

Multiple O-glycosylation sites on Notch function as a buffer against temperature-dependent loss of signaling

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SUMMARY

Mutations in *Drosophila rumi* result in a temperature-sensitive loss of Notch signaling. Rumi is a protein O-glycosyltransferase that adds glucose to EGF repeats with a C-X-S-X-P-C consensus sequence. Eighteen of the 36 EGF repeats in the *Drosophila* Notch receptor contain the consensus O-glycosylation motif. However, the contribution of individual O-glucose residues on Notch to the regulation of Notch signaling is not known. To address this issue, we carried out a mutational analysis of these glycosylation sites and determined their effects on Notch activity in vivo. Our results indicate that even though no single O-glucose mutation causes a significant decrease in Notch activity, all of the glucose residues on Notch contribute in additive and/or redundant fashions to maintain robust signaling, especially at higher temperatures. O-glucose motifs in and around the ligand-binding EGF repeats play a more important role than those in other EGF repeats of Notch. However, a single O-glucose mutation in EGF12 can be compensated by other O-glucose residues in neighboring EGF repeats. Moreover, timecourse cell aggregation experiments using a *rumi* null cell line indicate that a complete lack of Rumi does not affect Notch-Delta binding at high temperature. In addition, *rumi* fully suppresses the gain-of-function phenotype of a ligand-independent mutant form of Notch. Our data suggest that, at physiological levels of Notch, the combined effects of multiple O-glucose residues on this receptor allow productive S2 cleavage at high temperatures and thereby serve as a buffer against temperature-dependent loss of Notch signaling.

KEY WORDS: Notch signaling, O-glycosylation, *Drosophila*, EGF repeat, Recombineering

INTRODUCTION

The Notch signaling pathway plays key roles in numerous cell fate specification events throughout metazoan development (Fortini, 2009; Kopan and Ilagan, 2009; Tien et al., 2009). *Drosophila* Notch protein and its ligands, Delta and Serrate, are type I transmembrane proteins with multiple epidermal growth factor-like (EGF) repeats in their extracellular domain. Activation of the pathway occurs when ligands expressed on neighboring cells bind to the Notch receptor and trigger its proteolytic processing, which subsequently results in transcriptional regulation of downstream effectors. The EGF repeats of Notch are modified with several O-linked carbohydrates: O-fucose, O-GlcNAc (N-acetylglucosamine) and O-glucose (Moloney et al., 2000a; Shao et al., 2002; Acar et al., 2008; Matsuura et al., 2008). O-fucosylation of Notch, which is catalyzed by O-fucosyltransferase 1 (*O-fut1*), occurs at serine or threonine (S/T) residues in the consensus O-fucosylation motif C²-X-X-X-(S/T)-C³ (Shao et al., 2003). The O-fucose is then a substrate for 1,3-N-acetylglucosaminyltransferases encoded by *fringe* genes (Bruckner et al., 2000; Moloney et al., 2000b). Loss of *O-fut1* in *Drosophila* and of *Pofut1* in mice results in embryonic

lethality, with phenotypes similar to those observed upon the complete loss of Notch signaling (Okajima and Irvine, 2002; Okajima et al., 2003; Sasamura et al., 2003; Shi and Stanley, 2003). Elongation of O-linked fucose by Fringe is context specific and potentiates Notch-Delta signaling and inhibits Notch-Serrate signaling (Fleming et al., 1997; Panin et al., 1997; Bruckner et al., 2000; Hicks et al., 2000).

Notch proteins are also O-glycosylated at specific serine (S) residues of EGF repeats that contain the O-glycosylation consensus motif C¹-X-S-X-P-C² (Moloney et al., 2000a; Acar et al., 2008; Fernandez-Valdivia et al., 2011). Out of the 36 EGF repeats of *Drosophila* Notch, 18 contain a consensus O-glycosylation site (Fig. 1A). The functional importance of O-glycosylation in Notch signaling was demonstrated with the identification of the *Drosophila* protein O-glycosyltransferase Rumi as a temperature-dependent regulator of Notch signaling (Acar et al., 2008). RNAi-mediated knockdown of Rumi in *Drosophila* S2 cells causes a severe reduction in the level of O-glucose on Notch EGF repeats (Acar et al., 2008), suggesting that Rumi regulates Notch signaling by glycosylating Notch. However, the contribution of individual O-glucose residues on Notch to the regulation of Notch signaling is not known. Moreover, the proof that Notch is the biologically relevant substrate of Rumi requires the identification of functional sites of modification. To address these issues, we have performed in vivo structure-function studies on *Drosophila* Notch and found that Notch transgenes with O-glycosylation site mutations result in temperature-sensitive defects in Notch signaling. Our data indicate that all of the glucose residues on Notch contribute in additive and/or redundant fashions to maintain robust signaling as the temperature increases. Our data also indicate that although O-glycosylation is not required for ligand binding, O-glucose mutations in and around the ligand-binding domain exert a stronger effect on Notch function than other O-glucose mutations in Notch.

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MATERIALS AND METHODS

Drosophila strains

The following strains were used: *yw*, *N^{55e11}/FM7c*, *Kr-GAL4 UAS-GFP sn⁺*, *yw N⁵⁴¹⁹ FRT19A/FM7*, *Df(1)N⁸/FM7c*, *yw*; *FRT82B*, *FRT82B D^{prevF10} Ser^{RX82}/TM6B*, *Tb¹* (Micchelli et al., 1997), *UAS-Notch^{ΔNR-LexA}* (Lieber et al., 2002), *vas-int-ZH-2A*; *attVK22* (Venken et al., 2006), *nos-int-X*; *attP2* (Groth et al., 2004), *L/CyO*, *y⁺*, *yw Ubx-FLP Tub-GAL80 FRT19A*; *Act-GAL4 UAS-GFP^{mls}/CyO*, *y⁺*, *yw Ubx-FLP*; *FRT82B tub-GAL80 y⁺/TM6*, *Ubx*, *yw*; *FRT82B rumi^{Δ26}/TM6*, *Tb* (Acar et al., 2008), *N^{gt-wt}-attP2*, *N^{gt-wt}-attVK22*, *N^{gt-mut}-attVK22* (this study).

Molecular biology

To generate *Notch* genomic transgenes (see Fig. S1 in the supplementary material), we first retrieved a 40 kb fragment containing the *Notch* locus and its flanking sequences by recombineering-mediated gap repair (Liu et al., 2003) into the *attB-P[acman]-Ap^R* vector (Venken et al., 2006). *E. coli* SW102 cells (Warming et al., 2005) were used for recombineering. To introduce serine-to-alanine mutations into the *Notch-attB-P[acman]-Ap^R* construct, we performed what we call ‘GAP-repair mutagenesis’, which involves two rounds of recombineering (see Fig. S1 in the supplementary material). In the first round, we replaced the region of interest in the *Notch-attB-P[acman]-Ap^R* construct containing the EGF repeats to be mutagenized with a CAT-SacB cassette. For the second round of recombineering, we constructed a targeting vector that contains the same region of interest but harbors the desired *O*-glucose mutations flanked by the same homology arms used to target the CAT-SacB cassette into the *Notch-attB-P[acman]-Ap^R* construct. The mutations were made by site-directed mutagenesis (EGF4,5) or by gene synthesis (EGF10-35). In parallel, we generated a targeting construct containing the wild-type sequence of the EGF10-35 region by PCR. Targeting constructs with *O*-glucose mutations in smaller subsets of EGF repeats were constructed by restriction digestion and shuffling of fragments between the wild-type and mutant versions of the targeting vectors, or by site-directed mutagenesis. See Table S1 in the supplementary material for primer sequences.

Next, we performed recombineering between linearized *Notch-attB-P[acman]-Ap^R* containing the CAT-SacB cassette and the circular mutagenic targeting vector. Since the same homology arms were used in both constructs, GAP-repair recombination replaced the CAT-SacB cassette with the mutant targeting region. Once a positive clone was identified, the junctions to the *attB-P[acman]-Ap^R* vector and all exons were sequenced before injection.

Genetics

Generation of the *Notch* transgenes

A genomic source for the ΦC31 integrase (Bischof et al., 2007) was used to introduce the wild-type and mutant transgenes into the *VK22* docking site (57F5 on 2R) or the *attP2* docking site (68A4 on 3L, wild-type transgene only).

Gene dosage and rescue studies

yw/Y; *N^{gt-wt}/+* males were crossed to *yw* or *N^{+/FM7}* females and raised at the designated temperatures to obtain *N^{+/+}*, *N^{+/+}*; *N^{gt-wt}/+* and *N^{+/+}*; *N^{gt-wt}/+* females. *N^{gt-wt/gt-wt}* or *N^{gt-mut/gt-mut}* males were crossed to *N^{+/FM7}* females and raised at the designated temperatures to obtain *N^{+/Y}*; *N^{gt-wt}/+* and *N^{+/Y}*; *N^{gt-mut}/+* males, which were selected based on the absence of the *FM7* Bar eye phenotype.

Generation of MARCM clones

To generate *Notch* null MARCM clones, *yw Ubx-FLP Tub-GAL80 FRT19A*; *Act-GAL4 UAS-GFP^{mls}/CyO*, *y⁺* males were crossed to *yw N⁵⁴¹⁹ FRT19A/FM7c*, *Kr-GAL4 UAS-GFP sn⁺* or *yw N⁵⁴¹⁹ FRT19A/FM7c*, *Kr-GAL4 UAS-GFP sn⁺*; *N^{gt}/+* females. To generate clones overexpressing *Notch^{ΔNR-LexA}*, *yw Ubx-FLP*; *FRT82B tub-GAL80 y⁺/TM6*, *Ubx* females were crossed to the following males: *UAS-Notch^{ΔNR-LexA}/+*; *FRT82B/+*, *UAS-Notch^{ΔNR-LexA}/+*; *FRT82B D^{prevF10} Ser^{RX82}/+*, or *UAS-Notch^{ΔNR-LexA}/+*; *FRT82B rumi^{Δ26}/+*. Animals were raised at room temperature (21–23°C) until the second instar larval period and were then transferred to 30°C. Anti-LexA staining was used to confirm the expression of *Notch^{ΔNR-LexA}*.

Dissections, staining, image acquisition and processing

Dissection and staining were performed using standard methods. For surface staining of Notch, detergents were excluded from the protocol, as described previously (Baker and Yu, 1998; Wang and Struhl, 2004). Antibodies were mouse anti-NICD 1:1000, mouse anti-NECD 1:100 and mouse anti-Wingless 1:10 (DSHB); rabbit anti-LexA 1:1000 (MBL International); goat anti-mouse Cy3-conjugated and goat anti-mouse Cy5-conjugated 1:500 (Jackson ImmunoResearch Laboratories). Confocal images were scanned using a Leica TCS-SP5 microscope and processed with Amira5.2.2. Dissection, mounting and image acquisition for adult fly tissues were performed as described previously (Acar et al., 2008). Images were processed with Adobe Photoshop CS2 and were assembled in Adobe Illustrator CS2.

Cell aggregation and quantitative (q) RT-PCR assays

rumi^{-/-} cell lines were established from *rumi^{Δ26/Δ26}* embryos that simultaneously expressed an active form of Ras, Ras^{V12}, to promote the survival and proliferation of the cells (Simcox, A. et al., 2008). The control cells used in qPCR assays were established from embryos that expressed Ras^{V12} but were wild-type for *rumi*. Genomic PCR and western blotting confirmed that *rumi^{-/-}* cells are null for *rumi* (Simcox, A. A. et al., 2008). S2-DI cells were obtained from DGRC (Indiana University, Bloomington, IA, USA). For cell aggregation assays, 5×10^5 S2-DI cells (induced overnight with 0.7 mM CuSO₄) or S2 cells were mixed with 2.5×10^5 (1) *rumi^{-/-}* cells raised at 23°C (room temperature) or (2) *rumi^{-/-}* cells raised at 32°C in a total volume of 200 μl medium in a 24-well plate. Cells were then co-cultured and gently shaken at 150 rpm to allow aggregation. Images of aggregate formation were taken at 30-second intervals. Experiments were also repeated using a lower concentration of *rumi^{-/-}* cells (0.5×10^5). *E(spl)m3* and *rp49* (*HLHm3* and *RpL32* – FlyBase) mRNA expression in *rumi^{-/-}* and control cells (cultured at 23°C or 32°C) were assayed by qRT-PCR using TaqMan One-Step RT-PCR Master Mix and primers/probe sets from Applied Biosystems. Relative *E(spl)m3* mRNA levels were compared using the 2^{-ΔΔCT} method. *P*-values were determined by Student’s *t*-test.

RESULTS

A 40 kb *Notch* genomic transgene is functional in vivo

To determine the contribution of *O*-glucose residues on Notch to the regulation of Notch signaling we performed in vivo structure-function studies. The *Notch* (*N*) gene is dosage sensitive, and *rumi* mutations show a temperature-sensitive loss of Notch signaling. Therefore, to avoid the potential artifacts inherent to overexpression studies, we used recombineering (Copeland et al., 2001; Venken et al., 2006) and generated a 40 kb *Notch* genomic transgene in which the expression of Notch is driven by the endogenous promoter and enhancers (Fig. 1B).

Flies that carry a single copy of the *N^{gt-wt}* (*Notch* genomic transgene-wild type) show the Confluens phenotype (extra veins in the wing, Fig. 1D), which is exhibited by animals with a *Notch* duplication (Lyman and Young, 1993). In female *N^{+/+}* flies, thickening of the wing vein and wing margin loss are observed (Fig. 1E). However, *N^{+/+}*; *N^{gt-wt}/+* female flies show neither the haploinsufficient *N^{+/+}* phenotype nor the extra wing vein phenotype caused by the *N^{gt-wt}* transgene (Fig. 1F). Similar results were obtained for two independent insertions of the *N^{gt-wt}* and for several null alleles of *Notch* raised at 18–30°C (see Fig. S2 in the supplementary material; data not shown). These observations indicate that, at a genetic level, *N^{gt-wt}* behaves similarly to one copy of endogenous *Notch*.

Notch is an X-linked gene and hemizygous males harboring null alleles of *Notch* are embryonic lethal (Artavanis-Tsakonas et al., 1983). This lethality can be rescued by a single copy of the *N^{gt-wt}* at low and high temperatures and the rescued flies resemble wild-

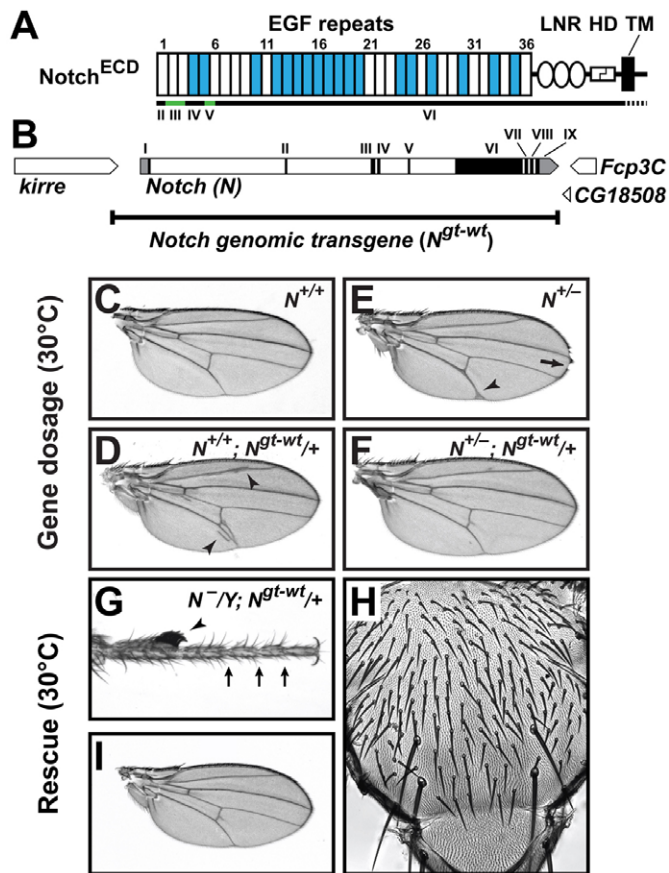


Fig. 1. A 40 kb *Notch* genomic transgene (N^{gt-wt}) behaves similarly to an endogenous copy of *Notch*. (A) The Notch extracellular domain (Notch^{ECD}) with 36 EGF repeats, three LNR domains and heterodimerization (HD) and transmembrane (TM) regions. EGF repeats with an *O*-glucosylation motif are in blue. Corresponding exons encoding the Notch^{ECD} are marked beneath. (B) The *Notch* genomic region (exons in black) and the 40 kb *Notch* genomic transgene (N^{gt-wt}). (C-F) Gene dosage experiments at 30°C. (C) A wild-type adult female *Drosophila* wing. (D) The Confluens phenotype with the addition of one copy of the N^{gt-wt} transgene inserted at the VK22 docking site. Arrowheads mark extra wing veins. (E) A *Notch*^{+55e11} haploinsufficient female wing with thickened veins (arrowhead) and wing margin loss (arrow). This phenotype can be rescued with one copy of N^{gt-wt} (F). (G-I) A *Notch*^{55e11}/Y hemizygous male rescued with one copy of the N^{gt-wt} transgene inserted at VK22 at 30°C, showing normal legs (G, arrowhead marks the sex comb, arrows mark the leg joints), normal bristle pattern on the thorax (H) and normal wings (I).

type males (Fig. 1G-I; data not shown). The N^{gt-wt} also rescues the lethality and zygotic phenotypes of $N^{-/-}$ females. Together, these observations indicate that the N^{gt-wt} is functional in vivo and can be used to assess the effects of *O*-glucose mutations on Notch signaling at various temperatures.

All EGF repeats with a C¹-X-S-X-P-C² consensus sequence that have been examined so far harbor an *O*-glucose (Hase et al., 1988; Nishimura et al., 1989; Moloney et al., 2000a; Acar et al., 2008; Matsuura et al., 2008; Bakker et al., 2009; Whitworth et al., 2010; Fernandez-Valdivia et al., 2011), indicating that this motif is highly predictive for *O*-glucosylation. Indeed, the presence of *O*-glucose on nearly all predicted sites on *Drosophila* Notch has been

experimentally confirmed (N. Rana and R. Haltiwanger, personal communication). To assess the contribution of *O*-glucosylation sites to the function of Notch, we introduced serine-to-alanine (S-to-A) mutations in the consensus sequence to abolish the addition of *O*-glucose. The S-to-A change is unlikely to result in misfolding of the EGF repeat, as some EGF repeats naturally contain a C¹-X-A-X-P-C² motif. We generated a series of *Notch* mutant transgenes with S-to-A mutations in various subsets of EGF repeats (Fig. 2). To minimize the expression variability among transgenes, we used ΦC31-mediated integration (Venken et al., 2006; Bischof et al., 2007) to insert each transgene into the same locus in the fly genome. We refer to the mutant *Notch* genomic transgenes as N^{gt-mut} , with numbers that correspond to the mutated EGF repeats; for example, $N^{gt-4..35}$ contains mutations in the EGF4-35 regions and $N^{gt-4,5}$ contains mutations in EGF4 and 5.

All *O*-glucose residues on Notch contribute to its function

To determine the contribution of the various Notch *O*-glucosylation sites to Notch signaling, we systematically tested the N^{gt-mut} transgenes for their ability to rescue the lethality and phenotypes of *Notch* null mutants at low, intermediate and high temperatures, and compared the resulting phenotypes with those caused by loss of *rumi*. At 30°C, *rumi*^{-/-} animals die at the late larval stage (Acar et al., 2008). At 25°C, some *rumi*^{-/-} animals reach adulthood but display a very severe loss of bristles on the thorax and a shortening of the legs (Fig. 3A,A'). Note that these flies die on the food after eclosion or trapped in the pupal case while eclosing, presumably owing to leg defects. As the temperature is decreased to room temperature (21-23°C), 76% of *rumi*^{-/-} adults ($n=25/33$) exhibit an intermediate loss of microchaetae (Fig. 3B) and 24% ($n=8/33$) show a severe loss of microchaetae (Fig. 3C). At 21-23°C, the legs are not short, but subtle leg joint defects are commonly observed (Fig. 3B',C'; $n=17/28$). When raised at 18°C, most *rumi*^{-/-} animals show small patches of microchaetae loss in anterior parts of the notum and a slight increase in the density of microchaetae, which suggests defects in lateral inhibition (see Fig. S3A in the supplementary material) (Acar et al., 2008). Some animals raised at this temperature only exhibit a mild lateral inhibition defect (see Fig. S3B in the supplementary material). No shortening of the legs is observed at 18°C, although most animals show minor defects in leg joints (see Fig. S3A',B' in the supplementary material). We used the microchaetae pattern on the thorax and the leg morphology as readouts of in vivo Notch activity in our rescue experiments.

A summary of the degree of bristle phenotype rescue by various *O*-glucose mutant *Notch* transgenes at different temperatures is shown in Fig. 2. We first tested $N^{gt-4..35}$, in which all 18 *O*-glucosylation sites are mutated. At 21-23°C, a number of rescued parate adults were observed, most of which showed an almost complete loss of microchaetae on the thorax (Fig. 3D; $n=6/7$). We observed variability at this temperature, as one of the rescued animals had some intact microchaetae (Fig. 2; data not shown). Note that macrochaetae patterning is not affected in the rescued animal (Fig. 3D), similar to *rumi*^{-/-} mutants (Fig. 3A-C) (Acar et al., 2008). The rescued animals commonly exhibit mild defects in leg joints (Fig. 3D'), but no leg shortening. At 18°C, the rescued animals show a mild loss of microchaetae and normal legs (see Fig. S3C,C' in the supplementary material). Since the only source of Notch in the rescued animals is from the $N^{gt-4..35}$ transgene, these results show that when all *O*-glucosylation motifs in Notch are mutated, the Notch protein remains largely functional at 18°C, but

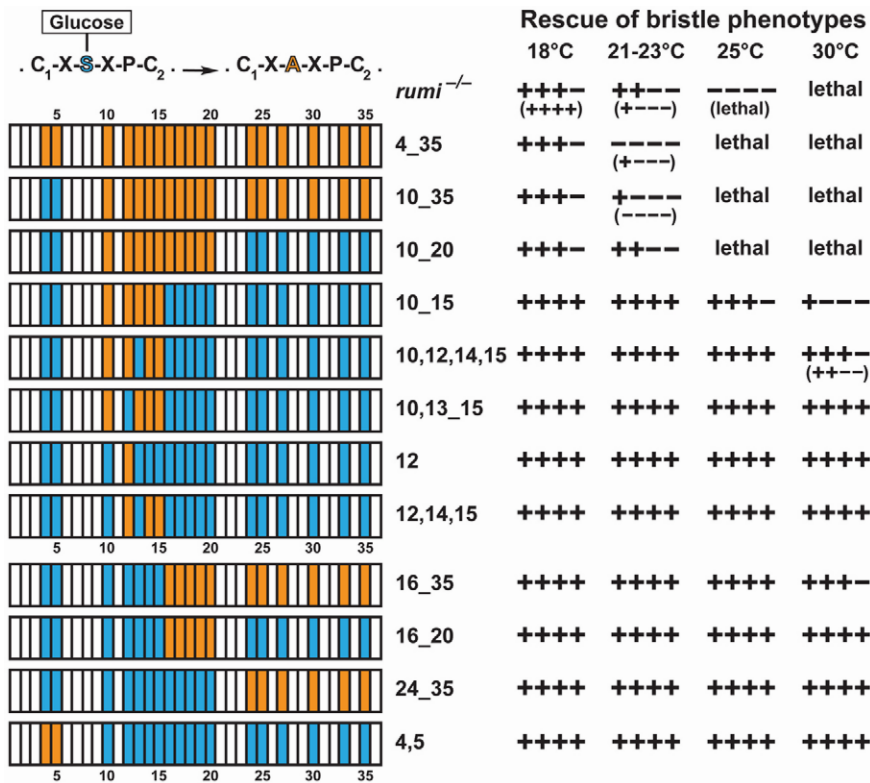


Fig. 2. The mutant *Notch* transgenes used in this study and a summary of results from the rescue studies.

Blue or orange boxes represent EGF repeats with a wild-type *O*-glucosylation motif or with an S-to-A mutation, respectively. Each mutant *Notch* genomic transgene (*N^{gt-mut}*) was tested for its ability to rescue a null allele of *Notch*. The extent of microchaetae rescue by each transgene is indicated, from '++++' indicating a normal microchaetae pattern in the rescued flies to '----' indicating an almost complete loss of microchaetae in the rescued flies. 'Lethal' refers to animals that did not reach the pharate adult stage. *rumi*^{-/-} phenotypes are shown for comparison. When phenotypes at a particular temperature are variable, the less common phenotype is shown in parentheses.

loses a significant level of its activity at 21-23°C. The similarities between the temperature-sensitive phenotypes of *N^{-/Y}; N^{gt-4_35}/+* and *rumi*^{-/-} animals indicate that the Notch protein is indeed a biologically relevant target of Rumi in flies. We note that some of the *N^{-/Y}; N^{gt-4_35}/+* phenotypes are slightly stronger than *rumi*^{-/-} phenotypes, possibly owing to subtle differences in the Notch protein level encoded by the *Notch* transgene compared with the endogenous *Notch* locus and/or differences in the genetic background. Nonetheless, our data indicate that the *Notch* genomic transgene provides a good model with which to assess the role of *O*-glucosylation sites in Notch signaling.

We next tested *Notch* genomic transgenes that contain mutations in subsets of EGF repeats. *N^{gt-4,5}*, with mutations in EGF4 and 5, fully rescues the lethality and phenotypes of *Notch* null mutants even at 30°C (Fig. 2; data not shown). Its complementary mutant transgene, *N^{gt-10_35}*, in which all *O*-glucosylation sites except for those in EGF4 and 5 are mutated, rescues *Notch* null alleles to pharate adulthood at 21-23°C. Four out of the nine rescued animals showed almost complete microchaetae loss (Fig. 2; data not shown) and the remainder showed a severe loss of microchaetae with some intact bristles (Fig. 3E). Leg joint defects without leg shortening were present in most of the rescued animals (Fig. 3E'). Overall, the *N^{-/Y}; N^{gt-4_35}/+* phenotypes seem to be more severe than the *N^{-/Y}; N^{gt-10_35}/+* phenotypes at 21-23°C. Together, these observations indicate that *O*-glucosylation of EGF4 and 5 is not essential for the function of Notch but contributes to Notch signaling in a redundant fashion.

We further dissected the role of the 16 *O*-glucosylation sites in EGF10-35. *N^{gt-24_35}*, with six *O*-glucose mutations between EGF24 and 35, fully rescues the lethality and phenotypes of *Notch* mutants at 30°C (Fig. 2; data not shown). The complementary transgene, *N^{gt-10_20}*, with mutations in EGF10 and EGF12-20, rescues *Notch* mutants to pharate adulthood at 21-23°C. However, the rescued

animals show an intermediate loss of microchaetae (Fig. 3F). Some rescued animals show leg joint defects (Fig. 3F'). Again, *N^{-/Y}; N^{gt-10_20}/+* show weaker phenotypes than *N^{-/Y}; N^{gt-10_35}/+* animals raised at the same temperature. Together, these data indicate an important role for the *O*-glucose motifs in EGF10-20 and strongly suggest that the six *O*-glucose residues in EGF24-35 contribute to signaling in a redundant fashion, even though they are not essential when other *O*-glucosylation sites are intact. Of note, at 18°C, *N^{gt-10_20}*, *N^{gt-10_35}* and *N^{gt-4_35}* show comparable abilities in rescuing the phenotypes of *Notch* mutants (see Fig. S3C-E' in the supplementary material). These observations suggest that, although the *O*-glucose motifs in EGF10-20 are the key targets of Rumi in the regulation of Notch signaling, *O*-glucose on EGF repeats outside of this region become important when the animals are raised at higher temperatures.

A combination of redundant and additive functions for *O*-glucose residues on Notch

N^{-/Y}; N^{gt-16_35}/+ flies reach pharate adulthood at 30°C and only show a mild loss of microchaetae (Fig. 2; data not shown). Moreover, *N^{gt-16_20}* fully rescues the bristle and leg phenotypes of *Notch* alleles at 30°C (Fig. 2; data not shown). These data suggest an important role for the *O*-glucosylation of EGF10-15. Indeed, at 30°C, *N^{-/Y}; N^{gt-10_15}/+* reach pharate adulthood but show a severe loss of bristles on the thorax, loss of leg joints and severe leg shortening (Fig. 4A,A'). When the rescued animals are raised at 25°C, they readily eclose, the severity of bristle loss is significantly decreased and the legs look normal (Fig. 4B,B'). At lower temperatures (18-23°C), the rescued animals show a normal bristle pattern and normal legs (Fig. 4C,C'; data not shown). These data indicate that a key aspect of Notch pathway regulation by Rumi is the addition of *O*-glucose to EGF10-15 of Notch. Since both *N^{-/Y}; N^{gt-10_15}/+* and *N^{-/Y}; N^{gt-16_35}/+* phenotypes are milder than *N^{-/Y};*

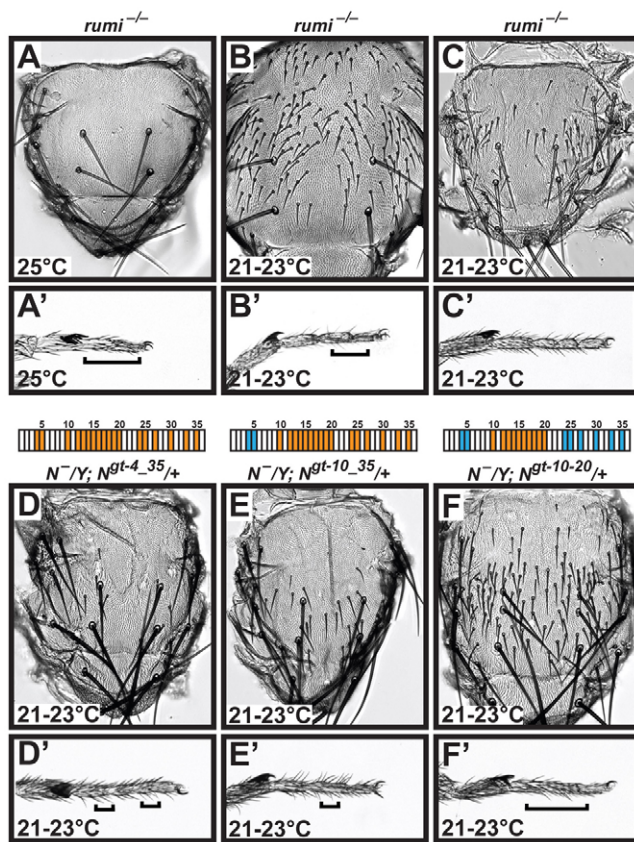


Fig. 3. O-glucose mutations in Notch cause bristle loss and leg abnormalities that recapitulate *rumi* mutations. (A–C') Bristle and leg phenotypes of flies homozygous for the protein-null allele *rumi*^{A26} (*rumi*^{-/-}). (D, D') *N*⁻/*Y*; *Ngt*^{4_35}/*+* males show very severe bristle loss and leg joint abnormalities at 21–23°C. (E, E') *N*⁻/*Y*; *Ngt*^{10_35}/*+* males show severe bristle loss and leg joint abnormalities at 21–23°C. (F, F') *N*⁻/*Y*; *Ngt*^{10_20}/*+* males show some bristle loss and leg joint abnormalities at 21–23°C. Brackets mark leg shortening and/or leg joint abnormalities.

Ngt^{10_35}/*+* phenotypes, we conclude that O-glucosylation sites in the EGF10–35 region regulate Notch signaling in an additive fashion.

To examine whether O-glucosylation of EGF10–15 is absolutely required for the function of Notch at the restrictive temperature, we tested the effects of increasing the dosage of the *Ngt*^{10_15} transgene on its ability to rescue *Notch* null phenotypes. As shown in Fig. 4D, D', two copies of the *Ngt*^{10_15} transgene significantly rescue the bristle and leg phenotypes of a *Notch* null allele at 30°C, although not completely because the rescued animals still display some leg joint defects and die as pharate adults. These observations strongly suggest that even at the restrictive temperature, the remaining 13 O-glucosylation sites in the *Ngt*^{10_15} transgene can compensate for the loss of O-glucose on EGF10–15 upon increasing the dosage of this transgene.

The temperature-sensitive decrease in the activity of the Notch protein encoded by the *Ngt*^{10_15} transgene might be due to the loss of O-glucose on a single EGF repeat or to the combined loss of O-glucose on multiple EGF repeats in this region. To distinguish between these alternatives, we reverted a single mutation in the ligand-binding EGF12 to the wild-type sequence (A to S). The *Ngt*^{10,13_15} transgene, with a wild-type O-glucosylation site in EGF12

but mutations in EGF10,13–15, is able to rescue the lethality and phenotypes of *Notch* mutants at 30°C (Fig. 4E, E', compare with 4A, A'). These results indicate that O-glucose on EGF12 is sufficient in the EGF10–15 region for Notch to function properly even at high temperature. This led us to test whether loss of O-glucose on EGF12 can mimic the loss of O-glucose on EGF10–15. Surprisingly, *Ngt*¹² fully rescues the lethality and phenotypes of *Notch* null mutants at 30°C (Fig. 4F, F'), suggesting that when other O-glucosylation motifs on Notch are intact, the O-glucose residue on the ligand-binding EGF12 is dispensable for Notch signaling at high temperature.

We also reverted the S-to-A mutation in EGF13 by generating the *Ngt*^{10,12,14,15} transgene. *N*⁻/*Y*; *Ngt*^{10,12,14,15}/*+* males raised at 30°C show a mild to moderate bristle loss on the thorax and occasional leg joint defects (Fig. 4G, G'). Comparison of these phenotypes with those of *N*⁻/*Y*; *Ngt*^{10_15}/*+* males raised at 30°C (Fig. 4A, A') indicates that a single O-glucose on EGF13 can partially restore the activity of *Ngt*^{10_15}. *Ngt*^{12,14,15}/*+* with restored O-glucosylation motifs in EGF10 and EGF13 fully rescues *Notch* null phenotypes, even when raised at 30°C (Fig. 4H, H'). Altogether, we conclude that even though no single O-glucose mutation decreases Notch signaling in our assays, O-glucose residues on Notch EGF repeats contribute both additively and redundantly to Notch signaling, especially at high temperatures.

O-glucose mutations suppress the Confluens Notch duplication phenotype at high temperatures

A genomic duplication containing the *Notch* locus results in the classical Confluens phenotype characterized by extra wing vein tissue (Welshons, 1971). Similarly, males with one copy of the *Ngt*^{wt} transgene show a Confluens phenotype (Fig. 5A–A''). This phenotype is also dosage sensitive, as males with two extra copies of *Ngt*^{wt} show an enhancement of the extra and expanded wing vein phenotypes (Fig. 5B–B'', arrowheads), with occasional blisters in the wing. The degree of wing vein expansion and the distribution of ectopic vein tissue are not altered when flies are raised at 18–30°C (Fig. 5A–B''), suggesting that at the phenotypic level the activity of wild-type Notch is not significantly affected by temperature changes in this range.

As described, *Ngt*^{10_15} contains five O-glucose mutations in the EGF10–15 region. Male flies with two extra copies of *Ngt*^{10_15} also show extra wing vein tissue and wing vein expansion (Fig. 5C–C''). However, as the temperature at which the flies are cultured increases, these phenotypes become less severe (Fig. 5C'', C'''). Male flies with two extra copies of *Ngt*^{10_35}, which contains 16 mutations in the EGF10–35 region, show the extra vein phenotype at 18–23°C (Fig. 5D, D') but not at higher temperatures (Fig. 5D'', D'''), indicating that O-glucose mutations render the Notch protein sensitive to temperature increase. Note that even at 18°C, the extent of extra veins caused by *Ngt*^{10_35} is not as severe as that caused by *Ngt*^{wt} (compare Fig. 5D with 5B), strongly suggesting that when the majority of O-glucose residues on Notch are lost, its activity is somewhat decreased even at low temperatures. These data are consistent with the mild loss of Notch signaling observed in *N*⁻/*Y*; *Ngt*^{10_35}/*+* and in *rumi*^{-/-} animals raised at 18°C (see Fig. S3 in the supplementary material) (Acar et al., 2008). Similar data were obtained for *Ngt*^{4_35}, in which all O-glucose motifs are abolished (data not shown). We conclude that O-glucose mutations decrease the activity of Notch in a temperature-dependent manner, and that there is an inverse correlation between the number of mutated O-glucose sites and the ability of the Notch protein to function at higher temperatures.

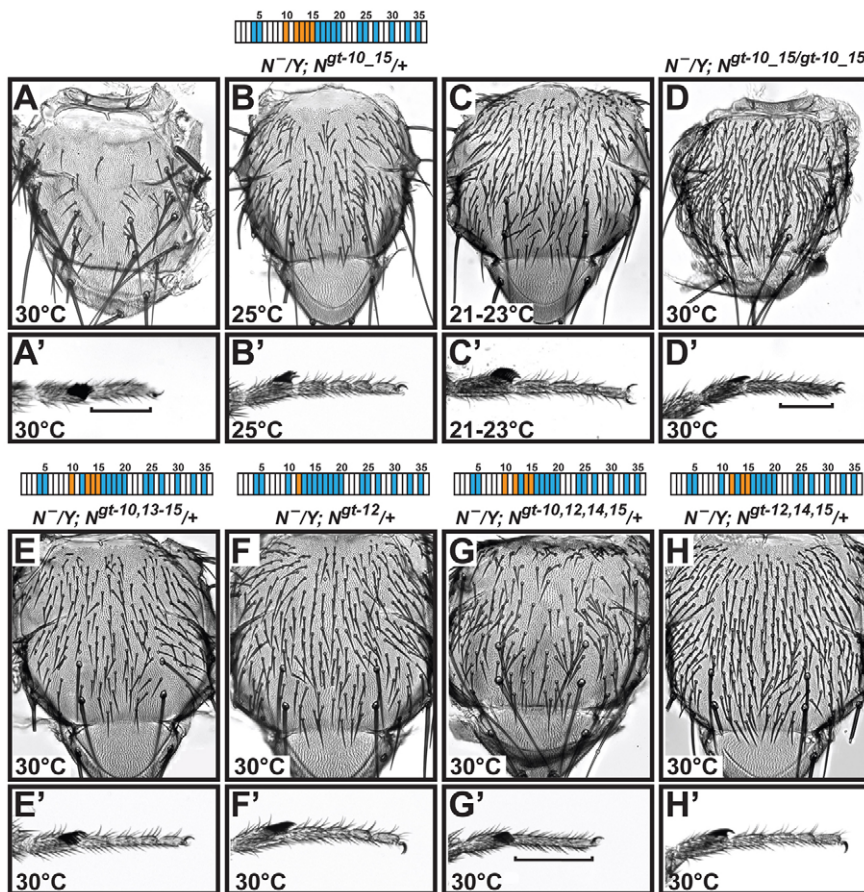


Fig. 4. O-glucose residues on EGF10-15 of Notch show a combination of additive and redundant functions. (A,A') At 30°C, N^{-}/Y ; $N^{gt-10-15}/+$ male flies show a severe loss of bristles and shortened legs with severe joint defects. (B,B') At 25°C, N^{-}/Y ; $N^{gt-10-15}/+$ males show a mild loss of bristles and normal legs. (C,C') At 21-23°C, $N^{gt-10-15}$ fully rescues the lethality and phenotypes of *Notch* null mutants. (D,D') The leg defects and microchaetae loss of the N^{-}/Y ; $N^{gt-10-15}/+$ males at 30°C are significantly improved by the addition of a second copy of the $N^{gt-10-15}$ transgene (compare with A,A'). (E-F') N^{-}/Y ; $N^{gt-10,13-15}/+$ (E,E') and N^{-}/Y ; $N^{gt-12}/+$ (F,F') males show a normal bristle pattern and normal legs at 30°C. (G,G') N^{-}/Y ; $N^{gt-10,12,14,15}/+$ males show mild bristle loss and abnormal leg joints at 30°C. (H,H') $N^{gt-12,14,15}$ fully rescues the phenotypes of a *Notch* null allele at 30°C. Brackets (A',D',G') mark leg shortening and leg joint abnormalities.

O-glucose mutations do not disrupt the endoplasmic reticulum exit or cell surface expression of Notch

The similarities between the phenotypes observed in *Notch* mutants rescued by the mutant *Notch* transgenes and in *rumi*^{-/-} mutants strongly suggest that loss of O-glucose on Notch EGF repeats is the bona fide reason for the temperature-dependent decline in the function of mutant Notch proteins. However, it is possible that the

lack of signaling at high temperature occurs because the Notch protein with multiple S-to-A mutations becomes severely misfolded, is trapped in the endoplasmic reticulum and therefore cannot traffic to the cell surface. To address this issue, we generated animals with mosaic clones of the *Notch*⁵⁴¹⁹ null allele with or without a copy of our N^{gt} transgenes and performed immunostainings to examine the distribution of the Notch protein. Since *Notch*⁵⁴¹⁹ is a protein-null allele (Fig. 6A,A'), the only source of Notch in *Notch*⁵⁴¹⁹ clones will

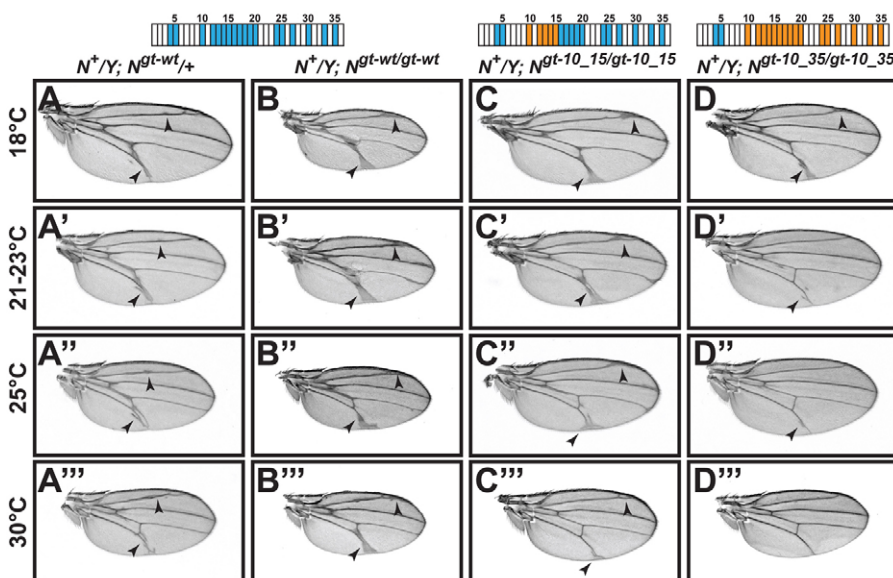


Fig. 5. O-glucose mutations decrease the activity of Notch in a temperature-dependent manner. Wings of adult male flies with wild-type *Notch* on the X chromosome and one or two copies of N^{gt-wt} (A-B'') or two copies of N^{gt-mut} (C-D'') transgenes inserted at the VK22 docking site on the second chromosome. (A-A'') One copy of N^{gt-wt} results in a Confluens phenotype (extra vein, arrowheads) at 18-30°C. (B-B'') Two copies of N^{gt-wt} cause an enhancement of the Confluens phenotype at 18-30°C. (C-C'') At 18°C, two copies of $N^{gt-10-15}$ show a Confluens phenotype comparable to that caused by two copies of N^{gt-wt} (compare B and C). The amount of extra vein tissue gradually decreases as the temperature is increased from low (18°C) to high (30°C). (D-D'') The extra vein phenotype caused by two copies of $N^{gt-10-35}$ at 18°C and 23°C is much milder than that caused by N^{gt-wt} and $N^{gt-10-15}$ (compare with B,B' and C,C'). At 25°C (D') and 30°C (D''), almost no Confluens phenotype is observed.

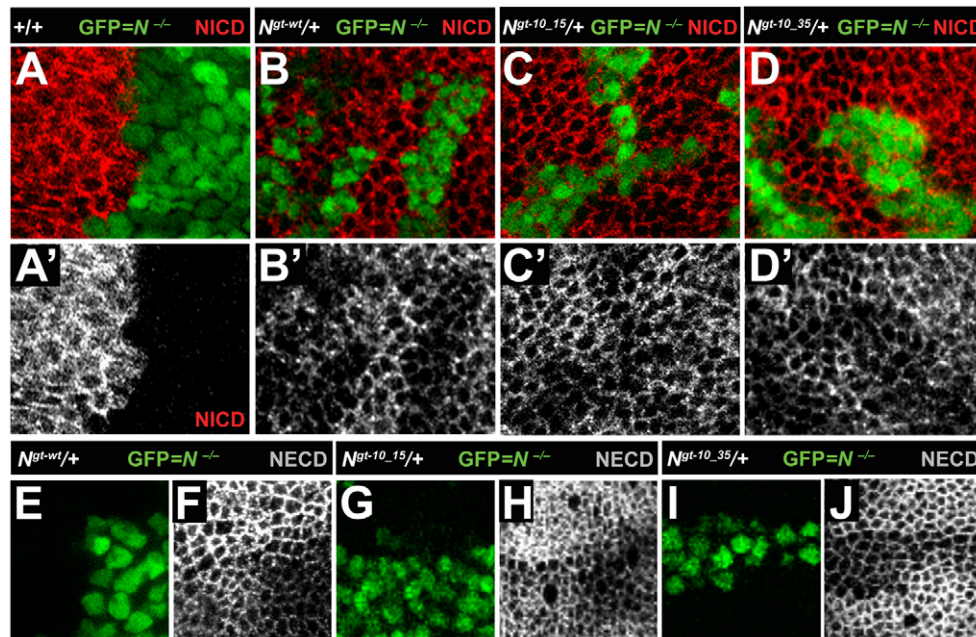


Fig. 6. O-glucose mutations do not alter the level of Notch expression or its traffic to the cell surface. Confocal images of wing imaginal discs of third instar *Drosophila* larvae raised at 30°C. Nuclear GFP (green) marks MARCM clones of the N^{5419} allele. (A-D') $N^{5419/5419}$ MARCM clones are generated in the absence (A,A') or presence (B-D') of one copy of wild-type (B,B') or mutant (C-D') *Notch* transgenes and stained with anti-NICD antibody (red in A-D, gray in A'-D'). (A,A') Lack of Notch staining in the clones indicates that N^{5419} is a protein-null allele. Notch proteins with mutations in EGF10-15 (C,C') or EGF10-35 (D,D') are expressed at levels, comparable to wild-type Notch (B,B'). (E-J) Surface expression of Notch. (F) A projection of three consecutive apical optical sections; (H,J) single apical optical sections; (E,G,I) basolateral optical sections from the same datasets as F,H,J, respectively.

be that expressed from N^{gt} transgenes. When raised at 30°C, *Notch*⁵⁴¹⁹ homozygous clones that carry one copy of the N^{gt-wt} transgene expressed Notch in a pattern similar to that of the neighboring cells (Fig. 6B,B'). As expected, the level of Notch in the neighboring tissues is higher, because in addition to one copy of the *Notch* transgene the heterozygous tissue and the twin spots have one or two copies of endogenous *Notch*, respectively. *O*-glucose mutant Notch proteins encoded by N^{gt-10_15} and N^{gt-10_35} show a staining pattern that is comparable to that generated by N^{gt-wt} at 30°C (Fig. 6C-D'). Similar expression patterns are observed at 18°C (data not shown). Immunostainings in the absence of detergent show that wild-type and *O*-glucose mutant Notch proteins encoded by N^{gt-wt} , N^{gt-10_15} , N^{gt-10_35} and N^{gt-4_35} can reach the cell surface with comparable efficiency (Fig. 6E-J; data not shown). We conclude that the significant decrease in the activity of *O*-glucose mutant Notch proteins at high temperature cannot be explained by impaired exit from the endoplasmic reticulum or impaired trafficking to the cell surface.

Timecourse cell aggregation studies indicate normal Notch-ligand binding upon complete loss of Rumi

Structural studies have suggested that *O*-glucose on EGF12 of human NOTCH1 is located on its ligand-interacting 'face' and might therefore modulate Notch-ligand binding (Cordle et al., 2008). Our data indicate an important role for the *O*-glucosylation motifs in and around the ligand-binding region of *Drosophila* Notch, raising the possibility that *O*-glucosylation of EGF12 might affect ligand binding. However, a soluble form of Notch expressed in S2 cells undergoing RNAi-mediated Rumi knockdown efficiently binds the surface of S2-Delta (DI) cells (Acar et al., 2008). Moreover, N^{gt-12}

can completely rescue a *Notch* null allele (Fig. 4E,E'), arguing against this scenario. To clarify this issue, we sought to determine whether full-length Notch expressed in the complete absence of Rumi shows a temperature-dependent decrease in ligand binding. We have established several independent *rumi*^{-/-} cell lines from the protein-null allele *rumi*^{Δ26} (Simcox, A. A. et al., 2008). These cells do not express Rumi, but express low levels of Notch and Delta (data not shown). qRT-PCR experiments indicate that the expression of the Notch target gene *E(spl)m3* in *rumi*^{-/-} cells is dramatically decreased at high temperatures (Fig. 7A), indicating that these cells recapitulate the temperature-dependent loss of Notch signaling observed in *rumi*^{-/-} animals.

To examine the effects of loss of Rumi on Notch-ligand binding, we co-cultured *rumi*^{-/-} cells with S2-Delta cells and followed the rate and size of the aggregates formed between these cells at low and high temperatures. When co-cultured with S2 cells, which do not express Notch ligands, *rumi*^{-/-} cells only make very small aggregates (Fig. 7B). However, when mixed with an identical number of S2-Delta cells, *rumi*^{-/-} cells quickly form aggregates that reach a much larger size than those observed in *rumi*^{-/-} S2 co-culture (Fig. 7B). Similar results were obtained using *rumi*^{-/-} cells that have been cultured overnight at 32°C (Fig. 7B). Quantification of the number of aggregates shows that the rate of aggregate formation between S2-Delta cells and *rumi*^{-/-} cells cultured at low and high temperatures is similar (see Fig. S4 in the supplementary material). Since the size of aggregates and the rate of aggregate formation closely correlate with the Notch-ligand binding strength (Ahimou et al., 2004), our data suggest that the temperature-dependent decrease in Notch signaling observed in the absence of Rumi cannot be explained by decreased binding between Notch and its ligands.

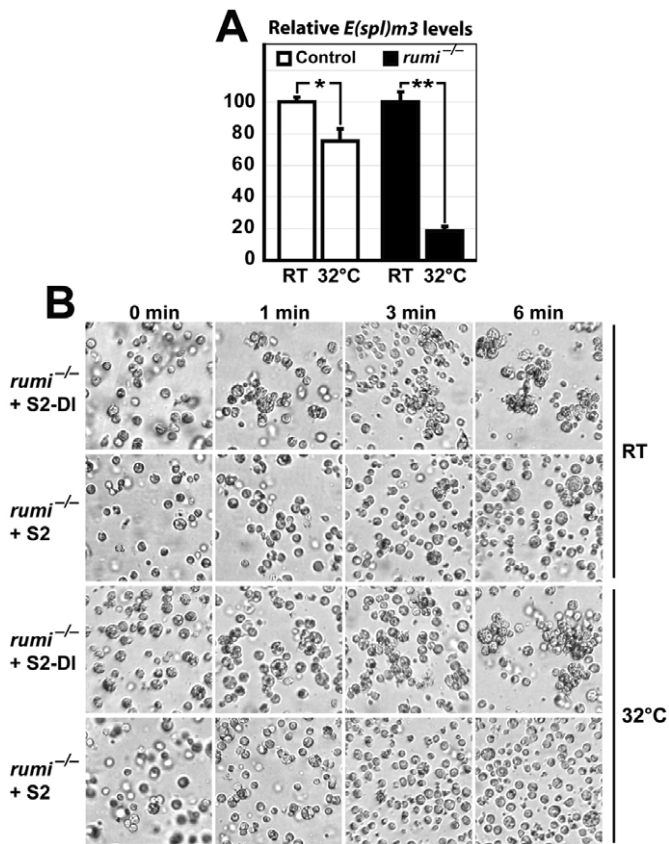


Fig. 7. Complete loss of Rumi does not affect the binding of endogenous Notch to Delta at the surface of neighboring cells.

(A) Relative mRNA level of *E(spl)m3* as measured by qRT-PCR in control and *rumi*^{-/-} *Drosophila* cells cultured at either room temperature (RT, 21–23°C) or 32°C. **P*<0.04, ***P*<0.00035. Error bars indicate s.e.m. (B) Cell aggregation assays between *rumi*^{-/-} cells (cultured at room temperature or 32°C) and S2-Delta (S2-DI) or S2 cells. Images of co-cultures at 0, 1, 3 and 6 minutes.

***rumi* suppresses the hyperactivation of Notch caused by the deletion of the LNR motif**

Deletion of the LIN-12/Notch (LNR) motif from Notch results in ligand-independent S2 cleavage and activation of Notch in *Drosophila* embryos and in mammalian cell lines (Lieber et al., 2002; Sanchez-Irizarry et al., 2004). Consistently, we find that overexpression of Notch^{ΔLNR-LexA} in MARCM clones of a wild-type chromosome results in strong activation of Notch signaling in the wing imaginal discs, as evidenced by imaginal disc overgrowth and induction of the Notch downstream target Wingless in these clones (Fig. 8A–B'). Activation of Notch^{ΔLNR-LexA} does not depend on the presence of ligands (Fig. 8C–D'). However, loss of *rumi* fully suppresses the Notch^{ΔLNR-LexA} gain-of-function phenotypes (Fig. 8E–F'). These observations suggest that *O*-glucosylation of the Notch EGF repeats is a prerequisite for S2 cleavage at high temperatures, independent of the function of ligands and the LNR motif.

DISCUSSION

Our studies indicate that the Notch receptor is the key target of the protein *O*-glucosyltransferase Rumi in the *Drosophila* Notch signaling pathway, as the temperature-sensitive loss of Notch

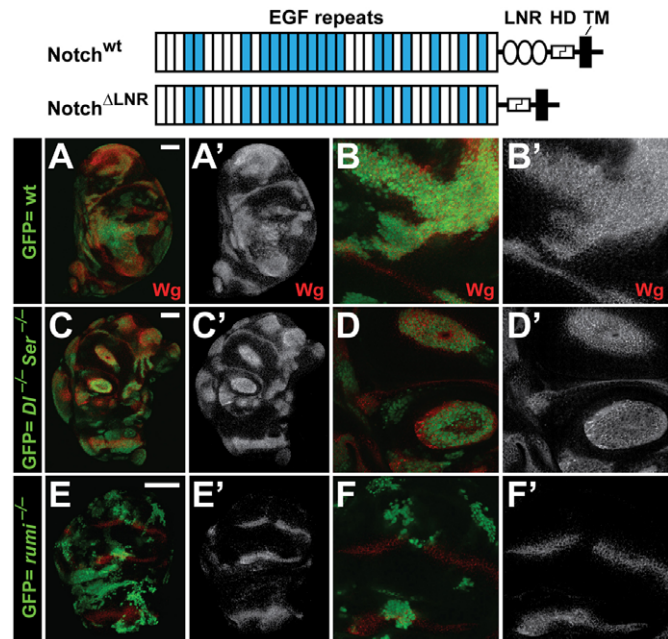


Fig. 8. Loss of *rumi* suppresses the gain-of-function phenotype resulting from the overexpression of Notch^{ΔLNR}. (A–F') Confocal images of wing imaginal discs of third instar *Drosophila* larvae raised at 30°C. Nuclear GFP (green) marks MARCM clones of wild type (A–B'), *Delta Serrate* (*Df Ser*) double mutant (C–D') or *rumi* mutant (E–F') overexpressing Notch^{ΔLNR}. Discs are stained with anti-Wingless (Wg) antibody (red in A–F, gray in A'–F'). (A,C,E) Projections of multiple optical sections; (B,D,E) higher magnifications of A,C,E, as projections of several sections.

signaling observed in *rumi* mutants can be recapitulated by mutations in the *O*-glucosylation motifs of Notch. In the mouse, a single knock-in mutation that abolishes the *O*-fucosylation of EGF12 of Notch1 results in decreased ligand binding of Notch1 and behaves as a hypomorphic allele (Ge and Stanley, 2008). Furthermore, overexpression studies in *Drosophila* indicate that a single *O*-fucose mutation in EGF12 significantly increases the activation of Notch by Serrate, most likely owing to an accompanying increase observed in the binding of Notch to Serrate (Lei et al., 2003). However, our data indicate that no single *O*-glucosylation motif, including that in EGF12, is essential for *Drosophila* Notch signaling. Even though *O*-glucose sites in EGF10–15 make a significant contribution to Notch signaling at high temperature, the *N/Y; Ngt-10-15/+* males only show Notch loss-of-function phenotypes at 25°C or higher and still reach the pharate adult stage at 30°C. These observations suggest a role for other *O*-glucose residues, in agreement with the mild decrease in the activity of the Notch *Ngt-16-35* transgene. The activity of *Ngt-10-20* is considerably less than that of *Ngt-10-15*, but mutating only the *O*-glucose sites on EGF16–20 does not affect the ability of *Ngt-16-20* to rescue the lethality and the bristle and leg phenotypes of a *Notch* null allele. These examples, together with similar comparisons between the various other mutant transgenes, indicate that all *O*-glucose residues contribute in additive and redundant fashions to ensure robust Notch signaling, especially at high temperatures.

We propose that rather than a local contribution to facilitate specific lectin-type interactions, the *O*-glucose residues on Notch EGF repeats function globally to maintain the Notch extracellular domain in a conformation that is permissive for signaling. Based

on our model, in wild-type flies the *O*-glucose residues on Notch act as a buffer to ensure robust Notch signaling, especially at high temperature. Several lines of evidence support this idea. First, Notch proteins with a smaller number of *O*-glucose mutations signal better and are more resistant to increased temperatures than Notch proteins with a greater number of mutations. Second, at low temperature, the function of Notch is less dependent on the number of *O*-glucose residues, as evidenced by the similarity of the *N⁻/Y; N^{gt-10_20}/+*, *N⁻/Y; N^{gt-10_35}/+* and *N⁻/Y; N^{gt-4_35}/+* phenotypes at 18°C (see Fig. S3 in the supplementary material). Third, increasing the dosage of *N^{gt-10_15}* can rescue the bristle and leg phenotypes of the *N^{55e11}* allele at 30°C, indicating that even though *O*-glucose residues on EGF10-15 play a prominent role in preventing the temperature-dependent loss of Notch signaling, a lack of *O*-glucose in this region can be compensated by *O*-glucose on other EGF repeats when the level of Notch^{gt-10_15} is increased.

Biochemical, X-ray crystallography and genetic experiments have established that deletion of the LNR motif from *Drosophila* and mammalian Notch proteins results in ligand-independent S2 cleavage and activation of Notch (Lieber et al., 2002; Sanchez-Irizarry et al., 2004; Gordon et al., 2007). It has been proposed that endocytosis of the Notch-bound ligand into the signal-sending cell applies a pulling force to the Notch extracellular domain and thereby leads, in a stepwise fashion, to LNR dissociation and heterodimer relaxation, which will ultimately expose the S2 cleavage site (Gordon et al., 2008). The complete suppression of the ligand-independent Notch^{ΔLNR-LexA} overexpression phenotypes in *rumi* MARCM clones (Fig. 8) suggests that the cross-talk between the EGF repeats and the heterodimerization region of Notch is not solely mediated by the LNR motif. The data further suggest that *O*-glucosylation of Notch by Rumi is required at a step that is common between ligand-dependent and ligand-independent forms of Notch activation. Mutations in the heterodimerization region of human NOTCH1 result in ligand-independent activation of NOTCH1 and thereby promote the development of T-cell acute lymphoblastic leukemia (Malecki et al., 2006; Chiang et al., 2008). Accordingly, decreasing NOTCH1 *O*-glucosylation using a Rumi inhibitor might offer a potential therapeutic avenue for this disease.

Based on these observations and the gradual increase in the severity of phenotypes caused by the loss of *rumi* or loss of *O*-glucose sites upon temperature increase, we propose that the ability of the Notch protein to undergo S2 cleavage gradually declines as the temperature increases. However, the broad distribution of *O*-glucose residues across the extracellular domain of Notch ensures that at the tissue and organismal levels, no significant decline in Notch signaling occurs at high temperatures and therefore wild-type flies raised at 30-32°C do not show *Notch* loss-of-function phenotypes. Of note, our qRT-PCR data on the control cells show a modest, yet statistically significant, decrease in *E(spl) m3* expression at higher temperatures, suggesting that the buffering role of *O*-glucose residues is not 100% efficient at the molecular level.

We have recently reported that a close homolog of fly Rumi is the primary, if not the only, protein *O*-glucosyltransferase in the mouse (Poglut1) (Fernandez-Valdivia et al., 2011). shRNA-mediated Rumi knockdown in mouse cell lines results in cellular and molecular phenotypes characteristic of loss of Notch signaling, including a severe decrease in the S3 cleavage of Notch1, without affecting the binding of Notch to the jagged 1 and delta-like 1 ligands (Fernandez-Valdivia et al., 2011). The number and distribution of the EGF repeats with a C¹-X-S-X-P-C² *O*-glucosylation motif are similar in vertebrate and fly Notch proteins

(Moloney et al., 2000a; Shao et al., 2002; Haines and Irvine, 2003), and mammalian Notch1 and Notch2 have been shown to harbor *O*-linked glucose (Moloney et al., 2000a; Bakker et al., 2009; Fernandez-Valdivia et al., 2011). Altogether, our previous and current observations suggest that the biologically relevant *O*-glucose residues on mammalian Notch proteins are likely to be broadly distributed in their extracellular domains.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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