Regulation of mammalian Notch signaling and embryonic development by the protein O-glucosyltransferase Rumi

Rodrigo Fernandez-Valdivia¹, Hideyuki Takeuchi², Amin Samarghandi³, Mario Lopez³, Jessica Leonardi³, Robert S. Haltiwanger² and Hamed Jafar-Nejad¹,³,⁴,⁵,⁶,*

SUMMARY

Protein O-glucosylation is a conserved post-translational modification that occurs on epidermal growth factor-like (EGF) repeats harboring the C¹-X-S-X-P-C² consensus sequence. The Drosophila protein O-glucosyltransferase (Poglut) Rumi regulates Notch signaling, but the contribution of protein O-glucosylation to mammalian Notch signaling and embryonic development is not known. Here, we show that mouse Rumi encodes a Poglut, and that Rumi¹⁺ mouse embryos die before embryonic day 9.5 with posterior axis truncation and severe defects in neural tube development, somitogenesis, cardiogenesis and vascular remodeling. Rumi knockdown in mouse cell lines results in cellular and molecular phenotypes of loss of Notch signaling without affecting Notch ligand binding. Biochemical, cell culture and cross-species transgenic experiments indicate that a decrease in Rumi levels results in reduced O-glucosylation of Notch EGF repeats, and that the enzymatic activity of Rumi is key to its regulatory role in the Notch pathway. Genetic interaction studies show that removing one copy of Rumi in a Jag¹⁺ (jagged 1) background results in severe bile duct morphogenesis defects. Altogether, our data indicate that addition of O-glucose to EGF repeats is essential for mouse embryonic development and Notch signaling, and that Jag1-induced signaling is sensitive to the gene dosage of the protein O-glucosyltransferase Rumi. Given that Rumi¹⁻ embryos show more severe phenotypes compared to those displayed by other global regulators of canonical Notch signaling, Rumi is likely to have additional important targets during mammalian development.

KEY WORDS: Notch signaling, O-glucosylation, Mouse, Jag1, EGF repeat, Drosophila

INTRODUCTION

Notch signaling is one of the pathways widely used during animal development and in the adult maintenance of a variety of tissues and cell types (Fortini, 2009; Kopan and Ilagan, 2009). Mutations in several components of this pathway play causative roles in various diseases (Ellisen et al., 1991; Joutel et al., 1996; Bulman et al., 2000; Eldadah et al., 2001; Garg et al., 2005; Lee et al., 2009), including a multisystem developmental disorder called the Alagille syndrome (Li et al., 1997; Oda et al., 1997). The Notch proteins and their ligands are type I transmembrane proteins with a number of epidermal growth factor-like (EGF) repeats in their extracellular domain. EGF repeats usually consist of ~40 amino acids, including six cysteine residues that form three disulfide bonds to generate the three-dimensional structure of the EGF repeat (Harris and Spellman, 1993). Several forms of O-linked carbohydrates decorate the EGF repeats of Drosophila and mammalian Notch proteins (Moloney et al., 2000; Matsuura et al., 2008): O-fucose, O-GlcNAc (N-acetylglucosamine) and O-glucose. Loss of the enzyme responsible for the addition of O-fucose to Notch (protein O-fucosyltransferase 1) results in embryonic lethality, with phenotypes similar to a global loss of Notch signaling in flies and in mice (Okajima and Irvine, 2002; Sasamura et al., 2003; Shi and Stanley, 2003). Therefore, Notch signaling seems to be the primary biologically relevant target of Ofut1/Pofut1 during embryonic development.

O-glucosylation occurs on Notch and other proteins with EGF repeats harboring a C¹-X-S-X-P-C² consensus motif, where S is the glucosylated serine and C¹ and C² are the first and second cysteine residues in the target EGF repeat (Harris and Spellman, 1993). Vertebrate and Drosophila Notch proteins harbor the largest number of EGF repeats with predicted O-glucosylation motifs, although other proteins, including Notch ligands and several coagulation factors, also contain a number of such motifs (Moloney et al., 2000). Mutations in Drosophila rumi, which encodes the fly protein O-glucosyltransferase, result in a temperature-sensitive loss of Notch signaling (Acar et al., 2008). Moreover, RNAi-mediated knockdown of Rumi in Drosophila S2 cells causes a severe reduction in the level of O-glucose on Notch EGF repeats. These observations established an important role for protein O-glucosylation in the regulation of Drosophila Notch signaling (Acar et al., 2008).

The glycosyltransferase activity of fly Rumi is mediated by the CAP10 domain, which is named after the cryptococcal protein CAP10 (Chang and Kwon-Chung, 1999). To examine whether the role of Rumi in the regulation of Notch signaling is conserved

¹Brown Foundation Institute of Molecular Medicine (IMM), The University of Texas Health Science Center, Houston, TX 77030, USA. ²Department of Biochemistry and Cell Biology, Institute for Cell and Developmental Biology, Stony Brook University, Stony Brook, NY 11794, USA. ³Program in Developmental Biology, Baylor College of Medicine, Houston, TX 77030, USA. ⁴Department of Biochemistry and Molecular Biology, Medical School, The University of Texas Health Science Center, Houston, TX 77030, USA. ⁵Program in Genes and Development, The University of Texas Graduate School of Biomedical Sciences, Houston, TX 77030, USA. ⁶Program in Biochemistry and Molecular Biology, The University of Texas Graduate School of Biomedical Sciences, Houston, TX 77030, USA.

*Author for correspondence (hamed.jafar-nejad@uth.tmc.edu)

Accepted 18 February 2011

© 2011. Published by The Company of Biologists Ltd
between flies and mammals, we initiated genetic, cell culture and biochemical studies on mouse genes encoding CAP10-containing proteins and found that only one of these homologs, mouse Rumi, has Poglut activity. Our data indicate that Rumi is required for embryonic development and Notch signaling in the mouse. Our data further suggest that Jag1-induced Notch signaling is sensitive to the gene dosage of mouse Rumi.

MATERIALS AND METHODS

Generation of the Rumi mutant mouse

The IST10323G11 (Texas A&M Institute for Genomic Medicine) (Hansen et al., 2008) heterozygous C57BL/6N ES cell clone with a gene trap insertion in intron 2 of Rumi was used to generate the Rumi mutant mouse (official name: Poglut
tg(IST10323G11)TIG6). For Southern blot analysis, genomic DNA was digested with AvrII, BglII or HindIII, and the blots were hybridized with a probe against the neomycin resistance gene (Fernandez-Valdivia et al., 2010). All mice were housed in temperature-controlled (22±2°C) rooms, with 12 hour light, 12 hour dark photocycle, and fed with rodent chow meal and fresh water, ad libitum. All surgical procedures as well as euthanasia protocols were approved by the Animal Welfare Committee of The University of Texas Health Science Center at Houston, and were in accordance with the procedures outlined in the ‘Guide for the Care and Use of Laboratory Animals’ (NIH publication 85-23).

Genotyping

Genomic DNA from tail tips, yolk sacs or ES cells was used for PCR genotyping. Paraformaldehyde-fixed early embryos were directly genotyped after immunohistochemical experiments. For primers, see Table S1 in the supplementary material.

Embryonic studies

Embryos were collected between E7.5 and E10.5. E8.0 embryos were fixed in 4% PFA in PBS, washed with PBS and pre-embedded in 1% low melting point agarose in PBS. Agarose blocks were dehydrated with alcohol series, cleared with xylenes and embedded in paraffin blocks. Embryo blocks were sectioned at 5 μm, and the sections stained with Hematoxylin and Eosin.

DBA lectin histochemistry and immunohistochemistry

DBA lectin histochemistry and Pecam1 staining were performed as described previously (McCright et al., 2002; Kuhnert et al., 2005). For Rumi staining, antigen was retrieved in a boiling solution of 10 mM sodium citrate (pH 6.0) for 20 minutes. The anti-Rumi antibody was raised in rabbits against the peptide RKQDGSKWKVFLD (see Fig. S2 in the supplementary material). Anti-Rumi and Cy3-conjugated goat anti-Rabbit secondary (Jackson ImmunoResearch Laboratories) antibodies were used at 1:250.

RT-PCR and qRT-PCR analysis

Total RNA was extracted using QIAzol and RNeasy kit (Qiagen). qRT-PCR was performed using 100 ng total RNA per well and TaqMan One-Step RT-PCR Master Mix (Applied Biosystems). Relative mRNA levels were compared using the 2-ΔΔCT method, with 18S as control. For Hes1 qRT-PCR in Neuro2A cells, 2 μg of total RNA per well were used. For RT-PCR, 1 μg of total RNA per lane was used to synthesize cDNA. For primers and primers/probe sets see Table S1 in the supplementary material.

Cell culture, Rumi knockdown, immunoblots and biotinylation assays

NMUli, HepG2, BNL-CL2, Neuro2A, HEK293T and C2C12 cells were cultured in DMEM (Thermo Fisher) supplemented with 10% FBS (Thermo Fisher). Transfections were performed in OPTI-MEM (Invitrogen), using either Fugene HD (Roche) or Lipofectamine 2000 (Invitrogen). pLKO.1 plasmids (Moffat et al., 2006) encoding shRNA-30 against mouse Rumi and non-target control shRNA-Na (Sigma) were used to generate stably transfected cells. Plasmids used for transient transfection include: mouse Rumi cDNA (pcDNA4-mRumi); EGFP-C2 (Clontech); pTracer; hRumi-FLAG; and pTracer-hRumi-FLAG-G169E mutant. Antibodies used in western blots are anti-mouse Rumi (1:500, this study), anti-α-Tubulin (1:2000, Santa Cruz Biotechnology), anti-GFP (1:4000, Abcam), anti-β-Actin-HRP (1:2000, Santa Cruz Biotechnology), anti-β-Actin (1:2000, Abcam) and anti-cleaved Notch1 (Val1744, 1:1000, Cell Signaling Technology). Biotinylation assays were performed as described previously (Mumm et al., 2000; Rampal et al., 2005) using the anti-Notch1 Nα polyclonal antibody (Lu and Lux, 1996).

Flow cytometry

Flow cytometry was performed on a BD FACS Aria II according to published protocols with slight modifications (Yang et al., 2005; Stahl et al., 2008). Purified rat DIII-human Fc (1 μg/ml), rat Jag1-human Fc (0.5 μg/ml) (Hicks et al., 2000) and human Fc (1 μg/ml) were preclutered with R-PE conjugated anti-human Fc for 1 hour at 4°C. The following antibodies were used: anti-Notch1 (10 μg/ml, R&D Systems); R-PE-conjugated F(ab’)2 anti-sheep IgG (1:100), sheep IgG (10 μg/ml), human Fc fragment (1 μg/ml) and R-PE-conjugated F(ab’)2 anti-human Fc fragment (1:100) from Jackson ImmunoResearch Laboratories.

Differentiation assays on Neuro2A cells

Neuro2A differentiation assays were performed as described previously (Franklin et al., 1999) with some modifications. At least three independent experiments were performed for each cell line/transfection. In each experiment, five independent fields of ~100 cells were used for quantification of the neurite extension per cell line/transfection.

Transgenic fly experiments

The ORF of the rat Jag1 was cloned into the pUAST-attB vector and integrated into the fly genome as described (Venken et al., 2006; Bischof et al., 2007). UAS-ratDll1-hA, UAS-mDLL3-FLAG (Geffers et al., 2007) and UAS-attB-ratJagged1 (this study) were used to overexpress mammalian ligands in mitotic clones of the null allele rumiΔ29 (Acar et al., 2008) or in mitotic clones of a wild-type chromosome (control) by using the MARCM technique (Lee and Luo, 2001) as described previously (Acar et al., 2008). Wing imaginal discs were dissected and fixed by using standard protocols and stained with α-Jag1 (1:100, Santa Cruz Biotechnology), α-Cut (1:500, DSHB) and/or α-FLAG (1:1500, Sigma) antibodies.

Enzymatic assays

Cells and livers were homogenized in approximately 5 volumes of TBS-Complete, and incubated on ice for 60 minutes. After centrifugation, the supernatants were used as protein extracts. Enzymatic assays for hRumi-FLAG and hRumi-G169E-FLAG were performed on cell lysates from transiently transfected HEK293T cells. Both O-glucosyltransferase and O-fucosyltransferase 1 assays were performed as previously described (Shao et al., 2002) with slight modifications.

Mass spectrometric analysis

A cDNA encoding a region of the extracellular domain of mouse Notch2 encompassing EGF repeats 13-18 (amino acids 468-699) was inserted into pSecTag2/Hygro vector (Invitrogen) so that the protein was expressed with a C-terminal Myc-6xHis tag. The expression vector was transiently transfected into C2C12-NT and C2C12-30 cells using PEI. Proteins were purified from the culture medium by Ni-NTA agarose affinity chromatography (Qiagen). Equivalent amounts of proteins from each cell line were separated by Nu-PAGE gradient gel (4-12%, Invitrogen), detected using a zinc-stain (BioRad), and bands were cut out and digested with Asp-N protease (Sigma) at 37°C for 16 hours as described (Nita-Lazar and Haltiwanger, 2006). The resulting (glyco)peptides were purified by Zip-Tip (Millipore) and analyzed by nanoLC-MS/MS on an Agilent XCT Ultra ion trap mass spectrometer with a CHIP-Cube interface as described (Leonhard-Melief and Haltiwanger, 2010). The relative quantities of the (glyco)peptides were analyzed by differential MS approach using the extracted ion chromatograms of each ion (Acar et al., 2008).

Statistics

Data are shown as mean±s.e.m. P values were determined by Student’s t-test or one-way ANOVA. For Neuro2A differentiation assays, one-way ANOVA was used to compare all groups to Neuro2A-NT, with Dunnet error protection at a 95% confidence interval.
RESULTS
Rumi is the sole enzyme in the mouse capable of adding O-glucosyl to EGF repeats

Drosophila Rumi has three homologs in mammals: Rumi [also known as CAP10-like protein 46 kDa (CLP46) and KTEL-containing 1 (KTEL1)], KDEL-containing 1 (KDEL1) and KDEL2 (Fig. 1A). Among these three proteins, mouse Rumi shares the highest homology with fly Rumi (56% overall identity; 66% identity in the CAP10 domain). The enzymatic and physiological roles of these proteins have not been reported (Kimata et al., 2000; Teng et al., 2006). Biochemical analysis and cross-species overexpression studies in flies indicate that the mouse/human Rumi is the only Drosophila Rumi homolog with similar enzymatic and functional characteristics (H.T., R.F.-V., H.J.-N. and R.S.H., unpublished). Therefore, we injected ES cells with gene-trapped Rumi alleles showing ~50% decrease in Rumi mRNA expression (Fig. 1C; see Fig. S1 in the supplementary material) into blastocysts and established a Rumi–/– strain (here referred to as Rumi or RumiGeo allele) from ES cell line IST10323G11. PCR sequencing and Southern blot with a Neo probe on genomic DNA confirmed a single gene trap insertion in intron 2 of the Rumi gene (Fig. 1B,D,E). qRT-PCR assays on P0 liver extracts of Rumi–/– newborns showed a 50% decrease in Rumi mRNA levels compared with wild-type littermates, demonstrating that the gene trap efficiently blocks the expression of Rumi mRNA (Fig. 2A). Moreover, immunostaining with a polyclonal anti-Rumi antibody (see Fig. S2 in the supplementary material) shows broad expression in wild-type and Rumi+/– embryos but no expression in Rumi–/– embryos (Fig. 2B,C; data not shown). We conclude that this allele in either null or a strong hypomorph.

To assess whether a decrease in Rumi levels affects the protein O-glucosyltransferase (Poglut) activity of mouse tissue extracts towards EGF repeats, we performed Poglut assays on liver extracts from newborn Rumi–/– animals and their wild-type littermates. We find that loss of one copy of Rumi results in ~50% decrease in the Poglut activity but with no significant change in Pofu1 activity (Fig. 2D), indicating that the effect observed on Poglut activity in Rumi+/– animals is specific. RT-PCR assays show that the other two mammalian homologs of Drosophila Rumi – KDEL1 and KDEL2 – are co-expressed with Rumi in neonatal mouse livers and in several mammalian liver cell lines (Fig. 2E). qRT-PCR on liver extracts indicates that KDEL1/KDEL2 mRNA are expressed at moderate levels but do not change in Rumi–/– compared with controls (Fig. 2A). Therefore, we conclude that these two proteins are not able to compensate for the loss of one copy of Rumi in the neonatal liver. Western blot analysis using our polyclonal anti-Rumi antibody indicates that Rumi is broadly expressed in neonatal and adult mouse tissues (Fig. 2F), in agreement with previous reports on the broad distribution of Poglut activity in rat tissues (Shao et al., 2002) and human Rumi mRNA in adult human tissues (Teng et al., 2006). Altogether, these data indicate that Rumi is the only Poglut enzyme in the mouse liver (and likely other tissues) able to add an O-linked glucose residue to EGF repeats with a C1-L-S-X-P-C2 consensus motif.

Early embryonic lethality and severe cardiovascular defects in embryos lacking Rumi

Rumi–/– animals are fertile and normal, and do not exhibit gross developmental abnormalities. From Rumi+/– intercrosses, wild-type and Rumi+/– heterozygous P0 progeny were obtained at a Mendelian ratio, but no newborn Rumi–/– mutants were obtained (Table 1). This observation suggests that Rumi is essential for mouse embryonic development. We therefore set timed pregnancies to study the embryonic phenotypes of Rumi–/– embryos. At E7.0–E7.5, Rumi–/– embryos could not be morphologically distinguished from their heterozygous and wild-type littermates (not shown). At E8.0, Rumi–/– embryos are characterized by an abnormally expanded neural plate that does not fold properly (Fig. 3C,F; compare with Fig. 3A,B,D,E). (D) Southern blot analysis with the NEO probe to verify unique insertion of the 6.5 kb VICTR76 gene trap vector in the Rumi locus. Insertion of the gene trap vector in Rumi results in a unique 8.0 kb band upon digestion with AvrI (A), BgIII (B) and HindIII (H) sites are depicted by vertical lines. (E) Multiplex PCR genotyping on genomic DNA with primers F1, R1 and R2 (5′ junction) and F1, R1 and F2 (3′ junction). (E) Southern blot analysis with the NEO probe to verify unique insertion of the 6.5 kb VICTR76 gene trap vector in the Rumi locus. Insertion of the gene trap vector in Rumi results in a unique 8.0 kb band upon digestion with AvrI (A), BgIII (B) and HindIII (H) digestion. Each release a unique fragment of ~5.8 kb from the mutant Rumi allele. Note the comparable numbers of digested genomic DNA loaded for C57 and RumiGeo genotypes.

Fig. 1. Generation of a loss-of-function allele of mouse Rumi.

(A) Structure of the mouse CAP10 proteins and their amino acid identity to Drosophila Rumi (dRumi). Blue boxes at N and C termini indicate the signal peptide and the ER-recycling signal, respectively. (B) The top panel shows the structure of the mouse Rumi locus. The vertical arrow shows the insertion site of the gene trap in intron 2 (ex, exon; SA, splice acceptor; pA, polyAdenylation signal; LTR, long terminal repeat; NEO, neomycin resistance gene probe). AvrlI (A), BgIII (B) and HindIII (H) sites are depicted by vertical lines. (C) qRT-PCR on RNA extracted from wild-type C57BL6 (C57) and the RumiGeo ES cell IST10323G11. Data are mean±s.e.m. (D) Multiplex PCR genotyping on genomic DNA with primers F1, R1 and R2 (5′ junction) and F1, R1 and F2 (3′ junction).
embryos (Fig. 3J,K), but not in Rumi−/+ embryos (Fig. 3L). At E8.5, Rumi−/+ embryos showed a neural plate that fails to fold properly, but continues to expand in the anterior part of the embryo (arrow in Fig. 3O; compare with Fig. 3M,N). In addition, the posterior parts of the embryo are completely lacking, and the allantois (bracket in Fig. 3O) continues to expand in the anterior part of the embryo (arrow in Fig. 3P). As cardiovascular phenotypes are one of the hallmarks of Notch signaling and neurite extension, we established Neuro2A cells stably transfected with shRNA-30 – which efficiently knocks down Rumi and decreases the Poglut activity in mouse cell lines (Fig. 5A,B). Further, we found a 79.6% decrease in Hes1 transcript levels in Neuro2A-30 cells compared with the Neuro2A-NT cells (Fig. 5A). Quantification of results from several independent differentiation assays indicates that ~45% of Rumi-knockdown Neuro2A cells harbor extensions longer than one cell diameter after 16-20 hours of differentiation (Fig. 5F,K). Only ~13% of control cells had such extensions (Fig. 5E,K). These results strongly suggest that Rumi regulates Notch signaling and neurite extension in Neuro2A cells.

To ensure that the effects observed in Neuro2A-30 cells are not due to off-target effects, we asked whether human Rumi (hRumi) – which is not affected by shRNA-30 (not shown) – can rescue the neurite phenotypes caused by mouse Rumi (mRumi) knockdown. We find that hRumi-FLAG, but not the empty vector, can rescue the increased neurite extension phenotype caused by shRNA-30 (Fig. 5G,H,K). To address whether Rumi plays a non-enzymatic role in mammalian cells, we overexpressed hRumi-G169E-FLAG,
which is enzymatically inactive (Fig. 5J), in Neuro2A-30 cells. hRumi-G169E-FLAG is not able to rescue the neurite outgrowth phenotype in Neuro2A-30 cells (Fig. 5I,K), even though hRumi-FLAG and hRumi-G169E-FLAG are expressed at comparable levels upon transient transfection (see Fig. S3 in the supplementary material). Altogether, these observations indicate that a decrease in the Poglut activity caused by mRumi knockdown significantly promotes neurite extension in Neuro2A cells, most probably owing to a decrease in Notch signaling.

We also assessed the effects of Rumi knockdown on Notch O-glycosylation and signaling in the C2C12 mouse myoblast cells. Stable transfection of C2C12 cells with shRNA-30 results in a 90% decrease in Poglut activity compared with control cells (Fig. 6A),
Fig. 5. Rumi knockdown promotes neurite extension in the Neuro2A neuroblastoma cell line. (A) qRT-PCR assays show a ~67% decrease in Rumi transcript levels (P<0.0006) and an 89.6% decrease in Hes1 levels (P<0.0016) in Neuro2A-30 cells compared with that in the Neuro2A-NT cells. (B) Poglut assays show a ~65% decrease in the enzymatic activity of Neuro2A-30 cells compared with Neuro2A-NT cells. Data in A,B are mean±s.e.m. (C,D) Differentiation assays at 4 hours on Neuro2A-NT and Neuro2A-30 cells. (E-I) Differentiation assays at 16-20 hours on Neuro2A-NT (E), Neuro2A-30 (F) and Neuro2A-30 cells transiently transfected with pTracer empty vector (G), pTracer-hRumi-FLAG (H) and pTracer-hRumi-G169E-FLAG (I). (J) Poglut assays indicate that hRumi-G169E-FLAG is enzymatically inactive. (K) Percentage of cells bearing neurites longer than one cell diameter after 16-20 hours of differentiation. Letters on the x-axis show the data for the corresponding panels. One-way ANOVA indicates that (E) is significantly different from all others except for (H) (P<0.0001). t-test indicates that the percentage of cells with neurites in Neuro2A-30 (F) is significantly different from Neuro2A-30-hRumi (H) (P=0.0008), but not from Neuro2A-30-G169E cells (I) (P=0.49). Data in J,K are mean±s.e.m.

Despite moderate to high level expression of Kdelc1 and Kdelc2 in these cells (data not shown). To examine the effects of Rumi knockdown on O-glucosylation of Notch EGF repeats, we performed differential mass spectrometry (Acar et al., 2008) on a Myc-6xHis-tagged fragment of the Notch2 extracellular domain harboring EGF13-18 expressed in C2C12-NT and C2C12-30 cells (Fig. 6B-E). The results suggest that the mNotch2 EGF repeats contain an O-linked xylose-xylose-glucose trisaccharide (see Fig. S4 in the supplementary material), which is identical to those found on EGF repeats from mNotch1 (Moloney et al., 2000; Bakker et al., 2009; Whitworth et al., 2010). A significant decrease in the O-glucosylated form and a corresponding increase in the unglucosylated form of peptides from EGF13 is present in the C2C12-30 cells compared with C2C12-NT cells (Fig. 6B-E). The reactivity of the anti-Notch antibody confirms this observation but indicates a 20% decrease in the level of Notch1 in the control sample (Fig. 6D), strongly suggesting that different Notch EGF repeats show different levels of O-glucose occupancy at endogenous levels of Rumi.

qRT-PCR assays for the Notch pathway effectors show a 92% decrease in Hey1 mRNA and a 59% decrease in Hes1 mRNA in C2C12-30 cells (Fig. 6F). We also performed western blots on protein extracts from C2C12-30 and C2C12-NT cells with anti-Val1744 – an antibody that specifically recognizes the γ-secretase cleavage product of Notch1 (Huppert et al., 2005). We find a dramatic reduction in the level of activated Notch1 in C2C12-30 cells (Fig. 6G). These data directly link the function of Rumi to the regulation of Notch signaling in C2C12 cells, and strongly suggest that the Poglut activity of Rumi is required for the activation of Notch1 in these cells.

To begin to address the mechanism of loss of Notch signaling in C2C12-30 cells, we asked whether Rumi regulates the surface expression and ligand binding of Notch in these cells. Biotinylation assays indicate that Notch1 reaches the surface of both C2C12-30 and C2C12-NT cells (Fig. 7A). Flow cytometry with an anti-Notch antibody confirms this observation but indicates a 20% decrease in the level of Notch1 at the surface of C2C12-30 cells compared with C2C12-NT cells (Fig. 7B). However, this modest decrease in Notch1 surface levels cannot explain the severe decrease in the level of Notch target expression and activated form...
Regulation of mammalian Notch by Rumi

Fig. 7. Notch ligand binding is not decreased upon Rumi knockdown. (A) Biotinylation assays on C2C12-NT and C2C12-30 cells show that Notch1 is present at the surface of both cell lines, as evidenced by the immunodetection of the N1ICD in the Avidin-bound samples. Data are representative from three independent experiments. β-Actin was used to indicate that intracellular proteins are not present in the Avidin-bound fraction, and also serves as a loading control for the input. TM-ICD indicates migration position of the transmembrane-intracellular fragment of Notch1. Asterisk indicates a non-specific C-terminal truncation of the TM-ICD, which is still exposed extracellularly and can be labeled by biotin. (B) Flow cytometry of Notch1 cell surface expression. Surface Notch1 was detected on both C2C12-NT and C2C12-30 cells (black dots to the right of the vertical line in the forward scatter plots). A modest yet significant difference in the mean fluorescence intensities (MFI) between C2C12-NT (MFI: 20580±340) and C2C12-30 (MFI: 16081±147) was observed (t=3, P=0.0003). Notch1 heterodimer removal by EDTA treatment resulted in negative immunolabeling. (C) Flow cytometry of ligand binding. Unlike the Fc negative control (a,d), both C2C12-NT and C2C12-30 cells strongly bind Jag1 (J1) and Delta-like1 (Dll1) (dark-gray histograms in b,c,e,f). No statistically significant difference was observed in their binding ability towards Jag1 (P=0.25) or Delta-like1 (P=0.92), as determined by comparison of the mean fluorescence intensities: C2C12-NT, J1 (MFI: 15819±435); C2C12-30, J1 (MFI: 17817±1423); C2C12-NT, Dll1 (MFI: 10402±491); C2C12-30, Dll1 (MFI: 10312±660). Incubation with EDTA Ca++ (J1-EDTA/Ca++ and Dll1-EDTA/Ca++) results in leftward shift of the histograms (light-gray histograms in b,c,e,f), indicating the specificity of the binding assays. Flow cytometry profiles are representative of three independent experiments.

of Notch1 upon Rumi knockdown. Moreover, flow cytometric analysis of ligand binding indicates that Rumi knockdown in C2C12-30 cells does not decrease the ability of Notch receptors to bind Jag1 and Delta-like1 ligands (Fig. 7C). Altogether, these data strongly suggest that Rumi primarily regulates Notch signaling at a step between ligand binding and S3 cleavage.

In addition to Notch receptors, most canonical ligands in mammals have between one to four Rumi target motifs (see Fig. S5 in the supplementary material and not shown) (Jafar-Nejad et al., 2010). To examine whether O-glucosylation is required for the function of mammalian Notch ligands, we performed cross-species transgenic analyses in Drosophila, where rumi mutations result in a complete loss of Notch signaling at 30°C (Acar et al., 2008). Overexpression of rat Delta-like 1 (Dll1) – with two predicted Rumi target EGF repeats – in rumi mutant clones in third instar wing imaginal discs raised at 30°C was able to induce Notch signaling in neighboring cells, as evidenced by the strong induction of the Notch downstream target Cut (Fig. 8A-B’). We performed similar experiments to examine whether the function of Jag1 and Dll3 depends on Rumi, but were not able to reach a conclusion, because these two mammalian ligands did not induce Notch signaling in wild-type or rumi” clones in Drosophila using this assay (see Fig. S5 in the supplementary material), in agreement with a previous report on Dll3 (Geffers et al., 2007). These data suggest that O-glucosylation by Rumi is not essential for the function of the rat Dll1.

Rumi regulates Jag1-induced Notch signaling in a dosage-sensitive manner in the mouse liver

To provide further in vivo evidence for the regulation of mouse Notch signaling by Rumi, we sought to determine whether decreasing the level of Rumi affects mouse Notch signaling in a Jag1” haploinsufficient background, as these animals are especially sensitive to alterations in the gene dose of other Notch
pathway components (Xue et al., 1999; McCright et al., 2001; McCright et al., 2002; Ryan et al., 2008). We crossed the Rumi$^{+/–}$ mice to animals heterozygous for the null allele Jagged1$^{DSL}$ (Xue et al., 1999) and analyzed liver sections of P0 animals double heterozygous for the null allele Jagged1$^{DSL}$ (Xue et al., 1999), and our Rumi allele and also their control littermates for binding to the Dolichos biflorus agglutinin (DBA) – a marker for bile duct epithelial cells (Watanabe et al., 1981). P0 livers of Rumi$^{+/–}$ mice showed patent bile ducts and numerous biliary cells around the portal veins, similar to their wild-type littersmates and animals heterozygous for Jagged1$^{DSL}$ or the null allele Notch2$^{d6L3}$ (McCright et al., 2006) (Fig. 9A-D). However, all of the five P0 Jag$^{+/–}$, Rumi$^{+/–}$ double heterozygous mice analyzed in our studies displayed bile duct paucity and a severe decrease in the number of biliary cells in the portal regions (Fig. 9E,F). As positive control, we generated P0 animals double heterozygous for Jagged1$^{DSL}$ and the null allele Notch2$^{d6L3}$. These mice also showed bile duct phenotypes comparable with Jag$^{+/–}$, Notch2$^{d6L3}$ (hypomorphic) (McCright et al., 2002) and Jag$^{+/–}$, Rumi$^{+/–}$ animals (compare Fig. 9G with 9E,F). Of note, P0 animals double heterozygous for Rumi and Notch2$^{d6L3}$ have patent bile ducts in the perportal regions (Fig. 9H,1). Anti-Rumi staining of wild-type E18.5 liver sections shows that some cells in the periportal regions express high levels of Rumi, in agreement with a dose-sensitive role in bile duct morphogenesis (Fig. 9G,K). These observations indicate that Rumi regulates mammalian Notch signaling in vivo, and that Jag1-induced signaling is sensitive to the gene dosage of Rumi.

**DISCUSSION**

Multiple lines of evidence indicate that, similar to its *Drosophila* homolog, mouse Rumi regulates Notch signaling. Notch signaling negatively regulates the number, length and branching of neurites in Neuro2A cells (Franklin et al., 1999; Mishra-Gorur et al., 2002; Ishikura et al., 2005). Therefore, increased neurite outgrowth and decreased Hes1 expression upon RNAi-mediated knockdown of Rumi in Neuro2A cells indicate a role for Rumi in the modulation of Notch signaling. In C2C12 cells, Rumi knockdown results in a significant decrease in Poglut activity and impaired Notch1 signaling, as evidenced by reduced levels of activated Notch1 ICD and Hey1 and Hes1 mRNA. Last but not least, removing one copy of Rumi results in bile duct defects in Jag$^{+/–}$ haploinsufficient animals. Together, these data provide strong evidence that mammalian Notch signaling is modulated by Rumi.

EGF repeats of both Notch1 (Moloney et al., 2000; Bakker et al., 2009; Whitworth et al., 2010) and Notch2 (Fig. 6) are O-glucosylated in mammalian cell lines. Despite the co-expression of moderate levels of KdelC1 and KdelC2 with Rumi, we observe a significant decrease in Poglut activity and the level of O-glucosylated Notch2 EGF repeats in Rumi knockdown C2C12 cells and a ~50% reduction in Poglut activity in Rumi$^{–/–}$ heterozygous livers. These data strongly suggest that of the three soluble ER proteins with a CAP10 domain found in mammals, Rumi is the only one able to add O-glucose to Notch proteins. The lack of compensation by KDEL1 and KDEL2 might be because these proteins lack a WXGG motif, which is thought to mediate the binding of clostridial O-glucosyltransferases to UDP-glucose (Busch et al., 2000) and is present in fly and mammalian Rumi proteins. Therefore, both in mouse cell lines and in the developing liver, the Notch loss-of-function phenotypes observed upon decreased Rumi levels correlate with decreased Poglut activity. The significant increase in neurite outgrowth observed in Rumi knockdown Neuro2A cells can be rescued by overexpression of hRumi but not with an enzymatically inactive mutant version of hRumi. These observations, together with our flow cytometry data and the severe decrease in Notch1 ICD in C2C12-30 cells strongly suggest that addition of O-glucose to Notch pathway components regulates mammalian Notch signaling at a step between ligand binding and S3 cleavage of Notch receptors.

Even though Notch1-4 quadruple knockout phenotypes are not known, comparison of the phenotypes displayed by Rumi$^{–/–}$ embryos with those of global regulators of canonical Notch signaling (Oka et al., 1995; Donoviel et al., 1999; Shi and Stanley, 2003) suggests that in addition to the Notch pathway components, other proteins are likely to depend on the function of Rumi during mouse embryonic development. We performed extensive database searches and identified 47 mouse proteins with EGF repeats harboring a consensus O-glucosylation motif (see Table S2 in the supplementary material). We found reports on mutant phenotypes for 38 of these targets, but to our knowledge none of these mutants exhibit the combination of phenotypes observed in Rumi$^{–/–}$ embryos (see Table S2 in the supplementary material and references therein). It is therefore likely that a combined defect in several targets of Rumi including the Notch pathway components is responsible for the early lethality and the phenotypes observed in Rumi$^{–/–}$ embryos.

The dosage-sensitive interaction observed between Jag1 and Rumi strongly suggests that when the level of Jag1 is limiting, optimal Jag1-induced signaling becomes sensitive to the degree of
O-glucosylation conferred by Rumi on one or more Notch pathway components. The high-level expression of Rumi in a subset of periporal cells suggests that in this context optimal Notch signaling requires high levels of O-glucose occupancy on Notch EGF repeats. Even though Notch2 plays a dominant role in bile duct morphogenesis, analysis of the three dimensional architecture of the intrahepatic bile ducts indicates that, in addition to Notch2, Notch1 and potentially other Notch receptors contribute to bile duct formation in a redundant fashion (Sparks et al., 2010). Accordingly, our observation that newborn Rumii/–/Notch2ΔD3Δ1 animals do not show bile duct phenotypes suggests that decreased glucosylation of several Notch receptors and/or the Jag1 protein itself contributes to the bile duct abnormalities in Jag1/+/Rumi/– double heterozygous animals. Mutations in JAG1 are identified in 94% of individuals with Alagille syndrome (Warthen et al., 2006). However, it is not uncommon to find other family members of an affected child with the same point mutation in JAG1 but with much milder symptoms, strongly suggesting that genetic and/or environmental modifiers play a significant role in the clinical presentation and prognosis of this disease (Li et al., 1997; Emerick et al., 1999). Our data suggest that human RUMI (POGLUTI) might represent a genetic modifier of JAG1 phenotypes in individuals with Alagille syndrome.

Acknowledgements
We thank Nadia Rana, Yi-Dong Li, Zhengmei Mao, Shinaokaku, Eva Zsigmond, Aleksey Domozhirov, Zizhen Wu, Jim Martin, David Haviland and Nathalie Brouard for technical assistance; Pamela Stanley and members of the Jafar-Nejad and Haltiwanger labs for discussions; Rafi Kopan, Bernadette Holdener and Tom Van Lee for comments on the manuscript; and Tom Gridley, Robert Jaekel, Thomas Klein, Pamela Stanley, Rafi Kopan and the Developmental Studies Hybriodna Bank for animals and reagents. We acknowledge support from the NIH (R01 GM084135 to H.J.-N. and R01GM061126 to R.S.H.), from The March of Dimes Foundation (Basil O’Connor Starter Scholar Research Award No. S-FY07-654 and Research Grant No. 1-FY07-362 to H.J.-N.) and from the Mizutani Foundation for Glycoscience (H.T.). Deposited in PMC for release after 12 months.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material for this article is available at Dev. Cell 16, e633-e647.


Liu, F. M. and lux, S. E. (1996). Constitutively active human Notch1 binds to the transcription factor CBFI and stimulates transcription through a promoter.


