O-Fucose Monosaccharide of Drosophila Notch Has a Temperature-sensitive Function and Cooperates with O-Glucose Glycan in Notch Transport and Notch Signaling Activation*

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Background: The requirement of O-fucose monosaccharide on Notch is not fully understood.

Results: Loss of O-fucose monosaccharide on Notch caused temperature-sensitive loss of Notch signaling.

Conclusion: O-Fucose monosaccharide of Notch has a temperature-sensitive function and cooperates with O-glucose glycan in Notch signal activation.

Significance: Our findings elucidate how different forms of glycosylation on a protein influence protein functions.

Notch (N) is a transmembrane receptor that mediates the cell-cell interactions necessary for many cell fate decisions. N has many epidermal growth factor-like repeats that are O-fucosylated by the protein O-fucosyltransferase 1 (O-Fut1), and the O-fut1 gene is essential for N signaling. However, the role of the monosaccharide O-fucose on N is unclear, because O-Fut1 also appears to have O-fucosyltransferase activity-independent functions, including as an N-specific chaperon. Such an enzymatic activity-independent chaperone could account for the essential role of O-fut1 in N signaling. To evaluate the role of the monosaccharide O-fucose modification in N signaling, here we generated a knock-in mutant of O-fut1 (O-fut1R245A knock-in), which expresses a mutant protein that lacks O-fucosyltransferase activity but maintains the N-specific chaperon activity. Using O-fut1R245A knock-in and other gene mutations that abolish the O-fucosylation of N, we found that the monosaccharide O-fucose modification of N has a temperature-sensitive function that is essential for N signaling. The O-fucose monosaccharide and O-glucose glycan modification, catalyzed by Rumi, function redundantly in the activation of N signaling. We also showed that the redundant function of these two modifications is responsible for the presence of N at the cell surface. Our findings elucidate how different forms of glycosylation on a protein can influence the protein’s functions.

Cell-cell signaling mediated by Notch (N) receptors plays crucial roles in many developmental processes in multicellular organisms (1–4). In humans, aberrant N signaling causes or is implicated in numerous diseases, including Alagille syndrome, spondylocostal dysostosis, cerebral autosomal dominant arthropathy with subcortical infarcts and leukoencephalopathy, multiple sclerosis, and various cancers (5–7).

N receptor family proteins have multiple EGF-like repeats in their extracellular domain (1–4), many of which can be modified with several types of O-linked glycosylation: O-fucose, O-glucose, O-xylene, and O-GlcNAc (8–12). N is O-fucosylated at serine or threonine residues in the C3–XXX(S/T)C4 consensus sequence that is present in various EGF-like repeats; for example, Drosophila has 36 EGF-like repeats, 23 of which are O-fucosylated (8, 13). This modification is mediated by protein O-fucosyltransferase 1 (O-Fut1 in Drosophila and protein O-fucosyltransferase 1 in mammals). β1,3-GlcNAc is further added to the O-fucose residue by Fringe (Fng) family proteins, which are β1,3-N-acetylgalactosaminyltransferases (14, 15).

O-fut1 and Pofut1 are apparently globally required for normal N signaling in Drosophila and mammals, respectively, in that their mutant phenotypes are similar to those caused by disrupted N signaling (16–18). In contrast, mutants of genes encoding Fng family proteins reduce N signaling in only a subset of contexts that involve N signaling (15, 19–21). Therefore, the GlcNAc modification by Fng, which regulates the interaction between N and its ligands, Delta (Dl) or Serrate, is required in a tissue-specific manner for various tissue boundary formations (22).

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O-Fut1 has two O-fucosyltransferase activity-independent functions (23–26). First, it is an endoplasmic reticulum (ER) resident protein and functions as a N-specific chaperon (23). Second, overexpressed O-Fut1 promotes the endocytosis of the N cell nonautonomously (25). Thus, it has been difficult to determine whether O-Fut1’s O-fucosyltransferase activity or its nonenzymatic functions (or both) are required in N signaling.

To address this issue, Okajima et al. (23) generated a genomic fragment, including a mutant O-fut1 locus, O-fut1R245A, which produces an O-Fut1 variant carrying an amino acid substitution in the binding site for GDP-fucose. O-Fut1R245A lacks O-fucosyltransferase activity in vitro, although it reportedly retains its N-specific chaperon activity (23). Most of the defects associated with the absence of O-fut1 are rescued by introducing the O-fut1R245A genomic locus, except for the phenotypes that are probably due to the disruption of Fng functions, which are tissue-specific and depend on the O-fucosylation of N (24). Based on these results, it was proposed that the O-fucose monosaccharide modification of N does not have a specific function in N signaling, although it is required for the Fng-dependent activation of N signaling. However, it was also reported that the ability of the O-fut1R245A genomic locus to rescue the O-fut1 null allele differs among transgenic lines, probably because the insertion site of the transgene affects the transcription efficiency of the integrated O-fut1R245A locus (23, 24).

To overcome this problem, here we generated a knock-in mutant of O-fut1R245A (O-fut1R245A knock-in) in Drosophila melanogaster. We used O-fut1R245A knock-in and other mutants affecting the O-fucosylation of Notch’s EGF-like repeats to examine the specific role of the monosaccharide O-fucose in N signaling. We also studied the genetic interaction between O-fut1R245A knock-in and rumi, which encodes an O-glucosyltransferase that adds O-glucose to the EGF-like repeats of N (27–30). Based on the presented results, we propose a model showing how multiple O-glycosylation sites in EGF-like repeats might affect the function of N.

**EXPERIMENTAL PROCEDURES**

Drosophila Strains—Canton-S was used as the wild-type strain. The following mutant alleles were used: O-fut116B, a null allele (17); fng13, a strong loss-of-function allele (31); Gmd178, a null allele (25); GmoSN1l(2)SH1931, a P-element insertion allele (32, 33); Df(2R)BSC783, a deficiency uncovering the Gmer locus (33); and rumi44, a null allele (27). The following UAS lines were used: UAS-NFL (a gift from S. Artavanis-Tsakonas), UAS-N^{AE} (34), UAS-NICD (35), and UAS-Lamp-HRP (36). Decapentaplegic (dpp)-Gal4 was described previously (37). 2xUbx-FLP was used to efficiently induce somatic mosaic clones (38). To induce germ line mosaic clones, ovoD G13 (17), ovoD FRT40A, and ovoD FRT80B (39) were used.

**Generation of the O-fut1R245A knock-in Fly—O-fut1R245A knock-in** is a knock-in mutation generated by a homologous recombination method described previously (40, 41). Two genomic fragments covering the O-fut1 locus, referred to as the left arm and right arm, were PCR-amplified. The left arm (5005 bp) was amplified using the primers 5’-CAACACGAGCAGGGCGATC-CCCA-3’ and 5’-ATTCTTTAATTATGATATAAATACA- AATA-3’, and it included the region from 4560 bp upstream of

the start of the O-fut1 5’UTR to 188 bp downstream of the end of the O-fut1 3’UTR. The right arm (4996 bp) was amplified using the primers 5’-TCTTTAGCTTTAATCCTAAAAGGATTT-3’ and 5’-CCGAATCCGGCAGCCATGA- AAC-3’, and it included the region from 189 bp downstream of the end of the O-fut1 3’UTR to 5115-bp downstream of the end of the O-fut1 3’UTR. The left arm fragment was inserted into the AscI site of the pT7 Blue vector (Novagen), and the right arm fragment was inserted between the SpIi and NotI sites of the pT7 Blue vector. The resulting constructs were pT7 Blue + left arm and pT7 Blue + right arm. To introduce a base substitution that would result in the amino acid replacement of arginine (Arg) at the 245th amino acid with alanine (Ala), an overlap extension PCR was performed using pT7 Blue + left arm and two primers, 5’-CATCTGGCCACGGATCTGATGGTG-3’ and 5’-ACCGTTGGCCAGATGAGTGC-CCAAA-3’. The right arm and mutated left arm were excised and cloned into an ends-out selectable recombinator vector, pW25, with a selectable marker, white (40, 41). This construct was introduced into the Drosofila genome by P-element-mediated transformation (41). Using the transgenic line obtained, homologous recombination was performed as described previously (40, 41). Briefly, pW25 contains two lox sites, which make it feasible to remove the white marker by Cre-mediated recombination (41). The white marker was removed as described previously (41), and the resulting lines were maintained as O-fut1R245A knock-in. The O-fut1 locus of the O-fut1R245A knock-in line was sequenced, and the mutation was confirmed.

**Generation of Somatic Mosaic Clones—Somatic clones of O-fut1R245A knock-in** and fng13 were generated by mitotic recombination in wing discs isolated from the larvae of y w 2xUbx-FLP; FRT13 O-fut1R245A knock-in/FRT13 Ubi-GFP and y w 2xUbx-FLP; fng13 FRT80B/Ubi-GFP FRT80B, y w 2xUbx-FLP; FRT82B rumi44/FRT82B Ubi-GFP, y w 2xUbx-FLP; FRT13 O-fut1R245A knock-in/FRT13 Ubi-GFP; FRT82B rumi44/FRT82B rumi44, using the FLP/FRT system, as described before (42). To express UAS-Lamp-HRP in wild-type or O-fut1R245A knock-in mutants, the following males were crossed to 2xUbx-FLP; FRT13 tub-Gal80/CyO; tub-Gal4/TM6B females, respectively: FRT13; UAS-Lamp-HRP/TM6B, and FRT13 O-fut1R245A knock-in/CyO; UAS-Lamp-HRP/TM6B. Until the larval stage, cultures were maintained at the indicated temperature (18, 25, or 30 °C).

**Epistasis Analysis Involving O-fut1R245A knock-in and Various N Derivatives Using the MARCM System**—The MARCM system was described previously (43). The following males were crossed to 2xUbx-FLP; FRT13 tub-Gal80/CyO; tub-Gal4, UAS-GFP/TM6B females to obtain flies with MARCM clones: FRT13 O-fut1R245A knock-in/CyO; UAS-N^{FL}/TM6B, FRT13/ CyO; UAS-N^{FL}/TM6B, FRT13 O-fut1R245A knock-in/CyO; UAS-N^{AE}/TM6B, FRT13/ CyO; UAS-N^{AE}/TM6B, FRT13 O-fut1R245A knock-in/CyO; UAS-N^{ICD}/TM6B, FRT13/CyO; UAS-N^{ICD}/TM6B.

**Generation of Germ Line Mosaic Clones to Remove the Maternal Contribution**—To obtain embryos homozygous for O-fut1R245A knock-in that originated from an O-fut1R245A knock-in homozygous germ line, y w hs-FLP/+; FRT13 ovoD/FRT13 O-fut1R245A knock-in females were crossed with FRT13
O-fut1$^{R245A}$ knock-in/CyO, wg-lacZ males. To obtain embryos homozygous for Gmd and lacking its maternal contribution, y w hs-FLP/++; ovo$^D$ FRT40A/Gmd$^{H78}$ FRT40A females were crossed with Gmd$^{H78}$ FRT40A/CyO, wg-lacZ males. To obtain embryos homozygous for Gmer and lacking its maternal contribution, y w hs-FLP/++; FRTG13 ovo$^D$/FRTG13 Gmo$^{24H}$ females were crossed with FRTG13 Gmer$^{24H}$/CyO, wg-lacZ males. To obtain embryos homozygous for fng and lacking its maternal contribution, y w hs-FLP/++; FRT80B/fng$^{13}$ FRT80B/H11001GmerSH/CyO, wg-lacZ males. To generate germ line mosaic clones, larvae were heat-shocked at 37 °C for 1 h 48–72 h after egg laying.

Western Blots—Western blotting was performed using a standard protocol (44). Wing discs of third-instar larvae were dissected and homogenized to prepare protein extracts. To detect Notch protein, 30 μg of protein extracts were resolved by electrophoresis on 4–15% Criterion TGX precast gels (Bio-Rad), and an anti-Notch intracellular domain antibody (1:5000 dilution, C17.9C6) (45) was used. As a loading control, α-tubulin was detected with an anti-α-tubulin antibody (1:2000 dilution, DM1A, Sigma) (46).

Immunostaining—Dissections and staining were performed using standard methods (44). The following antibodies were used: mouse anti-Notch intracellular domain (1:500; C17.9C6) (45); mouse anti-Notch extracellular domain (1:500, C458.2H) (46); mouse anti-Wg (1:250; 4D4) (48); mouse anti-Cut (1:250; 45); mouse anti-Notch extracellular domain (1:500, C458.2H) (46); mouse anti-Notch intracellular domain (1:500; C17.9C6) (45) was used. As a loading control, Rad), and an anti-Notch intracellular domain antibody (1:5000 (51); mouse anti-Pdi (1:500, 1D3, EnzoLife Sciences); rabbit anti-Rab7 (1:5,000) (51); rabbit anti-Rab11 (1:25, 9F8A9); rabbit anti-GFP (1:1,000; MBL, Nagoya, Japan); rat anti-GFP (1:1,000; Nacalai, Kyoto, Japan); rat anti-Elav (50) (1:25, 9F8A9); rabbit anti-Rab7 (1:5,000) (51); rabbit anti-Rab11 (1:1,000, 51); mouse anti-Pdi (1:500, 1D3, EnzoLife Sciences) (52); guinea pig anti-Senseless (1:500) (53); guinea pig anti-Hrs (36); guinea pig anti-O-Fut1 (1:1000) (23); and guinea pig anti-Boca (1:500) (54). Cy3-, Cy5-, and Alexa488-coupled secondary antibodies (1:500) were from Jackson ImmunoResearch and Molecular Probes. Images were acquired on an LSM5 Pascal or LSM700 microscope (Zeiss). All images were processed and assembled using ImageJ, Adobe Photoshop, and Adobe Illustrator.

Comparison of the O-Fucose and O-Glucose Sites in Various Notch Receptors—The Swiss-Prot and KEGG GENES databases were searched using the Motif search program or EGF-like repeats containing the consensus sequence for O-glycosylation between the first and second cysteines, and for O-fucosylation between the second and third cysteines (8, 13).

RESULTS

O-Fucosyltransferase Activity of O-fut1$^{R245A}$ knock-in Is Negligible in Vivo—In the previously reported Drosophila O-fut1$^{R245A}$ mutant, arginine is replaced by alanine at the 245th amino acid position of the deduced O-Fut1 protein (Fig. 1A) (23). This amino acid substitution is located in the GDP-fucose-binding motif and largely abolishes the O-fucosyltransferase activity in vitro (Fig. 1A) (23, 55, 56). We first sought to confirm that the O-fucosyltransferase activity of O-fut1$^{R245A}$ knock-in homozygotes survived until the third-instar larval stage (data not shown). In the wild-type wing discs of the third-instar larvae, N signaling is activated at the border between fng-expressing and fng-nonex-pressing regions, which correspond to the dorsal and ventral (D/V) compartments, respectively. Therefore, the expression of wingless (wg), a downstream gene of N signaling, is activated along the D/V compartment boundary (D/V boundary) (Fig. 1B) (57). This activation of N signaling depends on the selective binding of N with DI or Serrate, which is regulated by GlcNAc...
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Modification of the O-fucose on N’s EGF-like repeats by Fng (20). We found that wg expression was abolished in the wing discs isolated from O-fut1R245A knock-in homozygotes at the third instar cultured at 25 °C (Fig. 1C), probably because the fng function was disrupted in the absence of N’s O-fucosylation.

As reported previously, misexpressed fng driven by dpp-Gal4 at 25 °C resulted in the ectopic activation of wg in the ventral compartment of the wild-type wing, where endogenous fng is not expressed (n = 99) (Fig. 1, D and D’). However, when we misexpressed fng driven by dpp-Gal4 in the wing discs of O-fut1R245A knock-in homozygotes cultured at 25 °C, no wg expression was induced in any case examined (n = 52) (Fig. 1, E and E’). We confirmed that the expression pattern of dpp-Gal4 was almost the same in the wing discs of wild-type and O-fut1R245A knock-in homozygotes at this temperature (Fig. 1, D and E). This result suggested that in the O-fut1R245A knock-in homozygotes, the O-fucose modification of N was minimal, if it occurred at all.

O-fut1 is a maternal-effect gene (17). Therefore, to observe the phenotypes associated with the O-fut1R245A knock-in mutation, we needed to remove the maternal contribution of the wild-type O-fut1 gene from the female germ line. However, these females are heterozygous for O-fut1R245A knock-in because O-fut1R245A knock-in is a recessive lethal mutation (data not shown). Therefore, we obtained embryos homozygous for O-fut1R245A knock-in from females carrying germ line clones homozygous for O-fut1R245A knock-in, using a previously described method (17,58). In this study, we call these embryos O-fut1R245A knock-in/m.”

O-fut1R245A knock-in Homozygotes Obtained from Females Carrying the O-fut1R245A knock-in Homozygous Germ Line Show a Temperature-sensitive Neurogenic Phenotype—In the embryonic central nervous system, the number of neuroblasts segregated from the neuroectoderm is determined by “lateral inhibition” through N signaling (59). A disruption of N signaling causes a failure of lateral inhibition, resulting in neuronal hyperplasia, known as the neurogenic phenotype (59). We studied the nervous system development in O-fut1R245A knock-in/m embryos at 25 and 30 °C, in case O-fut1R245A had a temperature-sensitive property. Neurons were detected by anti-Elav antibody staining (Fig. 2, A–J). At 25 and 30 °C, the wild-type flies develop and reproduce normally, and their embryonic nervous system is also normal (Fig. 2, A, B, and K). In addition, the nervous system was normal in most of the O-fut1R245A knock-in/m embryos at 25 °C (Fig. 2, C and K; 1/38 embryos showed the neurogenic phenotype). However, at 30 °C, the O-fut1R245A knock-in/m embryos showed a highly penetrant neurogenic phenotype (Fig. 2, D and K; 15/15 embryos showed the neurogenic phenotype). This temperature-sensitive neurogenic phenotype has not been reported in previous studies using the transgenic O-fut1R245A genomic locus (23, 24).

To confirm that the temperature-sensitive neurogenic phenotype was not due to the degradation of O-Fut1R245A at 30 °C, we compared the protein levels of O-Fut1 and its derivative in wild-type and O-fut1R245A knock-in homozygous cells, respectively. We generated genetic mosaic wing discs composed of somatic clones homozygous for O-fut1R245A or O-fut1R245A knock-in/m.

FIGURE 2. Loss of O-fucose monosaccharide modification of N causes a neurogenic phenotype at 30 °C but not at 25 °C. A–J, lateral views of embryos stained with an anti-Elav antibody (white). A and B, wild-type embryos raised at 25 °C (A) and 30 °C (B). C and D, O-fut1R245A knock-in/m embryos raised at 25 °C (C) and 30 °C (D). E and F, GmdH78 embryos raised at 25 °C (E) and 30 °C (F). G and H, GmdH78 embryos raised at 25 °C (G) and 30 °C (H). I and J, fng embryos raised at 25 °C (I) and 30 °C (J). K, frequency of embryos (%) showing a normal central nervous system (orange) or a neurogenic phenotype (blue) at 25 and 30 °C. The number of embryos examined is shown at the top. Scale bars, 100 μm.
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O-Fucose Monosaccharide on the EGF-like repeats of N has a temperature-sensitive role in Notch signaling, which is independent of its further GlcNAc modification by Fng—O-Fucose Cooperates with O-Glucose Glycan in Notch Signaling

O-Fucose cooperates with O-glucose glycans in Notch signaling.

O-fucose monosaccharide plays an essential role in N signaling in Drosophila (25, 33).

We observed embryos homozygous for Gmd<sup>H78</sup> or Gmer<sup>H71</sup> lacking their maternal contributions (Gmd<sup>H78</sup>m/z and Gmer<sup>H71</sup>m/z). We found that most of the Gmd<sup>H78</sup>m/z (2/19 embryos showed the neurogenic phenotype) and Gmer<sup>H71</sup>m/z (0/26 embryos showed the neurogenic phenotype) embryos had a normal nervous system at 25 °C (Fig. 2, E, G, and K).

However, as found in the O-fut1<sup>R245A</sup> knock-in/m/z embryos, at 30 °C the neurogenic phenotype was highly penetrant in embryos with either the Gmd<sup>H78</sup>m/z (18/18 neurogenic embryos) or the Gmer<sup>H71</sup>m/z (23/25 neurogenic embryos) mutation (Fig. 2, F, H, and K). In these embryos, various fucose modifications, including the fucosylation of N-glycan, should be abolished. However, the collective results obtained from the O-fut1<sup>R245A</sup> knock-in/m/z, Gmd<sup>H78</sup>m/z, and Gmer<sup>H71</sup>m/z embryos indicated that the requirement for the monosaccharide O-fucose modification of N might be temperature-sensitive.

A contribution of fng to lateral inhibition has not been reported (22). However, to exclude the possibility that the temperature-sensitive neurogenic phenotype was caused by the lack of GlcNAc modification of O-fucose by Fng, we generated embryos homozygous for fng<sup>14</sup> and lacking its maternal contribution (fng<sup>13</sup>m/z). The fng<sup>13</sup>m/z embryos did not show the neurogenic phenotype at either 25 °C (n = 14) or 30 °C (n = 15) in any case examined (Fig. 2, I–K). This finding indicated that the absence of GlcNAc modification by Fng is irrelevant to the temperature-sensitive neurogenic phenotype observed in the O-fut1<sup>R245A</sup> knock-in/m/z, Gmd<sup>H78</sup>m/z, and Gmer<sup>H71</sup>m/z embryos. Together, these findings indicate that the O-fucose monosaccharide modification of N is essential for N signaling during lateral inhibition at 30 °C but not at 25 °C.

O-Fucose Monosaccharide Modification of N Is Generally Required to Activate N Signaling at 30 °C—We next examined whether the O-fucose monosaccharide modification of N is generally required for N signaling at 30 °C. For this analysis, we generated somatic mosaic clones homozygous for O-fut1<sup>R245A</sup> knock-in in the wing discs of third-instar larvae, using the FLP/FRT system (Fig. 4, B–C’). The expression of cut along the D/V boundary of wild-type wing discs is induced by N signaling (Fig. 4A). In the mosaic wing discs, cut was also ectopically expressed along the boundaries of the O-fut1<sup>R245A</sup> knock-in somatic homozygous clones (marked by the absence of GFP) located in the dorsal compartment, at 25 °C (Fig. 4, B and B’).

This phenotype was similar to that of somatic mosaic clones homozygous for fng<sup>13</sup> (Fig. 4, D–E’) at 25 and 30 °C (62). Therefore, the absence of monosaccharide O-fucose had a similar defect in cut activation at 25 °C as did the absence of GlcNAc on O-fucose. This result is consistent with the previous conclusion that O-fucose monosaccharide does not have a specific function in N signaling at 25 °C (24). However, at 30 °C, the ectopic expression of cut along the boundaries of somatic mosaic clones homozygous for O-fut1<sup>R245A</sup> knock-in was abolished (Fig. 4, C and C’), supporting the idea that the monosaccharide form of O-fucose plays an essential role in N signaling in...
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To obtain more evidence for this idea, we examined the formation of sensory organ precursors (SOPs) in the wing discs of third-instar larvae homozygous for O-fut1R245A knock-in. A single SOP is selected from each proneural cluster in the wing disc, where SOPs arise in a well-defined pattern at the third instar (63). In wild-type wing discs, the SOP formation pattern, observed by anti-Senseless (Sens) staining, was essentially the same from 18 to 30 °C (Fig. 4, F–H). In the wing discs of O-fut1R245A knock-in homozygotes, SOPs did not form along the D/V boundary, because this SOP formation depends on the expression of wg, which is induced by the fig-dependent activation of N signaling (Fig. 4, I–K) (62). At 18 and 25 °C, the number of SOPs elsewhere in the wing discs increased slightly (Fig. 4, I–J). However, these SOPs markedly increased in these wing discs at 30 °C (Fig. 4, K and K′). These results suggest that lateral inhibition was disrupted in the wing discs of O-fut1R245A knock-in homozygotes in a temperature-sensitive manner, as found in the embryonic central nervous system.

We also examined the SOP formation in the wing discs in the absence of Gmer function. A trans-heterozygote of GmerΔ41 and a deletion mutant uncovering the Gmer locus survived until the third-instar larval stage, when we could isolate the wing discs to study SOP formation (33). At 25 °C, the number of SOPs increased slightly in these wing discs, although they did not form along the D/V boundary due to the absence of N signaling there (Fig. 4, L and L′). Nevertheless, the SOPs, except for those located at the D/V boundary, noticeably increased at 30 °C (Fig. 4, M and M′). We also confirmed that the SOP number was not affected in somatic mosaic clones homozygous for fig13 in the wing discs of third-instar larvae, even at 30 °C (Fig. 4, N–N′). Based on these results, we concluded that the monosaccharide form of O-fucose is generally required for N signaling at 30 °C, although we could not exclude the possibility that some exceptions exist.

O-Fucose Monosaccharide Modification of N Functions Upstream of the Membrane-tethered Form of Activated N at 30 °C—To assess the function of the O-fucose monosaccharide modification of N’s EGF-like repeats, we performed a set of broken lines. F–M', SOPs in wing discs were detected by anti-Sens antibody staining. F–H', wild-type wing discs were isolated from larvae raised at 18 °C (F and F'), 25 °C (G and G'), or 30 °C (H and H'). Along the D/V boundary, the expression of sens, which is induced by fig-dependent N signaling, is shown by white brackets in F–H. I–M', SOPs in wing discs of O-fut1R245A knock-in homozygotes (I–K') and of heterozygotes between GmerΔ41 and a deletion uncovering the Gmer locus (GmerΔ41[Df (L–M')] are shown. Wing discs were isolated from larvae raised at 18 °C (I and I'), 25 °C (J, J', L, and L'), or 30 °C (K, K', M, and M'). F–M' show magnified views of the magenta squares in F–M, respectively. Arrowheads indicate SOPs in F–M'. Wing discs are outlined by broken lines. N–N', SOPs in wing discs with fig13 homozygote mosaic clones (detected by the absence of GFP, shown in green) were detected by anti-Sens antibody staining (magenta). Boundaries of mosaic clones are indicated by broken lines. Wing discs were isolated from larvae raised at 30 °C. N' shows merged images of N and N'. Scale bars, 20 μm for A–E, and 100 μm for F–N.

FIGURE 4. O-Fucose monosaccharide modification of N is generally required to activate N signaling at 30 °C. A–E, anti-Cut antibody staining (magenta) of wing discs with wild-type (A), O-fut1R245A knock-in (B–C'), or fig13 (D–E) mosaic clones. B–E, mosaic clones were identified by the absence of GFP (green). Wing discs were isolated from larvae raised at 25 °C (A, B, B', D, and D') or 30 °C (C, C', E, and E'). B', C', D', and E' show merged images of GFP and Cut staining from B to E. Boundaries of mosaic clones are indicated by broken lines. F–M', SOPs in wing discs were detected by anti-Sens antibody staining. F–H', wild-type wing discs were isolated from larvae raised at 18 °C (F and F'), 25 °C (G and G'), or 30 °C (H and H'). Along the D/V boundary, the expression of sens, which is induced by fig-dependent N signaling, is shown by white brackets in F–H. I–M', SOPs in wing discs of O-fut1R245A knock-in homozygotes (I–K') and of heterozygotes between GmerΔ41 and a deletion uncovering the Gmer locus (GmerΔ41[Df (L–M')] are shown. Wing discs were isolated from larvae raised at 18 °C (I and I'), 25 °C (J, J', L, and L'), or 30 °C (K, K', M, and M'). F–M' show magnified views of the magenta squares in F–M, respectively. Arrowheads indicate SOPs in F–M'. Wing discs are outlined by broken lines. N–N', SOPs in wing discs with fig13 homozygote mosaic clones (detected by the absence of GFP, shown in green) were detected by anti-Sens antibody staining (magenta). Boundaries of mosaic clones are indicated by broken lines. Wing discs were isolated from larvae raised at 30 °C. N' shows merged images of N and N'. Scale bars, 20 μm for A–E, and 100 μm for F–N.
epistasis analyses involving O-fut1R245A knock-in and various derivatives of N. We used the MARCM system (43) to produce full-length N (N\textsuperscript{FL}), a membrane-tethered form of activated N (N\textsuperscript{\Delta E}), and the N intracellular domain (NICD) in somatic mosaic clones homozygous for O-fut1R245A knock-in at 30 °C (Fig. 5). In wild-type wing discs, the overexpression of N\textsuperscript{FL}, N\textsuperscript{\Delta E}, or NICD resulted in the ectopic induction of cut at 30 °C (Fig. 5, A, C, and E). However, the overexpression of N\textsuperscript{FL} failed to induce ectopic cut expression in the somatic clones homozygous for O-fut1R245A knock-in at 30 °C (Fig. 5B). Thus, O-fut1R245A knock-in was epistatic to N\textsuperscript{FL} under this condition. By contrast, the

FIGURE 5. O-Fucose monosaccharide modification of N functions upstream of the membrane-tethered form of activated N. A–F, expression of \textit{cut} in the wing discs of third-instar larvae was detected by anti-Cut antibody staining (magenta in A–F). A and B, mosaic clones overexpressing full-length N (N\textsuperscript{FL}) (green in A and B) in wild-type (A) or O-fut1R245A knock-in homozygote mosaic clones (B). C and D, mosaic clones overexpressing a membrane-tethered form of activated N (N\textsuperscript{\Delta E}) (green in C and D) in wild-type (C) or O-fut1R245A knock-in homozygote mosaic clones (D). E and F, mosaic clones overexpressing the intracellular domain of N (nuclear form of activated N) (NICD) (green in E and F) in wild-type (E) or O-fut1R245A knock-in homozygote mosaic clones (F). Wing discs were isolated from larvae cultured at 30 °C. Scale bars, 100 μm.
overexpression of $N^{\Delta E}$ or NICD still induced the ectopic expression of cut in somatic mosaic clones homozygous for O-fut$^{R245A}$ knock-in at 30 °C (Fig. 5, D and F). Therefore, the two activated forms of $N$, $N^{\Delta E}$ and NICD, were epistatic to O-fut$^{R245A}$ knock-in under this condition. Based on these epistasis analyses, we speculated that the monosaccharide $O$-fucose modification of $N$ is required downstream of full-length $N$ and upstream of the S3 cleavages of $N$.

**O-Fucose Monosaccharide Modification of $N$ Is Required for the Proper Localization of $N$ at 30 °C**—To understand how the monosaccharide $O$-fucose modification of $N$ affects $N$ signaling, we compared $N$’s subcellular localization at 25 and 30 °C (Fig. 6). Therefore, the two activated forms of $N$, $N^{\Delta E}$ and NICD, were epistatic to O-fut$^{R245A}$ knock-in under this condition. Based on these epistasis analyses, we speculated that the monosaccharide $O$-fucose modification of $N$ is required downstream of full-length $N$ and upstream of the S3 cleavages of $N$.

**Table 1**

|                     | control at 25°C | control at 30°C | O-fut$^{R245A}$ at 25°C | O-fut$^{R245A}$ at 30°C | O-fut$^{486}$ at 25°C | O-fut$^{486}$ at 30°C | rumi$^{44}$ at 25°C | O-fut$^{R245A}$ rumi$^{44}$ at 25°C |
|---------------------|-----------------|-----------------|------------------------|------------------------|-----------------------|----------------------|-----------------|-------------------------------------|
| **ER (Boca)**       |                 |                 |                        |                        |                       |                      |                 |                                     |
| SA                  | -               | -               | +                      | +                      | +                     | -                    | +               | +                                   |
| medial              | 0.79±0.28       | 1.2±0.12        | 0.88±0.30              | 1.2±0.11               | 1.2±0.11              | 1.5±0.33             | 1.3±0.11        | 1.1±0.10                            |
| **ER (Pdi)**        |                 |                 |                        |                        |                       |                      |                 |                                     |
| SA                  | -               | -               | -                      | +                      | +                     | -                    | +               | +                                   |
| medial              | 1.3±0.055       | 2.0±0.44        | 1.5±0.078              | 2.1±0.14               | 1.2±0.21              | 2.4±0.27             | 1.4±0.17        | 1.8±0.48                            |
| **Golgi (GM130)**   |                 |                 |                        |                        |                       |                      |                 |                                     |
| 1.2±0.11            | 0.87±0.10       | 1.2±0.087       | 1.2±0.32               | 0.58±0.17              | 1.2±0.40              | 1.7±0.25             | 1.2±0.15        |                                     |
| **early endosome (Hrs)** | 6.7±1.9        | 13±2.4          | 8.9±1.7                | 23±2.8*                | 8.1±0.71              | 9.6±0.94             | 7.0±0.85        | 7.8±0.87                            |
| **late endosome (Rab7)** | 6.7±1.5        | 7.2±0.68        | 6.8±1.6                | 8.1±1.4                | 3.5±1.3               | 5.0±0.92             | 6.0±0.56        | 4.7±0.62                            |
| **recycling endosome (Rab11)** | 0.79±0.28     | 0.86±0.26       | 0.88±0.30              | 1.6±0.61               | 0.76±0.11             | 0.99±0.26            | 0.80±0.17        | 0.64±0.061                          |
| **lysosome (Lamp-HRP)** | 5.0±1.4        | 6.9±2.1         | 8.0±1.1                | 21±3.4*                | 11±2.4                | 11±2.6               | ND             | ND                                  |

To visualize N protein delivered to the epithelial cell surface at the sub-apical region, nonpermeabilized wing discs carrying...
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We next determined the intracellular compartments in which N accumulated in the somatic mosaic clones homozygous for O-fut1<sup>R245A</sup> knock-in at 30 °C. We identified the ER (Boca and Pdi), Golgi (GM130), early endosomes (Hrs), late endosomes (Rab7), recycling endosomes (Rab11), and lysosomes (Lamp) by immunostaining (25, 36, 51, 52, 54). In wing-disc epithelial cells, the ER was detected either as an indistinct and shapeless structure (Fig. 6, F–F′ and H–H′) or as dots (Fig. 6, G–G′ and I–I′). The former was detected mostly in the cells’ apical regions (Fig. 6, F–F′ and H–H′), and the latter in more basal regions (Fig. 6, G–G′ and I–I′). These structures were recognized by antibodies against two ER-marker proteins, Boca (Fig. 6, F–G′) and Pdi (Fig. 6, H–I′). To analyze the co-localization of N and markers for various intracellular compartments quantitatively, the percentage of vesicles positive for each marker that was also positive for N is shown in Table 1. At 30 °C, the distribution of accumulated N protein overlapped partly with the indistinct and shapeless ER in somatic mosaic clones homozygous for O-fut1<sup>R245A</sup> knock-in (white arrowhead in Fig. 6, F–F′ and H–H′, and Table 1), although hardly any N protein was detected in this structure in O-fut1<sup>R245A</sup> knock-in/+ (control) cells (Fig. 6, F–F′, and H–H′, and Table 1). However, there was no increase in N-protein level in the dot-structure ER suggesting that the intracellular accumulation of N could not be attributed to the GlcNAc modification of O-fucose (data not shown).

FIGURE 7. Co-localization of intracellular vesicular compartment markers with N in O-fut1<sup>R245A</sup> somatic mosaic clones. A–D′, wing discs with O-fut1<sup>R245A</sup> homozygote mosaic clones. Regions of mutants outlined by broken blue lines were double-stained with an anti-Boca (magenta in A, A′, B, and B′) or anti-Pdi (C, C′, D, and D′) antibody and an anti-N antibody (green in A, A′, B, B′, C, C′, D′, and D′). Wing discs were isolated from larvae raised at 25 °C. somatic mosaic clones homozygous for O-fut1<sup>R245A</sup> knock-in were stained with an antibody against the extracellular domain of N (Fig. 6, C–D′) (65). The amount of cell-surface N at the sub-apical plane was the same at 25 or 30 °C in cells homozygous for O-fut1<sup>R245A</sup> knock-in under this condition (Fig. 6, C–D′). Thus, even at 30 °C, N delivery to the cell surface was not disrupted in cells homozygous for O-fut1<sup>R245A</sup> knock-in, although N accumulated within these cells (Fig. 6, B and B′). However, intracellular and cell-surface N protein did not accumulate in somatic mosaic clones homozygous for fng<sup>112</sup> at 25 or 30 °C, suggesting that the intracellular accumulation of N could not be attributed to the GlcNAc modification of O-fucose (data not shown).
O-Fucose Cooperates with O-Glucose Glycan in Notch Signaling

not the dot-shaped ER structures may coincide with the loss of N signaling activity.

Although N is reported to accumulate in the ER in somatic clones homozygous for O-fut1R245A, a null mutant of O-fut1 (17), there are conflicting reports (23, 25). Here, we found that accumulated N partially overlapped with indistinct, shapeless ER structures, although there was no change of the amount of N in dot-shaped ER at 25 or 30 °C (Fig. 7, A–D′, and Table 1). This may account for the previous discrepancy in the ER accumulation of N reported in O-fut1R245A mutant cells. Nonpermeabilized staining did not detect N protein at the sub-apical cell-surface region in O-fut1R245A mutant epithelial cells at 25 or 30 °C; this agreed with previously reported findings at 25 °C (data not shown) (23, 25).

We also observed that N protein was detected more often in early endosomes (23 ± 2.8) and lysosomes (21 ± 3.4) in somatic mosaic clones homozygous for O-fut1R245A knock-in at 30 °C, compared with those at 25 °C or with control cells (13 ± 2.4 and 4.4 ± 0.59, respectively) (Fig. 7, E–F′ and Table 1). These differences were statistically significant (t test, p < 0.05). In contrast, there was no marked difference in N distribution in the Golgi, late endosomes, or recycling endosomes of these cells at either temperature (Fig. 7, G–I′ and Table 1). These data suggest that the O-fucose monosaccharide modification of N may be required for specific step(s) of N’s trafficking at 30 °C but not at 25 °C. In addition, the distinct defects in N’s trafficking found in O-fut1R245A knock-in mutant cells versus O-fut1R245A mutant cells suggest that the nature of the defect in these two mutants is different (Table 1). These observations further support our idea that the temperature-sensitive phenotype of O-fut1R245A knock-in is not due to a temperature-dependent loss of O-fut1 activity, associated with, for example, the breakdown of the O-Fut1R245A mutant protein (Fig. 3). To detect possible alterations in the N protein in the O-fut1R245A knock-in mutant, including changes in its stability and processing, we detected the N protein in O-fut1R245A knock-in homozygous wing discs from the third-instar larvae cultured at 25 and 30 °C, by Western blot (Fig. 8). However, we did not observe any marked differences in the amount or size of the N protein between the mutant and the wild-type wing discs (compare lanes 1 and 2 and lanes 3 and 4 in Fig. 8).

O-Fucose and O-Glucose Modifications of N Function Redundantly—The EGF-like repeats of N have consensus sequences not only for O-fucose but also for O-glucose modifications (27). O-Glucose is added by Rumi, a protein O-glucosyltransferase in Drosophila (27, 28). In the absence of rumi function, temperature-sensitive phenotypes associated with the loss of N signaling, which are similar to those of O-fut1R245A, are observed (27, 28). In somatic mosaic clones homozygous for rumi44, a null mutation, N accumulated intracellularly, which was also reminiscent of a defect in O-fut1R245A homozygous cells. Therefore, we thought that the O-fucose monosaccharide modification of N might function redundantly with the O-glucose glycan modification of N in N signaling. To test this possibility, we generated a rumi and O-fut1R245A knock-in double mutant and observed SOP formation in the wing discs of third-instar larvae. As described...
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FIGURE 10. Monosaccharide O-fucose and O-glucose glycan of N function redundantly in N signaling. A–B', anti-Cut antibody staining (magenta) of third-instar wing discs with mosaic clones homozygous for rumi44 (indicated by the absence of GFP, shown in green). The wing discs were isolated from larvae raised at 25 °C (A–A') or 30 °C (B–B'). C–C', anti-Cut antibody staining (magenta) of third-instar wing discs homozygous for rumi44 with mosaic clones homozygous for O-fut1R245A knock-in (indicated by the absence of GFP, shown in green). The wing discs were isolated from larvae raised at 25 °C.

FIGURE 11. Model of O-fucose and O-glucose on N and N accumulation in various mutants. A, schematic diagram showing the intracellular compartment, in which N (magenta) is strongly accumulated in wild-type and various mutant cells. EE, early endosome; lyso, lysosome; SA, sub-apical region. B, schematic model of the redundant roles of O-fucose monosaccharide and O-glucose glycan modifications of the EGF-like repeats of N in the proper folding of the EGF-like repeats (upper), which may affect the entire extracellular structure of the full-length N (lower).

above, the number of SOPs increased at 30 °C in wing discs homozygous for rumi44, but it was normal at 18 and 25 °C (Fig. 9, A–C'). This phenotype is very similar to that of O-fut1R245A knock-in wing discs (Fig. 4, I–K'). However, the number of SOPs increased in the wing discs homozygous for rumi44 and O-fut1R245A knock-in even at 18 and 25 °C, suggesting that N shown in green). The wing discs were isolated from larvae raised at 25 °C. The boundaries of mosaic clones are indicated by broken white lines. A', B', and C' show merged images of A, B, and C, respectively. D–H', permeabilized (D–D' and F–G') or nonpermeabilized (E–E' and H–H') wing discs were stained with antibodies against N's intracellular (D–D' and F–G') or extracellular domains (E–E' and H–H'). D–E', wing discs with mosaic clones homozygous for rumi44 (indicated by the absence of GFP, shown in green). F–H', wing discs of a rumi44 homozygote with mosaic clones homozygous for O-fut1R245A knock-in (indicated by the absence of GFP, shown in green in F, F', H, and H' or labeled “mutant” in G). Borders of mosaic clones are indicated by broken white lines. Scale bars, 100 μm for A–C' and 5 μm for D–H'.

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signaling was abolished under these conditions (Fig. 9, D–E'). These results suggest that the O-fucose monosaccharide and O-glucose glycans have a redundant function during lateral inhibition that controls the number of SOPs in the wing discs.

Similar redundancy in the function of the O-fucose monosaccharide and O-glucose glycans was observed in the activation of N signaling along the D/V boundary of the wing disc at the third instar. The expression of cut along the D/V boundary disappeared at 30 °C in somatic mosaic clones homozygous for rumi44 (Fig. 10, B–B'), but cut's expression was normal at 25 °C (Fig. 10, A–A'). The phenotype of the somatic mosaic clones homozygous for rumi44 at 30 °C was very similar to that of the somatic mosaic clones homozygous for O-fut1R245A knock-in at 30 °C (Fig. 4, C and C'). However, in somatic mosaic clones homozygous for rumi44 and O-fut1R245A knock-in, cut expression was abolished even at 25 °C (Fig. 10, C–C').

The O-fucose monosaccharide and O-glucose glycans on the EGF-like repeats of N also collaborated to increase the amount of N at the sub-apical plasma membrane. As reported previously, slight accumulations of N protein were found in cells homozygous for rumi44 alone, at 25 °C (Fig. 10, D–D'), although the amount of cell-surface N at the sub-apical region, detected by nonpermeabilized staining, was not altered in these cells (Fig. 10, E–E') (27). However, the N-protein accumulations were more prominent in intracellular compartments in somatic mosaic clones homozygous for rumi44 and O-fut1R245A knock-in (Fig. 10, F–F'). In these double mutant cells, the accumulated N partially co-localized with ER markers (Fig. 10, G–G'), and Table 1). Curiously, nonpermeabilized staining for N revealed that the cell-surface N was diminished in these cells (Fig. 10, H–H'), but it was unaffected in cells homozygous for either O-fut1R245A knock-in (Fig. 6, C–D') or rumi44 (Fig. 10, E–E') (27). Therefore, O-fucose monosaccharide and O-glucose glycans have redundant roles, and in their absence N was not delivered to or stably maintained at the plasma membrane.

DISCUSSION

The functions of the monosaccharide O-fucose on the EGF-like repeats of N have been vague. In Drosophila, the monosaccharide O-fucose was proposed to serve primarily as an acceptor for Fng, with O-Fut1 being required as a specific chaperon for N (23, 24). However, in mammalian cells, the O-fucose modification was reported to be required for the optimal activation of N signaling under certain conditions (55). More recently, Fng-independent functions of fucosylation in Drosophila N signaling were reported, although these effects are relatively mild or cell type-specific (66, 67). In this study, we revealed that the O-fucose monosaccharide, independent of GlcNAc modification by Fng, is essential for N signaling at 30 °C in various developmental contexts in Drosophila. Moreover, in the absence of modifications with both O-fucose monosaccharide and O-glucose glycan, N signaling was abolished even at 25 °C, although the loss of either modification alone caused this phenotype only at 29–30 °C (27). The loss of N signaling coincides with a severe reduction of N at the sub-apical plasma membrane and an accumulation of N in a particular fraction of the ER (Figs. 10, F–G' and 11A). These results suggest that O-fucose monosaccharide and O-glucose glycan modifications of N have redundant and collaborative roles in the activation of N signaling and the proper localization of N at the sub-apical plasma membrane (Fig. 11A). However, at this point we could not distinguish between whether N failed to reach the plasma membrane or it failed to be stably maintained on the membrane under this double mutant condition.
The biochemical roles of the O-fucose monosaccharide and O-glucose glycan modifications are still elusive. However, a previous NMR study revealed that the fucose moiety directly functions as a "bridge" in the formation of an antiparallel β-sheet in EGF-like repeat 12 of mouse Notch1, stabilizing its structure through an interaction between the fucose and the peptide backbone of the EGF-like repeat (68). Therefore, we speculate that structural destabilization of each EGF-like repeat may affect the folding of the full-length N (Fig. 11b). Deficient N folding may explain our observation that N accumulated in the ER of cells homzygous for O-fut1R245A knock-out at 30 °C (Fig. 11b). More severe folding defects may occur in the absence of both O-fucose monosaccharide and O-glucose glycan modifications (Fig. 11b). It was previously shown that O-fut1 and Rumi may be involved in quality control of the N protein, because they both only modify properly folded EGF-like repeats (69–71). Therefore, it is possible that full-length N with EGF-like repeats lacking O-fucose monosaccharide and O-glucose glycan modifications may escape from its chaperon, leading to misfolding of the full-length N. However, at this point, the mechanism by which the potential destabilization of EGF-like repeats influences the global structure of full-length N is unknown. Moreover, it was previously shown that disrupting the O-fucosylation site in the 12th EGF-like repeat of mouse Notch1 does not affect the amount of cell-surface Notch1, but instead it reduces Notch1's activation, suggesting that the O-fucose modification of the 12th EGF-like repeat does not play a role in Notch1's folding (72). Therefore, it is possible that the O-fucose modification on other EGF-like repeats is required for the folding of full-length N and that the O-fucose modification of N has multiple functions in N signaling.

Considering that the O-fucose monosaccharide and O-glucose glycan modifications of the EGF-like repeats may function to increase the heat stability of full-length N, it is tempting to speculate that some sites for these modifications are specifically conserved in animals with higher body temperatures (homeothermic) but not in those with lower temperatures (poikilothermic). However, a comparison of the consensus sequences for these modifications failed to find a correlation between body temperature and the evolutionary conservation of these sites (Fig. 12).

A number of protein motifs contain multiple glycosylation sites for different types of glycan modifications (73). In this study, we found that the O-fucose monosaccharide and O-glucose-glycan modifications of the EGF-like repeats of N have a redundant role, possibly in the folding of N. Thus, our results provide a case in which different types of glycosylation occurring at a protein motif may play a common and collaborative role.

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