Multiple O-glucosylation 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-glucosyltransferase that adds glucose to EGF repeats with a C-X-S-X-P consensus sequence. Eighteen of the 36 EGF repeats in the Drosophila Notch receptor contain the consensus O-glucosylation 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 glucosylation 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-glucosylation, 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 C1-X-S-X-X-(S/T)-C2 (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-glucosylated at specific serine (S) residues of EGF repeats that contain the O-glucosylation 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-glucosylation site (Fig. 1A). The functional importance of O-glucosylation in Notch signaling was demonstrated with the identification of the Drosophila protein O-glucosyltransferase 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 glucosylating 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-glucosylation 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-glucosylation 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, N55c1/FM7c, Kr-GAL4 UAS-GFP snr, yw N59/FRT19A/FM7, Df(1)N59/FM7c, yw; FRT82B, FRT82B Dv10 Spa+2/TM6B, Tb (MicheUi et al., 1997), UAS-NotchALNR-LexA (Lieber et al., 2002), vas-int-ZH-2A; attVK22 (Venken et al., 2006), nos-int-X; attP2 (Groth et al., 2004), L/CyO, yw; Ubx-FLP Tub-GAL80 FRT19A; Act-GAL4 UAS-GFP[6]CyO, yw; Ubx-FLP; FRT82B tub-GAL80 y+/TM6, Ubx, yw; FRT82B rumi26/TM6, Tb (Acar et al., 2008), N59-attP2, N59-attVK22, N59-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]-Ap8 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]-Ap8 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]-Ap8 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]-Ap8 construct, we performed what we call ‘GAP-repair mutagenesis’, which involves two rounds of recombineering (see Fig. S1 in the supplementary material).

Next, we performed recombineering between linearized Notch-attB-P[acman]-Ap8 containing the CAT-SacB cassette and the circular targeting vector. Since the same homology arms were used in both constructs, GAP-repair recombineering replaced the CAT-SacB cassette with the mutant targeting region. Once a positive clone was identified, the junctions to the attB-P[acman]-Ap8 vector and all exons were sequenced before injection.

Genetics

Generation of the Notch transgenes

A genomic source for the F3c1 integrase (Bischof et al., 2007) was used to introduce the wild-type and mutant transgenes into the Fk22 docking site (57F5 on 2R) or the attP docking site (68A4 on 3L, wild-type transgene only).

Gene dosage and rescue studies

yw; N59+e/f males were crossed to yw or N5/FM7 females and raised at the designated temperatures to obtain N59+e/f, N59+e/f and N59+e/f, N59+e/f females. N59+e/f or N59+e/f males were crossed to N5/FM7 females and raised at the designated temperatures to obtain N59+e/f and N59+e/f, N59+e/f 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[6]CyO, yw; FRT82B, FRT82B Dv10 Spa+2/TM6B, Tb (MicheUi et al., 1997), UAS-NotchALNR-LexA (Lieber et al., 2002), nos-int-ZH-2A; attVK22 (Venken et al., 2006), nos-int-X; attP2 (Groth et al., 2004), L/CyO, yw; Ubx-FLP Tub-GAL80 FRT19A; Act-GAL4 UAS-GFP[6]CyO, yw; Ubx-FLP; FRT82B tub-GAL80 y+/TM6, Ubx, yw; FRT82B rumi26/TM6, Tb (Acar et al., 2008), N59-attP2, N59-attVK22, N59-attVK22 (this study).

Dissections, staining, image acquisition and processing

Dissections 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

Rumi26 embryos were obtained for two independent insertions of the Notch genomic transgene in which the expression of Notch is driven by the UAS-NotchGAL4 UAS-GFPnls/CyO, y+.

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 N59e/f (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 N59e/f flies, thickening of the wing vein and wing margin loss are observed (Fig. 1E). However, N59e/f females show neither the haploinsufficient N59e/f phenotype nor the extra wing vein phenotype caused by the N59e/f transgene (Fig. 1F). Similar results were obtained for two independent insertions of the N59e/f 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, N59e/f 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 N59e/f at low and high temperatures and the rescued flies resemble wild-
**Type male** (Fig. 1G-I; data not shown). The N^{mut} also rescues the lethality and zygotic phenotypes of N^{−/−} females. Together, these observations indicate that the N^{mut} is functional in vivo and can be used to assess the effects of O-glucosylation mutations on Notch signaling at various temperatures.

All EGF repeats with a C^{1}-X-S-X-P-C^{2} 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 C^{1}-X-S-X-P-C^{2} 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^{mut}, with numbers that correspond to the mutated EGF repeats; for example, N^{N17-4.35} contains mutations in the EGF4-35 regions and N^{N17-4.3} 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^{N 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^{N17-4.35}, in which all 18 O-glucosylation sites are mutated. At 21-23°C, a number of rescued pharate 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. S3C) and a slight increase in the density of microchaetae (Fig. S3C'). 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^{N17-4.35} transgene, these results show that when all O-glucosylation motifs in Notch are mutated, the Notch protein remains largely functional at 18°C, but
losers a significant level of its activity at 21-23°C. The similarities between the temperature-sensitive phenotypes of N/Y; Ngt-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; Ngt-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. Ngt-4_35, 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 transgene, Ngt-10_20, in which all O-glucosylation motifs in EGF10-20 are intact. Of note, at 18°C, Ngt-10_35 and Ngt-16_35 show comparable abilities in rescuing 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; Ngt-4_35/+ phenotypes seem to be more severe than the N/Y; Ngt-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, Ngt-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, Ngt-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; Ngt-10_20/+ show weaker phenotypes than N/Y; Ngt-10_35/+ animals raised at the same temperature. Together, these data indicate an important role for the O-glucosylation 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, Ngt-10_20, Ngt-10_35 and Ngt-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; Ngt-10_35/+ flies reach pharate adulthood at 30°C and only show a mild loss of microchaetae (Fig. 2; data not shown). Moreover, Ngt-10_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; Ngt-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; Ngt-10_15/+ and N/Y; Ngt-10_35/+ phenotypes are milder than N/Y;
suggest that even at the restrictive temperature, the remaining 13 joint defects and die as pharate adults. These observations strongly not completely because the rescued animals still display some leg ligand-binding EGF12 to the wild-type sequence (A to S). The glucose on multiple EGF repeats in this region. To distinguish Ngt-10_15/+ males show very severe bristle loss and leg joint abnormalities at 21-23°C (Fig. 4G,G’). 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.

The temperature-sensitive decrease in the activity of the Notch protein encoded by the Ngr-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 Ngr-10_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, Ngr-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 Ngr-10,12,14,15+/+ transgene. N/Y; Ngr-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; Ngr-10,15+/+ males raised at 30°C (Fig. 4A,A’) indicates that a single O-glucose on EGF13 can partially restore the activity of Ngr-10,15, Ngr-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 (Wells, 1971). Similarly, males with one copy of the Ngr-10_35 transgene show a Confluens phenotype (Fig. 5A-A’). This phenotype is also dosage sensitive, as males with two extra copies of Ngr-10_35 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, Ngr-10_15/+ contains five O-glucose mutations in the EGF10-15 region. Male flies with two extra copies of Ngr-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 Ngr-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 Ngr-10_35 is not as severe as that caused by Ngr-10_15 (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; Ngr-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 Ngr-10_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. 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^{+/–}). (A,C) N/Y; Ngr-4_35/+ males show very severe bristle loss and leg joint abnormalities at 21-23°C. (B,E) N/Y; Ngr-10_35/+ males show severe bristle loss and leg joint abnormalities at 21-23°C. (F) N/Y; Ngr-10,20+/+ males show some bristle loss and leg joint abnormalities at 21-23°C. Brackets mark leg shortening and/or 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 Notch54l9 null allele with or without a copy of our Ngt transgenes and performed immunostainings to examine the distribution of the Notch protein. Since Notch54l9 is a protein-null allele (Fig. 6A,A′/H11032), the only source of Notch in Notch54l9 clones will

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; Npt-10,15/+ male flies show a severe loss of bristles and shortened legs with severe joint defects. (B,B′) At 25°C, N/Y; Npt-10,15/+ males show a mild loss of bristles and normal legs. (C,C′) At 21-23°C, Npt-10,15 fully rescues the lethality and phenotypes of Notch null mutants. (D,D′) The leg defects and microchaetae loss of the N/Y; Npt-10,15/+ males at 30°C are significantly improved by the addition of a second copy of the Npt-10,15 transgene (compare with A,A′). (E-F′) N/Y; Npt-10,12,15/+ (E,F′) and N/Y; Npt-12/+ (F′) males show a normal bristle pattern and normal legs at 30°C. (G-G′) N/Y; Npt-10,12,14,15/+ males show mild bristle loss and abnormal leg joints at 30°C. (H-H′) Npt-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.

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 Ngt-wt (A-B′/H11630) or two copies of Ngt-mut (C-D′/H11630) transgenes inserted at the VK22 docking site on the second chromosome. (A-A′) One copy of Ngt-wt results in a Confluens phenotype (extra vein, arrowheads) at 18-30°C. (B-B′) Two copies of Ngt-wt cause an enhancement of the Confluens phenotype at 18-30°C. (C-C′) At 18°C, two copies of Npt-10,15 show a Confluens phenotype comparable to that caused by two copies of Ngt-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 Npt-10,15 at 18°C and 23°C is much milder than that caused by Ngt-wt and Npt-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 N54l9 allele. (A-D) N54l954l9 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 N54l9 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 N54l9 transgenes. When raised at 30°C, Notch54l9 homozygous clones that carry one copy of the N54l9-null 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 N54l910.15 and N54l910.35 show a staining pattern that is comparable to that generated by N54l9 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 N54l9null, N54l910.15, N54l910.35 and N54l94.35 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 (Dl) cells (Acar et al., 2008). Moreover, N54l9 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-26 (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.
rumi suppresses the hyperactivation of Notch caused by the deletion of the LNR motif

Deletion of the LIN-12/Notch (LNR) motif from Notch proteins 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 NotchΔLNR-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/H11032). Activation of NotchΔLNR-LexA does not depend on the presence of ligands (Fig. 8C-D/H11032). However, loss of rumi fully suppresses the NotchΔLNR-LexA gain-of-function phenotypes (Fig. 8E-F/H11032). 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 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 NotchNgt-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; Ngt\(^{+/0}\), N/Y; Ngt\(^{+/15}\) and N/Y; Ngt\(^{+/-}\) phenotypes at 18°C (see Fig. S3 in the supplementary material). Third, increasing the dosage of Ngt\(^{+/15}\) can rescue the bristle and leg phenotypes of the N\(^{5+/5}\) 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\(^{+/15}\) 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\(^{+/LNR-LecA}\) 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-glycosylation 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-glycosylation 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 type flies raised at 30-32°C do not show the temperature increases. However, the broad distribution of the Notch protein to undergo S2 cleavage gradually declines as the temperature increases. However, the broad distribution of 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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References


(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.


Table S1. Mutagenesis primers used in this study

<table>
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<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>EGF12-Ser-For</td>
<td>CGAACATCAATGAGTGGCAATCGCATCCATGCGCCAGAACCAGG</td>
</tr>
<tr>
<td>EGF12-Ser-Rev</td>
<td>CCTCGTTCTGCAATGAGTGCGATGCGGATGCGAATTGCCCATCCATGCGCCAGAACCAGG</td>
</tr>
<tr>
<td>EGF13-Ser-For</td>
<td>CGACATTGAAGTGGCAATCGCATCCATGCGCCAGAACCAGG</td>
</tr>
<tr>
<td>EGF13-Ser-Rev</td>
<td>CCATCGTTCTGCAATGAGTGCGATGCGGATGCGAATTGCCCATCCATGCGCCAGAACCAGG</td>
</tr>
<tr>
<td>EGF10-Ser-For</td>
<td>GACGATGCGCTTCATCCTCGAATCGCCATG</td>
</tr>
<tr>
<td>EGF10-Ser-Rev</td>
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</tr>
<tr>
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<td>EGF12-Ala-Rev</td>
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