anti-Notch (C17.9C6) and anti-Myc (9E10).

RESULTS

O-fut1 is required for the endocytic transportation of Notch to the early endosome

Notch is distributed in a honeycomb pattern, which corresponds to the location of the adherens junctions, as judged by the localization of *D*E-cadherin, in the apical region of the wild-type wing disc epithelium (Fig. 1A, upper part; Fig. 1E) (Fehon et al., 1991; Oda et

al., 1994). However, in *O-fut1* homozygous mutant (*O-fut1*$^{-}$) cells, this localization was disrupted, and Notch accumulated in intracellular vesicles, which were distributed mostly in the apical cytoplasm, as reported previously (Fig. 1A-E) (Okajima et al., 2005). This Notch accumulation was cell autonomous in *O-fut1*$^{-}$ cells (Fig. 1B,C). It is proposed that this accumulation is caused by the quality control mechanism, which retains mis-folded Notch in the endoplasmic reticulum (ER) (Okajima et al., 2005). However, we previously showed that cell-surface Notch does not decrease significantly when O-fut1 is knocked down, in the *Drosophila* S2 cell line (Sasamura et al., 2003). Therefore, we decided to determine whether Notch was transported normally to the plasma membrane in *O-fut1*$^{-}$ cells in vivo.

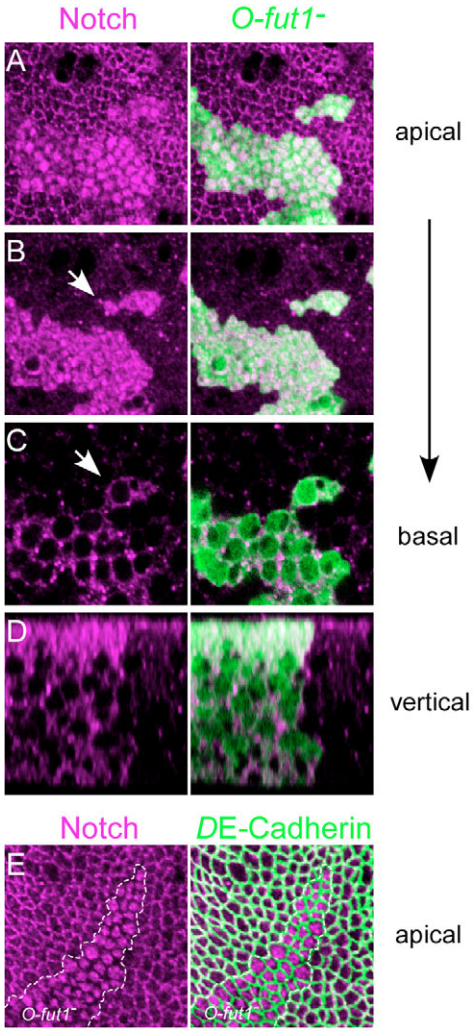

Fig. 1. Notch accumulated in *O-fut1*$^{-}$ cells. (**A-D**) *O-fut1* mutant clones, indicated by the expression of GFP (green), generated by the MARCM method in late third-instar wing discs, and stained with an anti-Notch antibody (C17.9C6, magenta). Apical (A), subapical (B, 1.2 μm beneath the apical level), basal (C, 6.6 μm) and vertical (D) sections of wing disc epithelium are shown. Strong Notch staining was detected in all *O-fut1*$^{-}$ cells. Arrows indicate a small clone surrounded by wild-type cells (B,C). (**E**) Notch failed to localize to the adherens junctions in *O-fut1*$^{-}$ clones. Late third-instar wing discs that contained *O-fut1*$^{-}$ somatic clones were stained with anti-*D*E-Cadherin (green), and anti-Notch antibodies (C17.9C6, magenta). The clone boundary is indicated by a white dashed line.

To detect the fates of the Notch receptors present at the cell surface, we incubated live wing discs from third-instar larvae in medium containing an antibody against the extracellular domain of Notch (rat1) (Le Borgne and Schweisguth, 2003). The antibody was detected in intracellular vesicles in wild-type cells after a 20 minute incubation (Fig. 2C-F, left part of each panel). However, the antibody was not incorporated into these vesicles in live Notch mutant cells, indicating that the antibody specifically labeled Notch under these conditions (Fig. 2A,B). In live *O-fut1*$^{-}$ cells under the same conditions, Notch was detected in intracellular vesicles that were mostly located in the apical region, although these Notch-containing vesicles were smaller and the staining was fuzzy compared with the Notch-containing vesicles in wild-type cells (Fig. 2C-F, at right). These results suggest that Notch was delivered to the plasma membrane in these cells.

We next studied the intracellular vesicles in wild-type and *O-fut1*$^{-}$ cells in more detail. In wild-type cells, most of the Notch-containing endocytic vesicles, visualized by live Notch staining, were labeled by the early endosome marker Hrs (Lloyd et al., 2002) (Fig. 2G-J, right part). In the *O-fut1*$^{-}$ cells, however, live-labeled Notch was not found in the Hrs-positive early endosomes (Fig. 2G-J, left). Furthermore, the live antibody staining for Notch in the intracellular vesicles of the *O-fut1*$^{-}$ cells was much stronger than in the wild-type cells after 10 hours, indicating that Notch was indeed incorporated into the cells by endocytosis but failed to be degraded (Fig. 2K). These results suggested that surface Notch was not transported to the early endosome in *O-fut1*$^{-}$ cells, thereby preventing the trafficking of Notch to the lysosomes, where Notch is degraded (Lu and Bilder, 2005).

Early and late endosomes in the wing disc epithelium can be visualized by fluorescent dextran added extracellularly to the live wing discs (Entchev et al., 2000). Using this system to follow the localization of Notch, we found that the dextran-positive vesicles also stained for Notch (95% of vesicles, n=77) in permeabilized wild-type cells (Fig. 2L, white arrowheads and upper left inset) (Hori et al., 2004). By contrast, in the *O-fut1*$^{-}$ cells, only 6% of the dextran-positive vesicles were also positive for Notch (n=47) (Fig. 2L, open arrowheads and lower right inset). Therefore, Notch failed to be transported to these endosomal compartments. Furthermore, the numbers of dextran-positive endocytic vesicles were equivalent in the wild-type and *O-fut1*$^{-}$ cells, indicating that the *O-fut1* mutation did not affect their formation (Fig. 2L). Together, these observations suggest that O-fut1 is required for the transportation of Notch from early endocytic vesicles to the early endosome. However, we could not identify the early endocytic vesicles in which Notch accumulated in the *O-fut1*$^{-}$ cells. None of the available markers for various endocytic compartments, such as Hook (early endosomes) (Chang et al., 2002), Rab11 (recycling endosomes) (Ullrich et al., 1996; Dollar et al., 2002), rab7-GFP (late endosomes) (Entchev et al., 2000) or ubiquitinylated proteins (aggresomes) (Kopito, 2000) showed co-labeling with Notch in the *O-fut1*$^{-}$ cells (data not shown).

Most Notch was not co-localized with established ER markers in *O-fut1*$^{-}$ cells

Next, we examined whether Notch co-localized with marker proteins for the ER. Protein disulfide isomerase (PDI) is generally involved in protein folding in the ER lumen and is important for quality control (Ferrari and Söling, 1999). A protein trap line, 74-1, expresses PDI-GFP under the control of the original *pdi* promoter and thus accurately reflects the spatiotemporal expression and localization of the PDI protein (Bobinnec et al., 2003). Under a

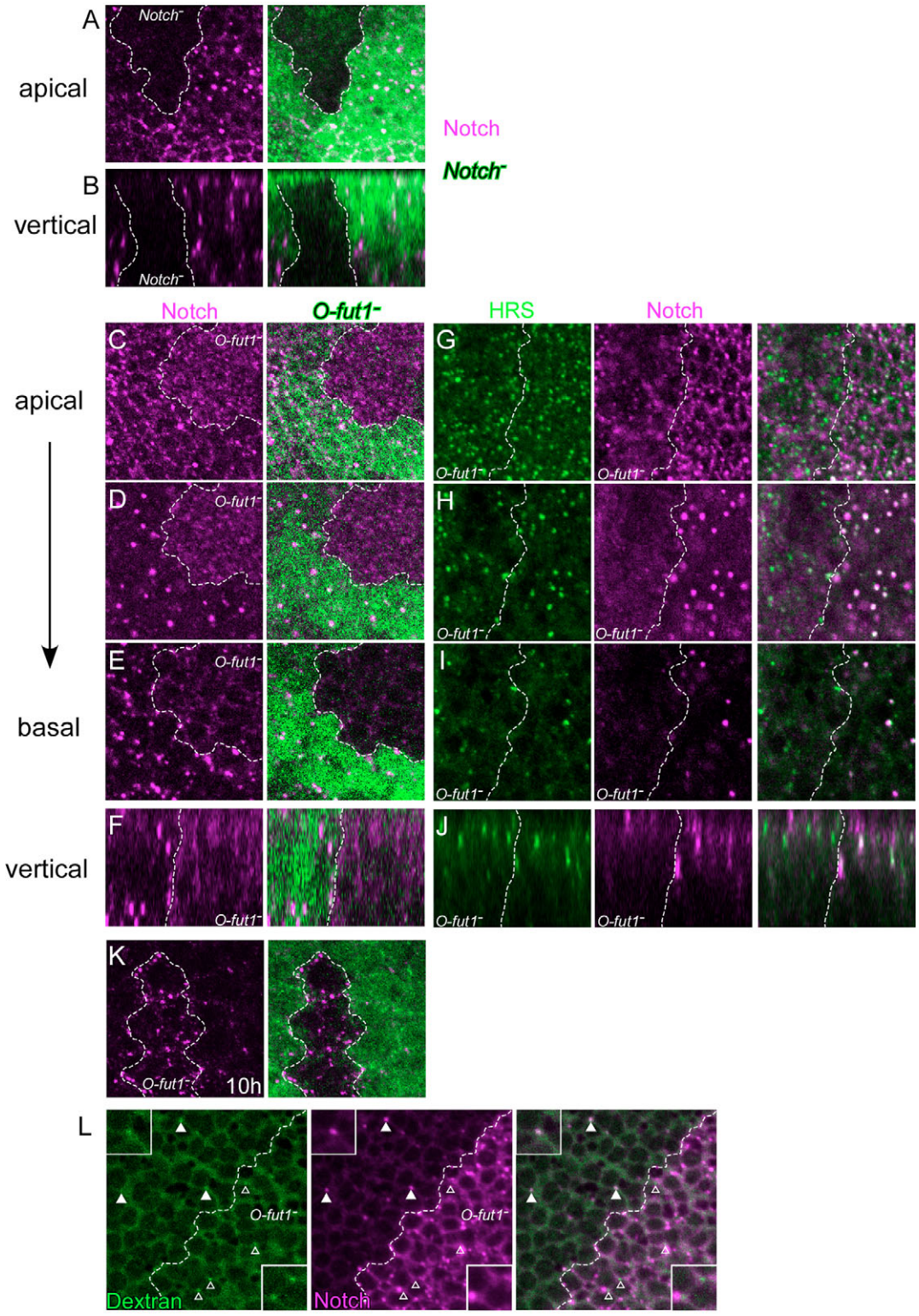

Fig. 2. Notch was endocytosed but not transported to early endosomes in *O-fut1*⁻ cells. (**A-K**) Live wing discs containing *Notch*⁻ (A,B) or *O-fut1*⁻ (C-K) clones were incubated with an anti-Notch extracellular antibody (rat1; magenta) to detect Notch on the plasma membrane, and allowed to endocytose Notch for 20 minutes (A-J) or 10 hours (K). Mutant cells were distinguished by the lack of GFP (green, A-F,K) or are labeled *O-fut1*⁻ (G-J). Wing discs were also stained with an anti-Hrs antibody (G-J, shown in green). Optical sections corresponding to the apical region (A,C,G) or to 2 μm (D,H) or 8 μm (E,I) beneath the apical level, and optical vertical sections (B,F,J) are shown. (**L**) Living wing discs with *O-fut1*⁻ clones (indicated by *O-fut1*⁻) incubated with fluorescent dextran (green) for 10 minutes, chased for 20 minutes at 25°C, and stained with an anti-Notch antibody (C17.9C6; magenta). Higher magnification of one or two dextran-positive vesicles in wild-type (arrowhead) and *O-fut1*⁻ (open arrowhead) cells are shown as insets in the upper left and lower right, respectively. The clone boundaries are demarcated by a white dashed line. All wing discs were isolated from late third-instar larvae.

standard confocal microscope, Notch appeared to mostly co-localize with PDI-GFP in *O-fut1*$^{-}$ cells, which was consistent with the previous report (Okajima et al., 2005) (Fig. 3A). However, higher resolution images obtained by deconvolution analysis revealed that this ER marker did not co-localize with the Notch-containing vesicles in *O-fut1*$^{-}$ cells (Fig. 3B,C). Similar results were obtained using ER-CFP as the ER marker (Fig. 3D,E). In the positive control, PDI-GFP was detected with the mouse anti-GFP primary antibody and two different fluorescent secondary antibodies (Fig. 3F), and the pattern of the two fluorescent signals was the same, confirming the resolution of our analysis. The Notch-containing vesicles were also negative for *cis*-Golgi (anti-KDEL receptor) and Golgi (anti-Golgi, *Drosophila*) markers (data not shown). Therefore, our results indicated that most Notch did not accumulate in the conventional ER. However, these results do not exclude the possibility that Notch accumulated in a specialized ER compartment (Huyer et al., 2004).

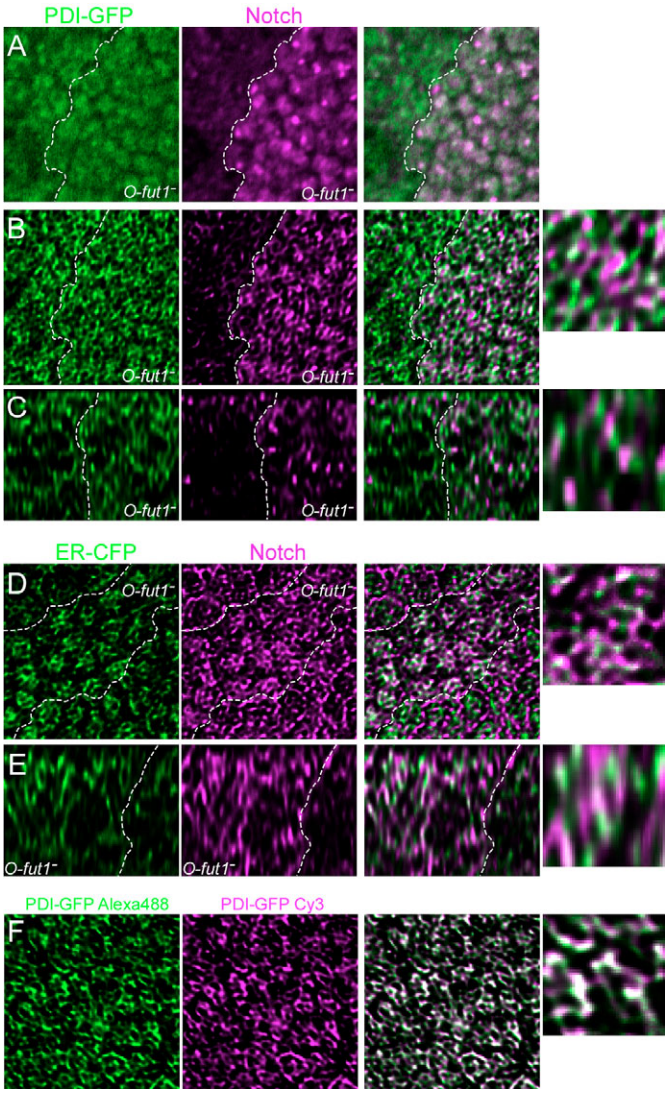

Fig. 3. Most Notch was not co-localized with ER markers in *O-fut1*$^{-}$ cells. (**A-E**) *O-fut1*$^{-}$ somatic clones (labeled *O-fut1*$^{-}$) generated in wing discs expressing ER marker PDI-GFP (A-C) or ER-CFP (driven by *sd-GAL4*; D,E), and stained with anti-Notch (C17.9C6, magenta) and anti-GFP (green) antibodies. (A) Normal confocal image showing the subapical region of the wing disc epithelium, where Notch accumulated in *O-fut1*$^{-}$ cells. (B) The resolution of A was improved using deconvolution. (C) Optical vertical section of B. (D) Deconvolution was used to improve the resolution of this image. (E) Optical vertical section of D. (**F**) A positive control for deconvolution analysis. Wing discs expressing PDI-GFP were incubated with a rat anti-GFP antibody, and stained simultaneously with two anti-rat secondary antibodies, conjugated to Alexa 488 or Cy3. All wing discs were isolated from late third-instar larvae. Right panels show merged images of the left and middle panels. Higher magnifications of *O-fut1*$^{-}$ cells are shown in the small panels at right (B-F). Clone boundaries are indicated by white dashed lines (A-E).

Enzymatic-activity-independent function of O-fut1 required for Notch endocytic trafficking

O-fut1 has both *O*-fucosyltransferase-activity-dependent and -independent functions (Okajima et al., 2005). Therefore, we investigated whether the novel function of O-fut1 in Notch endocytic trafficking depends on its enzymatic activity. As reported, an *O*-fucose-independent function of O-fut1 can be studied using a mutant of the *GDP-D-mannose 4,6-dehydratase* (*Gmd*) gene, which encodes the first enzyme of the de novo GDP-fucose synthesis pathway (Roos et al., 2002; Okajima et al., 2005). We generated two putative null-mutant alleles of *Gmd* (Fig. 4A). The concentration of GDP-fucose in lysates prepared from the third-instar larvae of *Gmd* mutants was determined biochemically (Fig. 4B). The GDP-fucose level in the Gmd^{H78} and Gmd^{H43} heterozygotes was approximately half that of wild-type larvae, and it was not detectable in the Gmd^{H78} and Gmd^{H43} homozygotes (Fig. 4B). These results, which are consistent with these two alleles being null mutations of *Gmd*, corroborate the belief that GDP-fucose is synthesized exclusively from GDP-mannose via the de novo pathway in *Drosophila* (Roos et al., 2002). As reported before, using the Gmd^{1} allele (Okajima et al., 2005), Notch signaling was abolished in the Gmd^{H78} wing discs (Fig. 4C,D). However, Engrailed (En) expression, which is confined to the posterior compartment of the wing disc, was normal in the Gmd^{H78} wing disc (Fig. 4E,F), supporting the idea that this defect was specific to the Notch signal.

To test whether the defect in Notch endocytic trafficking in *O-fut1*$^{-}$ cells was due to the failure of Notch *O*-fucosylation, we examined the endocytic transportation of Notch in the Gmd^{H78} background. By contrast to the result in *O-fut1*$^{-}$ cells (Fig. 2L), we found that Notch was mostly located in the dextran-positive vesicles (98%, n=87) in the *Gmd* mutant (Fig. 4G). In addition, Notch did not accumulate to abnormally high levels in the *Gmd* mutant wing discs, although the size of the Notch-containing endocytic vesicles was slightly greater than those observed in wild-type cells (Fig. 4H,I). Nevertheless, this result suggested that the defect in the endocytic trafficking in *O-fut1*$^{-}$ cells occurs independently of the *O*-fucose modification of Notch. To confirm that this defect was not due to a lack of *O*-fucosylation on Notch, we generated somatic *O-fut1*$^{-}$ clones in the Gmd^{H78} wing disc. We observed increased Notch protein levels in the *O-fut1*$^{-}$, Gmd^{H78}double-mutant cells (Fig. 4J, inside the white dotted line). Therefore, the knockout of *O-fut1* in the *Gmd* mutant still induced the abnormal accumulation of Notch (Fig. 4J), again indicating that this effect is independent of the enzymatic function of O-fut1.

O-fut1 promotes the degradation of Notch

Our results suggested that Notch was transported to the plasma membrane and internalized in *O-fut1*$^{-}$ cells, although it was not delivered to the early endosomes in these cells. In addition, our live-tissue labeling experiments showed that Notch became stable in endocytic vesicles after its incorporation by endocytosis in *O-fut1*$^{-}$ cells (Fig. 2K). Thus, it is likely that Notch failed to be delivered to

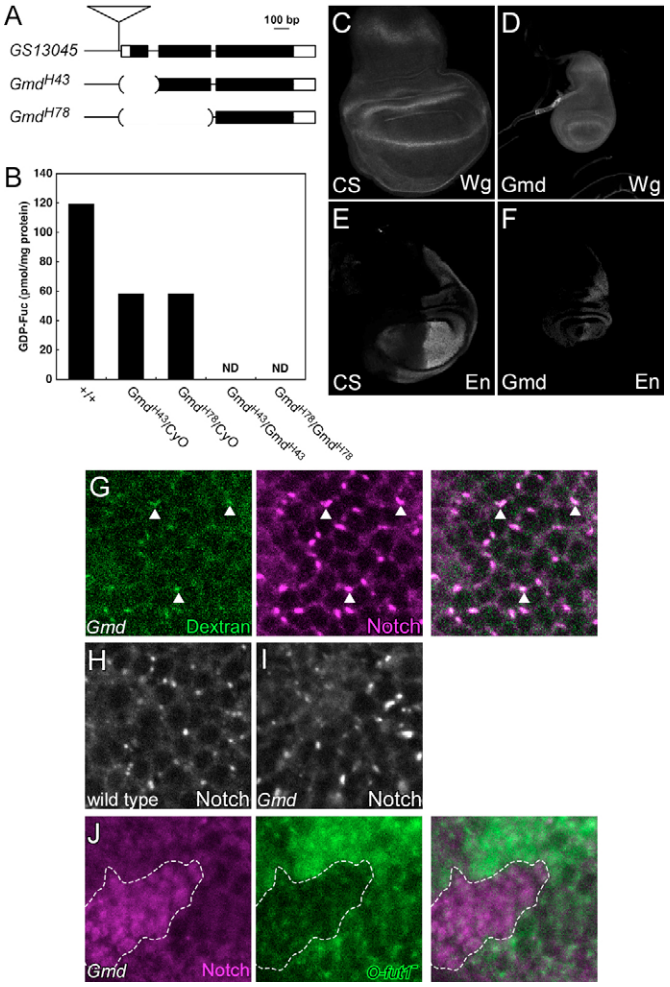

Fig. 4. O-fut1 was required for the endocytic transportation of Notch in a manner independent of its enzymatic activity. (**A**) Genomic organization of the *Gmd* locus. Exons are shown as boxes, and the predicted coding region is in black. The regions 3′ to the P-element insertion site of *GS13045* are deleted in Gmd^{H43} (~0.4 kb) and Gmd^{H78} (~0.8 kb). (**B**) Concentration of GDP-fucose in *Gmd* mutants. GDP-fucose in wild-type and heterozygous and homozygous *Gmd* mutant larvae was measured in duplicate. ND, not detected. (**C-F**) Wild-type (C,E) and Gmd^{H78} homozygous (D,F) wing discs stained with anti-Wg (C,D) and anti-En antibodies (E,F). (**G**) Uptake of fluorescent dextran by live Gmd^{H78} homozygous mutant wing discs after a 10 minute incubation and a 20 minute chase at 25°C. Dextran and Notch are shown in green and magenta, respectively. Some dextran-positive vesicles are indicated by arrowheads. (**H,I**) Notch staining in wild-type (H) and Gmd^{H78} (I) wing discs. (**J**) *O-fut1*$^{-}$ clones generated in Gmd^{H78} wing discs, then stained with anti-Notch (magenta) and anti-myc (clone marker, green) antibodies. The clone boundary is indicated by a white dashed line. All wing discs were isolated from late third-instar larvae.

the lysosomes, where it is normally degraded in these cells (Lu and Bilder, 2005). We therefore speculated that the turnover of Notch is reduced in the *O-fut1*$^{-}$ cells. To address this possibility, we examined the half-life of Notch in wild-type and *O-fut1*$^{-}$ cells. We used a heat-shock-inducible Notch-GAL4 fusion protein, N^{+}-GV3, which retains the wild-type function of Notch (Struhl and Adachi, 1998). The N^{+}-GV3, which is produced for a short period under the control of a heat-shock promoter, can be specifically detected by an anti-GAL4 antibody (Struhl and Adachi, 1998). Thus, we could trace the fates of this Notch protein against the background of continuously produced endogenous Notch (Hori et al., 2004). Thirty minutes after heat shock, N^{+}-GV3 was expressed uniformly throughout the wing disc (Fig. 5B). Although the N^{+}-GV3 was gradually degraded, there was more N^{+}-GV3 in the *O-fut1*$^{-}$ cells 6 and 12 hours after heat shock than in the surrounding wing disc cells (Fig. 5C,D).

As O-fut1 is thought to act extracellularly (Haines and Irvine, 2003), we next examined the requirement for the Notch extracellular domain for its stabilization in the *O-fut1*$^{-}$ clones. N^{EGF}-GV3, a *Drosophila* Notch derivative lacking the fifth to 23rd (of 36) Notch EGF-like repeats (Struhl and Adachi, 1998; Artavanis-Tsakonas et al., 1999), showed no increased stability in the *O-fut1*$^{-}$ clones (Fig. 5E). Therefore, the Notch EGF repeats seem to be essential for the stabilization of Notch in *O-fut1*$^{-}$ cells.

Because the half-life of Notch was prolonged in *O-fut1*$^{-}$ cells, we tested whether O-fut1 overexpression would promote Notch degradation. O-fut1 overexpression was driven by *UAS-O-fut1* under the control of *en-GAL4* (Fig. 5F-H). N^{+}-GV3 expression was induced by heat shock and detected with anti-GAL4 antibodies, as above. Although the anti-GAL4 antibody also recognized the GAL4 generated from *en-GAL4*, the GAL4 staining remained at the background level under our conditions (Fig. 5F). We found that N^{+}-GV3 was greatly decreased in the O-fut1-overexpressing cells compared with the wild-type cells 24 hours after heat shock (Fig. 5H). Therefore, as opposed to the loss of O-fut1 function, its overexpression promoted the degradation of Notch. O-fut1-G3, which has three amino acid substitutions in an essential motif for glycosyltransferase activity, lacks enzymatic activity [Nti-G3 in Sasamura et al. (Sasamura et al., 2003)]. Significantly, O-fut1-G3 promoted the degradation of Notch as efficiently as did wild-type O-fut1 (compare Fig. 5H,I), further supporting our idea that O-fut1 promotes the transportation of Notch to the lysosomes independent of Notch *O*-fucosylation.

Because a lack of O-fut1 activity results in the failure of Notch-ligand interactions (Sasamura et al., 2003; Okajima et al., 2003), it is difficult to study the consequences of the disturbance in Notch turnover using loss-of-function mutations of *O-fut1*. Therefore, to examine the possible role of the O-fut1-regulated Notch turnover in the developmental context, we resorted to overexpression studies. The overexpression of O-fut1 or O-fut1-G3 in wing discs caused wing-nicking and vein-thickening phenotypes (Fig. 5J-L) that were reminiscent of Notch loss-of-function phenotypes (de Celis and Garcia-Bellido, 1994). The expression of *wingless* (*wg*), a target of Notch signaling in the wing disc, was reduced significantly in the O-fut1-overexpressing wing disc (Fig. 5M,N) (Rulifson and Blair, 1995). Additional consistent evidence is the finding that O-fut1 overexpression represses Notch signaling in notal microchaete development (Okajima and Irvine, 2002). These results suggest that O-fut1 downregulates Notch signaling by promoting the degradation of Notch via an *O*-fucosylation-independent mechanism.

O-fut1 interacts with the extracellular domain of Notch

O-fut1 has a KDEL-like motif, a HEEL sequence, at its C-terminus, which probably acts as an ER-retention signal (Teasdale and Jackson, 1996). Indeed, it was reported that O-fut1 mostly localizes to the ER (Okajima et al., 2005). However, given that O-fut1 influences the endocytic trafficking of Notch, it is likely that at least some fraction of O-fut1 is transported to the plasma membrane and then incorporated into the cells. Garland cells are suitable to study the subcellular localization of proteins in vivo because of their large cytoplasm (Culi and Mann, 2003). In these cells, we found Myc-

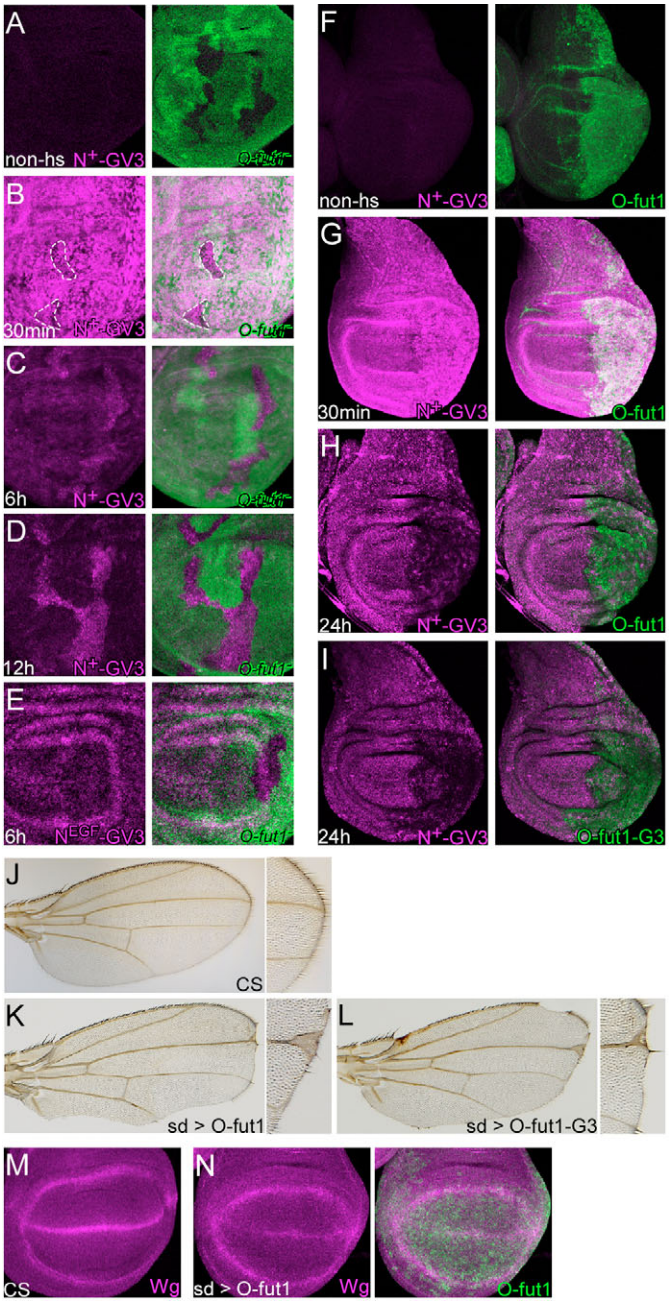

Fig. 5. Notch turnover is modulated by O-fut1. (**A-D**) The expression of N$^+$-GV3 (magenta) was induced by a 1 hour heat shock at 37°C (B-D) or was not induced (A), and the wing discs were subsequently incubated at room temperature for another 30 minutes (B), 6 hours (C) or 12 hours (D). *O-fut1*$^-$ clones are denoted by the absence of GFP (green). The clone boundaries are indicated by white dashed lines in (B). (**E**) Expression of a mutant form of N$^+$-GV3 (N^{EGF}-GV3, magenta), which lacks the fifth to 23rd EGF-like repeats, was induced by a 1 hour heat shock followed by a 6 hour incubation at room temperature. *O-fut1*$^-$ clones lack GFP (green). (**F-I**) O-fut1 (F-H) or O-fut1-G3 (I) driven by *en-GAL4* were overexpressed (green) in the posterior compartment of wing discs, and N$^+$-GV3 expression (magenta) was induced by a 3 hour heat shock at 37°C (G-I) or was not induced (F). Wing discs were dissected 30 minutes (G) or 24 hours (H,I) after heat shock. (**J-L**) Adult wings. (J) Wild-type. (K,L) Wing phenotype induced by the overexpression of wild-type O-fut1 (K) or O-fut1-G3 (L) driven by *sd-GAL4*. Higher magnification images of the wing margin are shown at right. (**M**,**N**) *wg* expression (magenta) was detected in the wild-type wing disc (M) or the wing disc overexpressing *O-fut1* (green) under the control of *sd-GAL4* (N). The right panel of N shows a merged image of Wg and O-fut1 expression. All wing discs were isolated from late third-instar larvae.

tagged O-fut1, which is otherwise wild type [O-fut1-myc; Nti-myc in Sasamura et al. (Sasamura et al., 2003)], in small vesicles, probably exocytic ones, that were not labeled with ER or Golgi markers (Fig. 6A,A′; data not shown). Some of the punctate O-fut1 staining co-localized with N$^+$-GV3 staining, which was located in vesicles distant from the peri-nuclear ER (Fig. 6B,B′, arrows). These results suggest that O-fut1 physically interacts with Notch during exocytosis and/or endocytosis in vivo. Indeed, O-fut1 forms a stable complex with Notch, as shown by immunoprecipitation, which has been proposed to account for the enzymatic-activity-independent functions of O-fut1 (Fig. 6C) (Okajima et al., 2005). We also found that a Notch derivative lacking the EGF-like repeats (Notch$^{\Delta EC}$) did not co-precipitate with O-fut1 (Fig. 6C). These results suggest that O-fut1 interacted with the EGF-like repeats of the Notch extracellular domain.

To test our hypothesis that O-fut1 functions during Notch endocytosis, we investigated whether extracellular O-fut1 was incorporated into cells by endocytosis and whether it could rescue the defects in Notch endocytosis associated with the knockdown of O-fut1 function. S2 cells expressing Notch were cultured with conditioned medium containing secreted Myc-tagged O-fut1 (O-fut1-ER$^-$). We detected the O-fut1-ER$^-$, which was added extracellularly, in vesicles in cells expressing Notch, but not in control cells (Fig. 6D). In the Notch-expressing cells, Notch and O-fut1-ER$^-$ were co-localized in the vesicles (Fig. 6D). In addition, the extracellularly added O-fut1 suppressed the defects in the endocytic transportation of Notch that were induced by the inhibition of O-fut1 function. In the control S2 cells, about 18% of the Notch-containing vesicles co-localized with Hrs (Fig. 6E,H). The co-expression of a double-stranded RNA corresponding to O-fut1 mRNA [O-fut1-IR; nti-IR in Sasamura et al. (Sasamura et al., 2003)] suppressed this co-localization to about 8.5% (Fig. 6F,H). Importantly, the extracellularly added O-fut1 was sufficient to rescue Notch co-localization with Hrs to 16.7% (Fig. 6G,H). Furthermore, we found that O-fut1-ER$^-$ was secreted more efficiently into the medium (data not shown) (Okajima et al., 2005) and accelerated the degradation of N$^+$-GV3 more strongly than native O-fut1 in vivo (Fig. 6I,J). In addition, the overexpression of O-fut1-ER$^-$ induced more severe Notch loss-of-function phenotypes than did overexpressed wild-type O-fut1 (Fig. 6K,L). Together, these results support the idea that the acceleration of Notch turnover, which is induced by extracellular O-fut1, leads to the downregulation of Notch signaling.

O-fut1 is required for the constitutive trafficking of Notch

Ligand binding can force a receptor to choose a specific trafficking path (Sorkin and Von Zastrow, 2002). Therefore, it is possible that extracellular O-fut1 is required for Notch-ligand interactions, which may trigger the transportation of Notch to the endosome. However, no accumulation of Notch was seen in the somatic clones of cells that were double mutants for *Delta* and *Serrate*, as

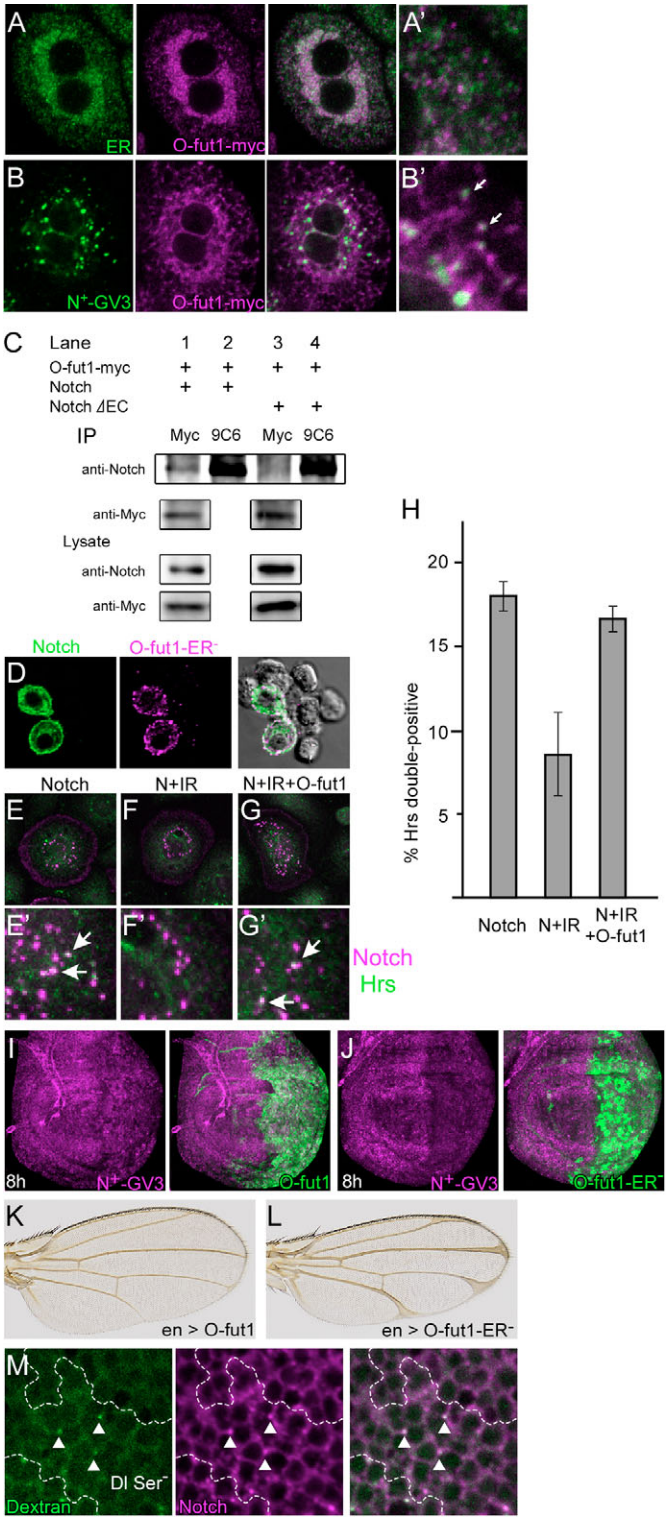

Fig. 6. O-fut1 is secreted and internalized by cells.
(**A**,**B**) Intracellular localization of O-fut1 was examined in Garland cells overexpressing either ER-CFP (A, green) or N^{+}-GV3 (B, green) and O-fut1-myc (A,B, magenta). ER-CFP and O-fut1-myc were driven by *da-GAL4*, and N^{+}-GV3 was induced by a 2 hour heat shock that ended 30 minutes before fixation. (**A′**,**B′**) Higher magnifications of A and B, respectively. Arrows indicate O-fut1 and Notch double-positive vesicles, which were distant from the peri-nuclear ER (B′). (**C**) S2 cells were transfected with the expression constructs for O-fut1-myc and Notch (lanes 1,2), or Notch$^{\Delta EC}$ (lanes 3,4). Lysates prepared from these cells were immunoprecipitated using anti-Myc (lanes 1,3) or anti-Notch (lanes 2,4) antibodies, and the blots were probed with the anti-Notch antibody (C17.9C6) or anti-Myc antibody (9E10). The expression of Notch and O-fut1 in the lysates was confirmed by western blot (Lysate, lower panels). (**D**) O-fut1 was incorporated into cells in a Notch-dependent manner. S2 cells expressing Notch (green) were incubated with the O-fut1-ER^{-} protein (magenta). The brightfield image is superimposed in the right panel. (**E-H**) The endocytosis defect in the O-fut1 knockdown cells was rescued by the addition of extracellular O-fut1. S2 cells expressing Notch (E) or Notch and O-fut1-IR (F,G) were incubated with anti-Notch antibody (rat1, magenta), fixed, and co-stained with anti-Hrs antibody (green). (G) Live cells expressing Notch and O-fut1-IR were incubated with O-fut1-ER^{-} for 20 minutes before the addition of anti-Notch antibody. (**E′-G′**) Corresponding higher magnification images of E-G. Arrows indicate Notch and Hrs double-positive vesicles. (H) Average ratio of Notch and Hrs double-positive vesicles shown as the percentage of Notch-positive vesicles. Mean ± s.d. from triplicate assays (more than 20 cells each) are shown. (**I**,**J**) The N^{+}-GV3 protein (magenta) 8 hours after heat shock for 1 hour in third-instar wing discs overexpressing O-fut1 (I) or O-fut1-ER^{-} (J) (green) driven by *en-GAL4*. (**K**,**L**) Adult wings. O-fut1 (K) and O-fut1-ER^{-} (L) were overexpressed under the control of *en-GAL4*, and the resulting wing phenotypes were examined. (**M**) Fluorescent dextran uptake by *Delta* and *Serrate* double-mutant cells in live wing discs after a 10 minute incubation and a 20 minute chase at 25°C. Clone boundary and dextran-positive vesicles are indicated by dotted lines and white arrowheads, respectively. All wing discs were isolated from late third-instar larvae.

previously reported (data not shown) (Okajima et al., 2005). In addition, Notch co-localized normally with dextran added extracellularly to these double-mutant cells, indicating there was no interference with the endocytic transportation of Notch (Fig. 6M). Therefore, O-fut1 is probably required for the constitutive vesicular transportation of Notch, rather than for its ligand-induced endocytic transportation.

DISCUSSION

O-fut1 is a novel extracellular component required for Notch endocytic trafficking

As a novel and enzymatic-activity-independent function of O-fut1, we propose that O-fut1 is required for the constitutive endocytic trafficking of Notch from the plasma membrane to the early endosome. Previously, it was shown that O-fut1 acts as a specific chaperon for Notch in an enzymatic-activity-independent manner (Okajima et al., 2005). Thus, O-fut1 has two distinct roles, neither of which depend on the *O*-fucosylation of Notch, during either the exocytotic or endocytic transportation of Notch.

As evidence for the chaperon activity of O-fut1, it was reported that Notch accumulates in the ER in *O-fut1*$^{-}$ cells (Okajima et al., 2005). The idea that O-fut1 acts as a chaperon was based on the assumption that its absence results in mis-folded Notch, which could be retained in the ER by quality control mechanisms. However, our results suggested that the level of apical surface Notch, which was recognized by live tissue staining with an anti-Notch antibody, was not reduced significantly. Therefore, at least some Notch is delivered to the plasma membrane in *O-fut1*$^{-}$ cells. This Notch transportation may be slower or less efficient in these cells, which could account for the previous observation that Notch did not reach the surface of O-fut1-depleted *Drosophila* S2 cells at a given time point (Okajima

et al., 2005). In addition, our high-resolution analysis revealed that Notch did not accumulate in vesicles that were positive for two well-characterized ER markers, although we could not exclude the possibility that Notch accumulates in a specific ER subdomain (Huyer et al., 2004). However, biochemical evidence indicates that O-fut1 promotes the proper folding of Notch (Okajima et al., 2005). Thus, we speculate that the two enzymatic-activity-independent roles of O-fut1 are not mutually exclusive and can take place simultaneously.

O-fut1 is required for the transportation of Notch to the early endosome

Our results showed that Notch failed to be delivered to the Hrs-positive early endosomes in *O-fut1*$^{-}$ cells. Therefore, we speculate that O-fut1 may be required for the early endosomal fusion of endocytic vesicles containing Notch, because Notch was internalized in small vesicles in *O-fut1*$^{-}$ cells, but was not transported to the early endosomes. Indeed, similar Notch accumulation in small vesicles is observed in cells with defective endosome vesicle fusion caused by a mutation in the *avl* gene (Lu and Bilder, 2005). However, in the present study, we were unable to determine the nature of the vesicles where Notch was accumulated in *O-fut1*$^{-}$ cells, because the available markers for ER, Golgi, early endosomes, recycling endosomes, late endosomes and lysosomes did not colocalize with the accumulated Notch.

Degradation of transmembrane proteins in the lysosome requires the proteins to be transported first to the early and then to the late endosome (Babst, 2005). Thus, our model predicts that the half-life of Notch is prolonged in *O-fut1*$^{-}$ cells, because Notch was not transported to the early endosome in these cells. Consistent with this model, we found that the half-life of Notch was extended in *O-fut1*$^{-}$ cells and reduced in cells overexpressing O-fut1. In addition, the overexpression of O-fut1 suppressed Notch signaling in vivo. Therefore, O-fut1 may play an important role in maintaining the appropriate Notch turnover ratio, which probably functions to downregulate Notch signaling in wild-type cells.

We also found that Notch did not accumulate in the cells that were double mutants for *Delta* and *Serrate*, suggesting that interactions between Notch and its ligands are not relevant to the O-fut1-dependent endocytosis of Notch. Therefore, the accumulation of Notch in *O-fut1*$^{-}$ cells is due to defects in constitutive endocytosis rather than in ligand-induced endocytosis. Taking these results together, we speculate that O-fut1 may be involved in sweeping unactivated and excess Notch from the plasma membrane under physiological conditions. Therefore, the lack of this O-fut1 function predictably results in the upregulation of Notch signaling. Our overexpression analysis of O-fut1 is consistent with this idea. However, it is difficult to examine this possibility using loss-of-function mutants of *O-fut1*, because O-fut1 is also essential for the interactions between Notch and its ligands (Sasamura et al., 2003; Okajima et al., 2003).

O-fut1 functions cell autonomously in the endocytic transportation of Notch

Although O-fut1 is known to be secreted when it is expressed in S2 cells (Okajima et al., 2005), we found that the function of O-fut1 was required in a cell-autonomous manner in vivo. The accumulation of Notch was observed in all *O-fut1*$^{-}$ cells in somatic clones, even in cells surrounded by wild-type cells (arrow in Fig. 1B,C). Therefore, under physiological conditions, O-fut1 is probably transported to the plasma membrane and then endocytosed only into the same cell. In wild-type cells, O-fut1 may continuously interact with Notch as it cycles from exocytic to endocytic pathways. The following three observations support this idea. First, O-fut1 forms a stable complex with the extracellular domain of Notch. Second, O-fut1 and Notch were occasionally found in the same exocytic vesicles. Third, O-fut1 added extracellularly was incorporated into the cells in a Notch-dependent manner. However, we also found that O-fut1 added to the medium was sufficient to restore the endocytosis of Notch in O-fut1 knockdown cells. Therefore, the interaction of O-fut1 and Notch, which occurs after Notch reaches the cell surface, is sufficient for the normal endocytic trafficking of Notch. However, it is presently unknown how extracellular interactions between Notch and O-fut1 affect the endocytic trafficking of Notch. The specific complex of the Notch extracellular domain and O-fut1 may influence the intracellular recognition machinery between Notch-containing early endocytic vesicles and the early endosomes, such as the tethering factors or Rab5 (Rodriguez-Boulan et al., 2005). Nevertheless, our results raise the interesting possibility that extracellular modification enzymes may be necessary for, or control, the endocytic transportation path of receptor proteins that are also their substrates.

We thank G. Struhl, A. K. Sato, H. J. Bellen, M. Gonzalez-Gaitan, N. Perrimon, the Bloomington Drosophila Stock Center, and the Developmental Studies Hybridoma Bank for providing fly stocks and antibodies. We also thank S. Artavanis-Tsakonas and A. Mukherjee for critical comments on the manuscript, and T. Okajima, K. D. Irvine, E. Spana, D. Bilder and N. Perrimon for support during the early stages of this work. This work was supported by grants-in-aid from the Japanese Ministry of Education, Culture, Sports and Science (K.M.), and PRESTO, JST (K.M.).

References

Artavanis-Tsakonas, S., Rand, M. D. and Lake, R. J. (1999). Notch signaling: cell fate control and signal integration in development. *Science* **284**, 770-776.
Babst, M. (2005). A protein's final ESCRT. *Traffic* **6**, 2-9.
Berdnik, D., Torok, T., Gonzalez-Gaitan, M. and Knoblich, J. A. (2002). The endocytic protein α-Adaptin is required for numb-mediated asymmetric cell division in *Drosophila*. *Dev. Cell* **3**, 221-231.
Bobinnec, Y., Marcaillou, C., Morin, X. and Debec, A. (2003). Dynamics of the endoplasmic reticulum during early development of *Drosophila melanogaster*. *Cell Motil. Cytoskeleton* **54**, 217-225.
Bray, S. and Furriols, M. (2001). Notch pathway: making sense of Suppressor of Hairless. *Curr. Biol.* **11**, R217-R221.
Brook, W. J. and Cohen, S. M. (1996). Antagonistic interactions between wingless and decapentaplegic responsible for dorsal-ventral pattern in the *Drosophila* Leg. *Science* **273**, 1373-1377.
Brückner, K., Perez, L., Clausen, H. and Cohen, S. (2000). Glycosyltransferase activity of Fringe modulates Notch-Delta interactions. *Nature* **406**, 411-415.
Chang, H. C., Newmyer, S. L., Hull, M. J., Ebersold, M., Schmid, S. L. and Mellman, I. (2002). Hsc70 is required for endocytosis and clathrin function in *Drosophila*. *J. Cell Biol.* **159**, 477-487.
Culi, J. and Mann, R. S. (2003). Boca, an endoplasmic reticulum protein required for wingless signaling and trafficking of LDL receptor family members in *Drosophila*. *Cell* **112**, 343-354.
de Celis, J. F. and Garcia-Bellido, A. (1994). Roles of the *Notch* gene in *Drosophila* wing morphogenesis. *Mech. Dev.* **46**, 109-122.
Dollar, G., Struckhoff, E., Michaud, J. and Cohen, R. S. (2002). Rab11 polarization of the *Drosophila* oocyte: a novel link between membrane trafficking, microtubule organization, and *oskar* mRNA localization and translation. *Development* **129**, 517-526.
Entchev, E. V., Schwabedissen, A. and González-Gaitán, M. (2000). Gradient formation of the TGF-β homolog Dpp. *Cell* **103**, 981-991.
Fehon, R. G., Kooh, P. J., Rebay, I., Regan, C. L., Xu, T., Muskavitch, M. A. and Artavanis-Tsakonas, S. (1990). Molecular interactions between the protein products of the neurogenic loci *Notch* and *Delta*, two EGF-homologous genes in Drosophila. *Cell* **61**, 523-534.
Fehon, R. G., Johansen, K., Rebay, I. and Artavanis-Tsakonas, S. (1991). Complex cellular and subcellular regulation of Notch expression during embryonic and imaginal development of *Drosophila*: implications for Notch function. *J. Cell Biol.* **113**, 657-669.
Ferrari, D. M. and Söling, H. D. (1999). The protein disulphide-isomerase family: unravelling a string of folds. *Biochem. J.* **339**, 1-10.
Fleming, R. J. (1998). Structural conservation of Notch receptors and ligands. *Semin. Cell Dev. Biol.* **9**, 599-607.
Giebel, B. and Wodarz, A. (2006). Tumor suppressors: control of signaling by endocytosis. *Curr. Biol.* **16**, R91-R92.

Goto, S., Taniguchi, M., Muraoka, M., Toyoda, H., Sado, Y., Kawakita, M. and Hayashi, S. (2001). UDP-sugar transporter implicated in glycosylation and processing of Notch. *Nat. Cell Biol.* **3**, 816-822.
Haines, N. and Irvine, K. D. (2003). Glycosylation regulates Notch signalling. *Nat. Rev. Mol. Cell Biol.* **4**, 786-797.
Herz, H. M., Chen, Z., Scherr, H., Lackey, M., Bolduc, C. and Bergmann, A. (2006). *vps25* mosaics display non-autonomous cell survival and overgrowth, and autonomous apoptosis. *Development* **133**, 1871-1880.
Hori, K., Fostier, M., Ito, M., Fuwa, T. J., Go, M. J., Okano, H., Baron, M. and Matsuno, K. (2004). *Drosophila* Deltex mediates Suppressor of Hairless-independent and late-endosomal activation of Notch signaling. *Development* **131**, 5527-5537.
Huyer, G., Longsworth, G. L., Mason, D. L., Mallampalli, M. P., McCaffery, J. M., Wright, R. L. and Michaelis, S. (2004). A striking quality control subcompartment in *Saccharomyces cerevisiae*: the endoplasmic reticulum-associated compartment. *Mol. Biol. Cell* **15**, 908-921.
Jékely, G. and Rørth, P. (2003). Hrs mediates downregulation of multiple signalling receptors in *Drosophila*. *EMBO Rep.* **4**, 1163-1168.
Johnson, R. L., Grenier, J. K. and Scott, M. P. (1995). *patched* overexpression alters wing disc size and pattern: transcriptional and post-transcriptional effects on *hedgehog* targets. *Development* **121**, 4161-4170.
Kidd, S., Lockett, T. J. and Young, M. W. (1983). The *Notch* locus of Drosophila melanogaster. *Cell* **34**, 421-433.
Kopito, R. R. (2000). Aggresomes, inclusion bodies and protein aggregation. *Trends Cell Biol.* **10**, 524-530.
Lai, E. C. (2004). Notch signaling: control of cell communication and cell fate. *Development* **131**, 965-973.
Le Borgne, R. and Schweisguth, F. (2003). Unequal segregation of Neuralized biases Notch activation during asymmetric cell division. *Dev. Cell* **5**, 139-148.
Le Borgne, R., Bardin, A. and Schweisguth, F. (2005). The roles of receptor and ligand endocytosis in regulating Notch signaling. *Development* **132**, 1751-1762.
Lee, T. and Luo, L. (1999). Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron* **22**, 451-461.
Ligoxygakis, P., Yu, S. Y., Delidakis, C. and Baker, N. E. (1998). A subset of *Notch* functions during *Drosophila* eye development require *Su(H)* and the *E(spl)* gene complex. *Development* **125**, 2893-2900.
Lloyd, T. E., Atkinson, R., Wu, M. N., Zhou, Y., Pennetta, G. and Bellen, H. J. (2002). Hrs regulates endosome membrane invagination and tyrosine kinase receptor signaling in *Drosophila*. *Cell* **108**, 261-269.
Lu, H. and Bilder, D. (2005). Endocytic control of epithelial polarity and proliferation in *Drosophila*. *Nat. Cell Biol.* **7**, 1132-1139.
Maitra, S., Kulikauskas, R. M., Gavilan, H. and Fehon, R. G. (2006). The tumor suppressors merlin and expanded function cooperatively to modulate receptor endocytosis and signaling. *Curr. Biol.* **16**, 702-709.
Matsuno, K., Ito, M., Hori, K., Miyashita, F., Suzuki, S., Kishi, N., Artavanis-Tsakonas, S. and Okano, H. (2002). Involvement of a proline-rich motif and RING-H2 finger of Deltex in the regulation of Notch signaling. *Development* **129**, 1049-1059.
Moberg, K. H., Schelble, S., Burdick, S. K. and Hariharan, I. K. (2005). Mutations in *erupted*, the *Drosophila* ortholog of mammalian tumor susceptibility gene 101, elicit non-cell-autonomous overgrowth. *Dev. Cell* **9**, 699-710.
Moloney, D. J., Panin, V. M., Johnston, S. H., Chen, J., Shao, L., Wilson, R., Wang, Y., Stanley, P., Irvine, K. D., Haltiwanger, R. S. et al. (2000). Fringe is a glycosyltransferase that modifies Notch. *Nature* **406**, 369-375.
Mukherjee, A., Veraksa, A., Bauer, A., Rosse, C., Camonis, J. and Artavanis-Tsakonas, S. (2005). Regulation of Notch signalling by non-visual beta-arrestin. *Nat. Cell Biol.* **7**, 1191-1201.
Mumm, J. S. and Kopan, R. (2000). Notch signaling: from the outside in. *Dev. Biol.* **228**, 151-165.
Noda, K., Miyoshi, E., Nakahara, S., Ihara, H., Gao, C. X., Honke, K., Yanagidani, S., Sasaki, Y., Kasahara, A., Hori, M. et al. (2002). An enzymatic method of analysis for GDP-L-fucose in biological samples, involving high-performance liquid chromatography. *Anal. Biochem.* **310**, 100-106.
Noda, K., Miyoshi, E., Gu, J., Gao, C.-X., Nakahara, S., Kitada, T., Honke, K., Suzuki, K., Yoshihara, H., Yoshikawa, K. et al. (2003). Relationship between elevated FX expression and increased production of GDP-L-fucose, a common donor substrate for fucosylation in human hepatocellular carcinoma and hepatoma cell lines. *Cancer Res.* **63**, 6282-6289.
Oda, H., Uemura, T., Harada, Y., Iwai, Y. and Takeichi, M. (1994). A *Drosophila* homolog of cadherin associated with armadillo and essential for embryonic cell-cell adhesion. *Dev. Biol.* **165**, 716-726.
Okajima, T. and Irvine, K. D. (2002). Regulation of Notch signaling by *O*-linked fucose. *Cell* **111**, 893-904.
Okajima, T., Xu, A. and Irvine, K. D. (2003). Modulation of Notch-ligand binding by protein *O*-fucosyltransferase 1 and Fringe. *J. Biol. Chem.* **278**, 42340-42345.
Okajima, T., Xu, A., Lei, L. and Irvine, K. D. (2005). Chaperone activity of protein *O*-Fucosyltransferase 1 promotes Notch receptor folding. *Science* **307**, 1599-1603.
Panin, V. M., Papayannopoulos, V., Wilson, R. and Irvine, K. D. (1997). Fringe modulates Notch-ligand interactions. *Nature* **387**, 908-912.
Patel, N. H., Martin-Blanco, E., Coleman, K. G., Poole, S. J., Ellis, M. C., Kornberg, T. B. and Goodman, C. S. (1989). Expression of *engrailed* proteins in arthropods, annelids, and chordates. *Cell* **58**, 955-968.
Rebay, I., Fleming, R. J., Fehon, R. G., Cherbas, L., Cherbas, P. and Artavanis-Tsakonas, S. (1991). Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multifunctional receptor. *Cell* **67**, 687-699.
Rodriguez-Boulan, E., Kreitzer, G. and Musch, A. (2005). Organization of vesicular trafficking in epithelia. *Nat. Rev. Mol. Cell Biol.* **6**, 233-247.
Roos, C., Kolmer, M., Mattila, P. and Renkonen, R. (2002). Composition of *Drosophila melanogaster* proteome involved in fucosylated glycan metabolism. *J. Biol. Chem.* **277**, 3168-3175.
Roy, S., Shashidhara, L. S. and VijayRaghavan, K. (1997). Muscles in the *Drosophila* second thoracic segment are patterned independently of autonomous homeotic gene function. *Curr. Biol.* **7**, 222-227.
Rulifson, E. J. and Blair, S. S. (1995). *Notch* regulates *wingless* expression and is not required for reception of the paracrine *wingless* signal during wing margin neurogenesis in *Drosophila*. *Development* **121**, 2813-2824.
Sakata, T., Sakaguchi, H., Tsuda, L., Higashitani, A., Aigaki, T., Matsuno, K. and Hayashi, S. (2004). *Drosophila* Nedd4 regulates endocytosis of Notch and suppresses its ligand-independent activation. *Curr. Biol.* **14**, 2228-2236.
Sasamura, T., Sasaki, N., Miyashita, F., Nakao, S., Ishikawa, H. O., Ito, M., Kitagawa, M., Harigaya, K., Spana, E., Bilder, D. et al. (2003). *neurotic*, a novel maternal neurogenic gene, encodes an *O*-fucosyltransferase that is essential for Notch-Delta interactions. *Development* **130**, 4785-4795.
Selva, E. M., Hong, K., Baeg, G. H., Beverley, S. M., Turco, S. J., Perrimon, N. and Häcker, U. (2001). Dual role of the *fringe connection* gene in both heparan sulphate and fringe-dependent signalling events. *Nat. Cell Biol.* **3**, 809-815.
Seugnet, L., Simpson, P. and Haenlin, M. (1997). Requirement for dynamin during Notch signaling in Drosophila neurogenesis. *Dev. Biol.* **192**, 585-598.
Shi, S. and Stanley, P. (2003). Protein *O*-fucosyltransferase 1 is an essential component of Notch signaling pathways. *Proc. Natl. Acad. Sci. USA* **100**, 5234-5239.
Sorkin, A. and Von Zastrow, M. (2002). Signal transduction and endocytosis: close encounters of many kinds. *Nat. Rev. Mol. Cell Biol.* **3**, 600-614.
Struhl, G. and Adachi, A. (1998). Nuclear access and action of Notch in vivo. *Cell* **93**, 649-660.
Teasdale, R. D. and Jackson, M. R. (1996). Signal-mediated sorting of membrane proteins between the endoplasmic reticulum and the golgi apparatus. *Annu. Rev. Cell Dev. Biol.* **12**, 27-54.
Thompson, B. J., Mathieu, J., Sung, H. H., Loeser, E., Rorth, P. and Cohen, S. M. (2005). Tumor suppressor properties of the ESCRT-II complex component Vps25 in *Drosophila*. *Dev. Cell* **9**, 711-720.
Toba, G., Ohsako, T., Miyata, N., Ohtsuka, T., Seong, K. H. and Aigaki, T. (1999). The gene search system: a method for efficient detection and rapid molecular identification of genes in *Drosophila melanogaster*. *Genetics* **151**, 725-737.
Ullrich, O., Reinsch, S., Urbe, S., Zerial, M. and Parton, R. G. (1996). Rab11 regulates recycling through the pericentriolar recycling endosome. *J. Cell Biol.* **135**, 913-924.
Vaccari, T. and Bilder, D. (2005). The *Drosophila* tumor suppressor *vps25* prevents nonautonomous overproliferation by regulating notch trafficking. *Dev. Cell* **9**, 687-698.
Wang, Y., Shao, L., Shi, S., Harris, R. J., Spellman, M. W., Stanley, P. and Haltiwanger, R. S. (2001). Modification of epidermal growth factor-like repeats with *O*-fucose. Molecular cloning and expression of a novel GDP-fucose protein *O*-fucosyltransferase. *J. Biol. Chem.* **276**, 40338-40345.
Wharton, K. A., Johansen, K. M., Xu, T. and Artavanis-Tsakonas, S. (1985). Nucleotide sequence from the neurogenic locus *Notch* implies a gene product that shares homology with proteins containing EGF-like repeats. *Cell* **43**, 567-581.
Wilkin, M. B., Carbery, A. M., Fostier, M., Aslam, H., Mazaleyrat, S. L., Higgs, J., Myat, A., Evans, D. A., Cornell, M. and Baron, M. (2004). Regulation of Notch endosomal sorting and signaling by *Drosophila* Nedd4 family proteins. *Curr. Biol.* **14**, 2237-2244.
Wodarz, A., Hinz, U., Engelbert, M. and Knust, E. (1995). Expression of crumbs confers apical character on plasma membrane domains of ectodermal epithelia of Drosophila. *Cell* **82**, 67-76.
Xu, T. and Rubin, G. M. (1993). Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development* **117**, 1223-1237.