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Rumi Is a CAP10 Domain Glycosyltransferase that Modifies Notch and Is Required for Notch Signaling

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SUMMARY

Notch signaling is broadly used to regulate cell-fate decisions. We have identified a gene, *rumi,* with a temperature-sensitive *Notch* phenotype. At 28°C–30°C, *rumi* clones exhibit a full-blown loss of Notch signaling in all tissues tested. However, at 18°C only a mild *Notch* phenotype is evident. In vivo analyses reveal that the target of Rumi is the extracellular domain of Notch. Notch accumulates intracellularly and at the cell membrane of *rumi* cells but fails to be properly cleaved, despite normal binding to Delta. Rumi is an endoplasmic reticulum-retained protein with a highly conserved CAP10 domain. Our studies show that Rumi is a protein *O*-glucosyltransferase, capable of adding glucose to serine residues in Notch EGF repeats with the consensus C^1-X-S-X-P-C^2 sequence. These data indicate that by *O*-glucosylating Notch in the ER, Rumi regulates its folding and/or trafficking and allows signaling at the cell membrane.

INTRODUCTION

Notch signaling is one of the most widely used signaling pathways in animals (Artavanis-Tsakonas et al., 1999). It is required for maintenance of the undifferentiated state, lateral inhibition, asymmetric cell divisions, vertebrate somitogenesis, cortical neurite outgrowth, and differentiation. Aberrant Notch signaling has been implicated in human diseases including cerebrovascular dementia (CADASIL) (Joutel et al., 1996), cancer (Bolos et al., 2007), as well as developmental disorders of liver, heart, skeleton, eye, and kidney (Li et al., 1997; Oda et al., 1997). It has also been shown to play important roles in stem cell biology (Carlson and Conboy, 2007).

The core components of the Notch pathway are the transmembrane ligands (Delta and Serrate in flies), receptor (Notch), and CBF1/Suppressor of Hairless/Lag-1 (CSL) transcription factors (Suppressor of Hairless in flies) (Lai, 2004; Schweisguth, 2004). Upon ligand binding, Notch is cleaved by an ADAM metalloprotease (Kuzbanian in flies), followed by an intramembranous cleavage mediated by the gamma-secretase complex (Brou et al., 2000; De Strooper et al., 1999; Mumm et al., 2000; Pan and Rubin, 1997; Struhl and Greenwald, 1999). The latter cleavage leads to translocation of the Notch intracellular domain (N^{ICD}) to the nucleus, where it binds CSL proteins to activate downstream effectors (Jarriault et al., 1995; Lecourtois and Schweisguth, 1995; Struhl and Adachi, 1998). In addition, there are many important proteins involved in the regulation of the pathway that function to regulate endocytosis, ubiquitination, intracellular trafficking, degradation, and glycosylation of various components (Haines and Irvine, 2003; Hori et al., 2004; Le Borgne et al., 2005).

The extracellular domain of Notch (N^{ECD}) is approximately 200 kDa and contains 36 tandem Epidermal Growth Factor-like (EGF) repeats. The EGF repeats undergo *O*-fucosylation and *O*-glucosylation (Moloney et al., 2000b). The *O*-fucosyltransferase-1 (Pofut1 in mammals, Ofut1 in flies) adds fucose (Shao and Haltiwanger, 2003; Wang et al., 2001) and is required for folding of Notch in the ER (Okajima et al., 2005), for Notch-ligand interaction (Okajima et al., 2003), and for intracellular trafficking of Notch (Sasaki et al., 2007; Sasamura et al., 2007). Interestingly, some of these roles do not seem to require enzymatic function (Okajima et al., 2005; Sasamura et al., 2007). Moreover, loss of *Ofut1* (*Pofut1* in mice) results in *Notch* loss-of-function phenotypes in flies and mice (Okajima and Irvine, 2002; Sasamura et al., 2003; Shi and Stanley, 2003). The fucose residue added to Notch by Pofut1 can be further modified by Fringe proteins, which add *N*-acetylglucosamine to *O*-fucose residues (Moloney et al., 2000a; Rampal et al., 2005; Shao et al., 2003). This modification alters the binding of Notch to Delta and Serrate and

regulates Notch signaling in specific contexts (Bruckner et al., 2000; Okajima et al., 2003; Panin et al., 1997).

Notch is also *O*-glucosylated at serine residues in the C^1-X-S-X-P-C^2 consensus present in some EGF repeats (Moloney et al., 2000b; Shao et al., 2002). Protein *O*-glucosylation is a rare modification that occurs on EGF repeats of a few proteins including coagulation factors VII and IX, protein Z, Delta-like protein, and Thrombospondin (Shao et al., 2002). Even though an enzymatic activity able to *O*-glucosylate EGF repeats is present in cell extracts from a variety of species (Shao et al., 2002), no specific protein has been identified that *O*-glucosylates Notch or any other protein. Although *Drosophila* Notch carries 19 putative *O*-glucosylation sites, many of which are evolutionarily conserved (Haines and Irvine, 2003; Moloney et al., 2000b; Shao et al., 2002), the in vivo role of *O*-glucosylation is unknown.

We have isolated a gene named *rumi* that causes a temperature-sensitive (ts) loss of bristles. Loss of *rumi* affects Notch signaling in all tissues tested. *rumi* encodes a soluble, ER protein with a CAP10 domain, which is involved in capsule formation and virulence in *Cryptococcus neoformans* (Chang and Kwon-Chung, 1999). Rumi has highly conserved homologs in species from yeast to human, but its role is unknown (Chang and Kwon-Chung, 1999; Teng et al., 2006). Our data indicate that Rumi regulates Notch signaling by modifying Notch in the ER, and that Rumi is a protein *O*-glucosyltransferase (Poglut). We propose that lack of *O*-glucosylation of Notch in *rumi* mutants results in a ts defect in Notch folding and signaling.

RESULTS

rumi Mutations Cause a Temperature-Dependent Loss of Notch Signaling

We performed a chemical mutagenesis screen to identify novel genes that affect adult bristle development (Jafar-Nejad et al., 2005) (Figure 1A). One of the complementation groups, named *rumi* (after a 13th century poet), showed bristle loss in mitotic clones when raised at 25°C (Figure 1B). However, when grown at 18°C, mutant clones did not show bristle loss (Figure 1C) but exhibited an increase in bristle density, suggesting a mild lateral inhibition defect (Figure 1D). To determine the cause of bristle loss, we stained *rumi* pupae raised at 25°C or 18°C for Cut, a protein that marks the nuclei of all cells of sensory clusters, and for ELAV, which marks neurons. As shown in Figures 1E and 1E′, all cells in a *rumi* sensory cluster raised at 25°C express ELAV, indicating a *Notch*-like cell-fate specification defect. However, *rumi* pupae raised at 18°C contain a single neuron in each sensory cluster (Figures 1F and 1F′), similar to wild-type (wt) pupae.

To provide a more direct link between *rumi* and Notch signaling, we performed genetic interaction experiments. Some *rumi* mutant animals reach adulthood at 25°C. These flies show a severe loss of microchaetae (Figure 1G). Adding one copy of $Notch^+$ restores most microchaetae at 25°C, indicating that the phenotype is sensitive to *Notch* dosage (Figure 1H). When raised at 18°C, *rumi* mutant animals do not show a bristle loss (Figure 1I), but removing a copy of *Notch* in these females results in a loss of microchaetae (Figure 1J). These data indicate that increasing the temperature results in a worsening of the *Notch* phenotype in *rumi* animals. Indeed, a complete loss of microchaetae in *rumi* animals raised at 29°C during early pupal stage cannot be rescued with an additional copy of *Notch* (Figure S1 available online).

To demonstrate that *rumi* affects lateral inhibition, we performed temperature shift experiments. Pupae harboring *rumi* clones were raised at room temperature, shifted to 28°C during lateral inhibition, and shifted back to 18°C during the asymmetric divisions (Figure 1K). Under this regimen, flies show a large excess of sensory bristles in mutant clones (Figure 1K). Hence, *rumi* regulates Notch signaling during lateral inhibition and asymmetric divisions of sensory precursors.

As shown in Figures 2A–2D, embryos lacking maternal and zygotic Rumi raised at 28°C have a neurogenic phenotype, similar to *Notch* embryos. Clonal analysis in the wing showed that ''inductive signaling'' (Lai, 2004) is also affected (Figure 2F, asterisks; Figures 2G–2J′). Moreover, genetic studies reveal a strong dosage-sensitive interaction between *rumi* and *Delta* in wing, eye, and leg development (Figures 2K–2M and S2). These data indicate that Rumi is a general regulator of Notch signaling.

rumi Encodes a CAP10-like Protein

We mapped *rumi* to *CG31152* (Figure 3A) (Zhai et al., 2003), which encodes a conserved protein (Figure 3C) with a signal peptide, a CAP10 domain, and a C-terminal KDEL ER-retention motif (Figure 3B). Allele *44* contains an in-frame deletion and allele *79* harbors a missense mutation, G189E (Figure 3B). All homo- and transheterozygous combinations of these alleles in combination with *Df(3R)Exel6192* produce viable progeny and exhibit a ts *Notch* phenotype.

The temperature sensitivity of the *rumi* alleles may be due to an abnormal Rumi protein that fails to function at high temperatures. Alternatively, *rumi*'s neighbor, *CG31139* (Figure 3A)—the only other fly gene encoding a CAP10 domain protein—may compensate in part for the lack of *rumi,* resulting in a ts phenotype. We therefore excised *P* element *EY00249* inserted 238 bp upstream of *CG31152* (Bellen et al., 2004). All deletions generated by imprecise excisions lack most of the *rumi* ORF (Figure 3A), and an antibody raised against Rumi failed to detect the protein in *Δ26/Δ26* animals, indicating that *Δ26* is a null allele (Figure 3D). All allelic combinations exhibit the ts phenotype and can be rescued with a *UAS-CG31152* or a genomic transgene only containing *CG31152* (Figure 3E and data not shown). These data indicate that the *partner of rumi* (*CG31139*) is not redundant. Hence, loss of *CG31152* is the cause of the loss-of-function phenotypes of *rumi* mutants and Rumi regulates a ts aspect of Notch signaling.

rumi Is Required in the Signal-Receiving Cell

To assess whether *rumi* is required in the signal-sending and/or receiving cell, we used the MARCM system (Lee and Luo, 2001) to overexpress Delta, Serrate, and Notch in *rumi* clones (28°C). If Rumi is essential for Delta or Serrate to induce Notch signaling in the neighboring cells, then expression of Delta and Serrate should not be able to induce Cut expression in cells along the border of the MARCM clones, as reported for *epsin* mutations (Wang and Struhl, 2004). As shown in Figures S3A–S3B′, overexpression of Delta or Serrate in *rumi* clones results in expression of Cut, suggesting that the signal-sending cell does not require

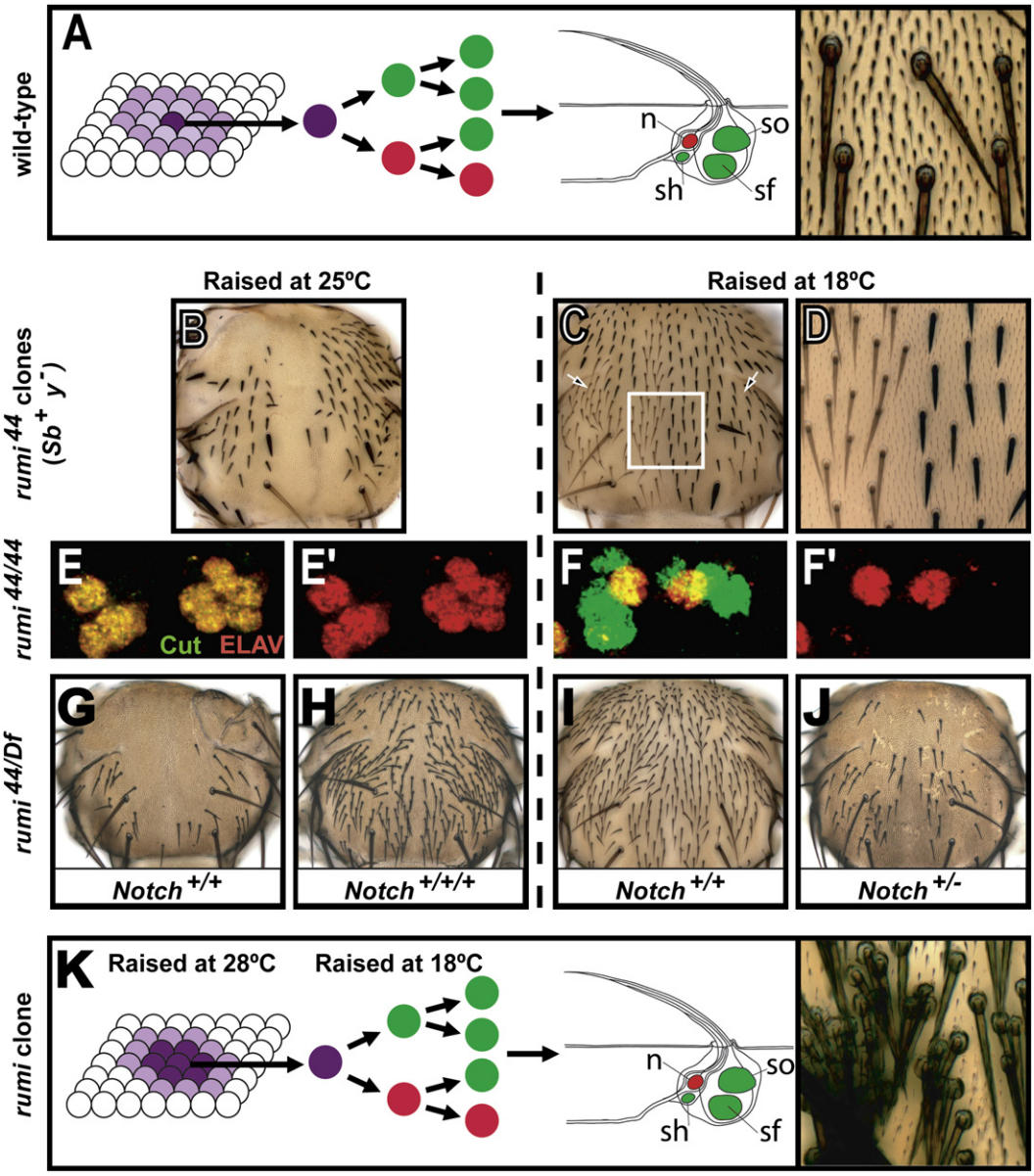

Figure 1. *rumi* Mutations Cause a ts *Notch* Phenotype

(A) Formation of an external sensory (es) organ from a single sensory organ precursor (SOP) cell is shown. Once the SOP is specified, it undergoes a series of asymmetric cell divisions to form the sensory organ structure. Only a single neuron forms in each sensory organ (red). The right picture shows wt microchaetae located on the thorax. n: neuron, so: socket cell, sh: sheath cell, sf: shaft cell. Bristle schematic adapted from Lai and Orgogozo (2004).

(B) Bristle development is impaired in *rumi* clones at 25°C. Heterozygote bristles are marked with *Sb* and y^+ markers. Note that not all bristles are lost at this temperature.

(C and D) Bristles form in a denser pattern in *rumi* clones raised at 18°C. Non-Sb and yellow bristles mark *rumi* clones (compare the regions marked by the two arrows in C). (D) is a close-up of the white square.

(E–F′) Cell-fate specification is impaired in the adult microchaetae lineages of *rumi* mutants raised at 25°C, as evidenced by presence of multiple $ELAV^+$ cells (red) per cluster (E and E′). Note that when raised at 18°C, only one of the Cut^+ cells (green) per cluster expresses ELAV (F and F′).

(G–J) Genetic interaction between *Notch* and *rumi*.

(G) Hemizygous $rumi^{44}$ flies that eclose at 25°C lose most of their microchaetae.

(H) Adding an extra copy of *Notch* rescues many of the lost bristles (compare G and H).

(I) At 18°C, *rumi* mutant flies do not exhibit a significant bristle loss.

(J) Flies heterozygous for *Notch* and hemizygous for $rumi^{44}$, however, lose many bristles at 18°C (compare I and J).

(K) Tufts of bristles form in *rumi* mutant clones in flies that are kept at 28°C during SOP specification and at 18°C during asymmetric cell divisions, indicating an important role for *rumi* during lateral inhibition.

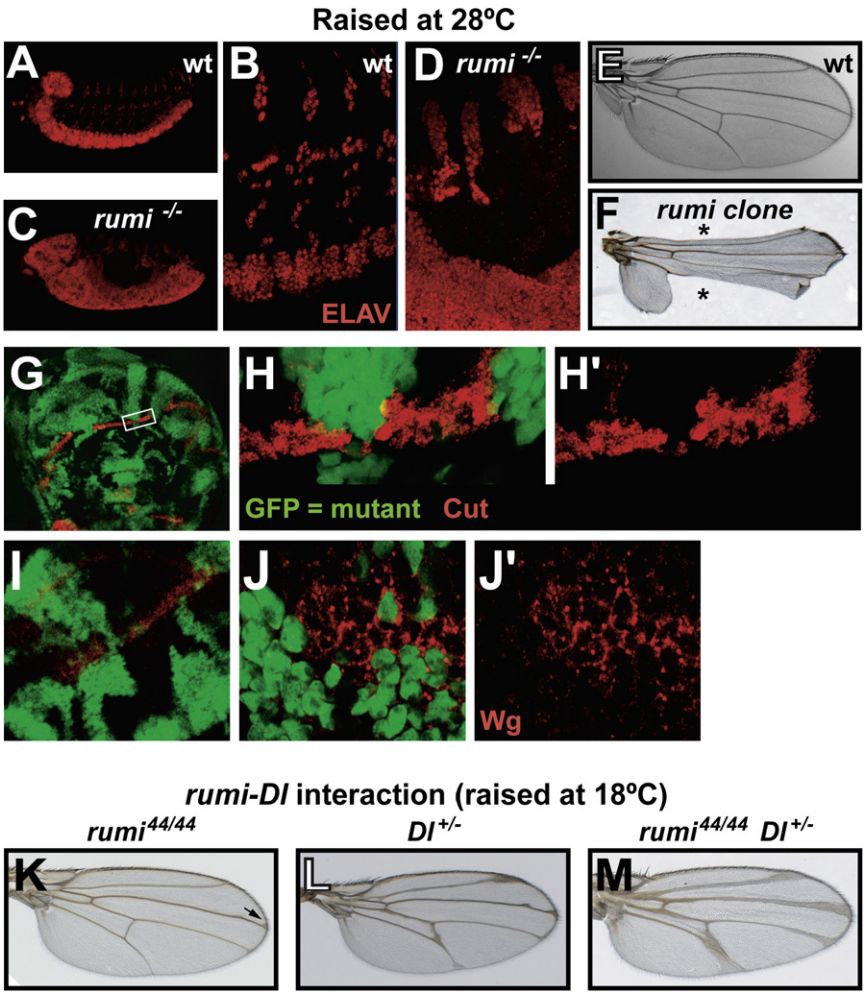

Figure 2. Loss of *rumi* Causes Loss of Notch Signaling in Various Contexts

(A–D) *rumi* embryos laid by homozygous *rumi* flies show a neurogenic phenotype at 28°C (compare A and B with C and D). Neurons are marked with ELAV (red).

(E and F) Wing margin development is impaired in *rumi* clones, causing Notching of the wings (asterisks in F). Panel F shows the wing of an adult who was subjected to the temperature-shift experiments described in Figure 1K.

(G–J′) Expression of the Notch downstream targets Cut (red in G–H′) and Wg (red in I–J′) is lost in *rumi* clones at the dorso-ventral boundary of the third instar wing imaginal disc at 28°C in a cell-autonomous manner. GFP (green) marks *rumi* mutant cells.

(K–M) Genetic interaction between *Delta* and *rumi*. All flies were raised at 18°C.

(K) Wings of the homozygous *rumi* flies exhibit a mild *Delta* phenotype (arrow).

(L) A wing of a fly that is heterozygous mutant for *Delta*.

(M) There is a synergistic increase in wing vein expansion in flies that are homozygous mutant for *rumi* and heterozygous for *Delta*, indicating that *rumi* and *Delta* genetically interact.

Rumi. Moreover, wing imaginal discs harboring *rumi* clones raised at 28°C and stained with anti-Delta or anti-Serrate show no alteration in the expression of these proteins (Figures S3C–S3D′). These data argue against a role for Rumi in the signal-sending cell and against a requirement for *rumi* for the function of Delta or Serrate.

To assess the function of Rumi in the signal-receiving cell, we performed similar experiments with full-length Notch (N^{FL}). When overexpressed in clones homozygous for a wt chromosome, N^{FL} induces Cut expression in proximity of the wing margin (Figures 4A and 4A′), as reported (Sasamura et al., 2003). However, N^{FL} failed to induce Cut expression in *rumi* clones (Figures 4B and 4B′). These observations indicate a requirement for Rumi in signal-receiving cells.

Binding of ligands to the N^{ECD} induces S2 cleavage of Notch by ADAM/TACE/Kuzbanian proteases (Brou et al., 2000; Lieber et al., 2002). This generates an active membrane-bound form of Notch, which undergoes S3 cleavage mediated by Presenilin and its binding partners (De Strooper, 2003; Struhl and Greenwald, 1999). To refine the step in the Notch transduction cascade in which Rumi is required, we overexpressed a membrane-bound, active version of Notch called N^{ECN} (Struhl et al., 1993) in *rumi* clones raised at 28°C and observed a robust induction of downstream targets (Figures 4C and 4C′). Since the activity of N^{ECN} depends on the Presenilin function, these data place the function of *rumi* upstream of the S3 cleavage of Notch in the signal-receiving cell and suggest that the N^{ECD} is the target of Rumi.

To address if Notch processing is impaired in *rumi* mutants, we performed western blots by using an anti-N^{ICD} antibody (Hu et al., 2002; Pan and Rubin, 1997). Reduction of Kuzbanian (Kuz) or Presenilin function alters the pattern of the Notch cleavage products detected by western blots of protein extracts prepared in a hypotonic, detergent-free lysis buffer. We tested protein extracts from wing discs and brains of late third instar wt and *rumi* mutant larvae reared at 18°C. One set was shifted to 28°C (third instars), whereas the other set was maintained at 18°C for 10 hr. The Notch cleavage product was detectable in wing disc extracts of both wt and *rumi* larvae kept at 18°C (see arrow in Figure 4D) but not in wing disc extracts of *rumi* mutants at 28°C (Figure 4D). Note that the full-length Notch protein serves as an internal control for protein loading. This is similar to what has been observed in wing discs that express a dominant-negative form of *kuz* (Pan and Rubin, 1997). Defects in Notch processing were also observed in brain extracts (Figure 4D). These results provide strong evidence that the *rumi* function is important for Notch processing.

We also performed RNAi experiments in *Drosophila* S2 cells raised at 28°C. RNAi-mediated knockdown of Rumi or Kuz results in loss of the upper cleavage product (Figure 4E, arrow)

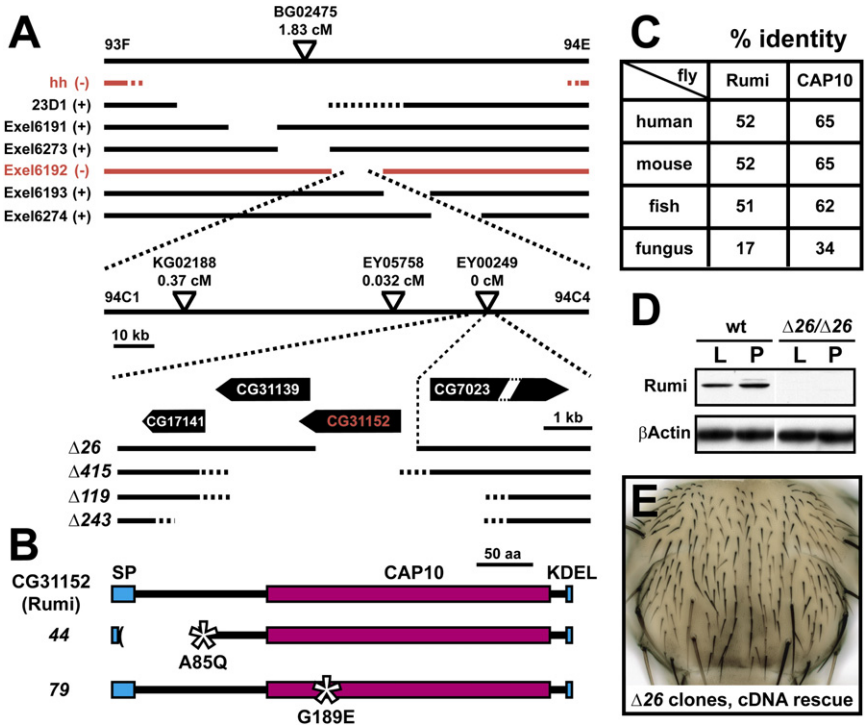

Figure 3. *rumi* Corresponds to *CG31152*

(A) The mapping strategy for *rumi*. The genomic region containing the *rumi* locus is shown. The broken lines depict deficiencies used to map *rumi*. *hh* and *Exel6192* deletions (red lines) failed to complement *rumi* alleles. The genetic distance between the *P* elements used for fine mapping and *rumi* is shown in centiMorgans (cM). Imprecise excision of *EY00249* generated the additional alleles that remove *rumi* (*CG31152*) alone or in combination with *CG31139*, the only other fly gene capable of encoding a CAP10 domain.

(B) *rumi* encodes a protein with a CAP10 domain, a KDEL ER-retention signal located at the C terminus, and a signal peptide (SP) located at the N terminus. Molecular lesions in alleles *44* and *79* are shown. Asterisks denote missense mutations.

(C) Rumi protein, especially the CAP10 domain, is highly conserved across different species.

(D) Western blotting on the protein extracts from wild-type (wt) and $rumi^{\Delta 26}$ larvae (L) and pupae (P) with a polyclonal α-Rumi antibody indicates that $rumi^{\Delta 26}$ is a protein null allele.

(E) A *UAS-rumi* transgene can rescue the bristle loss in *rumi* clones. The bristles rescued by the transgene are yellow.

normally observed in wt and control cells. Adding a Furin inhibitor does not alter the cleavage product pattern, consistent with the observation that the Furin-mediated S1 cleavage is not required for Notch signaling in flies (Kidd and Lieber, 2002). These data strongly suggest that Rumi is required for the function of Kuz at the restrictive temperature.

Notch Accumulates Intracellularly and at the Cell Surface in *rumi* Mutant Clones

Our data indicate that Rumi is retained in the ER by its C-terminal KDEL sequence and that ER retention is required for the function of Rumi in vivo (Supplemental Data and Figure S4) and that Rumi is required for proper folding of the N^{ECD}. Loss of Rumi may lead to accumulation of Notch in the ER or an inability of Notch to be recognized by proteins like Fringe, Delta, Serrate, or Kuz. Indeed, staining of third instar discs with anti-N^{ECD} shows an accumulation of Notch in *rumi* clones raised at 25°C but not at 18°C (Figures 5A–5B′). This increase is not due to an increase in *Notch* transcription (Figure S5).

To examine the subcellular localization of Notch in *rumi* clones, we stained with an α-N^{ICD} antibody. Notch accumulates throughout the cell in a cell-autonomous manner in *rumi* mutant cells (Figures 5C and 5C′). To ensure that *rumi* mutations do not disrupt apical-basal polarity, we examined the distribution of adherens junction marker E-Cadherin (Tepass et al., 1996). E-Cadherin is expressed at normal levels and is localized to adherens junctions in *rumi* clones (Figures 5D and 5D′), suggesting that accumulation and mislocalization of Notch are not due to polarity defects.

The above data suggest that lack of Rumi prevents proper trafficking and may affect surface expression of Notch at the restrictive temperature. To test this possibility, we used a no-detergent protocol to label the surface Notch with α-N^{ECD} (Wang and Struhl, 2004) and found that Notch accumulates at the surface of *rumi* mutant cells (Figures 5E and 5F). Moreover, the unfolded protein response is not induced in *rumi* clones, as evidenced by normal levels of HSC3(BiP) (Ryoo et al., 2007) (Figures S6A–S6A″). Finally, we do not observe an increase in the size of the ER in *rumi* clones (Figures S6B–S6B″). Together, these data indicate that accumulation of Notch in *rumi* clones is not due to ER entrapment, and that Notch is present at high levels at the surface of the *rumi* mutant cells.

Lack of Rumi Does Not Decrease Binding of Notch to Delta

Lack of Rumi may render Notch sensitive to temperature changes, and it may therefore be unable to bind its ligands at high temperatures. To address this issue, we first used a modified MARCM strategy (Wang and Struhl, 2004) to test whether increasing Delta levels in the signal-sending cell can overcome the inefficient reception of signal by *rumi* mutant cells. In this experiment, clones of wt cells overexpressing Delta flank homozygous mutant clones of *rumi*. As shown in Figures 5G–5G‴ overexpression of Delta results in induction of Cut in wt neighboring cells. However, despite the accumulation of Notch, *rumi* mutant cells fail to express Cut. Hence, overexpression of Delta in the signal-sending cell cannot suppress the *rumi* mutant phenotype in the signal-receiving cell.

To test if receptor-ligand interaction is impaired we used assays based on a secreted Notch-alkaline phosphatase (N-AP) fusion protein (Bruckner et al., 2000). We performed receptor-ligand interaction assays at room temperature and at 28°C.

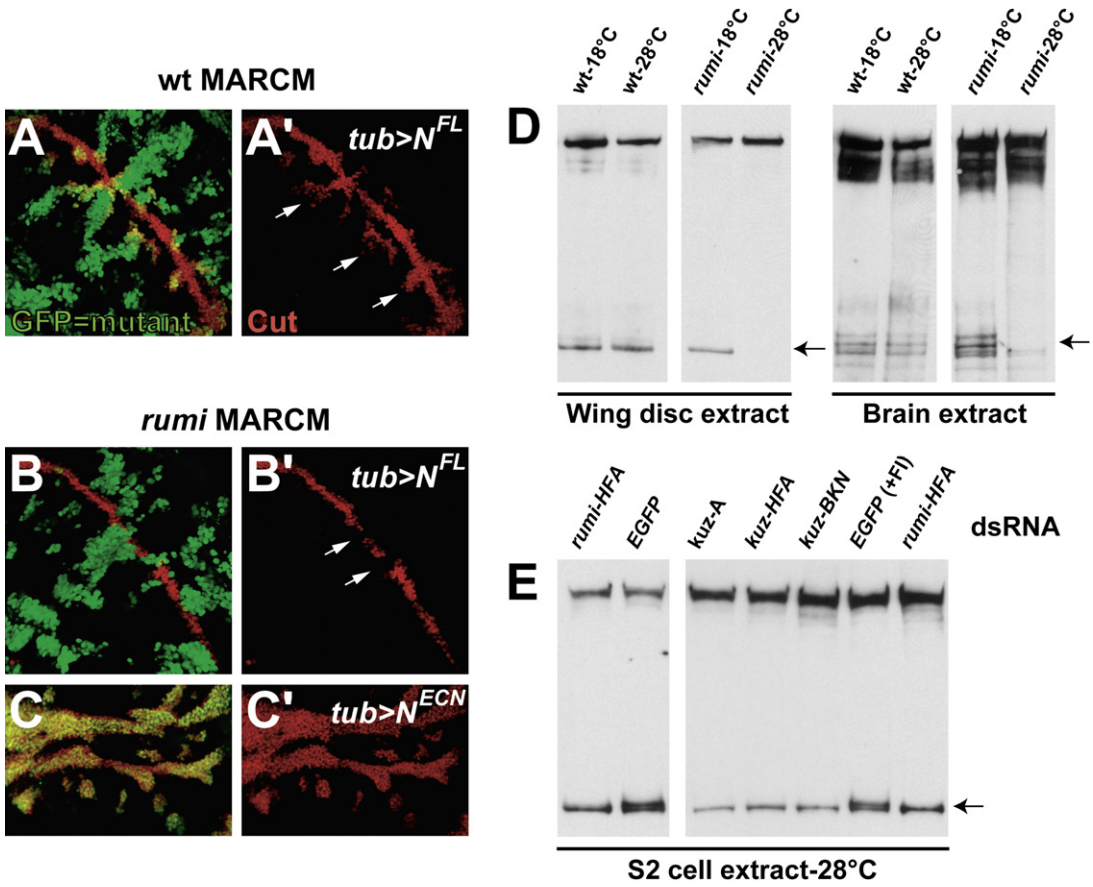

Figure 4. *rumi* Is Required in the Signal-Receiving Cell upstream of the S3 Cleavage

(A and A′) Ectopic expression of the full-length Notch (N^{FL}) induces aberrant Cut (red) expression in MARCM clones of a wt chromosome that are close to the dorso-ventral boundary (arrows in A′). GFP (green) marks the clones that ectopically express N^{FL} in (A) and (B).

(B and B′) Ectopic expression of N^{FL} in MARCM *rumi* clones does not induce Notch signaling. Note that in *rumi* mutant cells Cut (red) expression is lost in the prospective wing margin despite N^{FL} overexpression (arrows in B′).

(C and C′) Ectopic expression of N^{ECN} induces Cut (red) expression in *rumi* cells. GFP marks MARCM *rumi* clones that ectopically express N^{ECN}, suggesting that the Rumi function is required upstream of the S3 cleavage of Notch.

(D) Western blots showing that Notch processing is altered in the absence of Rumi function at the restrictive temperature. Anti-N^{ICD} antibody was used in all blots. The top bands in each blot correspond to full-length Notch, which is around 300 kDa. In wing disc extracts one predominant band that corresponds to Notch cleavage product is visible in both wild-type (wt) and *rumi* $^{\Delta 26/\Delta 26}$ (*rumi*) larvae kept at 18°C. This band is not detected in extracts of *rumi* larvae kept at 28°C for 10 hr prior to dissection (left arrow). In brain extracts four ~120 kDa fragments are visible in wt larvae and in *rumi* larvae that were kept at 18°C. The upper two bands, however, are lost in *rumi* larvae kept at 28°C (right arrow).

(E) RNAi-mediated knockdown of Rumi and Kuz results in similar Notch processing defects in S2 cells. Western blotting with anti-N^{ICD} on protein extracts from S2 cells raised at 28°C is used to determine the pattern of Notch cleavage. Control cells (*EGFP* dsRNA) show two cleavage products (arrow). Addition of a Furin inhibitor (FI) does not alter this pattern. However, treatment of S2 cells with dsRNA against Rumi or Kuz results in the loss of the upper cleavage product, strongly suggesting that the Kuz-mediated S2 cleavage of Notch is affected by the loss of Rumi at the restrictive temperature. Note that three different *kuz* dsRNAs produce the same results.

As shown in Figure S7, the binding of N-AP to Delta is not decreased by addition of *rumi* dsRNA to the N-AP-producing cells.

It has been recently shown that mutations in *lethal giant discs (lgd)* affect proper trafficking of Notch, causing ectopic activation of Notch in a ligand-independent manner (Childress et al., 2006; Gallagher and Knoblich, 2006; Jaekel and Klein, 2006). We therefore decided to carry out epistatic experiments between *lgd* and *rumi*. As shown in Figure S8, loss of *rumi* suppresses the ectopic activation of Notch in *lgd* mutant cells. Hence, loss of *rumi* affects the ligand-dependent and -independent Notch signaling.

Loss of Rumi Affects *O*-Glucosylation of the EGF Repeats of Notch

The CAP proteins (CAP10, 59, 60, and 64) are referred to as putative polysaccharide modifiers as they affect extracellular polysaccharide capsule formation (Okabayashi et al., 2007). Since Rumi contains a CAP10 domain (Figure 3) it may be a glycosyltransferase that modifies Notch. Since the enzymes involved in addition of *O*-glucose to Notch are unknown we examined whether Rumi plays a role in this process. Rumi was knocked down in S2 cells using RNAi. A portion of the N^{ECD} encoding

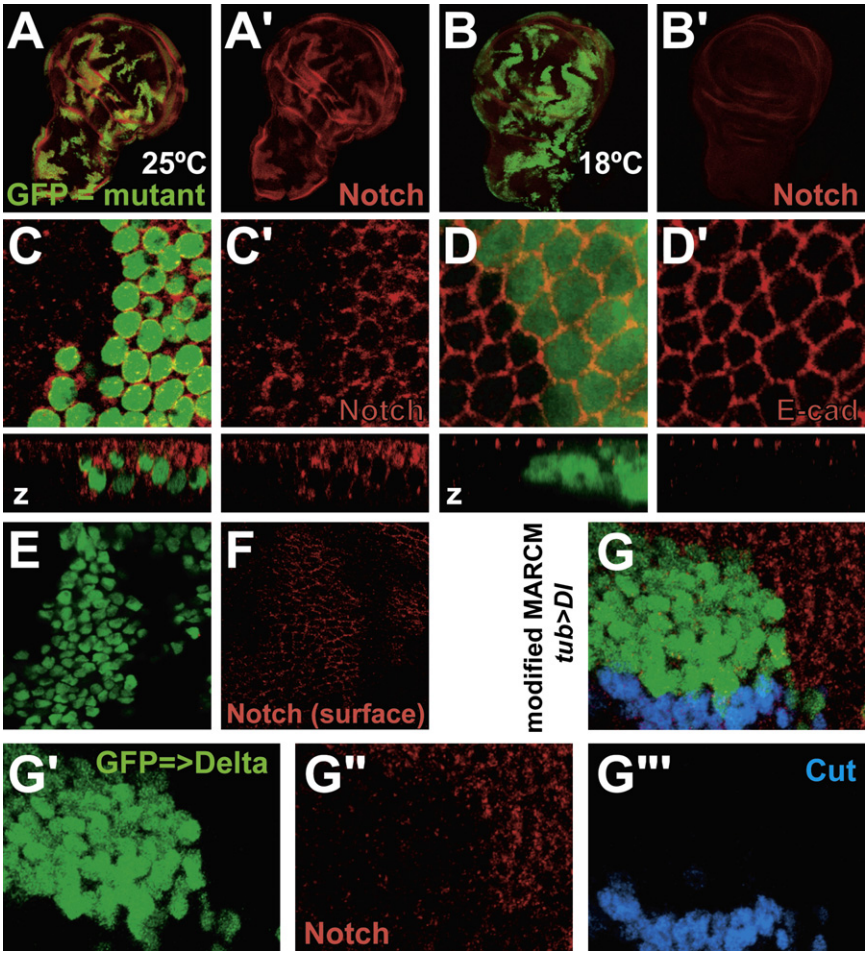

Figure 5. High Levels of Notch Accumulate inside and at the Membrane of *rumi* Mutant Cells at the Restrictive Temperature

(A–C′) *rumi* clones marked by GFP (green in A, B, and C) show an accumulation of Notch (red) when raised at 25°C (A, A′, C, C′) but not when raised at 18°C (B, B′). The accumulation is evident with antibodies against both N^{ECD} (A, A′) and N^{ICD} (C, C′). (A)–(B′) show third instar wing imaginal discs; (C) and (C′) show a close-up from a pupa 12 hr after puparium formation (APF). Note the cell-autonomous increase in Notch levels (C, C′). Also, optical z sectioning shows that in mutant cells Notch is not mainly in the apical regions anymore (z in C, C′).

(D and D′) E-Cadherin (E-cad, red) localization and levels do not change in *rumi* cells at 25°C.

(E and F) Notch protein levels at the cell membrane increase in *rumi* cells. Shown are two different sections of the wing disc of a third instar larva with MARCM *rumi* clones (green) raised at the restrictive temperature and fixed and stained with the α-N^{ECD} (red) antibody in the absence of detergent. (F) is close to the apical surface, and (E) is 700 nm basal to (F). Note the accumulation of Notch at apical regions (F) overlaying the clones marked by the nuclear GFP in (E). Absence of specific α-N^{ECD} staining in (E) indicates that the antibody has not entered the cell because of the lack of detergent.

(G–G‴) Ectopic overexpression of Delta in wt cells does not induce Notch signaling in adjacent cells that are mutant for *rumi,* despite the accumulation of Notch in these cells. Shown is an example of a modified MARCM clone located in the dorsal part of the wing pouch away from the wing margin (not shown), in which GFP (green) marks $rumi^{+/+}$ cells that overexpress Delta, and Notch accumulation (red) marks $rumi^{-/-}$ cells. Note that Delta from green cells is able to induce Cut expression (blue) in $rumi^{+/-}$ cells but not in $rumi^{-/-}$ cells.

EGF repeat 7 up to the transmembrane domain (EGF7-TM) was expressed in control and Rumi knockdown cells and purified from the medium. The presence of *O*-fucose and *O*-glucose glycans on EGF7-TM was then assessed using mass spectral analysis of tryptic peptides generated from EGF7-TM protein (Figure S9). No changes in *O*-fucosylation were detected. In contrast, reduction of *O*-glucose on several peptides from the Rumi RNAi samples was seen. Comparison of the relative amounts of a peptide from EGF repeat 14 in the two samples shows that while the glycopeptide can be detected in both samples, significantly less is seen in the Rumi knockdown sample (Figure 6B). Also, significant amounts of the unglycosylated peptide are seen only in the sample where Rumi was knocked down (Figure 6A). Rumi RNAi also caused reduction in the level of glycosylated form of EGF repeats 16, 17, 19, and 35 (Figure S10). These data strongly suggest that reduction in Rumi is causing a reduction in *O*-glucose levels on Notch.

Rumi Encodes a Protein *O*-Glucosyltransferase

To directly examine whether Rumi has Poglut activity toward EGF repeats, a FLAG-tagged version of Rumi was overexpressed in S2 cells, affinity-purified from cell extracts and media, and utilized in an in vitro Poglut assay (Shao et al., 2002). A factor VII EGF repeat containing an *O*-glucose consensus site was used as acceptor substrate, and UDP-[^{3}H]glucose as donor. Rumi samples showed Poglut activity compared to controls (Figure 6C). Chromatographic analyses confirmed that the product consisted of a glucose residue covalently attached to the factor VII EGF repeat in an *O*-linkage (Figure S11). These results show that Rumi is capable of adding a single glucose in *O*-linkage to an EGF repeat containing an *O*-glucose consensus sequence. Further assays showed that the Poglut activity was dependent on the amount of Rumi, the concentration of factor VII EGF repeat, and the concentration of UDP-glucose (Figures 6D–6F). Taken together, these results demonstrate that Rumi is a Poglut.

The Poglut Activity Mediated by Rumi Is Required for Proper Notch Signaling

Our data show that Rumi is a Poglut that adds glucose residues to EGF repeats of Notch and that Rumi function is essential for Notch signaling in a ts manner. Poglut activity may be required

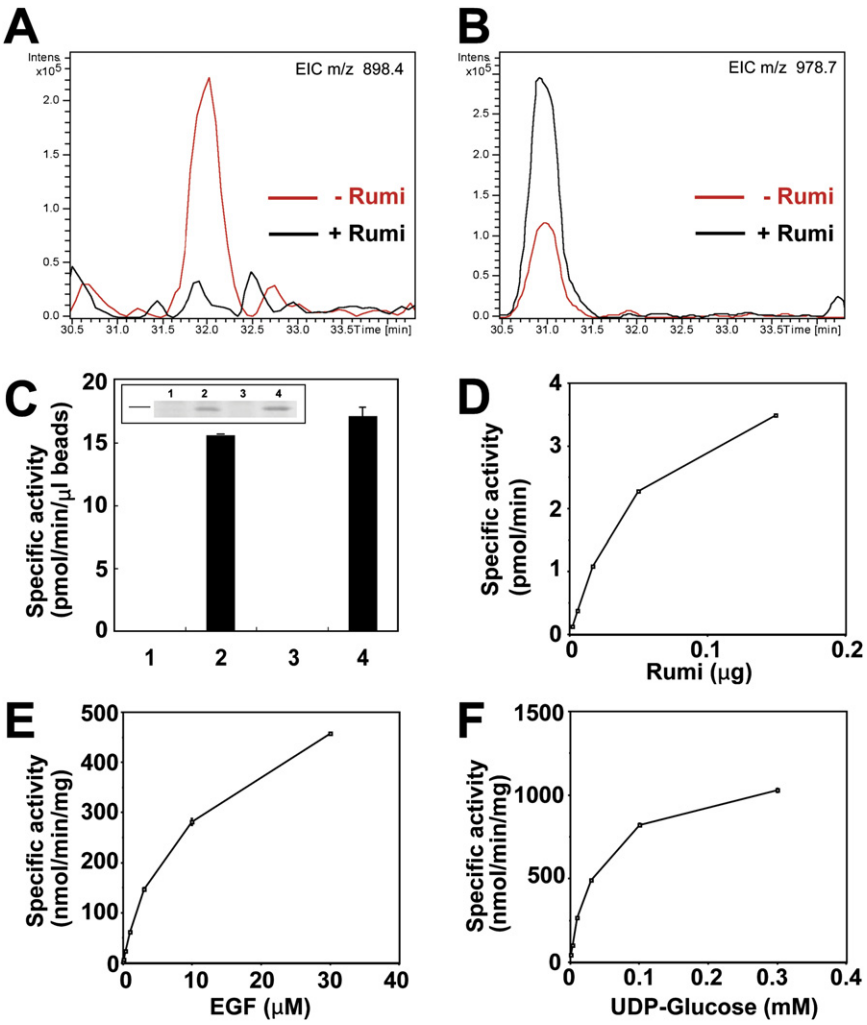

Figure 6. Rumi Is a Poglut

(A) The unglycosylated form of ^{561}CQINIDDCQSQPCR^{574} is present in the Rumi knockdown sample but not in the control sample. The MS data from both samples were searched for the doubly charged form of the peptide, m/z 898.4 (see Figure S9A for MS and MS/MS spectra at 32.0 min). The ion can be clearly seen eluting at 32.0 min in the Rumi knockdown sample (−Rumi), but it cannot be detected above the noise in the control sample (+Rumi).

(B) The *O*-glucosylated form of ^{561}CQINIDDCQSQPCR^{574} is more abundant in the control sample than in the Rumi knockdown sample. The MS data from both samples were searched for the doubly charged form of the glycopeptide, m/z 978.7 (see Figure S9B for MS and MS/MS spectra at 30.8 min). The ion can be seen in both samples at 30.8 min, but more is present in the control sample (+Rumi) than in the Rumi knockdown sample (−Rumi).

(C) Both cell extracts and culture media of Rumi-overexpressing S2 cells showed a Poglut activity in vitro. Inset: Coomassie staining after 10% SDS-PAGE of the equivalent amounts of the beads. Line indicates 50 kDa size marker. 1: cell extract from control cells, 2: cell extract from Rumi-overexpressing cells, 3: media from control cells, 4: media from Rumi-overexpressing cells.

(D) Poglut activity was dependent on the amount of the purified Rumi protein in vitro.

(E) Poglut activity of the purified Rumi protein was dependent on the concentration of factor VII EGF as acceptor substrate.

(F) Poglut activity of the purified Rumi protein was dependent on the concentration of UDP-glucose as donor substrate.

All Poglut assays were performed in duplicate. Error bars represent the range of duplicates.

in some contexts for Notch signaling, but Rumi may also function as a chaperone independently of its enzymatic function, as reported for Ofut1 (Okajima et al., 2005; Sasamura et al., 2007). To examine the importance of Notch *O*-glucosylation for signaling, we took advantage of one of our severe EMS-induced mutants, *rumi*^{79}, that has a G189E mutation (Figure 3B). We examined whether G189E possesses Poglut activity or not. Rumi-G189E is expressed at normal levels in *rumi*$^{79/79}$ flies (Figure 7A), and the protein is expressed and secreted as efficiently as the wt Rumi protein in S2 cells (Figure 7B). Moreover, the intracellular localization of Rumi-G189E in S2 cells is indistinguishable from wt Rumi (data not shown). These observations indicate that the G189E mutation does not impair Rumi expression or stability. However, Rumi-G189E showed no enzymatic activity (Figure 7C). These data do not support the presence of a key nonenzymatic role for Rumi, unlike what has been reported for Ofut1 (Okajima et al., 2005; Sasamura et al., 2007). The data also indicate that *O*-glucosylation mediated by Rumi is essential for Notch signaling. These conclusions are supported by in vitro and in vivo structure-function analyses of *rumi* (see Supplemental Data).

DISCUSSION

Loss of Rumi Causes a Loss of Notch Signaling in a ts Manner

In all contexts that we have examined, *rumi* is essential for Notch signaling in a ts manner, i.e., lateral inhibition, asymmetric division, and inductive signaling. Homozygous *rumi* animals are viable and fertile when kept at 18°C and exhibit a mild lateral inhibition defect and a modest Delta wing vein phenotype (Figures 1 and 2). *rumi* animals raised at 25°C show a very significant decrease in viability and fertility. At this temperature, there is a failure in the cell-fate specification process (Figure 1). At 28°C–30°C we observe a full-blown *Notch* phenotype in all tissues examined, and homozygous mutants can only reach the third instar stage because of the wt maternal component. The difference between the requirements for Rumi at 25°C and 28°C–30°C is also reflected in our genetic interaction studies, as an extra copy of *Notch* is able to improve Notch signaling in *rumi* mutants raised at 25°C (partial requirement) but not at 28°C–30°C (full requirement). Altogether these observations indicate that loss of *rumi* phenocopies loss of *Notch* in a temperature-dependent fashion.

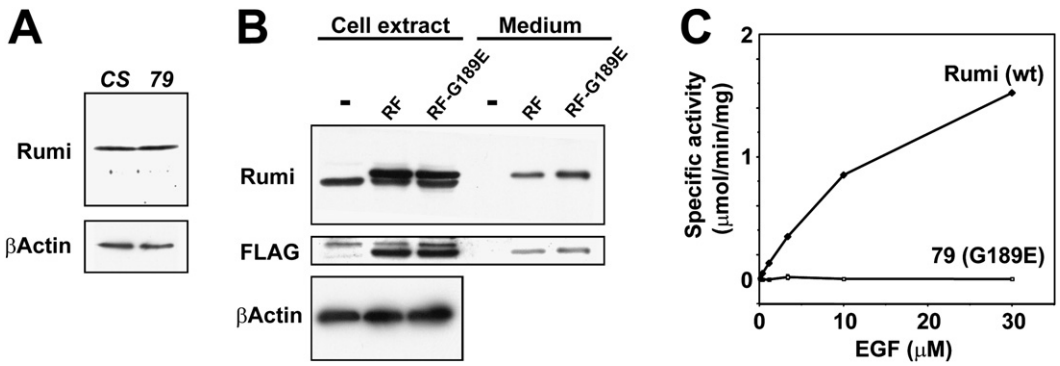

Figure 7. *O*-Glucosylation Mediated by Rumi Is Required for Notch Signaling

(A) Western blots showing the relative levels of Rumi and β-actin proteins in wild-type (*CS*) and homozygous *rumi*^{79} (*79*) larval protein extracts. Late third instar larvae were used to prepare protein extracts for each genotype. Note that the G189E mutation in *rumi*^{79} larvae does not affect the level of the Rumi protein.

(B) The G189E mutation does not affect the expression levels of Rumi in S2 cells. Western blots show the expression and secretion of Rumi-FLAG (RF) and Rumi-FLAG with the G189E mutation (RF-G189E) in S2 cells. RF and RF-G189E were expressed in S2 cells using *pUAST* and *pAc-Gal4* vectors. Note that the RF and RF-G189E proteins expressed in S2 cells run slightly slower than the endogenous Rumi protein due to the presence of the FLAG tag (see anti-Rumi blot). Endogenous Rumi is not expressed at levels that lead to its secretion in the medium. Some of the RF and RF-G189E proteins are secreted into the medium when expressed in S2 cells.

(C) The G189E mutation causes a complete loss of Poglut activity. Both wild-type and G189E Rumi proteins were overexpressed in S2 cells and purified from culture media by using the FLAG epitope as described in Experimental Procedures. The purified proteins were assayed for Poglut activity using increasing concentrations of the acceptor substrate, factor VII EGF repeat (wild-type Rumi, *diamonds*; G189E mutant, *open squares*). All assays were performed using 100 μM of UDP-glucose in duplicate. Error bars represent the range of duplicates.

Rumi Targets the Extracellular Domain of Notch

Multiple lines of evidence suggest that Rumi functions in the signal-receiving cell. Our MARCM experiments indicate that overexpression of Notch ligands in *rumi* mutant cells is able to induce signaling, suggesting that Rumi function is not required in the signal-sending cell (Figure S3). However, cells that are mutant for *rumi* are not able to receive the signal, even when ligands are overexpressed in adjacent cells (Figure 5). Of note, the only component of the Notch signaling pathway in flies with multiple *O*-glucosylation sites is the Notch protein itself. However, Delta, contains a single predicted site.

As we observed an upregulation of Notch protein in *rumi* mutant clones we hypothesized that Notch might be trapped in the ER and fail to reach the membrane at the restrictive temperature. However, we observe an accumulation of Notch at the surface of *rumi* mutant cells. In addition, we find a lack of an unfolded protein response (Patil and Walter, 2001; Ryoo et al., 2007) and a lack of expansion of the ER in *rumi* clones raised at the restrictive temperature (Figure S6). These data raised the possibility that Notch present at the cell surface may not interact with its ligands at the restrictive temperature. However, our data suggest that the Notch-Delta interaction is not decreased at 28°C (Figure S7), but rather that the cleavage of Notch at the membrane is impaired (Figure 4). Hence our data indicate that the S2 cleavage of Notch is impaired in *rumi* mutant signal-receiving cells.

Rumi Is a Poglut

Most proteins with a CAP10 domain contain a signal peptide and an ER retention signal. The CAP10 gene was first discovered in the fungus *Cryptococcus neoformans* (Chang and Kwon-Chung, 1999). The CAP proteins (CAP10, 59, 60, and 64) are referred to as putative polysaccharide modifiers as they affect extracellular polysaccharide capsule formation (Okabayashi et al., 2007). Our data indicate that knockdown of Rumi in S2 cells results in loss of *O*-glucosylation at serines in C^1-X-S-X-P-C^2 sites on numerous EGF repeats. No effects were seen on levels of *O*-fucosylation. In vitro assays with purified Rumi demonstrate that it can catalyze the transfer of glucose from UDP-glucose to an EGF repeat with the consensus sequence. Hence, Rumi encodes a Poglut. Rumi shares several common features with enzymes responsible for addition of *O*-fucose to EGF repeats and thrombospondin type 1 repeats (TSRs), Pofut1 and Pofut2, respectively. These proteins are soluble, ER localized, and only modify properly folded structures (EGF repeats for Pofut1, TSRs for Pofut2) (Luo et al., 2006a, 2006b; Wang and Spellman, 1998). Preliminary studies using crude lysates suggest that the mammalian form of the Poglut (presumably a Rumi homolog) can also distinguish folded from unfolded structures (Shao et al., 2002). The ER localization and ability to distinguish folded from unfolded structures suggests that all of these enzymes may function in folding and/or quality control.

Unlike Ofut1, which is reported to have important nonenzymatic functions (Okajima et al., 2005; Sasamura et al., 2007), our results indicate that the function of Rumi resides in the Poglut activity (Figure 7 and Supplemental Data). We therefore propose that preventing the addition of *O*-glucose to Notch causes a ts phenotype. We propose that the *O*-glucose glycans may function to hold the N^{ECD} in a stable conformation needed for proper function, especially at higher temperatures. For example, *O*-glucosylation of Notch might be a prerequisite for conformational changes in the N^{ECD} that are proposed to promote the S2 cleavage (Malecki et al., 2006; Parks et al., 2000). Alternatively, addition of *O*-glucose might be required for another posttranslational

modification. The importance of *O*-glucosylation of Notch is also supported by studies showing that elimination of individual *O*-glucosylation sites in mouse Notch1 impairs activation in cell-based Notch signaling assays (A. Nita-Lazar and R.S.H., unpublished data).

Lack of *O*-glucosylation at the restrictive temperature does not block the ER-to-membrane transport and ligand interactions but disrupts Notch cleavage. These data, together with accumulation of Notch intracellularly and at the cell membrane in *rumi* cells, suggest that lack of *O*-glucose modification causes a folding problem that impairs Notch function. Trafficking problems upstream of S3 cleavage have been documented to cause accumulation of Notch and ectopic activation of Notch signaling (Le Borgne, 2006). For example, loss of Lethal giant discs (Lgd), a protein required for proper trafficking of Notch, causes ectopic activation of Notch in a ligand-independent manner (Childress et al., 2006; Gallagher and Knoblich, 2006; Jaekel and Klein, 2006). Our data show that the loss of *rumi* suppresses the ectopic activation of Notch in *lgd* mutant cells (Figure S8), suggesting that the lack of *O*-glucosylation prevents the ligand-independent activation of Notch in the absence of Lgd.

In summary, our data uncover a mechanism for enzymatic regulation of Notch signaling in *Drosophila* by a Poglut and provide an in vivo model to study the role of *O*-glucosylation in developmental signaling. Given the evolutionary conservation of Notch signaling and the presence of conserved *O*-glucosylation motifs in other Notch proteins, addition of glucose may be required for proper folding and cleavage in many species.

EXPERIMENTAL PROCEDURES

Drosophila Strains and Genetics

The following strains were used in this study: *Canton-S, y w, Dl^{9P} e^{s}/TM3, Sb^{1}, N^{55e11}/FM7, Df(1)Notch^{8}/FM7c, w; Df(3R)Exel6192/TM6, Tb* (The Bloomington Stock Center), *y w; FRT82B pM Sb^{63} ry^{+} y^{+}/TM3, Ser* (Jafar-Nejad et al., 2005), *N^{ts1}; SM1, Dp(1;2)51b/+, UAS-Ser; D/TM6, Tb* (gift from B. Hassan), *hs-FLP, lgd^{1} FRT40A/CyO; FRT82B ubi-GFP/TM3, Sb^{1}* (Childress et al., 2006), *hs-FLP tub-GAL4 UAS-GFP^{nls}-6X-Myc* (Wang and Struhl, 2005), *y w; UAS-Dl^{30}, y w; UAS-N^{ECN}/CyO; MKRS/TM2, y w; UAS-N^{FL}/CyO; MKRS/TM2* (gift from G. Struhl), *dpp-GAL4* (Staehling-Hampton et al., 1994), *Notch-lacZ/FM6* (de Celis et al., 1997), *C684-GAL4* (Manseau et al., 1997), *y w; Ubx-FLP* (Emery et al., 2005), *Dl^{9P} rumi^{44}/TM3, Sb^{1}, y w; UAS-rumi, y w; UAS-rumi-FLAG, y w; UAS-rumi-FLAG-ΔKDEL, y w; rumi-FLAG rescue transgene, y w; C684-GAL4 FRT82B rumi^{79}, y w; C684-GAL4 FRT82B rumi^{44}, y w; FRT82B rumi^{79}, y w; FRT82B rumi^{44}, y w; FRT82B rumi^{Δ26}, y w Ubx-FLP tub-GAL4 UAS-GFP^{nls}-6X-Myc; FRT82B y^{+} tub-GAL80/TM6, Ubx, y w; L/CyO; FRT82B rumi^{Δ26} y^{+} tub-GAL80/TM6, Tb, y w Ubx-FLP Tub-GAL4 UAS-GFP^{NLS}-6X-Myc; FRT82B* (this study). For genetics, please see the Supplemental Data.

Immunostainings

Dissections and stainings were performed by using standard methods. For surface staining of Notch in *rumi* clones, detergents were excluded from the staining protocol, as described previously (Wang and Struhl, 2004). For α-Rumi antibody production and the list of other antibodies used in the study please see the Supplemental Data.

Receptor Ligand Interaction Assays and Production of Rumi Protein in S2 Cells

Receptor ligand interactions were performed as described previously (Bruckner et al., 2000; Childress et al., 2006). In these assays, a secreted hybrid protein comprised of the N^{ECD} and alkaline phosphatase (N-AP) was made in S2 cells treated with dsRNA against *EGFP* or *rumi*. Binding of N-AP to Delta present at the surface of S2-Delta cells was assessed by measuring the AP activity (for details see Supplemental Data). To make Rumi-FLAG protein, S2 cells were transfected with *pUAST-rumi-FLAG* and *pAc-Gal4*.

Analysis of *O*-Glucosylation of N^{ECD} in Rumi Knockdown S2 Cells

Analysis of *O*-glucosylation of tryptic peptides from EGF7-TM protein (see Supplemental Data) was performed by LC-MS/MS essentially as described (Nita-Lazar and Haltiwanger, 2006; Ricketts et al., 2007; Wang et al., 2007). Briefly, approximately 500 ng of EGF7-TM protein purified from the medium of control and Rumi knockdown cells were reduced, alkylated, separated by SDS-PAGE, and subjected to in-gel tryptic digestion. The resulting peptides were separated by reverse-phase HPLC and sprayed directly into an Agilent XCT ion trap mass spectrometer. Low-energy CID fragmentation was performed on the two most abundant ions in each MS scan. Unglycosylated peptides were identified by searching databases with the MS/MS data using the X! Tandem (Global Proteome Machine) search engine (http://h777.thegpm.org/tandem/thegpm_tandem.html) or by manually searching the MS data for ions matching predicted masses of tryptic peptides containing *O*-glucose consensus sequences. *O*-fucosylated peptides were identified by performing neutral loss scans of the data for ions losing 146 Da upon CID fragmentation. Similarly, *O*-glucosylated peptides were identified by performing neutral loss scans for ions losing 162 Da upon fragmentation. Relative amounts of individual molecular ions (representing either glycosylated or unglycosylated forms of specific peptides) in control or Rumi knockdown samples were compared by performing extracted ion searches of the MS data for the ion of interest.

Poglut Assay

Poglut assays were performed with slight modification as previously described (Shao et al., 2002). For details see Supplemental Data.

Western Blot Analyses and RNAi for Notch Cleavage Assays

Extract preparation and western blots were performed as described previously (Hu et al., 2002; Pan and Rubin, 1997; Ye et al., 1999). For *kuz* RNAi in S2 cells, three different dsRNAs were used. *pMT-Notch* construct was used to express Notch in S2 cells. Decanoyl-RVKR-CMK (Emd Biosciences) was used as the furin inhibitor at a final concentration of 50 μM.

Supplemental Data

Supplemental Data include Experimental Procedures, Results, and eleven figures and can be found with this article online at http://www.cell.com/cgi/content/full/132/2/247/DC1/.

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