Supporting Online Material For:

Chaperone Activity of Protein O-fucosyltransferase 1 Promotes Notch Receptor Folding

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Materials and Methods

DNA constructs

Previously described plasmids used include pMK33-fng:HisHA (S1), pRmHa-Notch (S2), and pRmHa-N:AP, pRmHa-DL:AP, pRmHa-SER:AP (S3). For S-tagged constructs of Ofut1, a 5’-AAAGAAAACCGCTGCTGCGAAAAAACTTTGAACGCCAGCAGCCAGCATGGACTCG-3’ sequence encoding the KETAAAKFERQHMDS S-tag peptide was inserted by PCR between the Bip sequence and the 22\textsuperscript{nd} amino acid of OFUT1 in plasmid pMT-Bip-Ofut1 (S4) to generate plasmid pMT-Bip-S-tag-Ofut1. To generate S-tag-Ofut1\[HEEL and S-tag-Ofut1\[SH constructs, the XhoI ER- stop OFUT1 primer (CCTGTTCTCGAGTACACGTTTGCTT) and XhoI OFUT1SHRV primer (CGACGGCTCGAGTTATGTTATTCATCATG), respectively, were used with a Fut535F primer (5’-GCTGCCAAGTGGCAGACCAAA-3’) and a plasmid encoding the wild type Ofut1 sequence as a template for PCR. The Eco47III/BglII fragment of the PCR products was then used to replace the corresponding fragment of the S-tag-Ofut1 wild type construct. For mouse Pofut1 constructs, a fragment corresponding to amino acids 27-393 was inserted between the BglII and XhoI sites of pMT-Bip-V5HisA to generate pMT-Bip-mPofut1. For R245K and R245A mutants, mutagenesis was carried out by PCR-based methods (S5) with Pfu Turbo DNA polymerase (Stratagene). To construct pRmHA-Ofut1, a BglII/XhoI fragment from pUAST-Ofut1 was inserted into BglII/Sall sites of pRmHa3b-GalNAcT-fringe, which resulted in
the replacement of GalNAcT-fringe fragment with Ofut1. The pRmHa-Ofut1:V5:His construct has been described previously (S6). To generate C-terminal V5:His tagged constructs of pRmHa3-Ofut1R245K, pRmHa3-Ofut1R245A and pRmHa3-Boca, the V5-His sequence from pMT-Bip-V5HisA (Invitrogen) was introduced in frame at the end of the coding sequence by PCR. GFP:KDEL was made by fusing a KDEL-encoding sequence in frame to C-terminal end of the GFP:V5 sequence from pMT-Bip-GFP:V5His vector (Invitrogen) by PCR using GFP-Fw primer (AGCGTGACCACATGTCCTCTTC) and ER-GFP-Rv primer (cagcgggtttaaactcAAGCTCTGGTCTCTCGCTaccgtACGCGTAGAATCGAGA) and the cloning a NotI/Pmel fragment of the PCR product into those sites in pMT-Bip-GFP-V5His to generate pMT-Bip-GFP:V5:KDEL. Site-specific mutagenesis of Notch was conducted as described previously (S7), using as primers ACGGGCACCAGTGTCGAGATCAATATCC (C599Y), CGGAATGGAGCTGCCTGTTCATGTTGC (EF23, S922A) CAGAACGGTGGAGCTCTGCTTCCAGATGGAT (EF24, T960V) CAGAACGGTGGAGCTCTGCTTCCAGATGGAT (EF25, T998V) TTAACGAGCTGCGCTTTCATGCGATG (EF26, S1037A) TTGAACGAGCCTGCTGCGAGCAGAAAA (EF27, T1074V) GAGAACGAGCGGCCTGCTGCGAGCAGAAAA (EF28, T1113V) TGCAAAATGCGCGCTGCAAGTACGCGATCAATATCC (EF29, S1273A). All mutations and construct junctions were confirmed by DNA sequencing.

Drosophila Stocks and cross
For RNAi experiments in wing discs, *ptc-Gal4 UAS-GFP* flies were crossed to *UAS-iOfut1[12.3]* flies (*S4*); progeny were kept at 30°C during larval stages. For marking endosomes, *UAS-rab5:GFP* and *UAS-rab7:GFP* transgenes were used (*S8*). As negative controls, *ptc-Gal4 UAS-GFP* flies were crossed to wild-type flies, and to flies expressing a fold-back construct for a different glycosyltransferase, CG8668 (*S9*) (Y. Lin and KI, unpublished).

Two independent insertions of *UAS-Ofut1^{R245A}* were isolated, *UAS-Ofut1^{R245A}[20.1]* on the second chromosome and *UAS-Ofut1^{R245A}[28.3]* on the third chromosome.

Clones of cells mutant for one gene and co-expressing another were generated by the MARCM method (*S7, 10, 11*). In brief, this involves repression of the expression of a gene of interest under UAS control by expression of the Gal80 transcriptional repressor. Stocks are designed such that the same mitotic recombination event that generates mutant clones also leads to loss of the Gal80 repressor, allowing expression of the gene of interest (in this case *Ofut1^{R245A}*).

Stocks used for this experiment were *y w hs-Flp[122] tub-Gal4 UAS-GFP:nls; FRT42B[G13] hs-p:Myc tub-Gal80[LL2]/CyO* females (*S12*), which were crossed to *FRT42B[G13] Ofut1^{4R6}/CyO (S13); UAS-Ofut1-R245A[28.3]/TM6B* males.

To generate *kuz* mutant clones, *w; FRT40A kuz^{2524} / CyO* males were crossed to *y w; hs-Flp[122]; 2x[hs-p:Myc] FRT40A* females. To generate *Dl Ser* double mutant clones, *y w hs-Flp[122] tub-Gal4 UAS-GFP:nls; UAS-y^{7}/CyO; FRT82B tub-Gal80[LL3]/TM6B* females were crossed to *FRT82B Dl^{rev^{10}} e Ser^{RX106}/TM6B*. To generate *Ps* mutant clones, *hs-Flp[122]; arm-LacZ FRT82B* flies were crossed to *y w hs-FLP[122]; P[w+]/Ps^{C1} FRT80B/TM6B*. To generate *Ofut1^{4R6* (null) mutant clones *y ; hs-Flp[122]; FRT42B[G13] Ofut1^{4R6}/CyO* females were crossed to *FRT42B[G13] bw sp* males. Progeny were heat-shocked at 38°C for 1h during the first instar to induce clones by Flp/FRT-mediated somatic recombination (*S14*).
Mutation of *Gmd*

*l(2)SH1606 (Gmd<sup>SH</sup>)* was obtained from S. Hou (<i>S15</i>). This line contains an insertion of a P transposable element just upstream of the *Gmd* translation start site. Comparison of homozygotes and hemizygotes suggests that it is a hypomorphic allele. To generate null alleles of *Gmd*, we took advantage of the observation that excision of P elements is often associated with deletion of flanking DNA (imprecise excision). *Gmd<sup>SH</sup>* flies were crossed to a source of transposase, and progeny were then screened for excision of the P insertion by loss of the white<sup>+</sup> marker gene, and then screened for imprecise excisions by Southern blotting and PCR. Analysis of the *Gmd<sup>l</sup>* isolate revealed that it deletes DNA including nucleotides 65 to 666 of the transcription unit, as well as all of the first and most of the second intron (The sequence across the deletion is ATGC<sup>64</sup> catgat gaataaatatatctaaggtaataactaa tccacctaa ctctttG<sup>667</sup> CCTG, where upper case indicates nucleotides in the wild-type GMD coding sequence, and underlining indicates nucleotides in the wild-type GMD intron. The other nucleotides are novel, and come from P element sequences or DNA repair. This removes DNA encoding amino acids 2 to 202, which are highly conserved from *C. elegans* to mammals. In addition, the deletion results in introduction of a stop codon after the first two amino acids (MP), and thus is expected to be a null allele. Genetically it is more severe than *Gmd<sup>SH</sup>* in terms of its influence on wing growth and its stage of lethality.

Cell culture and transfection

For staining and Western blotting experiments, S2 cells were transfected in 24-well tissue culture dishes with 1.6 µg of plasmids using 8 µL Cellfectin (Invitrogen). For AP secretion and ligand binding experiments, transfection was conducted as described previously (<i>S6</i>). Expression of
transgenes under metallothionein control was induced by treatment with with 0.7 mM CuSO₄ for 24h-36h. dsRNA treatment for RNAi experiments was performed as described previously (S4) unless otherwise noted.

**Cell and Tissue staining**

Antibodies used include mouse anti-α-Tubulin (DM1A, Sigma), mouse anti-HSP70 (Stressgen), mouse anti-KDEL (Stressgen), mouse anti-Golgi (Calbiochem), mouse anti-V5 (Invitrogen), rabbit anti-S-tag (Immunology Consultants Laboratory), mouse anti-Notch (C17.9C6, DSHB), rat anti-Notch (Rat8, S. Artavanis-Tsakonas), rabbit anti-Notch (Intracellular Notch, E. Giniger), rabbit anti-placental alkaline phosphatase (Zymed), mouse anti-placental alkaline phosphatase (Sigma), mouse anti-Crumbs (Cq4, DSHB), guinea pig anti-Boca (S16), mouse anti-FLAG M2 (Sigma), mouse anti-Delta antibody (C594.9B, DSHB), rat anti-Serrate, rat anti-DCAD2 (DSHB), mouse anti-Wingless (4D4, DSHB) and guinea pig anti-OFUT1 (S6). Cell surface staining in discs was also conducted with Alexa fluor 568-conjugated Concanavalin A (Con A) (Molecular Probe).

For imaginal disc staining, larva were dissected at late 3rd instar and subjected to immunofluorescent staining as described previously (S17). In situ hybridization was carried out as described previously (S18), using in vitro transcribed Notch (nucleotide 3883-5322) as a probe. This template was prepared by PCR using the following primers:

T3Notch3883F (T3 sequence is underlined)

5’-AATTAACCCTCACTAAAGGGGAGGGGCGACATCAACGAGTG CCTA-3’

T7Notch5322R (T7 sequence is underlined)

5’-TAATACGACTCACTATAGGGGACGCGCATGTGCCCGCTTTCTTTTG-3’

For S2 cell staining, cells were fixed for 10 min with 4% formaldehyde (Polysciences Inc.#18814), permeabilized with phosphate buffered saline (PBS, 10mM Sodium Phosphate,
2.7mM KCl, 137mM NaCl) containing 1% BSA and 0.1% TritonX-100 (PBST-BSA), and incubated with appropriate antibodies in PBST-BSA. For cell surface Notch staining (Fig. 2), following fixation, cells were instead washed with PBS and incubated with Rat anti-extracellular Notch (Rat8; 1:2000 dilution) in PBS for 40 min. After 3x5 min. washes with PBS, cells were then incubated with Alexa555 conjugated Goat anti-rat IgG (Molecular Probes; 1:50 dilution) in PBS for 30 min. After washing with PBS, cells were then permeabilized with PBST-BSA for 5 min, then stained with mouse anti-intracellular Notch Ab (C17.9C6; 1:1000) in PBST-BSA according to conventional methods. To better discriminate between subcellular markers in S2 cells (Fig. 3), S2 cells were plated on ConA-treated slides and allowed to spread for 1-2h (S19). Tissue culture slides (8 chamber; Falcon#354118) were treated by coating with 0.5 mg/ml of Con A (from Canavalia ensiformis; Sigma#L7647) for at least 2h.

**Western blotting**

For the experiment in Figure 2F, S2 cells were lysed in urea-SDS buffer (8 M urea, 5% SDS, 40 mM Tris-HCl pH 6.8, 0.1 mM EDTA and protease inhibitors) (S20) for preparation of total cell lysates. For other experiments, cell lysates were prepared in 1% Triton X-100 buffer (50 mM Hepes pH 7.0, 150 mM NaCl, 1% Triton X-100 and protease inhibitors). Samples were boiled with DTT and applied to 5-15 % gradient SDS-polyacrylamide gels, unless otherwise noted. Proteins were then transferred to Immobilon-P membrane (Millipore), and stained with primary antibodies as indicated in the figures. For detection, appropriate HRP-conjugated secondary antibodies (1: 50,000 dilution; Amersham Bioscinece) and SuperSignal West Femto Maximum Sensitivity Substrate (PIERCE) were used.

**Enzyme activity assays**
O-fucosyltransferase activity analysis using an EGF domain from Factor VII was performed as described previously (S4). For the assay in Fig. S3D, the concentrated culture media containing secreted OFUT1 was used as an enzyme source. For assays with V5-His tagged OFUT1 (Fig.S3B), OFUT1 was affinity-purified from the culture media using anti-V5 agarose beads (Sigma), and the enzyme-bound beads were used as the enzyme source.

O-fucosyltransferase activity analysis using N:AP as a substrate (Fig. 4E) was performed as follows: 100 OD/min activity of N:AP or Fc:AP (control) was purified on anti-human placental alkaline phosphatase Ab-conjugated agarose beads (Sigma) and incubated with affinity-purified OFUT1:V5His, Ofut1R245A:V5His or the same amount of BSA as a control. To enable detection of small amounts of activity, 0.1 mM of GDP-[14C]fucose was used without dilution with unlabeled GDP-fucose (unlike the assays on Factor VII EGF).

AP secretion assays

To assay the influence of OFUT1 and Fringe on secretion (Figs 5B, S4), pRmHA N:AP, Dl:AP or Ser:AP plasmids (S3) were transfected into S2 cells together with a copia-Renilla plasmid (S2I) or pMK33-firefly luciferase as an internal control. For the experiments with RNAi (Fig. 2), dsRNA for Ofut1 was co-transfected with plasmids so that the ratio between dsRNA and plasmid was 1:2. After 48h of incubation, protein expression was induce for 48h with CuSO4 as described above. To quantify the level of secreted AP in the culture media, endogenous AP activity was heat-inactivated by incubating at 65°C for 30 min, and AP activity was assayed using 1 mM 4-methylumbelliferyl phosphate (Molecular Probes) as a substrate in 1 M diethanolamine, 1 mM MgCl2, at 37°C for 1h and the fluorescent products were measured in a Fusion universal microplate analyzer (Packard). To normalize the transfection activity, sea pansy luciferase or firefly luciferase activity from cell lysates was measured using dual-luciferase reporter assay
system (Promega) or steady-glo luciferase assay system (Promega), respectively. Western blotting was also done, and confirmed that amounts of N:AP produced by cells were similar.

**Notch - OFUT1 binding assay**

pRmHa-Fc:AP, pRmHa-N:AP, pRmHa-Dl:AP or pRmHa-Ser:AP were co-transfected into S2 cells either with pRmHa-Boca:V5:His (control), pRmHa-Ofut1:V5:His or pRmHa-Ofut1^{R245A}:V5His. The culture media were recovered 20 h after induction and centrifuged to removed cell debris. The supernatant was then incubated at room temperature with monoclonal anti-human placental alkaline phosphatase-agarose (8B6; Sigma). After 1h, the beads were pelleted and briefly washed 3 times with HBSS. Protein on the beads was eluted with SDS-loading buffer with DTT and analyzed by Western blotting.

**Ligand binding Assay**

Cell-based ligand binding assays were conducted as described previously, with amounts of N:AP or mutant derivates equalized by normalization to AP activity (S3, 6).
Supplemental Figure legends

Figure S1. Additional characterization of Notch accumulation in OFUT1-depleted cells

Portions of wing imaginal discs are shown, with dorsal up and anterior left. Panels marked prime show separate channels of the same disc. Staining with an anti-Notch antibody in A-C and E-H reveals the localization and levels of Notch protein (magenta). A) Wing disc expressing a distinct glycosyltransferase unrelated to Notch signaling (CG8668) under ptc-Gal4 control (ptc-Gal4 UAS-GFP UAS-iCG8668[A]) demonstrates that the elevation of Notch levels is not a non-specific effect of ptc-Gal4, UAS-GFP, or expression of double stranded RNA. B) Disc with clones of cells mutant for Ofut1<sup>4R6</sup>, marked by absence of OFUT1 antibody staining (green). Notch levels are elevated within the clones (arrows), as in the RNAi situation (Fig. 1B). Vertical sections are shown at bottom. C) In a disc expressing iOfut1 under ptc-Gal4 control, apical Notch is localized in the ER instead of the adherens junctions in iOfut1 expressing cells (left of the dashes, see Fig. 1). Serrate (green) is expressed by dorsal cells and not by ventral cells, but its localization appears similar in both normal and iOfut1-expressing dorsal cells, and it localizes to the apical membrane. D) ptc-Gal4 UAS-Dl UAS-iOfut1 wing disc, stained with ConA (cyan) to mark the cell surface, and with antibodies against Delta (red). Delta was expressed under ptc-Gal4 control in this experiment because Notch signaling is required for Delta expression in the wing. ConA was used to mark the surface in this experiment because the best available antibodies for Delta and Notch are both made in mouse. Double staining reveals that Delta can be secreted to the cell surface. E-H) Show discs from animals expressing iOfut1 under ptc-Gal4 control, and are focused on the ventral edge of the disc near the A-P border. Notch accumulates in the ER in OFUT1-depleted cells (E), but it does not overlap with F) Golgi (green), G) Rab5:GFP (green), or H) Rab7:GFP (green). In (G) and (H) Rab:GFP fusion proteins are only detected in anterior (ptc-Gal4-
expressing) cells because they are under UAS control.

**Fig. S2 ER localization of Notch in S2 cells**

S2 cells transfected with expression vectors for Notch (red) and GFP:KDEL (green), and also stained for F-actin with phalloidin (blue). Actin staining marks the cell periphery.

**Figure S3. Additional characterization of R245A mutants**

A) Media and cell lysates were prepared from S2 cells transfected with vector alone (Mock), pRmHa-Ofut1 (OFUT1), or pRmHa-Ofut1R245A (OFUT1^{R245A}). Samples were subjected to SDS-PAGE on 10% polyacrylamide gels and stained with anti-OFUT1 antisera. The major upper band of OFUT1 in cell lysates co-migrates with secreted OFUT1. OFUT1^{R245A} exhibits the same profile, suggesting that its structural integrity is maintained. An additional minor band with similar mobility is also sometimes visible. B) Assays on a Factor VII substrate of OFUT1 activity in conditioned media from S2 cells (Con.) or S2 cells expressing wild-type (WT) or R245 mutant forms of OFUT1. A C-terminal V5 tag allows all three forms to be secreted, and Western blotting of conditioned media with anti-V5 (below) shows that all three forms are secreted with similar efficiency. C) Culture media of S2 cells transfected with vector alone (Mock), pMT-Bip-mPofut1 (Bip-mPofut1), or with pMT-Bip-mPofut1^{R245A} (Bip-mPofut1^{R245A}) were subjected to SDS-PAGE on 10% polyacrylamide gels and blotted to nitrocellulose membrane. Total protein was visualized with SYPRO Ruby protein blot stain (Molecular Probes). Band corresponding to mPofut1 is indicated (arrow); note the similar level of secretion between mPofut1 and mPofut1^{R245A}. D) Conditioned media from samples prepared as in (C) was concentrated and analyzed for O-FucT-1 activity on a Factor VII substrate.
**Fig. S4 Additional characterization of OFUT1 chaperone activity**

A) A co-immunoprecipitation experiments with V5 tagged-OFUT1 and Fc:AP, N:AP, DL:AP or SER:AP. The indicated proteins were co-expressed in S2 cells, and isolated from conditioned media. Input amounts of OFUT1 is shown by western blot (top) and of AP tagged proteins by AP assay. Co-immunoprecipitation (co-IP) is shown by the western blot at bottom. Binding of OFUT1 to DL:AP is detectable, but weaker than binding to N:AP. B) Relative secretion of N:AP when co-expressed with the indicated proteins in otherwise normal S2 cells, expressed as a normalized ratio of AP to luciferase activity. Overexpression of wild-type or mutant OFUT1 promotes Notch secretion, while control proteins (Boca and Fringe) do not. C) Binding of wild-type or mutant forms of N:AP, or as a negative control, Fc:AP, to Serrate-expressing (left of each pair of bars) or control S2 cells (right of each pair). AP fusions were transfected into wild-type (OFUT1-expressing) S2 cells, and where indicated cells were co-transfected with OFUT1^{R245A} or Boca. Equal amounts of protein (4000 mOD/min AP activity) were used in each case.

**Figure S5 Model for influence of OFUT1 on Notch folding**

A) We propose that wild-type OFUT1 binds transiently to individual, folded EGF domains of Notch in the ER. This binding helps to prevent inappropriate associations of those EGF domains with other regions of Notch during folding of the extracellular domain, thus allowing the appropriate tertiary structure to develop. OFUT1 also fucosylates these EGF domains, this might occur after folding (as depicted), or simultaneously with folding. B) In the absence of OFUT1, individual EGF domains can fold, but inappropriate interactions occur. These inappropriate interactions could interfere with the folding of other EGF domains, resulting in partially folded Notch, or result in the development of inappropriate tertiary structure even if all individual EGF domains fold correctly (mis-folded Notch), and in either case might also result in aggregated
Notch. C) OFUT1<sup>R245A</sup> can bind to Notch and promote its folding, but it can't fucosylate Notch.

REFERENCES FOR SUPPLEMENTARY MATERIAL

Okajima et al., Supplemental Figure S2
Supplementary Figure S4 Okajima et al.

- **A**
  - **Input**
  - **anti-V5 Western**
  - **OFUT1**

- **B**
  - **N:AP Secretion**
  - Bar chart showing relative AP activity

- **C**
  - **Serrate binding**
  - Graph showing bound AP activity
Okajima et al., Supplementary Fig S5

**A**

OFUT1$^+$

Folded Notch $\xrightarrow{\text{GDP} \triangle \text{GDP}}$ GDP-GDP

**B**

OFUT1$^-$

Mis-folded Notch

or

**C**

OFUT1$^{R245A}$

Unfolded Notch $\xrightarrow{\text{EGF domain}}$ EGF domain

Partially folded Notch $\xrightarrow{\text{OFUT1}}$ OFUT1

Mis-folded Notch $\xrightarrow{\text{Fucose}}$ Fucose

Folded Notch

**Key:**

- Unfolded Notch
- EGF domain
- Partially folded Notch
- OFUT1
- Mis-folded Notch
- Fucose