The balance between GMD and OFUT1 regulates Notch signaling pathway activity by modulating Notch stability

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INTRODUCTION

The Notch signaling pathway plays an important role in development and physiology. In Drosophila, Notch is activated by its Delta or Serrate ligands, depending in part on the sugar modifications present in its extracellular domain. O-fucosyltransferase-1 (OFUT1) performs the first glycosylation step in this process, O-fucosylating various EGF repeats at the Notch extracellular domain. Besides its O-fucosyltransferase activity, OFUT1 also behaves as a chaperone during Notch synthesis and is able to down regulate Notch by enhancing its endocytosis and degradation. We have reevaluated the roles that O-fucosylation and the synthesis of GDP-fucose play in the regulation of Notch protein stability. Using mutants and the UAS/Gal4 system, we modified in developing tissues the amount of GDP-mannose-deshydratase (GMD), the first enzyme in the synthesis of GDP-fucose. Our results show that GMD activity, and likely the levels of GDP-fucose and O-fucosylation, are essential to stabilize the Notch protein. Notch degradation observed under low GMD expression is absolutely dependent on OFUT1 and this is also observed in Notch Abruptex mutants, which have mutations in some potential O-fucosylated EGF domains. We propose that the GDP-fucose/OFUT1 balance determines the ability of OFUT1 to endocytose and degrade Notch in a manner that is independent of the residues affected by Abru\textit{ptex} mutations in Notch EGF domains.

SUMMARY

The Notch signaling pathway plays an important role in development and physiology. In Drosophila, Notch is activated by its Delta or Serrate ligands, depending in part on the sugar modifications present in its extracellular domain. O-fucosyltransferase-1 (OFUT1) performs the first glycosylation step in this process, O-fucosylating various EGF repeats at the Notch extracellular domain. Besides its O-fucosyltransferase activity, OFUT1 also behaves as a chaperone during Notch synthesis and is able to down regulate Notch by enhancing its endocytosis and degradation. We have reevaluated the roles that O-fucosylation and the synthesis of GDP-fucose play in the regulation of Notch protein stability. Using mutants and the UAS/Gal4 system, we modified in developing tissues the amount of GDP-mannose-deshydratase (GMD), the first enzyme in the synthesis of GDP-fucose. Our results show that GMD activity, and likely the levels of GDP-fucose and O-fucosylation, are essential to stabilize the Notch protein. Notch degradation observed under low GMD expression is absolutely dependent on OFUT1 and this is also observed in Notch Abru\textit{ptex} mutants, which have mutations in some potential O-fucosylated EGF domains. We propose that the GDP-fucose/OFUT1 balance determines the ability of OFUT1 to endocytose and degrade Notch in a manner that is independent of the residues affected by Abru\textit{ptex} mutations in Notch EGF domains.

Running title: GDP/OFUT1 balance regulates Notch stability.

Key words: Notch, GMD, O-fucosyltransferase, Drosophila.
condition, Notch is retained in the endoplasmic reticulum, and the resulting phenotypes are reminiscent of those caused by Notch null alleles (Okajima and Irvine, 2002; Okajima et al., 2005). In contrast, OFUT1 over-expression leads to Notch degradation, probably through the endocytic-lysosome pathway, which finally produces similar phenotypic outcomes (Sasamura et al., 2007). The effects observed in the OFUT1 gain and loss-of-function conditions are not dependent on altered O-fucosylation. Thus, the expression of an OFUT1 mutant protein lacking a functional catalytic domain is sufficient to rescue the endoplasmic retention of Notch observed in Ofut1-/- cells (Okajima et al., 2008). This mutant form binds to Notch and is as effective as normal OFUT1 in the induction of Notch degradation (Okajima et al., 2005; Sasamura et al., 2007). Consequently, the expression of OFUT1 in a Gmd mutant background, therefore in cells depleted of GDP-fucose, is enough for Notch to reach the plasma membrane, and the only phenotypes observed are those related to Fringe function (Okajima et al., 2008). Together, these observations suggest that Notch does not need to be O-fucosylated when it is involved in Fringe-independent processes.

Although Notch glycosylations have been extensively explored regarding Notch-ligand interactions (Shao et al., 2003; Xu et al., 2005; Xu et al., 2007) some intriguing observations require attention. First, so far the focus of O-fucosylation has been centered on Notch EGF repeats, even though they are also present in other proteins (i.e. Delta). Second, an additional O-fucosyltransferase enzyme (OFUT2) has been predicted in the Drosophila genome that could also function in these glycosylation processes (CG14789; Roos et al., 2002) and third, while Notch signaling is impaired in Gmd-/- mutant animals, a clear non-autonomous rescue is finally produced similar phenotypic outcomes. To understand the role of fucose modifications in Drosophila development and in Notch signaling in particular, we analyzed the consequences of modifying the levels of GMD,

MATERIALS AND METHODS

Drosophila melanogaster strains and phenotypic analysis

In a mutagenesis screening designed to identify genes affecting vein differentiation, we isolated one P-GS insertional (Toba et al., 1998) 14 bp upstream from the CG8890 (Gmd) transcription starting site (EP-800) (Fig. 1B; Molnar et al., 2006). We used the following Drosophila UAS lines: UAS-N 85, UAS-N 275. UAS-Ofut1, UAS-iOfut1 and the Gal4 lines pnr-Gal4, dll-Gal4, mb-Gal4, salPE-Gal4 (sal-Gal4 or salPE, Cruz et al., 2009), 638-Gal4 and 253-Gal4 (Callega et al., 1996; de Celis et al., 1999; Barrio and de Celis, 2004) and the P-lacZ reporter lines NlacZ and Espl (mb (de Celis et al., 1998). We also used the N Abruptex alleles Ax 1677 and Ax 151. Gmd deficient wing discs were obtained from Gmd-/-, Gmd-1/2 homozygous and Gmd-1/Gmd-2 heteroallelic mutant larva. Medium and large Gmd clones or mosaic nota were generated in the following genetic backgrounds using the Minute technique: hsFLP1.22; Gmd-/- FRT40A; M(2)z arm-lacZ FRT 40 and l(2)cl-L3 FRT40A/Gmd FRT40A; UAS-FLP/pnr-Gal4, respectively. The sal-Gal4 UAS-Gmd/SMa and sal-Gal4 EP-800/SMa were obtained by meiotic recombination. All stocks not described in the text can be found in Flybase (http://www.flybase.net/). All phenotypes were analyzed at 25°C, unless otherwise stated, and flies were mounted for microscopic examination in lactic acid-ethanol (1:1). Pictures were taken in an Axiohot microscope with a Spot digital camera and processed using Adobe Photoshop.

Generation of Gmd RNAi construct

The Gmd RNAi construct was amplified from the RE50314 clone using the following primers: forward 5'-CCCCGCTTAATTGCAATGTGC-3' and reverse 5'-GAATTCCGGACACCTGCG3'. The PCR product was cloned in the pSt Blue vector (Novagene) and sequenced. It was subcloned using the SacI and BamHI sites in the pHIBS vector (Nagel et al., 2002). Later the Notl/PstI fragment from the pSt Blue Gmd and Pstl/Xhol fragment from the pHIBS Gmd vector were directionally cloned in the pBK SK using the NotI and Xhol sites to obtain the hairpin construct. Finally the Gmd construct was introduced in the pUAST vector using the KpnI and NotI sites.

RT-PCR analysis

Actin and Gmd transcriptional profiles were analysed in third instar control (da-Gal4/+ ) and Gmd expressing larvae (Gmd+/+; da-Gal4/+). 10 larvae of each genotype were collected, total RNA extracted using Trizol reagent (Invitrogen) and cDNA synthesized using the ImProm-II kit (Promega) following manufacturer instructions. PCR analysis was performed using the following protocol: denaturation = 95°C 5 min; 27 cycles = 95°C 45 sec, 55°C 1 min and 72°C 40 sec; final elongation = 72°C 10 min. The following primers were used: Actin forward, 5'-GCCGGACTCGTGACTCTTCG-3' and reverse, 5'-GACGGAGATGTGGCCACCATCGTC-3'; Gmd forward, 5'-CCCCGCTTAATTGCAATGTGC-3' and reverse, 5'-GAATTCCGGACACCTGCG3'.

Drosophila immunohistochemistry and in situ hybridization

We used mouse monoclons anti-Necd, anti-Nintra, anti-Sc and anti-DI (Hybridoma bank), rabbit anti-Sal (dilution 1:200; Barrio et al., 1999), anti-senseless (dilution 1:100; a gift from Hugo Bellen) anti-Serb (dilution 1:200) and anti-bgal (dilution 1:200; Cappel). Secondary antibodies were from Jackson Immunological Laboratories (used at 1:200 dilution). Third instar imaginal discs were dissected, fixed and stained as described in de Celis (1997). Confocal images were captured using a BioRad and LSM 510 Zeiss confocal microscopes. In situ hybridization of imaginal discs was carried out as described in de Celis (1997). Sense and anti-sense digoxigenin-labeled RNA probes were prepared with T3 and T7 RNA polymerase using the RE50314 clone as template.

RESULTS

To understand the role of fucose modifications in Drosophila development and in Notch signaling in particular, we analyzed the consequences of modifying the levels of GMD,
the first enzyme in the de novo biosynthesis of GDP-fucose (Roos et al., 2002). We reasoned that changing locally the levels of GMD should result in variations in the levels of GDP-fucose in mutant cells. Accordingly, this might uncover the requirements of GDP-fucose during development, as well as in Notch synthesis and signaling, irrespective of the O-fucosyltransferase enzymes involved in the transfer of this sugar (OFUT1 or OFUT2) to their potential substrates.

Over-expression of GMD results in phenotypes similar to Notch loss-of-function

Gmd (CG8890) is located in the left arm of the second chromosome in the cytological position 25B5. Gene annotation (http://www.flybase.org/reports/FBgn0031661.html) predicts a single transcript of 1629 nt and a corresponding polypeptide of 395aa (Fig. 1A). Protein sequence comparison showed that GMD is highly conserved among different organisms, with amino acid identities of about 70% with its human and other invertebrate counterparts (data not shown). In situ hybridization analysis revealed that Gmd is expressed ubiquitously in the wing disc throughout larval development (Fig. 1B-D), although in third instar discs its expression is higher in the L3 and L4 proven territories, and appears reduced in the region corresponding to the future wing margin (arrows and arrowhead in Fig. 1D).

P-GS insertions allow the ectopic expression of downstream genes based on the Gal4/UAS binary system (Brand and Perrimon, 1993; Toba et al., 1999). We identified one of these insertions, the EP-800 line (Molnar et al., 2006), inserted 14 bp upstream of the Gmd transcription start site. The combination of EP-800 with a wing pouch specific driver (salPE-Gal4) induces Gmd expression at higher levels in this domain (Fig. 1E and F).

Analysis of adult phenotypes revealed that ectopic Gmd over-expression in the antenna-eye imaginal disc using the eyeless driver (EP-800/ey-Gal4) results in phenotypes reminiscent of Notch loss-of-function situations. Thus, over-expression of Gmd in this combination resulted in a reduction of the eye size and in ommatidial disorganization (Fig. 1G). As expected, the co-expression of an interference RNA directed against Gmd (Gmdi) cancelled the phenotypes generated by Gmd over-expression in this territory (Fig. 1G and G’), indicating that 1) Gmdi specifically and efficiently targets the Gmd message and 2) the eye phenotype observed in the EP-800/ey-Gal4 combination is in fact due to GMD over-expression. The ectopic expression of Gmd in the wing blade using two Gal4 lines expressed at different levels causes phenotypes ranging from strong reductions of wing size (EP-800/rub-Gal4; Fig. 2A and C) to wing notches (638-Gal4/+; EP-800/+; Fig. 2D and F). The expression of a dominant-negative form of Notch, consisting of its trans-membrane and extracellular domains, with these same Gal4 drivers produced very similar adult phenotypes (Fig. 2B and E). The expression of higher than normal levels of GMD in the central wing pouch domain using the salPE-Gal4 driver (Fig. 2I) reduces the size of the wing and alters the pattern of longitudinal veins (Fig. 2G). These alterations were efficiently rescued by the co-expression of a weak activated form of Notch (Fig. 2H), indicating that the increase in GMD function somehow interferes with Notch activation. Although we have not monitored the levels of GDP-fucose in these combinations, these results suggest that correct Notch signaling requires appropriate amounts of GMD and, possibly, of GDP-fucose.

To further confirm that GMD over-expression interferes with Notch signaling, we monitored the expression of Enhancer of split-mb and wingless, two Notch target genes, in the wing disc of these experimental animals. Over-expression of GMD in the wing pouch (salPE-Gal4/EP-800) interrupts the expression of wingless along the dorso-ventral boundary and reduces the expression of a E(spl)mb reporter (Fig. 2J, K for wg and L, M for E(spl)mb). In addition, GMD over-expression induces ectopic wingless expression in cells dispersed throughout the wing blade (Fig. 2K), as well as considerable cell death (data not shown). It is likely that this ectopic Wg signal is the consequence of the cell death induced by the high level of GMD expression (data not shown, Ryoo et al, 2004; Perez-Garijo et al, 2004).
RT-PCR analysis showed that expression of Gmd1 efficiently reduces Gmd message (Fig. 3F) and its induction in different tissues produced adult phenotypes analogous to those caused by reductions in Notch signaling. Thus, the loss of Gmd in the wing blade (638-Gal4; UAS-Gmd1/+ or sal-Gal4/UAS-Gmdi) or in the distal leg (dll-Gal4/UAS-Gmd1) affected vein width and tarsal joint formation, respectively (Fig. 3H, J and M). Notch signaling is also involved in the formation of the sensory organs in the thorax, affecting both the number of sensory organ precursors (SOP) and its differentiation (Schweisguth, 1995). Expression of Gmd1 in the thorax, using the apterus Gal4 driver (ap-Gal4/UAS-Gmd1), prevented the formation of most micro and macrochaetae, and the remaining bristles appeared duplicated or with two shafts and no sockets (Fig. 3K). This phenotype was observed when the expression of Gmd1 was driven in proneural clusters as well (253-Gal4/UAS-Gmd1, data not shown), and it is likely related to the known requirement of Notch in cell-fate assignation and asymmetric cell division during sensory organ formation (Guo et al., 1996).

Accordingly, in a Gmd mutant background (Gmd1/GmdH78), Scute accumulates at higher than normal levels in proneural clusters and several SOP were selected from each cluster (arrows in Fig. 3D). This effect is also detected in the adult, where additional sensory organs appeared in several positions of the thorax (arrows in Fig. 3E). The establishment of the wing margin was only affected when Gmd1 was expressed at higher levels by increasing the number of UAS-Gmd1 copies (638-Gal4; UAS-Gmd1/UAS-Gmd1; Fig. 3P) or when its expression was induced in flies heterozygous for a deficiency encompassing the Gmd locus (638-Gal4; UAS-Gmd1/Df(2L)sc19-4, BL-3813; Fig. 3N). In these conditions the wing margin develops severe notching and the bristles show shaft duplications (Fig. 3N and inset in P).

The loss of Gmd in the central wing pouch region (sal-Gal4/UAS-Gmd1) also interfered with Notch-target gene expression, reducing wg expression and Esplmb reporter activity in this domain (Fig. 3I, L, O and R). To confirm the impairment in Notch signaling detected upon Gmd knockdown we co-expressed Gmd1 with an activated form of Notch (N*Notch) in the wing blade territory using the 638-Gal4 driver. In this situation (638-Gal4; UAS-Gmd1/UAS-N*Notch) the phenotype produced by Gmd1 was almost completely reversed (Fig. 3Q), further implicating Gmd function in events upstream of Notch activation. Interestingly, the reduction of Gmd levels impaired both Fringe-dependent (tarsal joint and wing margin formation) and Fringe-independent processes (sensory organ formation and vein thickness). Since Fringe modulates Notch signaling through O-linked fucose modification of Notch EGF repeats, the global requirement of GMD function implies a role for GDP-fucose synthesis independent of Fringe activity. In summary, the reduction in GMD levels specifically modifies Notch signaling and it does so interfering with every aspect of Notch function in a similar way as reductions of the fucosyltransferase OFUT1 (Okajima and Irvine 2002; Sasamura et al., 2003).

A GMD-dependent process stabilizes the Notch protein

The fact that the phenotypes caused by knocking down Gmd expression were suppressed by an activated version of Notch suggests that GDP-fucose is required either for receptor activation or for its synthesis. Previously, it has been
shown that decreasing the levels of OFUT1 influences Notch trafficking and subcellular localization, causing its retention and accumulation in the endoplasmic reticulum (Okajima et al., 2005). To identify whether Gmd reductions cause similar changes in Notch trafficking, we monitored the localization of Notch in wing discs where Gmd expression was either reduced (sal-Gal4/UAS-Gmdi) or increased (sal-Gal4/EP-800) in the central wing pouch region. Surprisingly, we found that reducing GMD expression strongly diminished the levels of Notch at the apical membrane in this territory (Fig. 4A-D). This reduction was not the consequence of a failure of the antibody to detect unmodified Notch proteins, because it was also observed using antibodies that recognize both the extracellular (Fig. 4B) as well as the Notch intracellular domain (Fig. 4C). Furthermore, the reduction in Notch protein was independent of the transcription of the Notch gene, since the reduction of Gmd does not alter the expression of a Notch reporter consisting in a P-lacZ insertion in Notch that reveals Notch transcription (Supplementary Fig. S1). Finally, the effects produced by the reduction of GMD in Notch levels seem to be specific to this receptor, because other proteins with EGF motifs susceptible to be O-fucosylated, such as Delta or Crumbs, are detected at normal levels and with a normal distribution in discs with reduced Gmd levels (Supplementary Fig. S1 and data not shown).

In order to establish whether Notch was accumulating in a subcellular compartment distinct from the apical membrane, we analyzed the expression of Notch and the apical marker Scribble in confocal transverse sections of wing discs expressing the Gmdi construct in the central wing pouch region (sal-Gal4/UAS-Gmdi). In these sections, we failed to detect Notch accumulation in any subcellular compartment, and only a very weak signal was detected at the apical cell membranes (Fig. 4D). The failure to detect Notch was not the result of a general disturbance in the apico-basal polarity of the cell, as other proteins, like Scribble, Delta, Discs large and Crumbs were normally localized at apical regions of Gmdd-expressing cells (Fig. 4D and data not shown). Therefore, we suggest that the normal processing of Notch during its transport to the apical cell membrane fails when the level of GMD is lowered, or alternatively, that Notch endocytosis and its subsequent

Figure 3. Gmd knockdown generates Fringe-dependent and independent Notch phenotypes. (A, B, C) Wg expression (green) in Gmd1 homozygous mutant wing disc (A) and in two different wing discs with Gmd1 clones (labeled by the absence of Gal in B and C). Expression of Wg at the dorso-ventral boundary is rescued by surrounding wild type cells (B, marked by Gal expression, red) and only in very large mutant clones the loss of Gmd1 eliminates Wg expression (arrowhead in C). (D) Senseless and Scute expression in the notum region of wing imaginal discs from control (left) and Gmd1/Gmd1 (right) mutant larva. Anterior posterior (APA), posterior dorso-central (PDC) and posterior scutellar (PSC) proneural cluster are indicated (arrow). Note the presence of extra SOPs in the Gmd proneural clusters. (E) Dorsal thorax of a regional mosaic Gmd mutant animal. Mitotic recombination was induced expressing FLP in the notum with the Pannier-Gal4 driver (U2;cl-L3 FRT40A/Gmd1 FRT40A; UAS-FLP/Pnr-Gal4). Note the reduction in bristle number and the appearance of an extra macrochaetae in the APA domain (arrowhead). (F) RT-PCR analysis of control (+/+; da-Gal4/+; and Gmdi-expressing third instar larvae (UAS-Gmdi; da-Gal4/+). (G, H, J, K, M, N, P, Q) Adult phenotypes. (I, L, O, R) Confocal images of wing discs of third larva stage. (G) Control wild type wing. (H) Expression of Gmdi in the wing blade produces wing margin defects and vein thickening (arrowhead, 638-Gal4/+; UAS-Gmdi/+). (I) Expression of Gmdi in the spalt domain produces vein thickening (sal-Gal4/UAS-Gmdi). (K) Gmdi expression in the thorax inhibits bristles formation and generates shaft duplications (arrow). (M) Gmdi impairs joints formation in the distal leg (arrow, dll-Gal4/UAS-Gmdi). (N) Stronger notching and vein thickening is obtained when Gmd1 is expressed in a Gmd mutant background (arrowhead, 638-Gal4/+; UAS-Gmdi/Df(2L)sc19-4). (P) A similar wing phenotype is produced increasing the amount of Gmdi expressed (638-Gal4/+; UAS-Gmdi/UAS-Gmdi). In these cases shaft duplications are clearly observed in the sensory organs of the wing margin (inset). (O) Expression of Gmdi with the N intra construct, N 85.1, fully reverts the margin and vein thickening phenotypes caused by loss of Gmd (638-Gal4; UAS-Gmdi/UAS-N 85.1). (I, L) Expression of Wg at the dorso-ventral boundary (green) is lost in the distal wing of sal-Gal4, UAS-Gmdi (L) compared with control disc (I). (O, R) the expression of the Notch-activity reporter E(spl)mb-cdc2 (green in O and R) is reduced by Gmdi in sal-Gal4, UAS-Gmdi/E(spl)mb-cdc2 discs (R) compared to wild type controls (O).
degradation are augmented under these conditions. Ectopic expression of GMD also affected Notch localization (Fig. 4E). However, in these discs apico-basal polarity was severely disturbed and the epithelial integrity is lost as evidenced by the extrusion of dying cells. These observations suggest a more general effect of GMD excess in protein trafficking that could be caused by lowering the amount of GDP-L-mannose, the substrate of GMD, which plays key roles in the secretory pathway.

An OFUT1/GDP-fucose sequential mechanism controls Notch stability

OFUT1 activity is required for Notch protein trafficking from the ER to the cell membrane. To perform this function, the O-fucosyltransferase activity of OFUT1 is not essential (Okajima et al., 2008), and therefore it has been proposed that OFUT1 acts as a chaperone that prevents inappropriate folding of Notch (Okajima et al., 2005). Thus, OFUT1 reduction leads to Notch accumulation at the ER, blocking its processing and apical localization and, in consequence, preventing its potential activation (Okajima et al. 2005). In agreement with previous results, expression of an Ofut1 interference RNA (iOfut1) in the central wing pouch domain (sal-Gal4/UAS-iOfut1) increases Notch accumulation in intracellular domains, causing a reduction in Notch activity and consequently Notch loss-of-function phenotypes in the adult wing (arrows Fig. 5A, B and B’; Okajima and Irvine, 2002). Likewise, loss of GMD function also reduces Notch signaling and causes a vein thickening phenotype, although in this case the defect is caused by a decrease in Notch protein levels (arrows Fig. 5C, D and D’). Co-expression of the two RNAi constructs, iOfut1 and Gmdi, also resulted in adult wing phenotypes compatible with reductions of Notch signaling (Fig. 5E), but notably, in this case Notch protein accumulated as in iOfut1-expressing discs (compare Fig. 5F-F’ with B-B’). These results suggest that the GMD-dependent process related to Notch stability occurs after the traffic of Notch from the ER to the Golgi apparatus and that OFUT1 is essential for this route of Notch degradation. These observations indicate that an unbalance of GDP-fucose in the cell facilitates Notch degradation, likely through an endocytic-lysosome pathway assisted by OFUT1, further supporting the notion that OFUT1, acting from the extracellular milieu, directs the endocytosis and degradation of Notch (Sasamura et al., 2007).

Figure 4. Expression of Gmdi reduce Notch protein levels. (A) Confocal image and the corresponding Z section of a control third instar wing disc stained for Notch (green) and Scribble (red) proteins. Both proteins localize preferentially at the membrane apical region. (B, C) Expression of Gmdi in the central wing disc region (B, sal-Gal4, UAS-Gmdi) or in a central stripe (dpp-Gal4 UAS-GFP/UAS-Gmdi) decreases the amount of Notch protein detected using two different antibodies directed against the extracellular domain (Ne, green in B) or the intracellular domain (Ni, red in C). (D) Notch (green) is not accumulated in intracellular compartments in cell expressing the Gmdi construct. The localization of Scribble (red) is not altered in this condition (sal-Gal4, UAS-Gmdi). (E) Over-expression of GMD in the central wing disc region (sal-Gal4/ EP-800) disrupts the epithelial structure.

Supplementary Figure S1. The reduction of GMD does not affect Notch transcription or Delta expression. (A, A’, A’’) Expression of Gmdi in the Spalt domain (Spalt, red) does not alter the expression of the Notch P-lacZ insertion reporter (NlacZ; green). (B, B’, B’’) Delta (green) localization and stability are not modified by the reduction of GMD in the central wing imaginal disc region (Spalt, red; sal-Gal4 UAS-Gmdi/+).
**Abruptex alleles are subject to OFUT1 and GMD-dependent control**

To define the EGF domains involved in the GMD- and OFUT1-dependent mechanism that controls Notch levels, we analyzed the effect of changing the amount these proteins in Abruptex mutant backgrounds. Abruptex alleles are caused by point mutations within EGF repeats 24 to 29 of the Notch extracellular domain and modify Notch activation in a ligand-dependent manner (Kelley et al., 1987; de Celis et al., 1994; Brennan et al., 1997; de Celis and Bray, 2000). In addition, *in vitro* binding assays showed that the Abruptex region specifically interacts with the ligand binding region of Notch, and this has been proposed to serve as a barrier for the ligand-induced activation of Notch (Pei and Baker, 2008).

*Ax*1672 viable hemizygotes show higher levels of Notch signaling, specifically affecting wing growth and vein development (Fig. 6A). *Gmdi* expression using the *nubbin*-Gal4 driver in the wing blade region of *Ax*1672 (*Ax*1672; *nub*-Gal4/ UAS-*Gmdi*), as well as in *Ax*381 background (data not shown), completely suppressed the *Ax* gain-of-function phenotype. Moreover, the veins appeared thickened, suggesting that reductions in GMD function produce a Notch deficiency even in the presence of hyper-activable Notch proteins (Fig. 6B). As in Notch wild-type conditions, the levels of *Ax* mutant proteins are also decreased when *Gmdi* is expressed in the central wing pouch domain (*Ax*1672; *sal*-Gal4/UAS-*Gmdi*; Fig. 6E-F), demonstrating that the GMD-dependent control mechanism is independent of the EGF domains mutated in these *Ax* alleles. A similar reduction in Notch activity was observed knocking-down *Ofut1* function in *Ax* backgrounds (*Ax*1672;*sal*-Gal4/ UAS-*iOfut1*; Fig. 6C). Conversely, the over-expression of GMD using the *sal*-Gal4 driver in *Ax* mutants enhances the loss of vein phenotype produced by the *Ax* allele (*Ax*1672; *sal*-Gal4/EP800; arrows indicating L3 and L5 in Fig. 6D), and in this case the mutant Notch protein appeared at higher than normal levels and in a punctuate distribution (Fig. 6G).

**DISCUSSION**

Notch is a glycosylated transmembrane receptor with central roles during development (Artavanis-Tsakonas et al., 1999). The Notch protein suffers several glycosylation steps that affect Notch-ligand interactions (Xu et al., 2005; Bray, 2006;...
Xu et al., 2007). Classical sugar modifications occur in the ER and Golgi apparatus by a battery of glycosyltransferases (Helenius and Aebi, 2001). So far, two of these enzymes have been involved in Notch synthesis. The O-fucosyltransferase neurotic/Ofut1 (Sasamura et al., 2003; Okajima and Irvine, 2002), which potentially transfers fucose to the EGF domains (Panin et al., 2002; Shao et al., 2003; Xu et al., 2005) and the β1,3N-acetylglucosaminyltransferase Fringe, which elongates this priming moiety by adding N-acetylglucosamine units (Lei et al., 2003; Shao et al., 2003).

A recent report has uncovered a quality control mechanism that affects Notch synthesis, which unexpectedly, relies on a non-enzymatic activity of OFUT1 (Okajima et al., 2005; Okajima et al., 2008). Besides this function, Sasamura et al. (2007) showed that OFUT1 acting from the extracellular environment was able to down regulate Notch by enhancing its endocytosis towards the degradative pathway. In addition, our results show that it is the balance of GDP-fucose and OFUT1 what modulates Notch signaling by regulating the amount of functional Notch protein at the cell surface.

The phenotypes caused by changes in GMD activity are related to loss of Notch signaling

Gmd null alleles and OFUT1 mutant forms have been used to assess the specific dependency of Notch signaling on O-fucosylation (Okajima et al., 2005; Sasamura et al., 2007; Okajima et al., 2008). This has lead to the conclusion that O-fucosylation is exclusively required for Fringe-dependent processes (Okajima et al., 2008). We found that over-expression of Gmd leads to a reduction of Notch activity. This effect is reverted by a weak constitutively active form of Notch, indicating that GMD over-expression interferes upstream of Notch activation. GMD is the enzyme that performs the first step in the synthesis of GDP-fucose in Drosophila (Roos et al., 2002). In accordance to this, no GDP-fucose is detected in Gmd null mutant embryos (Sasamura et al., 2007) and wing discs from these animals show no expression of seg at the dorso-ventral boundary (Micchelli et al., 1997; Okajima and Irvine, 2002; Okajima et al., 2008). Clonal analysis has shown a non-cell-autonomous requirement of Gmd, preventing the analysis of GMD function in mosaics. By expressing the Gmd RNAi construct in large wing domains, we were able to analyze the cellular consequences of Gmd and GDP-fucose reductions. In this background there is a strong reduction of Notch protein levels that was not detected in mosaic Gmd tissues (data not shown). Remarkably, the adult phenotypes produced by lowering GMD affected both Fringe-dependent and independent processes, and these effects were completely reverted by the expression of a Notch intracellular construct, indicating that under low GDP-fucose Delta/Serrate activation of Notch is impaired.

An appropriate balance between GMD activity and OFUT1 modulates Notch stability

Our GMD knockdown conditions reduce Notch protein levels without noticeable changes in Notch gene expression. Delta levels or apico-basal polarity. Thus, appropriate levels of GDP-fucose are specifically essential for Notch stability, but not for the accumulation or function of other proteins that contain EGF domains. The observation that Notch is not retained in the ER in GMD-depleted cells additionally confirms that OFUT1 working as a chaperone, and not O-fucosylation, is necessary for Notch trafficking (Okajima et al., 2005; Okajima et al., 2008). But how is Notch destabilized under our experimental conditions? Sasamura et al. (2008) have recently identified a second role of OFUT1 in Notch signaling that might explain our observations. They showed that OFUT1 acting from the extracellular space enhances Notch endocytosis promoting its degradation (Sasamura et al., 2007). Interestingly, loss of Gmd fails to promote Notch degradation when the levels of OFUT1 are lowered, indicating that OFUT1 is required for Notch degradation in conditions of low levels of GDP-fucose. Okajima et al., (2008) showed that in Drosophila S2 cells, where OFUT1 is endogenously expressed, the treatment with a dsRNA against Gmd produces a reduction in the secretion of Notch. These observations suggest us that under acute GDP-fucose restrictions, there is an increment in the ability of OFUT1 to remove Notch from the cell surface leading to its degradation. Our in vivo analysis directly implicates the level of GDP-fucose in the ability of OFUT1 to induce the removal of Notch from the cell surface.

In our view, during Notch synthesis, OFUT1 sequentially associates and facilitates the folding of the Notch extracellular domain. The O-fucosylation performed by OFUT1 stabilizes the conformation of particular EGF domains allowing the progression of Notch through the secretory pathway (Okajima et al., 2005; Okajima et al., 2008). The association of Notch with OFUT1 decreases after O-fucosylation and, consequently, only small amounts of OFUT1 reach the plasma membrane. In this scenario, the endocytosis and degradation promoted by extracellular OFUT1 are kept at low levels (Sasamura et al., 2007). However, if O-fucosylation is not correctly executed, for instance due to the lack of GDP-fucose, higher levels of OFUT1 could progress to the plasma membrane in association with Notch, inducing its rapid endocytosis and posterior degradation. Such a mechanism might ensure that only correctly modified Notch proteins are retained in the membrane ready for ligand interactions.

Which Notch domains are involved in GMD/OFUT1 stabilization?

Several EGF repeats have been implicated in Notch-ligand binding and Fringe activity (Xu et al., 2007). One of them, the Abruptex region, has been shown to be part of intramolecular contacts involving EGF 11-12 and EGF 22-27 that competitively modulates Notch-ligand interactions (Pei and Baker, 2008). The low stability of Notch protein observed with our Gmdi construct and its retention in OFUT1-depleted cells are also observed in Abruptex backgrounds (Axβ172 and Axβ61). The suppression of the hyperactivation properties of these alleles suggests that the OFUT1-dependent mechanism for Notch downregulation proposed here and the previously reported OFUT1-assisted folding of Notch (Okajima et al., 2005) are also operative in these Notch mutants. Thus, our observations indicate that the EGF domains mutated in Abruptex alleles are not involved in the structural roles of OFUT1 during Notch trafficking. It is worth mentioning that increments in GMD levels augmented the Notch gain-of-function phenotype and protein accumulation in Ax background, while the same treatment resulted in a reduction of Notch activity in normal discs. The opposite responses of Ax and wild type Notch proteins to higher than normal levels of GMD suggest that a
limiting aspect of the hyperactivity observed in Ax proteins is dependent on the presence of GDP-fucose. Thus under high GDP-fucose Ax proteins seem to be less susceptible to OFUT1-induced endocytosis and degradation than wild type Notch. Alternatively, GMD over-expression could reduce the amount of GDP-L-mannose, the substrate of GMD, which is required in a control and sorting mechanism operating in the secretory pathway (Helenius and Aebi, 2001). Perhaps Ax proteins and not wild type Notch partially bypass this GDP-L-mannose-dependent control mechanism resulting in the activation or blockage of Notch signaling, respectively.

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