A doctoral thesis

Dual roles of *O*-glucose glycans redundant with monosaccharide *O*-fucose on Notch in Notch trafficking

Research Supervisor

Dr. Kenji Matsuno

Department of Biological Sciences, Osaka University,

1-1 Machikaneyama, Toyonaka, Osaka 560-0043, Japan

Kenjiroo Matsumoto

Department of Biological Sciences, Osaka University,

1-1 Machikaneyama, Toyonaka, Osaka 560-0043, Japan

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CHAPTER ONE: SUMMARY

Notch is a transmembrane receptor that mediates cell-cell interactions and controls various cell-fate specifications in metazoans. The extracellular domain of Notch contains multiple epidermal growth factor (EGF)-like repeats. At least five different glycans are found in distinct sites within these EGF-like repeats. The function of these individual glycans in Notch signaling has been investigated, primarily by disrupting their individual glycosyltransferases. However, we are just beginning to understand the potential functional interactions between these glycans. Monosaccharide *O*-fucose and *O*-glucose trisaccharide (*O*-glucose-xylose-xylose) are added to many of the Notch EGF-like repeats. In *Drosophila*, Shams adds a xylose specifically to the monosaccharide *O*-glucose. We found that loss of terminal dixylose of *O*-glucose-linked saccharides had little effect on Notch signaling. However, our analyses of double mutants of *shams* and other genes required for glycan modifications revealed that both the monosaccharide *O*-glucose and the terminal dixylose of *O*-glucose-linked saccharides function redundantly with the monosaccharide *O*-fucose in Notch activation and trafficking. The terminal dixylose of *O*-glucose-linked saccharides and the monosaccharide *O*-glucose were required in distinct Notch-trafficking processes: Notch transport from the apical plasma membrane to adherence junctions, and Notch export from the endoplasmic reticulum, respectively. Therefore, the monosaccharide *O*-glucose and terminal dixylose of *O*-glucose-linked saccharides have distinct activities in Notch trafficking, although the lack of these activities is compensated for by the presence of monosaccharide *O*-fucose. Given that various glycans attached to a protein motif may have redundant functions, our results suggest that these potential redundancies may lead to a serious underestimation of glycan functions.

CHAPTER TWO: INTRODUCTION

Cell-cell signaling is essential for development and homeostasis of multicellular organism

During development of multicellular organisms, a fertilized egg is transformed into the complex structure of embryo. Embryogenesis requires precisely regulated cell proliferation, cell differentiation, cell migration, and apoptosis. In general, these behaviors of cells are strictly controlled through the intrinsic and extrinsic mechanisms. The extrinsic mechanisms involve cell-cell interactions and subsequent cell signaling events inside of the cells. Besides the functions in development, cell-cell interactions also play essential roles in homeostasis. Cell-cell interactions are often mediated by ligand and receptor interactions. Ligands can be secreted and diffused intracellularly, which enables it to bind to the receptors. Alternatively, transmembrane ligands bind to and activate the receptor presented at the cell surface of neighboring cells. Notch receptor plays important roles in the latter case and regulates a broad range of cell fate specifications through the local cell-cell interaction.

The first *Notch* allele was found in *Drosophila* in Thomas Morgan laboratory in 1913 (Morgan et al., 1916; Morgan., 1917). The name of *Notch* is from its phenotype, since heterozygote of *Notch* mutant has notched wing phenotype (Mohr et al., 1919; Daxter., 1920). Poulson discovered that *Notch* mutant embryos lack endodermal and mesodermal tissues, while ectodermal tissues produce nervous system at the expense of epidermal cells (Poulson., 1937). Now this phenotype is called “neurogenic phenotype” (Lehmann et al., 1983) (Fig. 1). Neurogenic phenotype is caused by the failure of “lateral inhibition,” that is mediated by Notch signaling. Lateral inhibition prevents proneural cells that neighbor a neuroblast from choosing the neuroblast-fate during neuroblast segregation. Thus, in the absence of Notch signaling, proneural cells differentiate into neuroblast at the expense of epidermoblasts (Hartenstein and Posakony., 1989) (Fig. 1). Neuroblast cells subsequently become various types of neuronal linage cells, for example, sensory organ precursors (SOPs) (Fig. 1). In

Drosophila, genetic screens to isolate the mutants that showed neurogenic phenotype led to the identification of genes encoding core Notch signaling components (Fortini., 2009) (Fig. 2). It was found that almost all components of Notch signaling pathway are evolutionarily conserved in the metazoan (Fortini., 2009). In human, defects of the Notch signaling pathway cause various diseases, such as Alagille syndrome (Li et al., 1997; Oda et al., 1997; Artavanis-Tsakonas., 1997; Onouchi et al., 1999; Ahn et al., 2015), CADASIL (Joutel et al., 1996; Joutel et al., 1997; Mizuno., 2012; Mizuno., 2013), and various types of cancers (Taketo et al., 2011; Acar et al., 2016; Iitani et al., 2016).

Notch signaling activation

Notch protein is a type I transmembrane receptor (Kidd et al., 1989; Artavanis-Tsakonas et al., 1983; Gordon et al., 2007). The structure of Notch is well conserved from *Drosophila* to human (Artavanis-Tsakonas et al., 1983; Takeuchi et al., 2014). The ligand of Notch are Delta and Serrate, which are also type I transmembrane proteins (Jarriault et al., 1998; Fortini., 2009; Luca et al., 2015). Direct physical interaction between the extracellular domains of Notch and Delta or Serra is essential for the activation of Notch signaling (Luca et al., 2015) (Fig. 2). The Notch-ligand binding induce structure change of Notch and cleavage of Notch intracellular domain by Kuzbanian/ADAM10 (S2 cleavage) and γ-secretase (S3 cleavage) (Wong et al., 1997; Ye et al., 1999; Struhl et al., 1999; De et al., 1999; Qi et al., 1999; Mumm et al., 2000; Lieber et al., 2002), and the intracellular domain of Notch are liberated to the cytoplasm (Struhl et al., 1999; De et al., 1999; Qi et al., 1999) (Fig. 2). Finally, the liberated Notch intracellular domain is translocated to the nucleus (Schrieter et al., 1998; Ye et al., 1999; Struhl et al., 1999; De et al., 1999; Qi et al., 1999) and make a complex with co-activator of transcription to activate the expression of the downstream targets genes of its (Bray., 1998; Bray., 2006; Bray

and Bernar., 2010) (Fig. 2).

Glycan modification of Notch

Many proteins have glycan chain modifications, and these glycan modifications are essential for many aspects of protein functions (Fig. 3) (Varki et al., 1993; Haltuwanger and Lowe., 2004; Moremen et al., 2012; Pinho et al., 2013; Xu and Esko., 2014; Hart., 2014; ;Taniguchi and Kizuka., 2015). Glycan modifications of proteins are occurred in the lumen of endoplasmic reticulum (ER) or Golgi, except for *O*-GlcNac modification (Kollmann et al., 2009; Hoseki et al., 2010; Hart et al., 2011; Xu and Ng., 2015) (Fig. 3). Thus, almost all transmembrane proteins and secreted proteins are glycosylated (Fig. 4) (Dennis et al., 2009; Moremen et al., 2012). It is shown that glycan modifications of transmembrane receptors, including Notch, play important roles in the maturation and transportation of these proteins, and physical interactions with their ligands (Fig. 3) (Fig. 4) (Moloney et al., 2000; Yamamoto et al., 2012; Moremen et al., 2012; Pinho et al., 2013; Vasudevan and Haltiwanger., 2014; Luca et al., 2015; Haltom et al., 2015).

The Notch-receptor family typically contains 36 epidermal growth factor (EGF)-like repeats, which bind to transmembrane ligands belonging to Delta or Serrate family (Chillakuri et al., 2012) (Fig. 5). An EGF-like repeat has a characteristic structure and contains one Ca^{2+} and six cysteine residues that form three disulfide bonds in an EGF-like structure (Fig. 5). At least five different glycan modifications have been found on the Notch EGF-like repeats: *N*-linked glycan modifications (Goto et al., 2001) and four *O*-linked glycan modifications: *O*-fucose glycan (Okajima and Irvine.,2002; Sasamura et al., 2003; Moloney et al., 2000; LeBon et al., 2014), *O*-glucose glycan (Acar et al., 2008; Lee et al., 2013), *O*-GlcNAc (Okajima et al., 2008; Matsura et al., 2008; Sakaidani et al., 2010; Sakaidani et al.,

2011), and *O*-GalNAc (Boskovski et al., 2013) (Fig. 5). A potential *O*-xylosylation of the EGF-like repeats has also been observed *in vitro* (Takeuch et al., 2011) (Fig. 5). The individual modifications have been analyzed to determine their specific functions and form a model of how glycosylation regulates receptor functions (Okajima et al.,2002; Sasamura et al., 2003; Moloney et al., 2000; Acar et al., 2008; Lee et al., 2013; Sakaidani et al., 2011; Boskovski et al., 2013; Takeuch et al., 2011). However, the functional interactions between these various glycans are not well understood.

The functions of *O*-fut1 and *O*-fucosylation

Serine/Threonine residue of Cys-X-X-X-X-(Ser/Thr)-Cys sequence on Notch EGF-like repeats has *O*-fucose glycan modification (Wang et al., 2001; Panin et al., 2001; Haltiwanger and Stanley., 2002.). The *O*-fucose glycan modification is composed of disaccharide, "EGF-fucose-GlcNAc" in *Drosophila*. First *O*-fucose is attached by *O*-fucosyltransferase 1 (*O*-fut1), and Fringe that is a β1,3 *N*-acetylglucosaminyltransferase adds GlcNAc to this fucose (Moloney et al., 2000; Okajima et al., 2002). *O-fut1* gene is ubiquitously expressed, and *O-fut1* gene is essential for Notch signaling activation because its null mutant phenotypes are similar to those caused by *Notch*-null mutant (Sasamura et al., 2003). In contrast, *fringe* is expressed region-specifically, and *fringe* mutants reduce Notch signaling in only a subset of contexts that involve Notch signaling (Singh et al., 2000; Moloney et al., 2000; LeBon et al., 2014). The GlcNAc modification of monosaccaride *O*-fucose led to the changes in the affinity between Notch and its ligands (Moloney et al, 2000; Okajima et al., 2003; LeBon et al., 2014; Taylor et al., 2014; Luca et al., 2015) (Fig. 6). Notch with the monomeric *O*-fucose modified binds to Delta- and Serrate-type ligands equally well (Fig. 6A) (Okajima et al., 2003; Sasamura et al., 2004). However, when Notch has the *O*-fucose-GlcNAc disaccharide modified, Notch selectively

binds to Delta-type ligand but not Serrate-type ligand (Fig. 6B) (Moloney et al., 2000; Okajima et al., 2002; Okajima et al., 2003; LeBon et al., 2014). This regulation is conserved from *Drosophila* to mammals, although some exceptions were reported in mammals (Shimizu et al., 2000; Wang et al., 2001; Martinez-Duncker et al., 2003; Shi et al., 2005; Shi et al., 2006). Combination with *cis*-inhibition of Notch activity by Serrate-type ligands, the activation of Notch signaling restrictedly occurs along the boundary between *fringe*-expressing and *fringe*-non-expressing regions (Fig. 6) (Barrantes., 1999; Hicks., 2000; Cole et al., 2000; Moloney et al., 2000; Yaron et al., 2012; Fleming., 2013; LeBon et al., 2014). Therefore, the monosaccharide *O*-fucose modification is required for an acceptor for the GlcNAc modification by Fng in a context-dependent manner (Moloney et al., 2000).

Besides the *O*-fucosyltransferase activity, *O*-Fut1 has two *O*-fucosyltransferase activity-independent functions. The first is *O*-Fut1 has a Notch-specific chaperon function (Okajima et al., 2005; Okajima et al., 2008). It was shown that Notch is accumulated in the ER, and the secretion of the extracellular domain of Notch is reduced in *O-fut1* knockdown cells (Okajima et al., 2005). However, mouse Notch1 is presented at the cell-surface in *POFUT1* (mammalian *O-fut1* ortholog) null-mutant cells, although it is accumulated in the ER (Okumura and Saga., 2008). Thus, *O*-Fut1 chaperon activity requirement may be different among species. Second is an enzymatic activity independent function of O-Fut1. The *O*-Fut1 enzymatic activity independent function is required for normal endocytosis of Notch. Notch protein can be detected by an antibody which against the extracellular domain of Notch EGF-like repeats at the cell membrane (Klueg and Muskavitch., 1999). The cell membrane Notch was not significantly reduced in *O-fut1* null-mutant cells *in vivo* (Sasamura et al., 2007). Notch failed to be transported from cell membrane to endosome in *O-fut1* null-mutant cells. However, an addition of the *O*-Fut1 protein into cell-culture medium promotes the endocytosis of Notch in

O-fut1 knockdown cells (Sasamura et al., 2007). Interestingly, Notch1 endocytic pathways are changed from clathrin-dependent pathway to caveolin-dependent pathway in *POFUT1* null-mutant cells in mouse, and Notch is accumulated in caveolin-positive vesicles in *POFUT1* null-mutant cells (Hasegawa et al., 2012). Therefore, it is possible that Notch endocytosis activity of *O*-Fut1 may be conserved from mammalian to *Drosophila*, although it is still unknown whether the POFUT1 function in endocytosis depends on POFUT1 *O*-fucosyltransferase activity or not in mammalian. However, although analyses of *O-fut1* mutants in *Drosophila* and mouse revealed that *O-fut1* is essential for Notch signaling in these species, it was difficult to determine whether monosaccharide *O*-fucose is essential for Notch signaling, because *O*-fut1 has its enzymatic activity-independent roles, which may fully account for the essential role of *O-fut1* gene in Notch signaling. To address this issue, an *O-fut1* mutant that results in amino acid-substitution in its GDP-fucose binding site was generated, which is called $Ofut1^{R245A}$ (Okajima et al., 2005). The 245th arginine of *O*-Fut1 is essential for binding to GDP-fucose, which is the fucose donor of *O*-fucose modification (Okajima et al., 2005). It was shown that $Ofut1^{R245A}$ encodes a mutant protein that largely lacks *O*-fucosyltransferase activity *in vitro* (Okajima et al., 2005). However, $Ofut1^{R245A}$ promote the export of Notch extracellular domain as efficient as a wild-type *O*-fut1, suggesting $Ofut1^{R245A}$ maintains its Notch-specific chaperone activity (Okajima et al., 2005). It was found that $Ofut1^{R245A}$ knock in mutant shows neurogenic phenotype, reminiscent of Notch mutant phenotype, and accumulate Notch in the ER at 30 ºC but not at 25 ºC (Ishio et al., 2015). Thus, monosaccharide *O*-fucose is essential for general Notch signaling activation at 30 ºC but not at 25 ºC (Ishio et al., 2015).

The function of the monosaccharide *O*-glucose and terminal dixylose of *O*-glucose-linked saccharides

Serine residue of Cys-X-Ser-X-(Pro/ Ala)-Cys sequence on EGF-like repeats has *O*-glucose glycan modification (Acar et al., 2008). The structure of the *O*-glucose glycan is a trisaccharide, "EGF-glucose-xylose-xylose" (Acar et al., 2008; Lee et al., 2013). First, *O*-glucose is attached by Rumi, and Shams adds xylose to this *O*-glucose. *rumi*-mutant shows phenotype associate with missing of Notch signaling at 28 ºC, but not at 18ºC (Acar et al., 2008). Thus, the monosaccharide *O*-glucose is probably essential for Notch signaling at 28 ºC. Notch is accumulated in the ER and apical cell membrane at 28 ºC in *rumi*-mutant (Acar et al., 2008). However, binding affinity between Notch and Delta is normal at 28 ºC in *rumi* mutant cells (Leonaldi et al., 2012). Thus, it was suggested that the monosaccharide *O*-glucose is essential for the S2 cleavage of Notch at 28 ºC (Acar et al., 2008). Shams adds xylose to the monosaccharide *O*-glucose (Lee et al., 2013). As a result, the *O*-glucose trisaccharide becomes the trisaccaride of "EGF-glucose-xylose-xylose". In *shams*-mutant cells, accumulation of Notch on apical cell membrane and hyper activation of Notch signaling are observed (Lee et al., 2013). Conversely, ectopic expression of *shams* in wing disc cells led to reducing of Notch signaling activation (Lee et al., 2013). These results suggest that the terminal dixylose of *O*-glucose-linked saccharides of Notch negatively regulates Notch signaling activation (Lee et al., 2013).

As found in *O*-fut1, glycosyltransferase could have an enzymatic activity-independent function (Okajima et al., 2005). Thus, Shams may also have enzymatic activity-independent function. Thus, to analyze the function of terminal dixylose of *O*-glucose-linked saccharides of Notch, the terminal dixylose of the *O*-glucose glycan should be removed by disrupting other gene required for xylosylation of *O*-glucose. Therefore, in this study, I focused on a synthetase of UDP-xylose. UDP-xylose is xylose donor of the xylosylation of *O*-glucose-linked saccharides of Notch. UDP-xylose is synthesized from UDP-GlcNAc,

which is catalyzed by UDP-xylose synthetase (Uxs). In chapter four, analyses of a *Uxs* mutant are presented.

Redundant function of the monosaccharide *O*-fucose and *O*-glycan glycan on Notch

The monosaccharide *O*-fucose and *O*-glycan glycan are simultaneously found in many of EGF-like repeats of Notch (Fig. 5). In addition, *O-fut1* and *rumi* mutants show similar temperature-sensitive neurogenic phenotype (Acar et al., 2008; Ishio et al., 2015). Therefore, it was thought that monosaccharide *O*-fucose and *O*-glycan glycan may have a redundant role in Notch signaling. Single mutants of *Ofut1*^{R245A}or *rumi* did not show neurogenic phenotype at 25 ºC. However, in double mutant of *Ofut1*^{R245A}and *rumi* showed neurogenic phenotype even at 25 ºC. Therefore, the monosaccharide *O*-fucose and *O*-glucose glycan on Notch have redundant role in Notch signaling activation (Ishio et al., 2015).

The *O*-glycan glycan on Notch is the trisaccharide. Although the monosaccharide *O*-fucose and *O*-glucose trisaccharide have redundant role in Notch signaling, it is still unknown which part of *O*-glycan glycan functions redundantly with the monosaccharide *O*-fucose is unknown. Therefore, in this study, I determined the moieties of *O*-glycan glycan displaying the redundant roles with the monosaccharide *O*-fucose in Notch signaling activation and the trafficking of Notch. These studies are described in chapter five.

CHAPTER THREE:
EXPERIMENTAL PROCEDURES

Drosophila strains

We used Canton-S as the wild-type strain. The following mutant alleles were used: *O-fut1*^{4R6} ; the null allele of *O-fut1*(Sasamura et al., 2006) , *O-fut1*^{R245A} (Ishio et al., 2015); the previously described *sham*-null allele *shams*^{34} (Lee et al., 2013); the *Uxs*-null allele *Uxs*^{1} (this study); the *UDP-xylose synthase* (*Uxs*)-deletion mutant *Df(2R)BSC783* (Bloomington 27355); and the *rumi*-null allele *rumi*^{44} (Acara et al., 2008).

Drosophila genetics

The following stocks were established and used for genetic crosses in this study

1. *w; FRTG13 O-fut1*^{R245A} */CyO Actin-GFP*
2. *w; FRTG13 O-fut1*^{4R6} */CyO Actin-GFP*
3. *y w; FRT82B shams*^{34} */TM6B Tb*
4. *y w;FRT82B rumi*^{44}*/TM6B Tb*
5. *y w;FRT2A Uxs*^{1}*/TM6B Tb*
6. *Efr 19A/FM'Actin-GFP x XX/Y*
7. *Gfr/ TM6B Tb*
8. *Efr 19A/FM'Actin-GFP; Gfr/ TM6B Tb x XX/Y; Gfr/ TM6B Tb*
9. *w; FRTG13 O-fut1*^{R245A} */CyO, Actin-GFP; FRT82B shams*^{34} */TM6B Tb*
10. *w; FRTG13 O-fut1*^{R245A} */CyO, Actin-GFP; FRT82B rumi*^{44} */TM6B Tb*
11. *w; FRTG13 O-fut1*^{R245A} */CyO, Actin-GFP; FRT82B Uxs*^{1}*/TM6B Tb*
12. *w; FRTG13 O-fut1*^{R245A} */CyO, Actin-GFP; FRT82B Uxs*^{1}*/TM6B Tb*
13. *w; FRTG13 Ubi-GFP /CyO, Actin-GFP; FRT82B shams*^{34} */TM6B Tb*
14. *w; FRTG13 Ubi-GFP /CyO, Actin-GFP; FRT82B rumi*^{44} */TM6B Tb*
15. *y w Ubx-FLP;FRTG13 Ubi-GFP*

16. *y w Ubx-FLP; FRT2A Ubi-GFP*

17. *y w Ubx-FLP; FRT82B Ubi-GFP*

18. *y w Ubx-FLP; FRTG13 Ubi-GFP; FRT82B rumi^{44} /TM6B Tb*

19. *y w Ubx-FLP; FRTG13 Ubi-GFP; FRT82B shams^{34} /TM6B Tb*

20. *, y w Ubx-FLP FRT19A*

21. *Sco/CyO Actin-GFP; MKRS/TM6B Tb*

22. *Sco/Cyo Actin-GFP*

23. *P{EPgy2}CG7979EY00136*

24. *Dr/TMS Delta2-3*

25. *y w UAS-dicer2; Apterous-Gal4/ CyO Action-GFP*

26. *w; TM3 Sb/ TM6 Tb*

27. *w; patched(ptc)-Gal4, UAS-GFP/ CyO Action-GFP*

28. *w; apterous(ap)-Gal4, UAS-GFP/ CyO Action-GFP*

29. *w; hedgehog(hh)-Gal4, UAS-GFP/ CyO Action-GFP*

30. *w; scabrous(sca)-Gal4, UAS-GFP/ CyO Action-GFP*

31. *w ;GMR-Gal4, UAS-GFP/ CyO Action-GFP*

32. *w; UAS-HA-Uxs/ TM6B Tb*

33. *w; UAS-inxxin2-inverted repeat(IR) /TM6B Tb*

34. *w; UAS-Uxs-IR /CyO Actin-GFP*

35. *w; UAS-Notch-IR /CyO Actin-GFP*

36. *w; UAS-Uxs-IR /CyO Actin-GFP; UAS-inxxin2-IR/TM6B Tb*

37. *w; UAS-O-xylosyltransferase(oxt)-IR /CyO Actin-GFP*

I used FLP-FRT system to generate somatic mosaic clone (Fig. 7) (Lacroix et al., 2011). FLP recombinase mediates site-specific recombination between FRT (FLP

recombinase target) sites (Lacroix et al., 2011). FLP-mediated recombination between FRT sites in each homologous chromosome generates mitotic clones homozygous for a mutant in cells heterozygous for it (Lacroix et al., 2011). Somatic clones of *O-fut1*^{R245A}, *rumi*^{44}, and *shams*^{34} were generated by FLP-FRT recombination in the wing discs of third-instar larvae of

1. *y w Ubx-FLP; FRTG13 O-fut1*^{R245A}*/FRTG13 Ubi-GFP*
2. *y w Ubx-FLP; shams*^{34} *FRT82B/Ubi-GFP FRT82B*
3. *Ubx-FLP; Ubi-GFP; FRT82B/ rumi*^{44}*, FRT82B,*
4. *y w Ubx-FLP; FRTG13 O-fut1*^{R245A}*/FRTG13 Ubi-GFP; FRT82B shams*^{34}*,*
5. *y w Ubx-FLP; FRTG13 O-fut1*^{R245A}*/FRTG13 Ubi-GFP; FRT80B rumi*^{44}

I used GAL4- upstream activating sequence (UAS) system to overexpress genes listed below in this study (Fig. 8). I used Gal4-UAS system in the wing discs of third-instar larvae of

1. *w UAS-dicer2; ap-Gal4/UAS-Uxs IR*
2. *w UAS-dicer2; ap-Gal4/UAS-Uxs IR; UAS-inx2 IR/+*
3. *w UAS-dicer2; ap-Gal4/+; UAS-inx2 IR/+*
4. *w; ptc-Gal4/ UAS-Uxs1-HA/ UAS-GFP; Uxs*^{1}
5. *w; ap-Gal4/UAS-Notch IR*
6. *w; ap-Gal4/UAS-Uxs IR*
7. *w; ap-Gal4/+; UAS-oxt IR*
8. *w; sca-Gal4/+; UAS-oxt IR*
9. *w; GMR-Gal4/+; UAS-oxt IR*
10. *w; hh-Gal4/+; UAS-oxt IR*

Generation of *Uxs*^{1}

To generate a *Uxs*-deletion mutant, I imprecisely excised a P-element inserted in the *Uxs* locus of *P{EPgy2}CG7979EY00136* (Bloomington stock number 15001) using a standard procedure (Rincon-Limas et al., 1999). I mobilized the P-element by crossing *P{EPgy2}CG7979EY00136* with *w*; Dr^1/TMS Delta2-3* (Bloomington stock number 1016). I took *P{EPgy2}CG7979EY00136/TMS Delta2-3* flies and crossed it with *w; TM3 Sb/ TM6 Tb.* I established about 2000 potential deletion-mutant lines. Genomic DNA was purified from these potential deletion-mutant lines, and deletions were detected by PCR using the forward primer 5'-GAGCTGTAACCTGCAAGAAGTC-3' and the reverse primer 5'-CACATTTCTGGATCTCAGCTAG-3'.

Construction of UAS-*Uxs-HA*

A double-stranded oligo nucleotides encoding *Hemagglutinin* (*HA*)-tag were added to the 3'-end of *Uxs* cDNA (Drosophila Genomics Resource Center stock number LD39939) by PCR using a forward primer (5'-GAATTCGCCACCATGACTGCCACCAAAAAG-3') and a reverse primer (5'-CTAAGCGTAATCTGGAACATCGTATGGGTACGGTGTATGTGTATCAAAGTACTT-3'). After confirmation of DNA sequence, the resulting PCR fragment was subcloned into the NotI and EcoRI sites of pUASTattP vector which have UAS promoter (Bischof et al., 2007).

Injection of vector

Transgenic fly lines were established by a standard procedure (Bischof et al., 2007). First, I injected UAS-*Uxs-HA* Plasmid DNA (250ng/μl) dissolved in injection buffer (0.1mM NaHPo4 (pH6.8), 5mM KCl) into stage1-4 embryos of y^1 w^{1118}; *PBac{y^+-attP-9A}VK00022* by glass needle (eppendorf). These embryos were incubated in moisture chamber. Hatched larvae were

collected and cultured at 25° C. Adult files obtained were crossed with *w^{1118}; TM3, Sb*/ TM6, Tb**.

Immunostaining

Drosophila wing discs were immunostained as described previously (Ishikawa et al., 2010), except for the 3G10 and 2H antibody staining. Briefly, wing discs were dissected from third instar larva in PBS and fixed in PLP fixing solution (2% paraformaldehype, 0.01M $NaIO_4$, 0.075M lysine, 0.037M sodium phosphatase pH 7.2) for 40 minutes at room temperature. Wing discs were washed three times in PBS-DT (0.3% sodium deoxycholate, 0.3% Triton X-100 in PBS) and incubated in PBS-DT with primary antibody at 4˚C overnight. Wing discs were washed three times in PBS-DT and incubated in PBS-DT with each secondary antibody at room temperature for two hours. Wing discs were washed three times in PBS-DT and observed it by LSM700. For 3G10 antibody staining, fixed wing discs were incubated with 400 mU of heparinase III (NEB) for 4 hours at 37˚C (Takei et al., 2004; Ishikawa et al., 2010). For staining with 2H (C458.2H) antibody, recognizing the Notch extracellular domain, fixed wing discs were washed in PBS (without detergent) and incubated with the 2H antibody for 2 hours at room temperature. Those wing discs are washed by PBS and fixed again. Wing discs were washed in PBS-0.5% Tween 20 (0.5% Tween 20 in PBS) and stained with an anti-GFP antibody. Finally, wing discs were washed in PBS-0.5% Tween 20 and treated with a secondary antibody. The following antibodies were used at the indicated concentrations: mouse anti-NECD monoclonal antibody, (1:100; C458.2H (2H); the Developmental Studies Hybridoma Bank (DSHB)) (Ishikawa et al., 2010), mouse anti-NICD monoclonal antibody (1:1000; C17.9C6; DSBC) (Ishio et al., 2015), rat anti-NICD polyclonal antibody (1:1000; gifted by Dr. Spyros Artavanis-Tsakonas) (Ishio et al., 2015), mouse anti-Wg monoclonal

antibody (1:20; 4D4; DSBC) (Ishio et al., 2015) , rat anti-Cadherin monoclonal antibody (1:20, 7E8A10; DSBC) (Ishio et al., 2015), guinea pig anti-Senseless polyclonal antibody (1:1000; gifted by Dr. Hugo Bellen) (Ishio et al., 2015), rabbit anti-GFP polyclonal antibody (1:5000, MBL), and Alexa 488-, Cy3-, and Cy5-conjugated affinity-purified donkey secondary antibodies (Ishio et al., 2015) (1:500; Jackson). Epitope of the 2H antibody is EGF repeats 12-20 in the extracellular domain of Notch, and epitope of C17.9C6 is the intracellular domain of Notch.

***In situ* hybridization**

A *wg* cDNA, described before, was used as a template to synthesize RNA probes (Kuroda et al., 2013; Shimizu et al., 2014; Aoyama et al., 2014). T7 and SP6 promoters were added to 5'- and 3'-end of the *wg* cDNA by PCR using a forward primer (5'-AAATAATACGACTCACTATAGGGATGCGTGGAAAACTTACAAG -3') and a reverse primer (5'-AAACTATAGTGTGTCACCTAAATCGCATTCGATTTTTCTGC-3'). Digoxiginin-labeled *wg* RNA probe were synthesized from this template using DIG RNA labeling mix (Roch). SP6 and T7 RNA polymerases were used for making anti-sense and sense probes, respectively.

BLAST analysis

The potential sites of *O*-fucosylation and *O*-glucosylation were searched in proteins encoded in *Drosophila* genome. First, protein coding sequences encoding EGF-like repeats homologous to Notch EGF-like repeats (http://flybase.org/cgi-bin/getseq.html?source=dmel&id=FBgn0004647&chr=X&dump=PrecompiledFasta&targetset=translation) were searched by using NCBI Protein Basic Local Alignment Search Tool

(BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and RefSeq pratein datebase. Crumbs, Cubin, Delta, Serrate, Uninflatable, Dumpy, Eye shut, Weary, Slit, faulty attraction, Kugelei, Draper, LDL receptor protein 1, ankyrin 2, Fat , C901, CG31999, Tenascin accessory, CG3104, Shifted, Tankyrase, CG8526, Mind bomb 1, and Relishthe proteins were found. Second, the consensus sequence of *O*-fucosylation and *O*-glucosylation were searched in these proteins. by BLAST analyses.

CHAPTER FOUR: The function of terminal dixylose of *O*-glucose-linked saccharides of Notch

The terminal dixylose of *O*-glucose-linked saccharides negatively regulates Notch signaling in a tissue-specific manner (Lee et al.,2013), as shown by loss- and gain-of-function analyses of the *shams* gene, which encodes *O*-glucose-specific xylosyltransferase (Lee et al.,2013). However, this negative regulation may not be due to its xylosyltransferase activity, since the *O*-fucosyltransferase *O*-fut1 plays an essential role in Notch signaling that is independent of its enzymatic activity (Okajima et al., 2005, Sasaki et al., 2007, Sasamura et al., 2007). Thus, before addressing the functions of the terminal dixylose of *O*-glucose-linked saccharides, it is important to confirm that phenotypes associated with *shams* mutations are indeed due to the absence of the terminal dixylose of *O*-glucose-linked saccharides. Therefore, I attempted to abolish Notch's xylosylation by mutating a different gene in *Drosophila*.

CG7979 encodes a *Drosophila* UDP-xylose synthase

UDP-xylose, which is essential for xylosylation as the xylose donor, is synthesized from UDP-glucuronic acid (Eames et al., 2010) (Fig. 10). In mammals, UDP-xylose synthesis fully depends on *UDP-xylose synthase 1*, which encodes their only UDP-xylose synthase (Bakker et al., 2009). The *Drosophila* ortholog of *UDP-xylose synthase 1* is *UDP-xylose synthase* (*Uxs*), the only gene in the *Drosophila* genome that encodes UDP-xylose synthase (Gotting et al., 2000). The *Drosophila Uxs* gene, previously registered as *CG7979*, is designated here as *Uxs* (Gotting et al., 2000). To generate potential *Uxs*-null mutants, I used imprecise P-element excision. Excising the P-element from the {EPgy2}CG7979^{EY00136} line produced the *Uxs*-deletion mutant *Uxs*^{1} (Fig. 11). The *Uxs*^{1} mutant has a 1201-base pair deletion of the *Uxs* genomic DNA locus, which removes its putative initiation codon and more than half of its coding region, corresponding to the N-terminal half of the Uxs protein (including a nucleotide-binding site) (Gotting et al., 2000) (Fig. 11). The molecular changes

in the *Uxs*^{1} mutant suggest that it is a null-mutant allele of *Uxs*.

To analyze the defects associated with this *Uxs* mutation, we observed phenotypes of *Uxs*^{1} mutant. To avoid observing phenotypes associated with potential background mutations, we analyzed trans-heterozygotes of *Uxs*^{1} and *Deficiency* (*Df*) *f(3L)Exel6112*, a previously reported deletion mutant uncovering the *Uxs* locus. These phenotypes were essentially the same as those of the *Uxs*^{1} homozygote, further indicating that *Uxs*^{1} is a null allele. I also compared these phenotypes with *shams* mutants, in which the *O*-glucose attached to the Notch EGF-like repeats is not xylosylated (Lee et al., 2013). I found that *Uxs*^{1} was a recessive lethal mutant, although pharate adults were occasionally obtained (Fig. 12B, F, and J). In contrast, homozygous *sham*-null mutants are viable, as previously described (Fig. 12C, G, and K), indicating that the *Uxs*^{1}-mutant defects are more severe than those in *shams* mutants (Lee et al., 2013). I found that all of the *Uxs*^{1}-mutant pharate adults examined (n=14) were missing bristles on the dorsal head capsule (Fig. 12B); this phenotype was also found in the *shams* mutants (n=22) (Fig. 12C), as previously observed (Lee et al., 2012). This overlap in phenotypes between *Uxs*^{1} and *shams* mutants appears consistent with our hypothesis that both genes are required for the xylosylation of *O*-glucose on Notch EGF-like repeats. In addition, the *Uxs*^{1} mutants (n=14) had a rough-eye phenotype and were missing scutellum bristles (Fig. 12F and J); these defects were not present in the *shams* mutants (n=22) (Fig. 12G and K). I speculated that these phenotypes might result from defects in glycosaminoglycan (GAG) synthesis, which requires UDP-xylose (Ishikwa et al., 2010). GAG synthesis begins with the *O*-xylosylation of core proteins by *O*-xylosyltransferase (Couso et al., 1994). Thus, GAG synthesis is probably abolished in the *Uxs*^{1} mutant due to the absence of UDP-xylose. I detected GAG by a specific antibody against the 3G10 epitope, which is exposed after GAG is digested by heparitinase (The and Perimon, 2000; Takei et al., 2004). GAG staining was

largely abolished in all of the wing discs in trans-heterozygotes of *Uxs*^{1} and *Df(3L)Exel6112* (n=23), (Fig. 13C-D) compared to wild-type wing discs (n=27) (Fig. 13A-B). Mutants of other genes required for GAG synthesis, such as *tout-velu* and *sulfateless*, are also recessive lethal at the same stage to *Uxs*^{1}, which is consistent with our idea that GAG synthesis is disrupted in *Uxs*^{1} mutant (Takei et al., 2004).

Oxt is another galactosyltransferase required for GAG synthesis (Ueyama et al., 2008). Although *oxt* mutant has not been reported, knock-down of *oxt* by RNA interference (RNAi) was reported. As reported before, knock-down of *oxt* resulted in rough eye phenotype (n=26) (Fig. 12L) (Ueyama et al., 2008). Here, we found that knock-down of *oxt* gave loss of bristle phenotype in the scutellum (n=21) (Fig. 12H). However, we did not observed the missing bristles on the dorsal head capsule by knock-down of *oxt* (n=28) (Fig. 12D). Thus, with the limitation of RNA interference analysis, these results suggest that bristle loss in scutellum and rough eye may be due to the disruption of GAG synthesis as found in *Uxs*-mutant or *oxt* knock-down flies, whereas loss of terminal dixylose of *O*-glucose-linked saccharides may be responsible for bristles missing on the dorsal head capsule as found in *Uxs*- or *shams*-mutant flies.

To confirm that the mutation of *Uxs* gene is responsible for the absence of GAG, I overexpressed *UAS-Uxs-HA* in the wing discs of *Uxs*^{1} homozygote. Their overexpression even in the limited part of a wing disc, which was driven by *patched* (*ptc*)-*Gal4*, was sufficient to restore the GAG staining in a whole wing disc of *Uxs*^{1} mutant, suggesting a cell-autonomous function of *Uxs* (n = 16) (Fig. 14A-A''). However, the Uxs proteins is restrictedly detected in the region where the overexpression of *UAS-Uxs-HA* was driven, indicating that Uxs protein does not diffuse intercellularly (n = 16) (Fig. 14B-B''). Previous study showed that GDP-fucose is delivered intercellularly among the epithelial cells through

the gap junction (Ayukawa et al., 2012). Therefore, I speculated that UDP-xylose is also supplied intercelluialry through the gap junction. In wild type wing discs, local knock down of either *Uxs* or *innexin2* (*inx2*), encoding a component of gap junction, by RNA interference did not affect the GAG staining (*Uxs* KD: n = 18) (*inx2* KD: n = 18) (Fig. 15A-B and C-D). However, simultaneous knock-down of *Uxs* and *inx2* resulted in the marked reduction of the GAG staining within the region where these two genes are knocked down (n = 19) (Fig. 15E-F). Therefore, UDP-xylose is transported intercelluialry through the gap junction, suggesting that gap junction-mediated intracellular transport is probably a general property of nucleotide-sugars.

Wg signaling is decreased in the *Uxs* mutant

Previous analysis of the *shams* mutant showed that the xylosylation of *O*-glucose on the EGF-like repeats of Notch negatively regulates Notch signaling in a tissue-specific manner, although the *shams* mutant phenotype suggested that the hyper-activation of the Notch signaling was subtle (Lee et al., 2013). Thus, I used the *Uxs* mutant to re-examine potential roles of *O*-glucose xylosylation in other developmental processes controlled by Notch signaling. The expression of *wingless* (*wg*), which is activated by Notch signaling along the boundary of the dorsal and ventral compartments in the third-instar wing disc (Frise et al., 1996), was analyzed by *in situ* hybridization in a *Uxs*-mutant trans-heterozygote of Uxs^{1} and *Df(3L)Exel6112* (Fig. 16B and E). Although the *wg* expression may have been slightly reduced in the *Uxs*-mutant wing discs (Fig. 16B), the pattern of expression was not markedly different from wild type (Fig. 16A), indicating that the Notch-signaling activity was not severely affected in any of the wing discs examined (n=19) (compare Fig. 16A and B). Similarly, *wg* expression was slightly reduced in the region of wing disc where *Oxt* gene was

knocked-down by RNAi (n=36) (Fig. 16C). In addition, the Wg protein was slightly reduced and distributed more diffusely in the wing discs of all of *Uxs*-mutant (n=23) and 77% of *oxt* knock-down wing discs (n=31) (Fig. 16C and F). Similar abnormalities in Wg protein distribution were reported in mutants with defective GAG synthesis (Port et al., 2008). In addition, the array of SOPs expressing *senseless* (*sens*), which depends on Wg signaling, along the boundary of the dorsal and ventral compartments was interrupted in these wing discs (Port et al., 2008; Franch-Marro et al., 2008) (compare Fig. 17A, B, and C and see arrowheads in Fig. 17B and C). Therefore, Wg signaling is decreased in the *Uxs*-mutant and *oxt* knock-down wing disc, probably due to disrupted GAG synthesis. This reduction in Wg signaling may also account for the apparent slight reduction in *wg* expression I observed in the *Uxs* mutant and *oxt* knock-down wing discs (Port et al., 2008; Franch-Marro et al., 2008).

CHAPTER FIVE: Distinct functions of monosaccharide *O*-glucose and the terminal dixylose of *O*-glucose-linked saccharides in the absence of monosaccharide *O*-fucose

_O_-fucose and the terminal dixylose of _O_-glucose-linked saccharides on Notch EGF-like repeats play redundant roles in Notch-signaling activation

The number of SOPs in wing discs is restricted by lateral inhibition though Notch signaling (Lehmann et al., 1983). Knocking down *Notch* significantly increased the number of SOPs, which subsequently formed SA neurons (compare Fig. 19A and B and see Fig. 18). However, the number of SOPs in *Uxs* or *shams*-mutant wing discs was comparable to that in wild type at 25ºC (permissive temperature) (compare Fig. 17A, Fig. 17B, and Fig. 19C see Fig. 18). Therefore, Notch signaling is not notably altered in the absence of the terminal dixylose of *O*-glucose-linked saccharides. However, our group previously found that monosaccharide *O*-fucose and *O*-glucose trisaccharide on Notch EGF-like repeats have redundant roles in Notch trafficking and Notch-signaling activation (Ishio et al., 2015). Thus, although the Notch protein synthesized in the *O-fut1*^{R245A} mutant lacks the *O*-fucose modification, the Notch protein is folded within the scope of the *O*-fut1 chaperone-like function. The number of SOPs was not significantly affected in *O-fut1* ^{R245A} homozygote wing discs at 25ºC) (Fig. 19E and see Fig. 18), although the number increased markedly at 30ºC (non-permissive temperature) (Fig. 19F and see Fig. 18), as previously reported (Ishio et al., 2015). *rumi*–mutant also shows temperature sensitive phenotype (Acar et al., 2008). The number of SOPs was not significantly affected in *rumi* homozygote wing discs at 25ºC (Fig. 19G and see Fig. 18), although the number increased markedly at 30ºC, as previously reported (Acar et al., 2008; Ishio et al., 2015). However the number increased markedly in *O-fut1* ^{R245A} and *rumi* double homozygote wing discs at 25ºC (Fig. 20D) (Ishio et al., 2015). Thus, although the loss of only the terminal dixylose of *O*-glucose-linked saccharides did not significantly affect Notch signaling, it was possible that the concurrent loss of monosaccharide *O*-fucose and the terminal dixylose of *O*-glucose-linked saccharides could cause defects in Notch signaling. To address this possibility, we examined the effect of this xylosylation on Notch signaling in the absence of *O*-fucosylation. In this study, to compare effects on Notch signaling

under the same conditions, all of the other experiments were conducted at 25ºC. In the *O-fut1*^{R245A} and *Uxs*^{1} double-homozygote wing discs, the number of SOPs was not affected at 25ºC (Fig. 20A and see Fig. 18). However, in *O-fut1*^{R245A} homozygotes combined with *Uxs*^{1} heterozygotes, the number of SOPs increased significantly at 25ºC (Fig. 20B and Fig. 18). This discrepancy can be explained if the Wg signal-dependent induction of SOP fate, which occurs prior to its lateral inhibition, was disrupted in the *Uxs*^{1} homozygote at 25ºC. In this case, SOP fate might be induced normally in the *Uxs*^{1} heterozygote wing discs, but a subsequent failure of the typical Notch-signal-dependent lateral inhibition increases the number of SOPs. This idea was further supported by our analysis of *O-fut1*^{R245A} mutant wing discs in combination with *shams*-mutant wing discs, in which Wg signaling is normal (Lee et al., 2013). The number of SOPs increased significantly in the wing discs of a *O-fut1*^{R245A} and *shams* double homozygote at 25ºC (Fig. 20C and Fig. 18). These findings indicated that the terminal dixylose moiety of *O*-glucose glycans and the *O*-fucose monosaccharides on Notch function redundantly in Notch-signaling activation.

Monosaccharide *O*-fucose and the terminal dixylose of *O*-glucose-linked saccharides function redundantly in Notch trafficking

To investigate the cause of the reduction in Notch signaling associated with the concurrent loss of *O*-fucose and of the terminal dixylose of *O*-glucose-linked saccharides, I examined Notch trafficking. *O*-fucose and *O*-glucose glycan contribute to normal Notch trafficking in a temperature-dependent manner; Notch trafficking is defective at 30ºC but largely normal at 25ºC (Lee et al., 2013; Ishio et al., 2015). Using the FLP/FRT system, I generated somatic mosaics of *O-fut1* ^{R245A} or *shams*-mutant cells, or *O-fut1*^{R245A} and *shams* double-mutant cells; the mutant cells were identified by the absence of *GFP* expression in the wing discs. In these experiments, Notch protein localized to the apical plasma membrane, adherens junctions

(AJs), and endoplasmic reticulum (ER) was analyzed at 25ºC (Fig. 21). Notch at the apical plasma membrane was specifically detected by an anti-Notch extracellular domain antibody (NECD) in non-permeabilized wing discs (Okajima et al., 2005). Notch localized normally to the apical plasma membrane in mutant cells of either *O-fut1* ^{R245A} (n=23) or *shams* (n=24) in all cases (Fig. 22A-A'' and Fig. 23A-A''). However, in *O-fut1* ^{R245A} and *shams* double-mutant cells, Notch showed excessive accumulation at the apical plasma membrane in all cases (n=19) (Fig. 24A-A''). In permeabilized epithelial cells, Notch protein detected by an anti-Notch intracellular domain antibody (NICD) localized predominantly to the AJs, as reported previously (Sasamura et al., 2006; Sasaki et al., 2007). In these experiments, AJs were detected by staining with an anti-*Drosophila* E-Cadherin (*D*E-cad) antibody (Sasamura et al., 2006; Sasaki et al., 2007). Notch's localization to the AJs was not markedly affected in either *O-fut1* ^{R245A} (n=28) or *shams* (n=23) mutant cells (Fig. 22B-B'''and 23B-B'''). However, Notch protein was severely reduced in the AJs of all double-mutant cells examined (n = 24), whereas the D*E*-cad staining appeared normal, suggesting that the AJ formation was not disrupted under these conditions (Fig. 24B-B'''). Thus, monosaccharide *O*-fucose and the terminal dixylose of *O*-glucose-linked saccharides function redundantly in localizing Notch to the AJs. It has been suggested that Notch is first transported to the plasma membrane after synthesis, and is then relocated to the AJs through transcytosis (Sasamura et al., 2006; Sasaki et al., 2007). Thus, I speculated that Notch's relocation from the apical plasma membrane to the AJs might be prevented in the double-mutant cells. In contrast, Notch protein in the ER, detected by an anti-Protein disulfate isomerase (Pdi) antibody staining, appeared normal in *O-fut1* ^{R245A} (n=26) or *shams*-mutant (n=28) cells and in *O-fut1*^{R245A} and *shams* double-mutant cells (n=22) (Fig. 22C-C''', 23C-C''', and 24C-C'''). Thus, Notch was normally exported from the ER in the absence of monosaccharide *O*-fucose and the terminal dixylose of *O*-

glucose-linked saccharides (Fig. 24C-C’’’).

Distinct functions of monosaccharide *O*-glucose and the terminal dixylose of *O*-glucose-linked saccharides in the absence of monosaccharide *O*-fucose

In the *shams* mutant, terminal dixylose of *O*-glucose-linked saccharides is missing from the *O*-glucose glycan on the EGF-like repeats of Notch, although monosaccharide *O*-glucose is still present. Thus, I next examined whether Notch trafficking is affected differently by the absence of the terminal dixylose of *O*-glucose-linked saccharides versus the absence of monosaccharide *O*-glucose when Notch is not *O*-fucosylated. I generated somatic mosaics of *rumi*-mutant cells in which *O*-glucosylation of the Notch EGF-like repeats was abolished at 25ºC. In these cells, there was no marked difference in Notch detected at the apical plasma membrane (n=31), at the AJs (n=42), or at the ER (n=26) (Fig. 25A-C’’’). In contrast, in *O-fut1* ^{R245A} and *rumi* double-mutant cells, in which both monosaccharide *O*-fucose and *O*-glucose were missing, Notch was not detected at the apical plasma membrane (N=18) or AJs (n=20) at 25ºC in any of the cases examined (Fig. 26A-A’’ and B-B’’’). Thus, it is likely that the delivery of Notch to the plasma membrane fails under these conditions. I also observed that in all cases examined (n=22), Notch accumulated in a portion of the ER in these double-mutant cells (Fig. 26C-C’’’). Thus, monosaccharide *O*-fucose and *O*-glucose share a redundant but essential function in exporting Notch from the ER. Interestingly, our results showed that in the absence of monosaccharide *O*-fucose, Notch-trafficking defects caused by the absence of the terminal dixylose of *O*-glucose-linked saccharides were different from those caused by the absence of monosaccharide *O*-glucose. Thus, although both the terminal dixylose of *O*-glucose-linked saccharides and monosaccharide *O*-glucose function redundantly with monosaccharide *O*-fucose in Notch trafficking and Notch activation, their

functions are distinct; the terminal dixylose of *O*-glucose-linked saccharides is required for relocating Notch from the apical plasma membrane and monosaccharide *O*-glucose is essential for exporting Notch from the ER when monosaccharide *O*-fucose is absent. Thus, the roles of the terminal dixylose of *O*-glucose-linked saccharides and monosaccharide *O*-glucose are distinct (Fig. 27). However, both of these roles are complemented by monosaccharide *O*-fucose when present

CHAPTER SIX: DISCUSSION

Multiple glycan modifications on a protein may have redundant roles

The EGF-like repeats in Notch contain multiple glycosylation sites to which specific and distinct sugars are added (Moloney et al., 2000; Goto et al., 2001; Okajima et al.,2002; Sasamura et al., 2003; Acar et al., 2008; Matsura et al., 2008; Okajima et al., 2008; Sakaidani et al., 2010; Sakaidani et al., 2011; Boskovski et al., 2013; Lee et al., 2013; LeBon et al., 2014). Most studies of the specific functions of these glycans have drawn conclusions based on the consequences of the loss of a single sugar or a part of a single glycan chain. However, when multiple glycans have redundant functions, it is difficult to fully assess their functions using current approaches. In this study, I found that the terminal dixylose of *O*-glucose-linked saccharides and the monosaccharide *O*-fucose on Notch EGF-like repeats play redundant roles in Notch trafficking and activation.

A temperature-sensitive requirement of monosaccharide *O*-fucose in Notch signaling and transport has been observed in *Drosophila* (Ishio et al., 2015). At a non-permissive temperature (30 °C), the monosaccharide *O*-fucose modification becomes essential for Notch signaling, and Notch lacking this modification accumulates in the ER (Ishio et al., 2015); this effect is not observed at a permissive temperature (25 °C) (Ishio et al., 2015). Similarly, in the absence of the terminal dixylose of *O*-glucose-linked saccharides, as observed in *Uxs* or *shams* mutants, Notch signaling and transport was normal at 25 °C (Fig. 17A-B and Fig. 19F-G). However, importantly, the concurrent loss of the terminal dixylose of *O*-glucose-linked saccharides and of monosaccharide *O*-fucose significantly reduced Notch signaling (Fig. 19A and Fig. 20C). However, some Notch signaling was still present, since the number of SOPs increased even more markedly when the *Notch* gene was knocked down (Fig. 19A and Fig. 19C). Nevertheless, it is clear that monosaccharide *O*-fucose and the terminal dixylose of *O*-glucose-linked saccharides function redundantly in Notch-signaling activation.

Multiple roles of Notch *O*-glucose glycan

I found that in the absence of monosaccharide *O*-fucose, the terminal dixylose of *O*-glucose-linked saccharides was required for relocating Notch from the apical plasma membrane to the AJs, while monosaccharide *O*-glucose was essential for exporting Notch from the ER (Fig. 24C-C’’’, Fig. 26C-C’’’, and Fig. 27). These results suggest that the *O*-glucose trisaccharide has multiple functions in Notch trafficking, and that these distinct functions are complemented by monosaccharide *O*-fucose. Currently, the nature of these complex relationships between structure and function remains unclear. The *O*-fucose glycan on Notch’s EGF-like repeats has multiple roles in Notch signaling (Moloney et al., 2000; Okajima et al.,2002; Sasamura et al., 2003). Fringe adds GlcNAc to the monosaccharide *O*-fucose, thereby modifying Notch’s affinities for its ligands (Moloney et al., 2000; Okajima et al.,2002; Sasamura et al., 2003; Taylor et al., 2014; Luca et al., 2015). Monosaccharide *O*-fucose plays a sole and essential role in exporting Notch from the ER at a non-permissive temperature (Ishio et al., 2015). Furthermore, in FGF signaling, FGF binds the GAG chain, which regulates its own signaling activity (Forsten-Williams et al., 2005; Pan et al., 2006). Removing specific regions of the GAG chain causes distinct defects in FGF signaling, suggesting that various GAG-chain regions have distinct biochemical roles (Forsten-Williams et al., 2005; Pan et al., 2006). Similarly, it is possible that monosaccharide *O*-glucose and the terminal dixylose of *O*-glucose-linked saccharides perform distinct biochemical functions to control Notch trafficking.

Redundant role of monosaccharide *O*-fucose and the terminal dixylose of *O*-glucose-linked saccharides may be involved in Notch transcytosis

When both monosaccharide *O*-fucose and the terminal dixylose of *O*-glucose-linked

saccharides were absent, Notch specifically accumulated at the apical plasma membrane and failed to localize to the AJs (Fig. 24A-A'' and Fig. 27). Thus, the reduction in Notch-signaling activity may be attributed to this mislocalization of Notch. However, the mechanism of this mislocalization is still unknown. It has been suggested that Notch is not directly transported to the AJs, but is instead relocated from the apical plasma membrane to the AJs by transcytosis (Sasamura et al., 2006; Sasaki et al., 2007). Thus, the terminal dixylose of *O*-glucose-linked saccharides and the monosaccharide *O*-fucose may be crucial in facilitating the transcytosis of Notch. There are other cases in which the transcytosis of membrane proteins is required for relocation to their final position (Watoson et al., 1999; Abad-Rodriguez et al., 2015). In neurons, the transcytosis of tropomyosin-related kinase A (TrkA) from the somatodendritic membrane is required for its specific localization to the axonal membrane (Watoson et al., 1999; Abad-Rodriguez et al., 2015). TrkA has a terminal *N*-acetyllactosamine on an *N*-linked glycan modification; galectin-4 binds the N-acetyllactosamine and promotes the transcytosis of TrkA to the axonal membrane (Watoson et al., 1999; Abad-Rodriguez et al., 2015). However, the molecular mechanisms of Notch transcytosis are not clear at present.

Are redundant roles of different glycan modifications general phenomena?

In this study, I demonstrated that two different *O*-glycan modifications on the EGF-like repeats of Notch have redundant roles. In addition to Notch EGF-like repeats, EGF-like repeats in many other proteins have monosaccharide *O*-fucose and *O*-glucose modifications. For example, Blood coagulation factor V and VII have monosaccharide *O*-fucose and *O*-glucose modifications, and these modifications were found in the X-ray crystal structure of blood coagulation Factor VII (Natsuka et al., 1988; Hase et al., 1988; Hase and Ikenaka 1988; Nishimura et al., 1989; Hase et al., 1990; Kuraya and Hase., 1992). Monosaccharide *O*-

fucose were also found in Delta-like 1 (DLL1) and Delta-like 3 (DLL3) (Serth et al., 2015). The *O*-fucosylation of DLL1 is dispensable for ligand function *in vitro* (Müller et al., 2014), however the *O*-fucosylation of DLL3 is required for its function during somitogenesis *in vivo* (Serth et al., 2015). Recently, monosaccaride *O*-glucose were found in Eye-shut and Crumbs (Haltom et al., 2014).

In this study, I found that EGF-like repeats of many *Drosophila* proteins also simultaneously contain consensus sequences of *O*-fucosylation or *O*-glucosylation. As shown in Table1, Crumbs, Cubilin, Dumpy, Eyeshut, Delta, Serrate, Weary, Fat, Kugelei (Fat2), CG31999, LDL receptor protein 1, Arrow, Lipophorin receptor 1, Lipophorin receptor 2, Slit, Faulty attraction, Uninflatable, and Tenascin accessory have these consensus sequences simultaneously. Although at this point, potential redundancy of these two *O*-glycan modifications makes difficult to study their functions. However, based on the results in this study, I speculate that monosaccharide *O*-fucose and *O*-glucose modifications redundantly play specific functions in the trafficking and maturation of these proteins.

Since glycans in other protein motifs with multiple glycosylation sites may also function redundantly, analyzing glycan function by disrupting individual glycans may not be very informative. It is possible that a significant portion of information about the functions of glycan modifications is missing from previous studies that were based on the mutation analysis of single genes encoding a glycosyltransferase. To resolve this issue, it may be necessary to develop a systematic approach to disrupting multiple glycan structures.

CHAPTER SEVEN: REFERENCES

1. Abad-Rodriguez, J., and Diez-Revuelta, N. (2015) Axon glycoprotein routing in nerve polarity, function, and repair. *Trends in biochemical sciences* **40**, 385-396
2. Acar, M., Jafar-Nejad, H., Takeuchi, H., Rajan, A., Ibrani, D., Rana, N. A., Pan, H., Haltiwanger, R. S., and Bellen, H. J. (2008) Rumi is a CAP10 domain glycosyltransferase that modifies Notch and is required for Notch signaling. *Cell* **132**, 247-2582.
3. Acar, A., Simoes, B. M., Clarke, R. B., and Brennan, K. (2016) A Role for Notch Signalling in Breast Cancer and Endocrine Resistance. *Stem cells international* **2016**, 2498764
4. Ahn, K. J., Yoon, J. K., Kim, G. B., Kwon, B. S., Go, J. M., Moon, J. S., Bae, E. J., and Noh, C. I. (2015) Alagille syndrome and a JAG1 mutation: 41 cases of experience at a single center. *Korean journal of pediatrics* **58**, 392-397
5. Aoyama, N., Yamakawa, T., Sasamura, T., Yoshida, Y., Ohori, M., Okubo, H., Iida, E., Sasaki, N., Ueda, R., and Matsuno, K. (2013) Loss- and gain-of-function analyses of vacuolar protein sorting 2 in Notch signaling of Drosophila melanogaster. *Genes & genetic systems* **88**, 45-57
6. Artavanis-Tsakonas, S. (1997) Alagille syndrome--a notch up for the Notch receptor. *Nature genetics* **16**, 212-213
7. Artavanis-Tsakonas, S., Muskavitch, M. A., and Yedvobnick, B. (1983) Molecular cloning of Notch, a locus affecting neurogenesis in Drosophila melanogaster. *Proceedings of the National Academy of Sciences of the United States of America* **80**, 1977-1981
8. Ayukawa, T., Matsumoto, K., Ishikawa, H. O., Ishio, A., Yamakawa, T., Aoyama, N., Suzuki, T., and Matsuno, K. (2012) Rescue of Notch signaling in cells incapable of

GDP-L-fucose synthesis by gap junction transfer of GDP-L-fucose in Drosophila. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 15318-15323

9. Bakker, H., Oka, T., Ashikov, A., Yadav, A., Berger, M., Rana, N. A., Bai, X., Jigami, Y., Haltiwanger, R. S., Esko, J. D., and Gerardy-Schahn, R. (2009) Functional UDP-xylose transport across the endoplasmic reticulum/Golgi membrane in a Chinese hamster ovary cell mutant defective in UDP-xylose Synthase. *The Journal of biological chemistry* **284**, 2576-2583
10. Bar-Peled, M., Griffith, C. L., and Doering, T. L. (2001) Functional cloning and characterization of a UDP- glucuronic acid decarboxylase: the pathogenic fungus Cryptococcus neoformans elucidates UDP-xylose synthesis. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 12003-12008
11. Barrantes, I. B., Elia, A. J., Wunsch, K., Hrabe de Angelis, M. H., Mak, T. W., Rossant, J., Conlon, R. A., Gossler, A., and de la Pompa, J. L. (1999) Interaction between Notch signalling and Lunatic fringe during somite boundary formation in the mouse. *Current biology : CB* **9**, 470-480
12. Bischof, J., Maeda, R. K., Hediger, M., Karch, F., and Basler, K. (2007) An optimized transgenesis system for Drosophila using germ-line-specific phiC31 integrases. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 3312-3317
13. Boskovski, M. T., Yuan, S., Pedersen, N. B., Goth, C. K., Makova, S., Clausen, H., Brueckner, M., and Khokha, M. K. (2013) The heterotaxy gene GALNT11 glycosylates Notch to orchestrate cilia type and laterality. *Nature* **504**, 456-459
14. Bray, S. (1998) Notch signalling in Drosophila: three ways to use a pathway.

Seminars in cell & developmental biology **9**, 591-597

15. Bray, S. J. (2006) Notch signalling: a simple pathway becomes complex. *Nature reviews. Molecular cell biology* **7**, 678-689
16. Bray, S., and Bernard, F. (2010) Notch targets and their regulation. *Current topics in developmental biology* **92**, 253-275
17. Chillakuri, C. R., Sheppard, D., Lea, S. M., and Handford, P. A. (2012) Notch receptor-ligand binding and activation: insights from molecular studies. *Seminars in cell & developmental biology* **23**, 421-428
18. Cohen, S. M. (1990) Specification of limb development in the Drosophila embryo by positional cues from segmentation genes. *Nature* **343**, 173-177
19. Cole, L. K., Le Roux, I., Nunes, F., Laufer, E., Lewis, J., and Wu, D. K. (2000) Sensory organ generation in the chicken inner ear: contributions of bone morphogenetic protein 4, serrate1, and lunatic fringe. *The Journal of comparative neurology* **424**, 509-520
20. Couso, J. P., and Martinez Arias, A. (1994) Notch is required for wingless signaling in the epidermis of Drosophila. *Cell* **79**, 259-272
21. Dang, D. T., and Perrimon, N. (1992) Use of a yeast site-specific recombinase to generate embryonic mosaics in Drosophila. *Developmental genetics* **13**, 367-375
22. De Strooper, B., Annaert, W., Cupers, P., Saftig, P., Craessaerts, K., Mumm, J. S., Schroeter, E. H., Schrijvers, V., Wolfe, M. S., Ray, W. J., Goate, A., and Kopan, R. (1999) A presenilin-1-dependent gamma-secretase-like protease mediates release of Notch intracellular domain. *Nature* **398**, 518-522
23. Dexter, J. S. (1920) ALBINO VERTEBRATES. *Science (New York, N.Y.)* **52**, 130-131

24. Diederich, R. J., Matsuno, K., Hing, H., and Artavanis-Tsakonas, S. (1994) Cytosolic interaction between deltex and Notch ankyrin repeats implicates deltex in the Notch signaling pathway. *Development (Cambridge, England)* **120**, 473-481

25. Eames, B. F., Singer, A., Smith, G. A., Wood, Z. A., Yan, Y. L., He, X., Polizzi, S. J., Catchen, J. M., Rodriguez-Mari, A., Linbo, T., Raible, D. W., and Postlethwait, J. H. (2010) UDP xylose synthase 1 is required for morphogenesis and histogenesis of the craniofacial skeleton. *Developmental biology* **341**, 400-415

26. Ellgaard, L., and Helenius, A. (2001) ER quality control: towards an understanding at the molecular level. Current opinion in cell biology 13, 431-437

27. Fehon, R. G., Kooh, P. J., Rebay, I., Regan, C. L., Xu, T., Muskavitch, M. A., and Artavanis-Tsakonas, S. (1990) Molecular interactions between the protein products of the neurogenic loci Notch and Delta, two EGF-homologous genes in Drosophila. *Cell* **61**, 523-534

28. Fernandez-Valdivia, R., Takeuchi, H., Samarghandi, A., Lopez, M., Leonardi, J., Haltiwanger, R. S., and Jafar-Nejad, H. (2011) Regulation of mammalian Notch signaling and embryonic development by the protein O-glucosyltransferase Rumi. *Development (Cambridge, England)* **138**, 1925-1934

29. Fleming, R. J., Hori, K., Sen, A., Filloramo, G. V., Langer, J. M., Obar, R. A., Artavanis-Tsakonas, S., and Maharaj-Best, A. C. (2013) An extracellular region of Serrate is essential for ligand-induced cis-inhibition of Notch signaling. *Development (Cambridge, England)* **140**, 2039-2049

30. Fortini, M. E. (2009) Notch signaling: the core pathway and its posttranslational regulation. *Developmental cell* **16**, 633-647

31. Forsten-Williams, K., Chua, C. C., and Nugent, M. A. (2005) The kinetics of FGF-2

binding to heparan sulfate proteoglycans and MAP kinase signaling. *Journal of theoretical biology* **233**, 483-499

32. Franch-Marro, X., Wendler, F., Guidato, S., Griffith, J., Baena-Lopez, A., Itasaki, N., Maurice, M. M., and Vincent, J. P. (2008) Wingless secretion requires endosome-to-Golgi retrieval of Wntless/Evi/Sprinter by the retromer complex. *Nature cell biology* **10**, 170-177

33. Frise, E., Knoblich, J. A., Younger-Shepherd, S., Jan, L. Y., and Jan, Y. N. (1996) The Drosophila Numb protein inhibits signaling of the Notch receptor during cell-cell interaction in sensory organ lineage. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 11925-11932

34. Gordon, W. R., Vardar-Ulu, D., Histen, G., Sanchez-Irizarry, C., Aster, J. C., and Blacklow, S. C. (2007) Structural basis for autoinhibition of Notch. *Nature structural & molecular biology* **14**, 295-300

35. Goto, S., Taniguchi, M., Muraoka, M., Toyoda, H., Sado, Y., Kawakita, M., and Hayashi, S. (2001) UDP-sugar transporter implicated in glycosylation and processing of Notch. *Nature cell biology* **3**, 816-822

36. Gottardi, C. J., and Gumbiner, B. M. (2004) Distinct molecular forms of beta-catenin are targeted to adhesive or transcriptional complexes. *The Journal of cell biology* **167**, 339-349

37. Gotting, C., Kuhn, J., Zahn, R., Brinkmann, T., and Kleesiek, K. (2000) Molecular cloning and expression of human UDP-d-Xylose:proteoglycan core protein beta-d-xylosyltransferase and its first isoform XT-II. *Journal of molecular biology* **304**, 517-528

38. Guo, M., Jan, L. Y., and Jan, Y. N. (1996) Control of daughter cell fates during

asymmetric division: interaction of Numb and Notch. Neuron 17, 27-41

39. Haltom, A. R., Lee, T. V., Harvey, B. M., Leonardi, J., Chen, Y. J., Hong, Y., Haltiwanger, R. S., and Jafar-Nejad, H. (2014) The protein O-glucosyltransferase Rumi modifies eyes shut to promote rhabdomere separation in Drosophila. *PLoS genetics* **10**, e1004795

40. Hart, G. W. (2014) Three Decades of Research on O-GlcNAcylation - A Major Nutrient Sensor That Regulates Signaling, Transcription and Cellular Metabolism. *Frontiers in endocrinology* **5**, 183

41. Hase, S., and Ikenaka, T. (1988) [Structure and analyses of sugar chains]. *Rinsho byori. The Japanese journal of clinical pathology* **Spec No 79**, 1-12

42. Hase, S., and Ikenaka, T. (1990) Estimation of elution times on reverse-phase high-performance liquid chromatography of pyridylamino derivatives of sugar chains from glycoproteins. *Analytical biochemistry* **184**, 135-138

43. Hase, S., Natsuka, S., Oku, H., and Ikenaka, T. (1987) Identification method for twelve oligomannose-type sugar chains thought to be processing intermediates of glycoproteins. *Analytical biochemistry* **167**, 321-326

44. Hase, S., and Ikenaka, T. (1988) [Structure and analyses of sugar chains]. *Rinsho byori. The Japanese journal of clinical pathology* **Spec No 79**, 1-12

45. Hase, S., Kawabata, S., Nishimura, H., Takeya, H., Sueyoshi, T., Miyata, T., Iwanaga, S., Takao, T., Shimonishi, Y., and Ikenaka, T. (1988) A new trisaccharide sugar chain linked to a serine residue in bovine blood coagulation factors VII and IX. *Journal of biochemistry* **104**, 867-868

46. Hase, S., Natsuka, S., Oku, H., and Ikenaka, T. (1987) Identification method for twelve oligomannose-type sugar chains thought to be processing intermediates of

glycoproteins. *Analytical biochemistry* **167**, 321-326

47. Hase, S., and Ikenaka, T. (1990) Estimation of elution times on reverse-phase high-performance liquid chromatography of pyridylamino derivatives of sugar chains from glycoproteins. *Analytical biochemistry* **184**, 135-138

48. Ishikawa, H. O., Ayukawa, T., Nakayama, M., Higashi, S., Kamiyama, S., Nishihara, S., Aoki, K., Ishida, N., Sanai, Y., and Matsuno, K. (2010) Two pathways for importing GDP-fucose into the endoplasmic reticulum lumen function redundantly in the O-fucosylation of Notch in Drosophila. *The Journal of biological chemistry* **285**, 4122-4129

49. Ishio, A., Sasamura, T., Ayukawa, T., Kuroda, J., Ishikawa, H. O., Aoyama, N., Matsumoto, K., Gushiken, T., Okajima, T., Yamakawa, T., and Matsuno, K. (2015) O-fucose monosaccharide of Drosophila Notch has a temperature-sensitive function and cooperates with O-glucose glycan in Notch transport and Notch signaling activation. *The Journal of biological chemistry* **290**, 505-519

50. Itatani, Y., Sonoshita, M., Kakizaki, F., Okawa, K., Stifani, S., Itoh, H., Sakai, Y., and Taketo, M. M. (2016) Characterization of Aes nuclear foci in colorectal cancer cells. *Journal of biochemistry* **159**, 133-140

51. Haltom, A. R., and Jafar-Nejad, H. (2015) The multiple roles of epidermal growth factor repeat O-glycans in animal development. *Glycobiology* **25**, 1027-1042

52. Hasegawa, K., Okamura, Y., and Saga, Y. (2012) Notch signaling in Sertoli cells regulates cyclical gene expression of Hes1 but is dispensable for mouse spermatogenesis. *Molecular and cellular biology* **32**, 206-215

53. Hicks, C., Johnston, S. H., diSibio, G., Collazo, A., Vogt, T. F., and Weinmaster, G. (2000) Fringe differentially modulates Jagged1 and Delta1 signalling through Notch1

and Notch2. *Nature cell biology* **2**, 515-520

54. Hummel, T., and Klambt, C. (2008) P-element mutagenesis. *Methods in molecular biology (Clifton, N.J.)* **420**, 97-117

55. Jafar-Nejad, H., Acar, M., Nolo, R., Lacin, H., Pan, H., Parkhurst, S. M., and Bellen, H. J. (2003) Senseless acts as a binary switch during sensory organ precursor selection. *Genes & development* **17**, 2966-2978

56. Jarriault, S., Le Bail, O., Hirsinger, E., Pourquie, O., Logeat, F., Strong, C. F., Brou, C., Seidah, N. G., and Isra l, A. (1998) Delta-1 activation of notch-1 signaling results in HES-1 transactivation. *Molecular and cellular biology* **18**, 7423-7431

57. Joutel, A., Corpechot, C., Ducros, A., Vahedi, K., Chabriat, H., Mouton, P., Alamowitch, S., Domenga, V., Cecillion, M., Marechal, E., Maciazek, J., Vayssiere, C., Cruaud, C., Cabanis, E. A., Ruchoux, M. M., Weissenbach, J., Bach, J. F., Bousser, M. G., and Tournier-Lasserve, E. (1996) Notch3 mutations in CADASIL, a hereditary adult-onset condition causing stroke and dementia. *Nature* **383**, 707-710

58. Joutel, A., Vahedi, K., Corpechot, C., Troesch, A., Chabriat, H., Vayssiere, C., Cruaud, C., Maciazek, J., Weissenbach, J., Bousser, M. G., Bach, J. F., and Tournier-Lasserve, E. (1997) Strong clustering and stereotyped nature of Notch3 mutations in CADASIL patients. *Lancet (London, England)* **350**, 1511-1515

59. Kamimura, K., Koyama, T., Habuchi, H., Ueda, R., Masu, M., Kimata, K., and Nakato, H. (2006) Specific and flexible roles of heparan sulfate modifications in Drosophila FGF signaling. The Journal of cell biology 174, 773-778

60. Kidd, S., Baylies, M. K., Gasic, G. P., and Young, M. W. (1989) Structure and distribution of the Notch protein in developing Drosophila. *Genes & development* **3**, 1113-1129

61. Kono, M., Suganuma, M., Takama, H., Zarzoso, I., Saritha, M., Bodet, D., Aboobacker, S., Kaliaperumal, K., Suzuki, T., Tomita, Y., Sugiura, K., and Akiyama, M. (2015) Dowling-Degos disease with mutations in POFUT1 is clinicopathologically distinct from reticulate acropigmentation of Kitamura. The British journal of dermatology 173, 584-586

62. Kuraya, N., and Hase, S. (1992) Release of O-linked sugar chains from glycoproteins with anhydrous hydrazine and pyridylamination of the sugar chains with improved reaction conditions. *Journal of biochemistry* **112**, 122-126

63. Kuroda, J., Nakamura, M., Yoshida, M., Yamamoto, H., Maeda, T., Taniguchi, K., Nakazawa, N., Hatori, R., Ishio, A., Ozaki, A., Shimaoka, S., Ito, T., Iida, H., Okumura, T., Maeda, R., and Matsuno, K. (2012) Canonical Wnt signaling in the visceral muscle is required for left-right asymmetric development of the Drosophila midgut. Mechanisms of development 128, 625-639

64. Lacroix, C., Giovannini, D., Combe, A., Bargieri, D. Y., Spath, S., Panchal, D., Tawk, L., Thiberge, S., Carvalho, T. G., Barale, J. C., Bhanot, P., and Menard, R. (2011) FLP/FRT-mediated conditional mutagenesis in pre-erythrocytic stages of Plasmodium berghei. *Nature protocols* **6**, 1412-1428

65. LeBon, L., Lee, T. V., Sprinzak, D., Jafar-Nejad, H., and Elowitz, M. B. (2014) Fringe proteins modulate Notch-ligand cis and trans interactions to specify signaling states. *eLife* **3**, e02950

66. Lee, T. V., Sethi, M. K., Leonardi, J., Rana, N. A., Buettner, F. F., Haltiwanger, R. S., Bakker, H., and Jafar-Nejad, H. (2013) Negative regulation of notch signaling by xylose. *PLoS genetics* **9**, e1003547

67. Lendahl, U. (1998) A growing family of Notch ligands. *BioEssays : news and reviews*

in molecular, cellular and developmental biology **20**, 103-107

68. Leonardi, J., Fernandez-Valdivia, R., Li, Y. D., Simcox, A. A., and Jafar-Nejad, H. (2011) Multiple O-glucosylation sites on Notch function as a buffer against temperature-dependent loss of signaling. *Development (Cambridge, England)* **138**, 3569-3578

69. Lieber, T., Kidd, S., and Young, M. W. (2002) kuzbanian-mediated cleavage of Drosophila Notch. *Genes & development* **16**, 209-221

70. Li, L., Krantz, I. D., Deng, Y., Genin, A., Banta, A. B., Collins, C. C., Qi, M., Trask, B. J., Kuo, W. L., Cochran, J., Costa, T., Pierpont, M. E., Rand, E. B., Piccoli, D. A., Hood, L., and Spinner, N. B. (1997) Alagille syndrome is caused by mutations in human Jagged1, which encodes a ligand for Notch1. *Nature genetics* **16**, 243-251

71. Luca, V. C., Jude, K. M., Pierce, N. W., Nachury, M. V., Fischer, S., and Garcia, K. C. (2015) Structural biology. Structural basis for Notch1 engagement of Delta-like 4. *Science (New York, N.Y.)* **347**, 847-853

72. Martinez-Duncker, I., Mollicone, R., Candelier, J. J., Breton, C., and Oriol, R. (2003) A new superfamily of protein-O-fucosyltransferases, alpha2-fucosyltransferases, and alpha6-fucosyltransferases: phylogeny and identification of conserved peptide motifs. *Glycobiology* **13**, 1c-5c

73. Matsuura, A., Ito, M., Sakaidani, Y., Kondo, T., Murakami, K., Furukawa, K., Nadano, D., Matsuda, T., and Okajima, T. (2008) O-linked N-acetylglucosamine is present on the extracellular domain of notch receptors. *The Journal of biological chemistry* **283**, 35486-35495

74. Micchelli, C. A., The, I., Selva, E., Mogila, V., and Perrimon, N. (2002) Rasp, a putative transmembrane acyltransferase, is required for Hedgehog signaling.

Development (Cambridge, England) 129, 843-851

75. Mizuno, T. (2012) [Diagnosis, pathomechanism and treatment of CADASIL]. *Rinsho shinkeigaku = Clinical neurology* **52**, 303-313

76. Mizuno, T. (2013) [Pathomechanisms and treatment of CADASIL]. *Brain and nerve = Shinkei kenkyu no shinpo* **65**, 811-823

77. Mohr, O. L. (1919) Character Changes Caused by Mutation of an Entire Region of a Chromosome in Drosophila. *Genetics* **4**, 275-282

78. Muller, J., Rana, N. A., Serth, K., Kakuda, S., Haltiwanger, R. S., and Gossler, A. (2014) O-fucosylation of the notch ligand mDLL1 by POFUT1 is dispensable for ligand function. PloS one 9, e88571

79. Poulson, D. F. (1937) Chromosomal Deficiencies and the Embryonic Development of Drosophila

80. Melanogaster. *Proceedings of the National Academy of Sciences of the United States of America* **23**, 133-137

81. Moloney, D. J., Panin, V. M., Johnston, S. H., Chen, J., Shao, L., Wilson, R., Wang, Y., Stanley, P., Irvine, K. D., Haltiwanger, R. S., and Vogt, T. F. (2000) Fringe is a glycosyltransferase that modifies Notch. *Nature* **406**, 369-375

82. Moremen, K. W., Tiemeyer, M., and Nairn, A. V. (2012) Vertebrate protein glycosylation: diversity, synthesis and function. *Nature reviews. Molecular cell biology* **13**, 448-462

83. Morgan, W. P. (1916) THE TREATMENT OF WOUND INFECTIONS. *British medical journal* **1**, 685-688

84. Mummery-Widmer, J. L., Yamazaki, M., Stoeger, T., Novatchkova, M., Bhalerao, S., Chen, D., Dietzl, G., Dickson, B. J., and Knoblich, J. A. (2009) Genome-wide

analysis of Notch signalling in Drosophila by transgenic RNAi. *Nature* **458**, 987-992

85. Mumm, J. S., Schroeter, E. H., Saxena, M. T., Griesemer, A., Tian, X., Pan, D. J., Ray, W. J., and Kopan, R. (2000) A ligand-induced extracellular cleavage regulates gamma-secretase-like proteolytic activation of Notch1. *Molecular cell* **5**, 197-206

86. Natsuka, S., Himeno, M., Hase, S., Ito, H., Ueda, T., Kato, K., and Ikenaka, T. (1988) Structures of sugar chains of a p-nitrophenyl acetate-hydrolyzing esterase from the microsomes of rat liver. *Journal of biochemistry* **103**, 986-991

87. Nolo, R., Abbott, L. A., and Bellen, H. J. (2001) Drosophila Lyra mutations are gain-of-function mutations of senseless. *Genetics* **157**, 307-315

88. Pan, Y., Woodbury, A., Esko, J. D., Grobe, K., and Zhang, X. (2006) Heparan sulfate biosynthetic gene Ndst1 is required for FGF signaling in early lens development. *Development (Cambridge, England)* **133**, 4933-4944

89. Port, F., Kuster, M., Herr, P., Furger, E., Banziger, C., Hausmann, G., and Basler, K. (2008) Wingless secretion promotes and requires retromer-dependent cycling of Wntless. *Nature cell biology* **10**, 178-185

90. Poulson, D. F. (1937) Chromosomal Deficiencies and the Embryonic Development of Drosophila Melanogaster. *Proceedings of the National Academy of Sciences of the United States of America* **23**, 133-137

91. Oda, T., Elkahloun, A. G., Pike, B. L., Okajima, K., Krantz, I. D., Genin, A., Piccoli, D. A., Meltzer, P. S., Spinner, N. B., Collins, F. S., and Chandrasekharappa, S. C. (1997) Mutations in the human Jagged1 gene are responsible for Alagille syndrome. *Nature genetics* **16**, 235-242

92. Onouchi, Y., Kurahashi, H., Tajiri, H., Ida, S., Okada, S., and Nakamura, Y. (1999) Genetic alterations in the JAG1 gene in Japanese patients with Alagille syndrome.

Journal of human genetics **44**, 235-239

93. Okajima, T., and Irvine, K. D. (2002) Regulation of notch signaling by o-linked fucose. *Cell* **111**, 893-904

94. Okajima, T., Xu, A., and Irvine, K. D. (2003) Modulation of notch-ligand binding by protein O-fucosyltransferase 1 and fringe. *The Journal of biological chemistry* **278**, 42340-42345

95. Okajima, T., Xu, A., Lei, L., and Irvine, K. D. (2005) Chaperone activity of protein O-fucosyltransferase 1 promotes notch receptor folding. *Science (New York, N.Y.)* **307**, 1599-1603

96. Okajima, T., Matsuura, A., and Matsuda, T. (2008) Biological functions of glycosyltransferase genes involved in O-fucose glycan synthesis. *Journal of biochemistry* **144**, 1-6

97. Okajima, T., Reddy, B., Matsuda, T., and Irvine, K. D. (2008) Contributions of chaperone and glycosyltransferase activities of O-fucosyltransferase 1 to Notch signaling. BMC biology 6, 1

98. Okamura, Y., and Saga, Y. (2008) Pofut1 is required for the proper localization of the Notch receptor during mouse development. *Mechanisms of development* **125**, 663-673

99. Panin, V. M., Shao, L., Lei, L., Moloney, D. J., Irvine, K. D., and Haltiwanger, R. S. (2002) Notch ligands are substrates for protein O-fucosyltransferase-1 and Fringe. *The Journal of biological chemistry* **277**, 29945-29952

100. Pinho, S. S., and Reis, C. A. (2015) Glycosylation in cancer: mechanisms and clinical implications. *Nature reviews. Cancer* **15**, 540-555

101. Rincon-Limas, D. E., Lu, C. H., Canal, I., Calleja, M., Rodriguez-Esteban, C., Izpisua-Belmonte, J. C., and Botas, J. (1999) Conservation of the expression and

function of apterous orthologs in Drosophila and mammals. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 2165-2170

102. Port, F., Kuster, M., Herr, P., Furger, E., Banziger, C., Hausmann, G., and Basler, K. (2008) Wingless secretion promotes and requires retromer-dependent cycling of Wntless. *Nature cell biology* **10**, 178-185

103. Qi, H., Rand, M. D., Wu, X., Sestan, N., Wang, W., Rakic, P., Xu, T., and Artavanis-Tsakonas, S. (1999) Processing of the notch ligand delta by the metalloprotease Kuzbanian. *Science (New York, N.Y.)* **283**, 91-94

104. Ray, M., and Lakhotia, S. C. (2015) The commonly used eye-specific sev-GAL4 and GMR-GAL4 drivers in Drosophila melanogaster are expressed in tissues other than eyes also. Journal of genetics 94, 407-416

105. Ryder, E., Ashburner, M., Bautista-Llacer, R., Drummond, J., Webster, J., Johnson, G., Morley, T., Chan, Y. S., Blows, F., Coulson, D., Reuter, G., Baisch, H., Apelt, C., Kauk, A., Rudolph, T., Kube, M., Klimm, M., Nickel, C., Szidonya, J., Maroy, P., Pal, M., Rasmuson-Lestander, A., Ekstrom, K., Stocker, H., Hugentobler, C., Hafen, E., Gubb, D., Pflugfelder, G., Dorner, C., Mechler, B., Schenkel, H., Marhold, J., Serras, F., Corominas, M., Punset, A., Roote, J., and Russell, S. (2007) The DrosDel deletion collection: a Drosophila genomewide chromosomal deficiency resource. *Genetics* **177**, 615-629

106. Sakaidani, Y., Furukawa, K., and Okajima, T. (2010) O-GlcNAc modification of the extracellular domain of Notch receptors. *Methods in enzymology* **480**, 355-373

107. Sakaidani, Y., Nomura, T., Matsuura, A., Ito, M., Suzuki, E., Murakami, K., Nadano, D., Matsuda, T., Furukawa, K., and Okajima, T. (2011) O-linked-N-acetylglucosamine on extracellular protein domains mediates epithelial cell-matrix

interactions. *Nature communications* **2**, 583

108. Sasaki, N., Sasamura, T., Ishikawa, H. O., Kanai, M., Ueda, R., Saigo, K., and Matsuno, K. (2007) Polarized exocytosis and transcytosis of Notch during its apical localization in Drosophila epithelial cells. *Genes to cells : devoted to molecular & cellular mechanisms* **12**, 89-103

109. Sasamura, T., Sasaki, N., Miyashita, F., Nakao, S., Ishikawa, H. O., Ito, M., Kitagawa, M., Harigaya, K., Spana, E., Bilder, D., Perrimon, N., and Matsuno, K. (2003) neurotic, a novel maternal neurogenic gene, encodes an O-fucosyltransferase that is essential for Notch-Delta interactions. *Development (Cambridge, England)* **130**, 4785-4795

110. Sasamura, T., Ishikawa, H. O., Sasaki, N., Higashi, S., Kanai, M., Nakao, S., Ayukawa, T., Aigaki, T., Noda, K., Miyoshi, E., Taniguchi, N., and Matsuno, K. (2007) The O-fucosyltransferase O-fut1 is an extracellular component that is essential for the constitutive endocytic trafficking of Notch in Drosophila. *Development (Cambridge, England)* **134**, 1347-1356

111. Schroeter, E. H., Kisslinger, J. A., and Kopan, R. (1998) Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. *Nature* **393**, 382-386

112. Serth, K., Schuster-Gossler, K., Kremmer, E., Hansen, B., Marohn-Kohn, B., and Gossler, A. (2015) O-fucosylation of DLL3 is required for its function during somitogenesis. PloS one 10, e0123776

113. Simcox, A. A., Austin, C. L., Jacobsen, T. L., and Jafar-Nejad, H. (2008) Drosophila embryonic 'fibroblasts': extending mutant analysis in vitro. *Fly* **2**, 306-309

114. Shimizu, H., Woodcock, S. A., Wilkin, M. B., Trubenova, B., Monk, N. A., and Baron, M. (2014) Compensatory flux changes within an endocytic trafficking network

maintain thermal robustness of Notch signaling. *Cell* **157**, 1160-1174

115. Shi, S., Stahl, M., Lu, L., and Stanley, P. (2005) Canonical Notch signaling is dispensable for early cell fate specifications in mammals. *Molecular and cellular biology* **25**, 9503-9508

116. Shi, S., and Stanley, P. (2006) Evolutionary origins of Notch signaling in early development. *Cell cycle (Georgetown, Tex.)* **5**, 274-278

117. Singh, N., Phillips, R. A., Iscove, N. N., and Egan, S. E. (2000) Expression of notch receptors, notch ligands, and fringe genes in hematopoiesis. *Experimental hematology* **28**, 527-534

118. Stanley, P., and Okajima, T. (2010) Roles of glycosylation in Notch signaling. *Current topics in developmental biology* **92**, 131-164

119. Struhl, G., and Greenwald, I. (1999) Presenilin is required for activity and nuclear access of Notch in Drosophila. *Nature* **398**, 522-525

120. Takei, Y., Ozawa, Y., Sato, M., Watanabe, A., and Tabata, T. (2004) Three Drosophila EXT genes shape morphogen gradients through synthesis of heparan sulfate proteoglycans. *Development (Cambridge, England)* **131**, 73-82

121. Taketo, M. M. (2011) Reflections on the spread of metastasis to cancer prevention. *Cancer prevention research (Philadelphia, Pa.)* **4**, 324-328

122. Takeuchi, H., Fernandez-Valdivia, R. C., Caswell, D. S., Nita-Lazar, A., Rana, N. A., Garner, T. P., Weldeghiorghis, T. K., Macnaughtan, M. A., Jafar-Nejad, H., and Haltiwanger, R. S. (2011) Rumi functions as both a protein O-glucosyltransferase and a protein O-xylosyltransferase. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 16600-16605

123. Takeuchi, H., and Haltiwanger, R. S. (2014) Significance of glycosylation in Notch

signaling. *Biochemical and biophysical research communications* **453**, 235-242

124. Taniguchi, N., and Kizuka, Y. (2015) Glycans and cancer: role of N-glycans in cancer biomarker, progression and metastasis, and therapeutics. *Advances in cancer research* **126**, 11-51

125. Taylor, P., Takeuchi, H., Sheppard, D., Chillakuri, C., Lea, S. M., Haltiwanger, R. S., and Handford, P. A. (2014) Fringe-mediated extension of O-linked fucose in the ligand-binding region of Notch1 increases binding to mammalian Notch ligands. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 7290-7295

126. The, I., and Perrimon, N. (2000) Morphogen diffusion: the case of the wingless protein. *Nature cell biology* **2**, E79-82

127. Thuringer, F., Cohen, S. M., and Bienz, M. (1993) Dissection of an indirect autoregulatory response of a homeotic Drosophila gene. *The EMBO journal* **12**, 2419-2430

128. Ueyama, M., Takemae, H., Ohmae, Y., Yoshida, H., Toyoda, H., Ueda, R., and Nishihara, S. (2008) Functional analysis of proteoglycan galactosyltransferase II RNA interference mutant flies. The Journal of biological chemistry 283, 6076-6084

129. Vasudevan, D., and Haltiwanger, R. S. (2014) Novel roles for O-linked glycans in protein folding. *Glycoconjugate journal* **31**, 417-426

130. Vasudevan, D., Takeuchi, H., Johar, S. S., Majerus, E., and Haltiwanger, R. S. (2015) Peters plus syndrome mutations disrupt a noncanonical ER quality-control mechanism. Current biology : CB 25, 286-295

131. Watson, F. L., Porcionatto, M. A., Bhattacharyya, A., Stiles, C. D., and Segal, R. A. (1999) TrkA glycosylation regulates receptor localization and activity. *Journal of*

neurobiology **39**, 323-336

132. Wong, P. C., Zheng, H., Chen, H., Becher, M. W., Sirinathsinghji, D. J., Trumbauer, M. E., Chen, H. Y., Price, D. L., Van der Ploeg, L. H., and Sisodia, S. S. (1997) Presenilin 1 is required for Notch1 and DII1 expression in the paraxial mesoderm. *Nature* **387**, 288-292

133. Wang, Y., Shao, L., Shi, S., Harris, R. J., Spellman, M. W., Stanley, P., and Haltiwanger, R. S. (2001) Modification of epidermal growth factor-like repeats with O-fucose. Molecular cloning and expression of a novel GDP-fucose protein O-fucosyltransferase. *The Journal of biological chemistry* **276**, 40338-40345

134. Xu, C., and Ng, D. T. (2015) Glycosylation-directed quality control of protein folding. *Nature reviews. Molecular cell biology* **16**, 742-752

135. Xu, D., and Esko, J. D. (2014) Demystifying heparan sulfate-protein interactions. *Annual review of biochemistry*

136. Yamamoto, S., Charng, W. L., Rana, N. A., Kakuda, S., Jaiswal, M., Bayat, V., Xiong, B., Zhang, K., Sandoval, H., David, G., Wang, H., Haltiwanger, R. S., and Bellen, H. J. (2012) A mutation in EGF repeat-8 of Notch discriminates between Serrate/Jagged and Delta family ligands. *Science (New York, N.Y.)* **338**

137. Yaron, A., and Sprinzak, D. (2012) The cis side of juxtacrine signaling: a new role in the development of the nervous system. *Trends in neurosciences* **35**, 230-239

138. Ye, Y., Lukinova, N., and Fortini, M. E. (1999) Neurogenic phenotypes and altered Notch processing in Drosophila Presenilin mutants. *Nature* **398**, 525-529

CHAPTER EIGHT:

Paper and review

1. Ayukawa, T., **Matsumoto**, K., Ishikawa, H. O., Ishio, A., Yamakawa, T., Aoyama, N., Suzuki, T., and Matsuno, K. (2012) Rescue of Notch signaling in cells incapable of GDP-L-fucose synthesis by gap junction transfer of GDP-L-fucose in Drosophila. *Proc. Natl. Acad. Sci. USA.* **109**, 15318-15323
2. **Kenjiroo Matsumoto**, Akira Ishio, Kenji Matsuno (2014) *O*-Fucose Glycan in *Drosophila* Notch Signaling. **Glycoscience: Biology and Medicine,** 1-7
3. **Kenjiroo Matsumoto**, Akira Ishio, Kenji Matsuno (2014) 糖鎖の新機能開発・応用ハンドブック(Japanese book)
4. **Matsumoto, K,** Ayukawa, T., Ishio, A., Sasamura, T., Yamakawa, T., and Matsuno, K. (2015) Dual roles of *O*-glucose glycan redundant with monosaccharide *O*-fucose on Notch in Notch trafficking. *J. Biol. Chem.* (掲載予定)

CHAPTER NINE:
CONFERENCE PRESENTATIONS

1. Mitsutoshi Nakamura, **Kenjiroo Matsumoto**, Yuta Iwamoto, Ryo Hatori, Kenji Matsuno (2013) Reduced cell number in the hindgut epithelium disrupts hindgut left-right asymmetry in a mutant of pebble, encoding a RhoGEF, in *Drosophila* embryos. The 54th Annual *Drosophila* Research Conference, 3-7 April, Washington, DC, USA
2. Takuma Gushiken, **Kenjiroo Matsumoto**, Ryo Hatori, Tomoko Yamakawa, Takeshi Sasamura, Kenji Matsuno (2013) Identification of novel maternal neurogenic genes that are potential components of Notch signaling in *Drosophila,* The 2nd Asia-Pacific *Drosophila* Research Conference, 13-16 May, Seoul, South Korea
3. Takuma Gushiken, **Kenjiroo Matsumoto**, Takahiro Seto, Ryo Hatori, Shunsuke Shimaoka, Tomoko Yamakawa, Takeshi Sasamura, Kenji Matsuno (2013) Identification of novel maternal neurogenic genes that are potential components of Notch signaling in *Drosophila,* The 54th Annual *Drosophila* Research Conference, 3-7 April, Washington, DC, USA
4. Takuma Gushiken, **Kenjiroo Matsumoto**, Ryo Hatori, Tomoko Yamakawa, Takeshi Sasamura, Kenji Matsuno (2013) Identification of novel maternal neurogenic genes that are potential components of Notch signaling in *Drosophila,* The 46th Japanese Society of Developmental Biology annual meeting,28-31 May, Shimane, Matsue, Japan **(Reviewed, Poster presentation)**
5. Takuma Gushiken, **Kenjiroo Matsumoto**, Ryo Hatori, Tomoko Yamakawa, Takeshi Sasamura, Kenji Matsuno (2013) Functions of chromatin remodeling factor Okra / Rad54 in Notch signaling, The 65th Annual Meeting of the Japan Society for Cell Biology, 19-21 June, Matsue, Tottori, Japan **(Reviewed, Oral presentation)**
6. Takuma Gushiken, **Kenjiroo Matsumoto**, Ryo Hatori, Tomoko Yamakawa, Takeshi Sasamura, Kenji Matsuno (2013) Identification of novel maternal neurogenic genes that are potential components of Notch signaling in *Drosophila,* 7th Japanese Notch meeting,

February, Mishima, Shizuoka, Japan **(Reviewed, Oral presentation)**

7. **Kenjiroo Matsumoto** and Kenji Matsuno, The role of glycan modifications of Notch receptor in Notch signaling (2013) The 3th Model organism meeting, September 21, Toyonaka, Osaka, Japan **(Reviewed, Oral presentation)**
8. **Kenjiroo Matsumoto**, Akira Ishio, and Kenji Matsuno (2014) Roles of glycan modifications

   of Notch receptor in *Drosophila* Notch signaling, The 55th Annual *Drosophila* Research Conference, 3-7 April, Sandi ego, USA
9. **Kenjiroo Matsumoto**, Shoko Nishihara, and Kenji Matsuno (2014) The role of glycan modification of Notch receptor in Notch signaling, The 15th KANSAI glycol science forum, April 24, Osaka city, Osaka, Japan **(Reviewed, Oral presentation)**
10. **Kenjiroo Matsumoto**, Shoko Nishihara, and Kenji Matsuno (2014) The role of glycan modification of Notch receptor in Notch signaling, Bilaterial NTHU-OH Life Science Conference 2014, May 16, National Tsing-Hua University, Hsinchu City, Taiwan **(Reviewed, Oral presentation)**
11. **Kenjiroo Matsumoto**, Akira Ishio, and Kenji Matsuno (2014) Roles of glycan modifications

    of Notch receptor in *Drosophila* Notch signaling, The 11th Japanese *Drosophila* Research Conference, June 4-6, Kanazawa, Ishikawa, Japan
12. **Kenjiroo Matsumoto** and Kenji Matsuno (2015) The role of glycan modifications of Notch receptor in *Drosophila* Notch signaling, Finnish-Japanese joint symposium on Morphogenesis and Signaling, March 3-4, Helsingfors, Finland
13. **Kenjiroo Matsumoto** and Kenji Matsuno (2015) *O*-Fucose monosaccharide on Notch corporate with xylose modification of *O*-glucose on Notch in Notch transport and signaling,

The 16th KANSAI glycol science forum, May 16, Toyonaka, Osaka, Japan **(Reviewed, Oral presentation)**

14. **Kenjiroo Matsumoto** and Kenji Matsuno (2015) *O*-Fucose monosaccharide on Notch corporate with xylose modification of *O*-glucose on Notch in Notch transport and signaling, The 34th Japanese Carbohydrate Symposum, July 31- August 2, Ueno, Tokyo, Japan **(Reviewed, Poster presentation)**

CHAPTER TEN:

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CHAPTER ELEVEN:
FIGRES AND TABLE

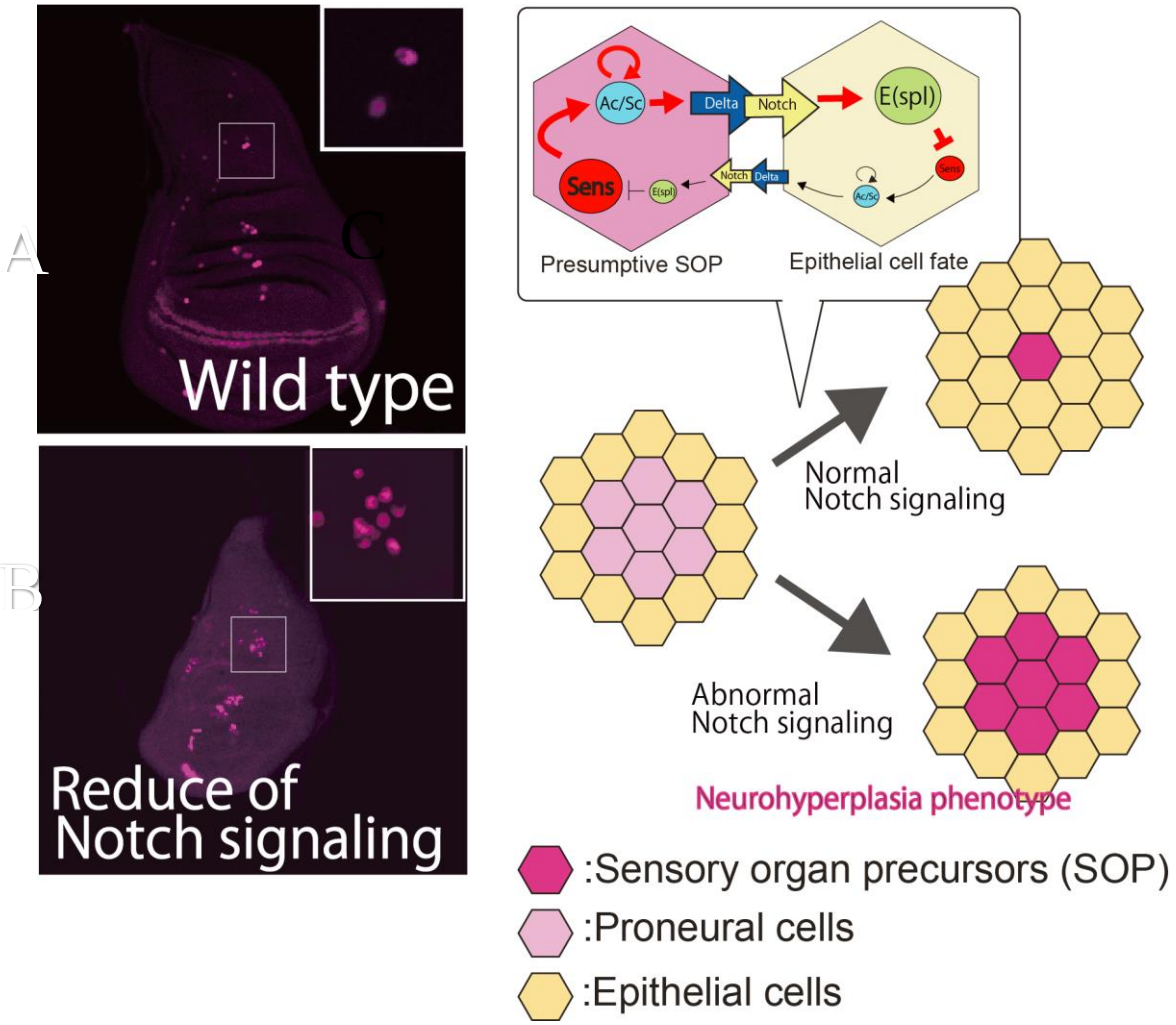

Fig. 1: Notch signaling is essential for SOPs development, and defect of Notch signaling causes hyperplasia of SOPs phenotype

(A-B) Sensory organ precursors (SOPs) in wing discs were detected by anti-Sens antibody staining (magenta). Wing discs from a wild type (A) and mutant in which Notch signaling is reduced (B). Insets show magnified views of the areas in white squares, which include SA-neuron precursors, derivatives of SOPs that will form a specific neuron. (C)Scheme of SA neuron development. In epithelial cells, cells that have potential to differentiate neuronal cells are selected as cluster of proneural cells. One proneural cell starts to highly express Delta and activates Notch signaling in surrounding cells. This cell differentiates into SOP, and the

surrounding proneural cells become epithelial cells. This process is called “lateral inhibition.” The number of SA neurons are increased when Notch signaling are diminishing.

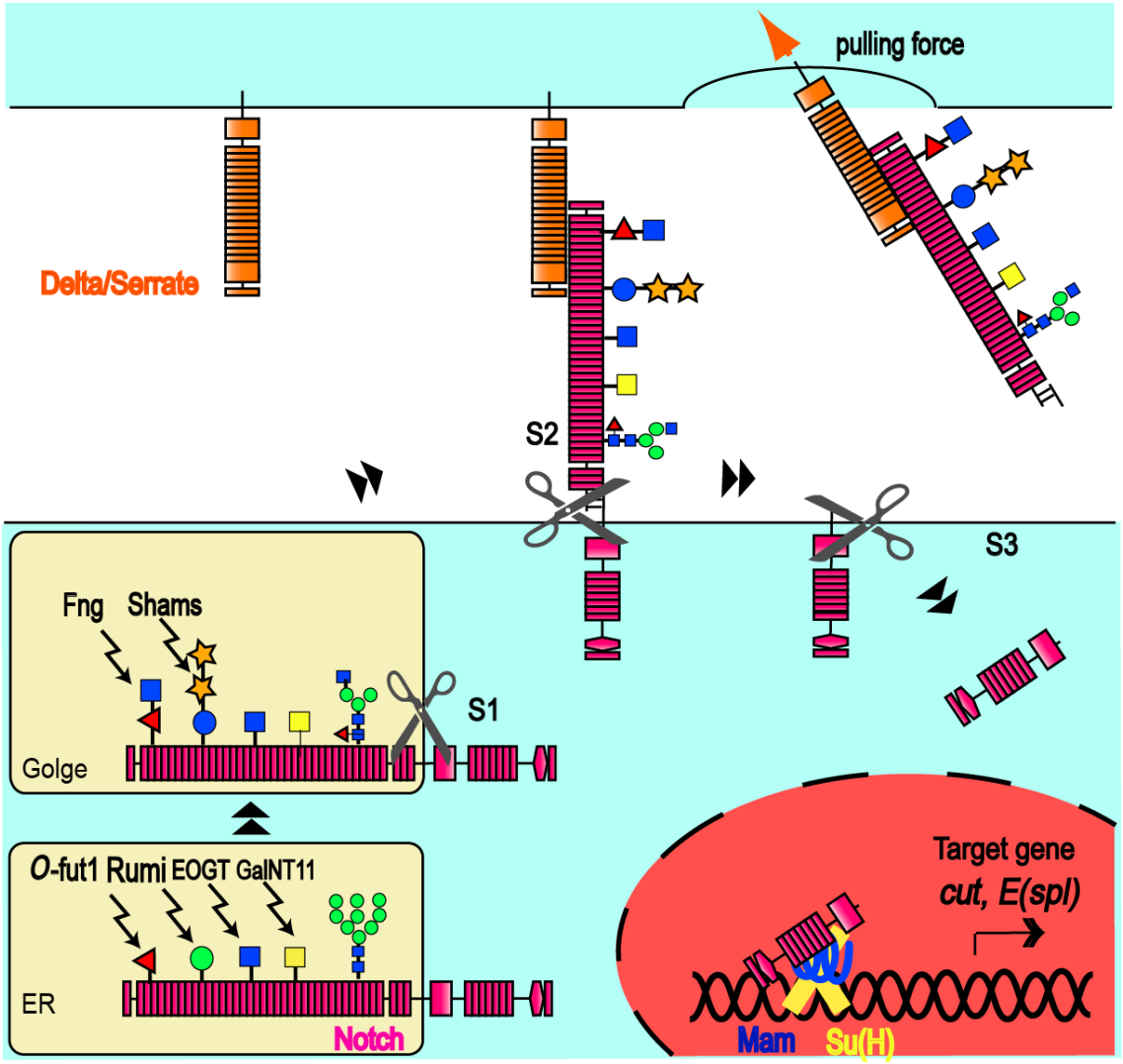

Fig. 2: Schema of Notch signaling activation

Notch is a single-span trans-membrane receptor protein and, its ligands, Delta and Serrate, are also single-span trans-membrane proteins. Notch signal mediates direct cell-cell interaction and is necessary for many cell-fate decisions. This figure represents a scheme of Notch signaling activation. First, Notch is glycosylated in ER and Golgi. After glycan modifications of Notch, Notch is translocated to the cell membrane. Notch binds to its ligand, either Delta or Serrate. After Notch-ligand binding, Notch intracellular domain is cleaved, and the intracellular domain is translocated to the nucleus. Consequently, the downstream target genes of Notch signaling are activated.

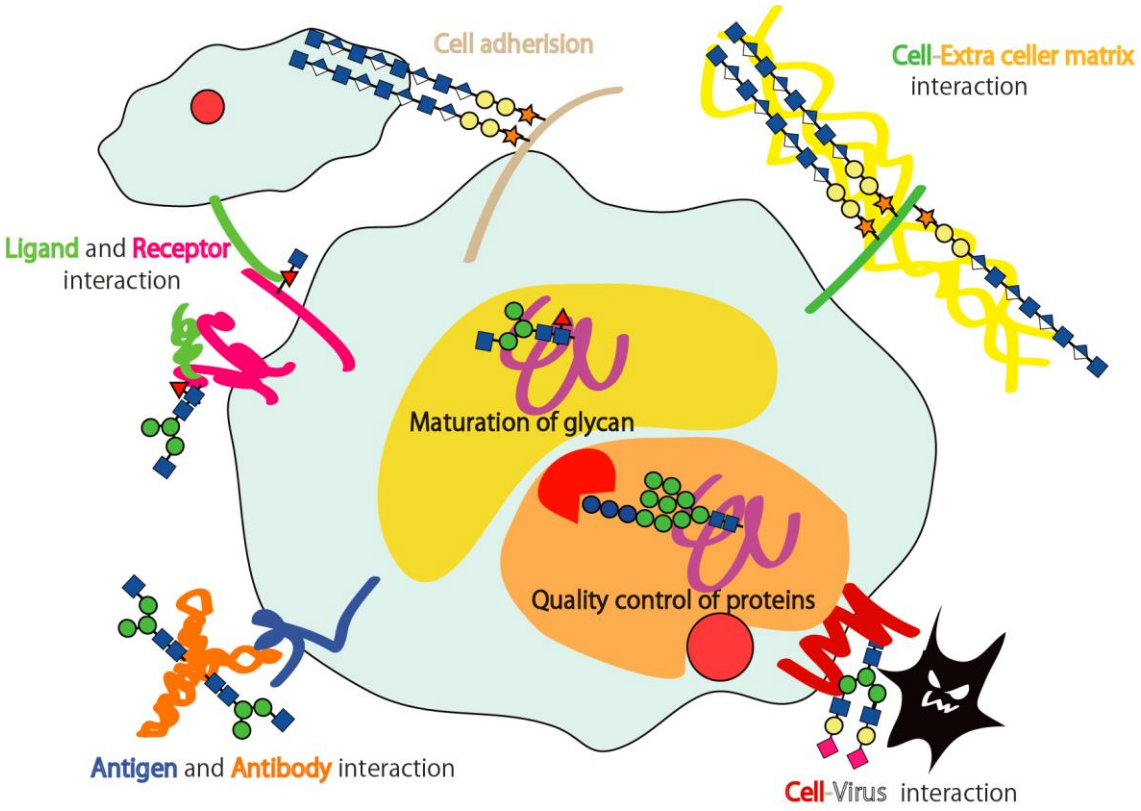

Fig. 3: Glycan chains are essential for many aspects of protein functions.

For example, protein quality control, antigen-antibody interaction, ligand-receptor interaction, cell-adhesion, cell-extracellular matrix interaction, and cell-virus interaction depend on glycosylation. Many of these phenomena are mediated by membrane proteins that have glycan modifications.

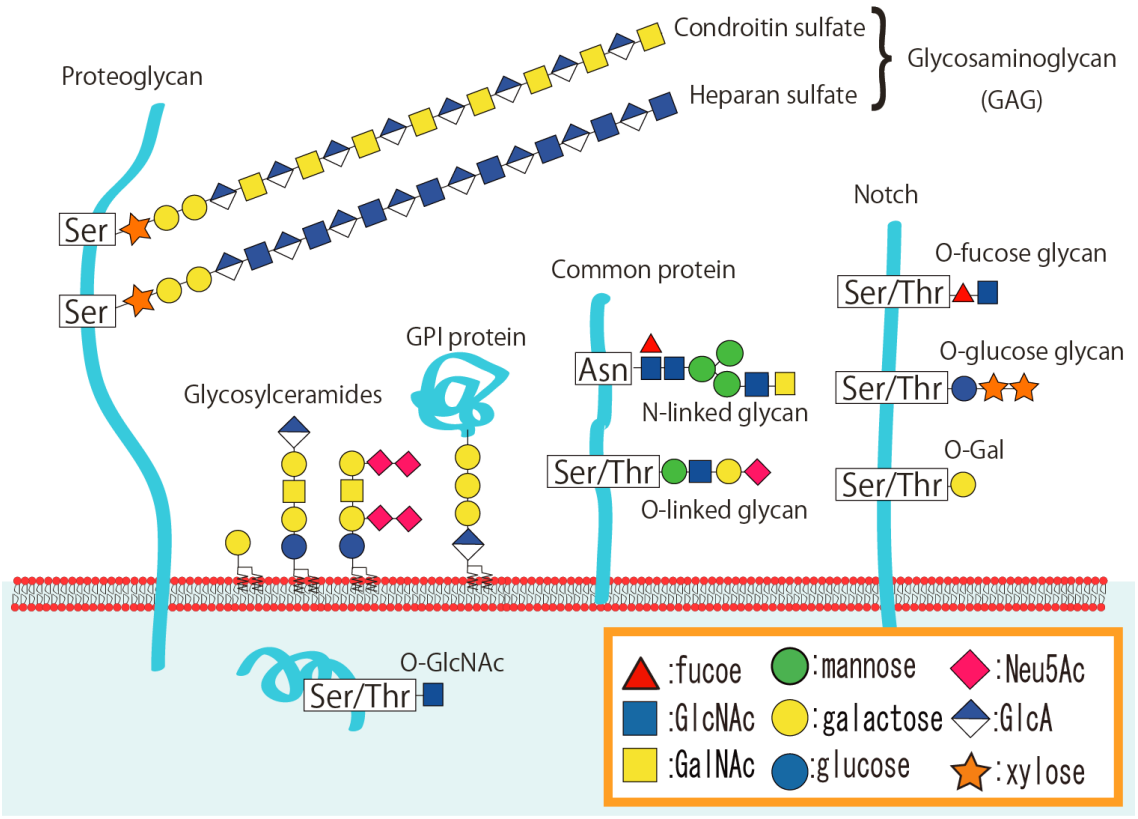

Fig. 4: Various membrane proteins have glycan chains

Most of membrane proteins are glycosylated. Glucosamine glycan, GPI uncured protein, N-linked glycan, and *O*-linked glycan are schematically shown.

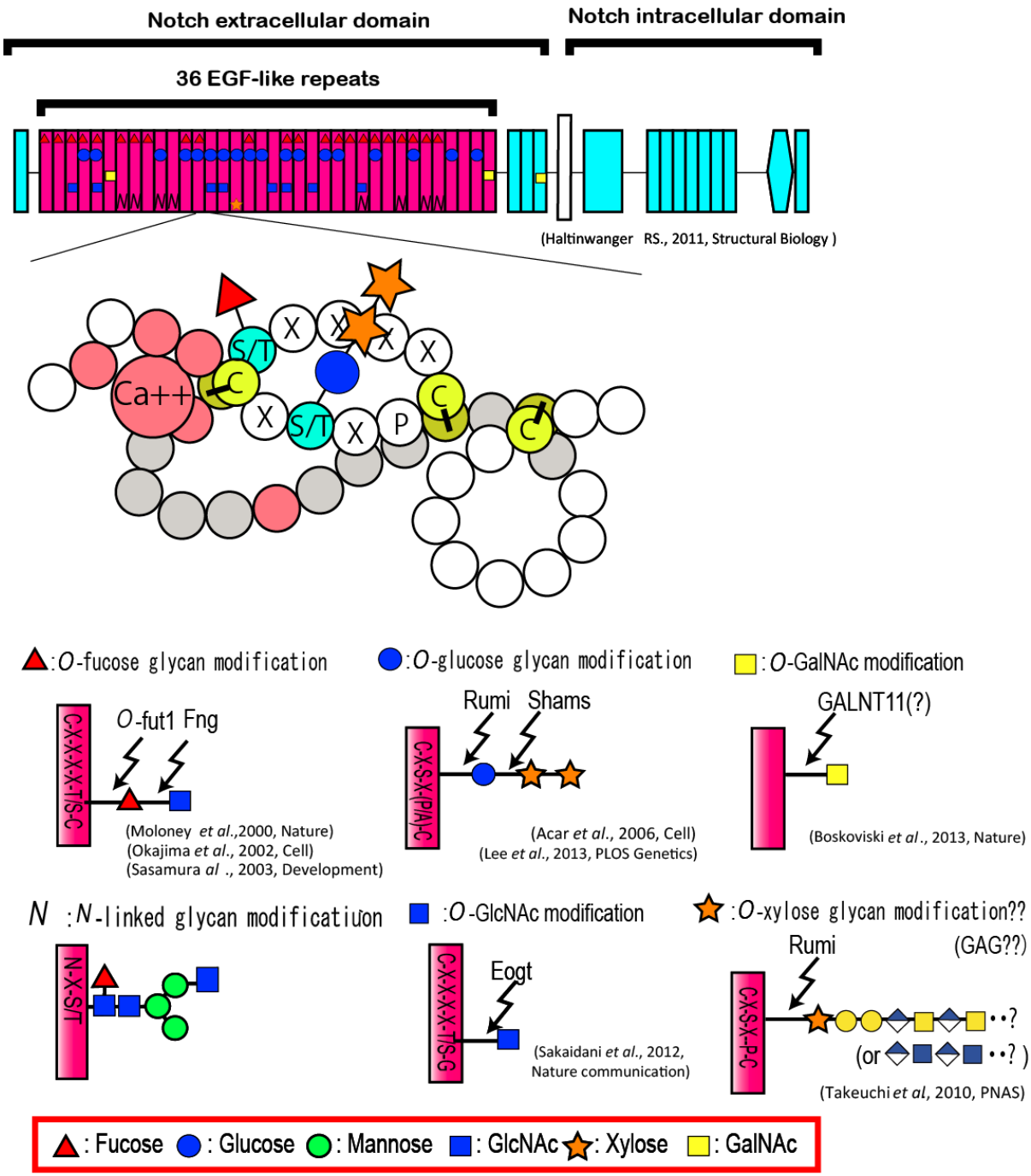

Fig. 5: Glycan modification of Notch

This is Notch extracellular domain and intracellular domain. Notch have 36 EGF-like repeat structure in its extracellular domain and there are many glycan modifications in this structure. 6 glycan modification are known glycan modification. *O*-fucose glycan, *O*-glucose glycan, *O*-GalNAc modification, *N*-linked glycan modification, and *O*-GlcNAc modification. And

Notch may have *O*-xylose modification.

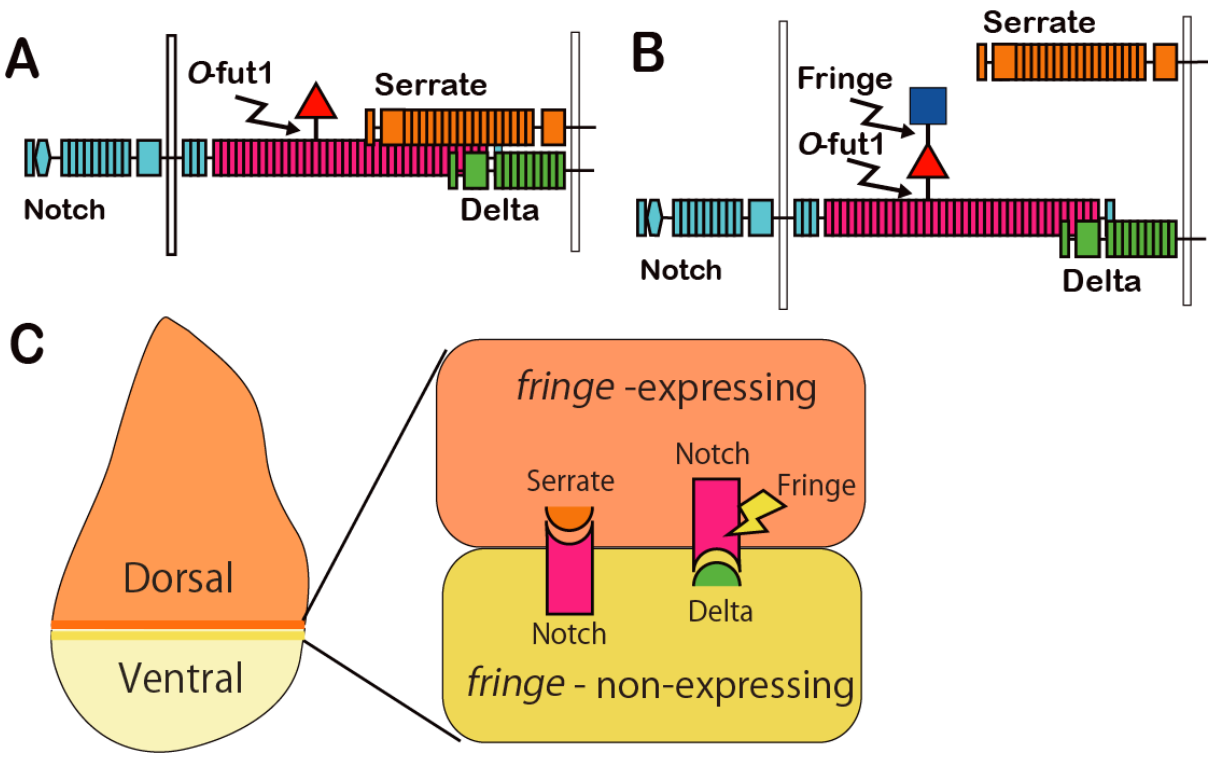

Fig. 6: The function of *O*-fucose glycan modification on Notch in *Drosophila* wing imaginal discs

(A)*O*-fucosylation of Notch is catalyzed by *O*-fut1, and the O-fucosylated Notch equally binds to two ligands, Delta and Serrate. (B) Fringe adds GlcNAc to the *O*-fucose, and Notch with this disaccharide chain binds to Delta but not to Serrate. Due to the *O*-fucose-GlcNAc disaccharide modification of Notch EGF-like repeats, Notch signal is activated along the boundary between *fringe*-expressing and *fringe*-non-expressing tissues (C).

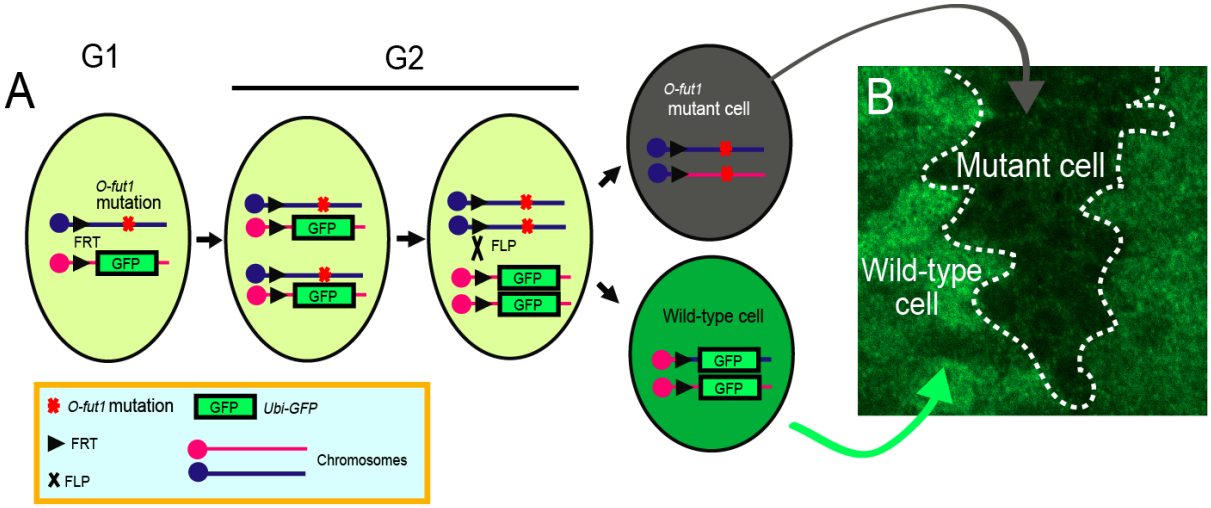

Fig. 7: FRP-FRT system

(A and B) FLP-FRT system was used to generation genetic mosaic in *Drosophila*. (A) A schematic diagram demonstrating how somatic mosaic of *O-fut1* mutant cells was formed using FLP-FRT system. Larvae carrying a FRT-sequence and the flip (FLP), which is FRT-sequence specific recombinant enzyme, were generated. Somatic recombination is occurred on FRT sequence mediated by FLP in somatic cell division phase. (B) *O-fut1* homozygote cells in *O-fut1* heterozygote animals (A and B). *O-fut1* homozygote cells lack GFP expression, which is the marker of wild type chromosome (A). Thus, GFP-negative cells are *O-fut1* homozygote cells.

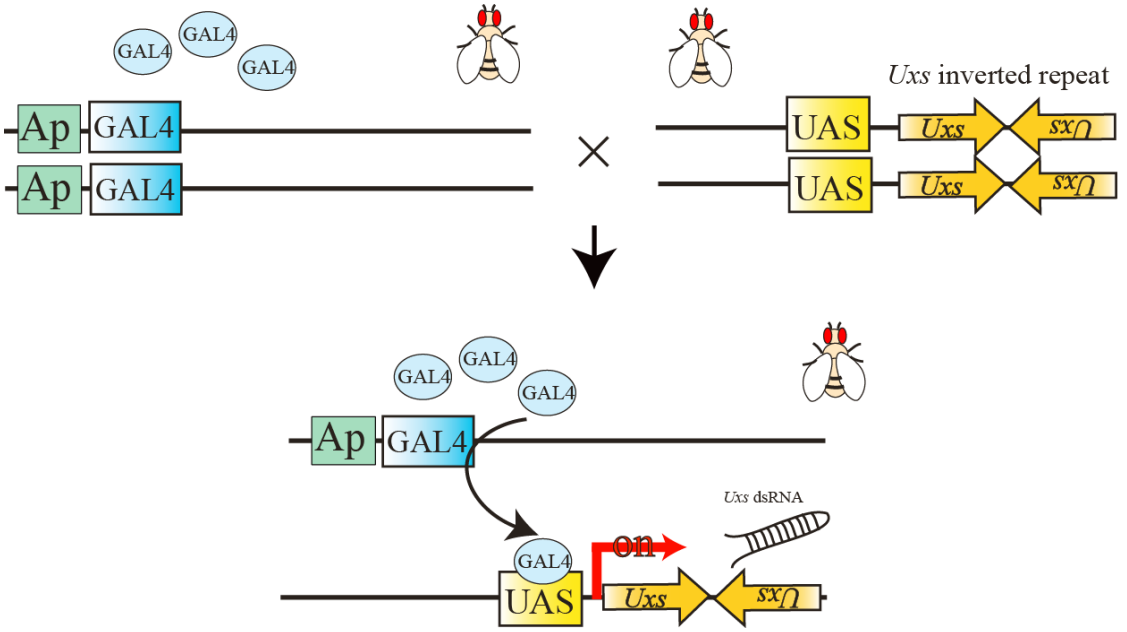

Fig. 8:GAL4-UAS system

GAL4-UAS system is commonly used for forced expression of genes *in vivo*. Gal4 is a yeast transcriptional factor that binds and activates to UAS promoter. The Gal4 binding to UAS led to the activation of an arbitrary gene that is inserted into the downstream of UAS promoter. For example, *Gal4* is expressed under the control of *apterous* (*ap*) gene promoter (AP) in *ap-Gal4* line. *UAS-Uxs-IR* line carries inverted repeat sequence of *Uxs* in the downstream of *UAS* promoter. Progenies obtained by genetics cross between these two lines carry *ap-GAL4* and *UAS-Uxs-IR* line (bottom) overexpresses *Uxs-IR* in the tissues expressing Gal4 driven by *ap-GAL4*.

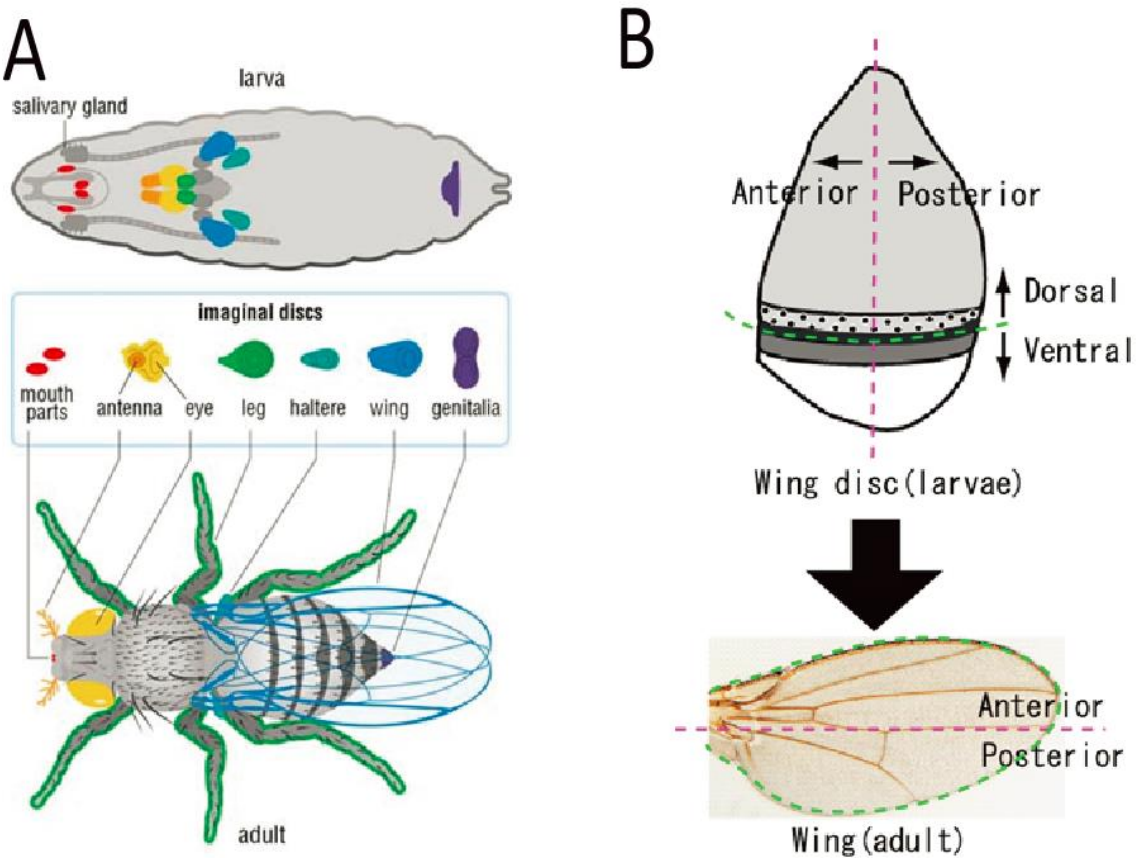

Fig. 9: *Drosophila* wing imaginal discs

(A) *Drosophila* larva has various imaginal discs. *Drosophila* adult organs are delivered from these imaginal discs, as indicated by colors. (B) *Drosophila* adult wings (bottom) are derived from larval wing imaginal discs (top). Dorsal/Ventral boundary of *Drosophila* wings is a signaling center controlling the development of wing and formed at the third instar larva stage. Notch signaling is activated is along Dorsal/Ventral compartment.

(This figure is cited from "Essential developmental biology" and "Wolpert developmental biology")

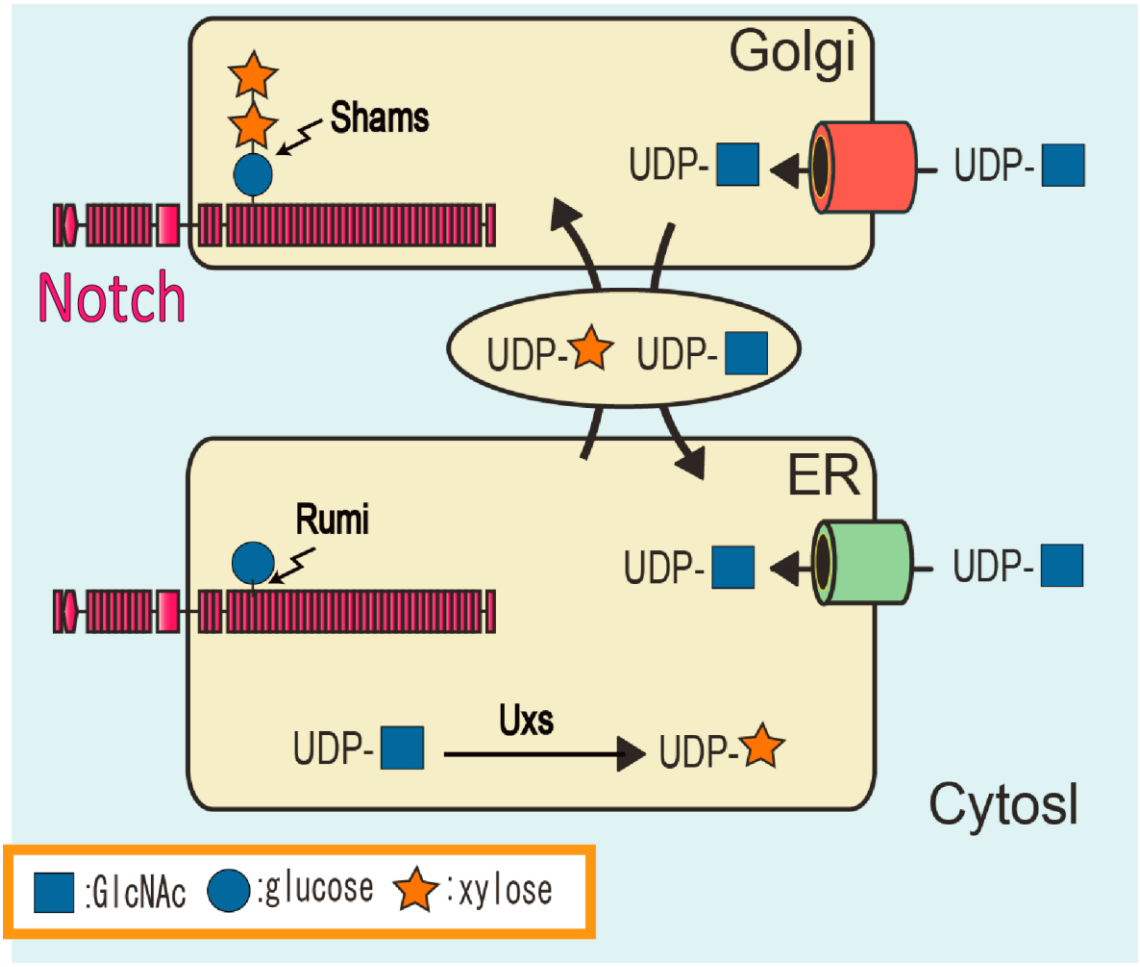

Fig. 10: Supply pathway of UDP-xylose to Notch

O-glucose glycan modification is composed of 3 glycan ”EGF-glucose-xylose-xylose”. First glucose (blue round) is attached by Rumi and Shams add xylose (orange star) to this glucose. This is supply pathway of xylose to Notch. Donor of terminal dixylose of *O*-glucose-linked saccharides is UDP-xylose. UDP-xylose is synthesided from UDP-GlcNAc catalyzed by Uxs.

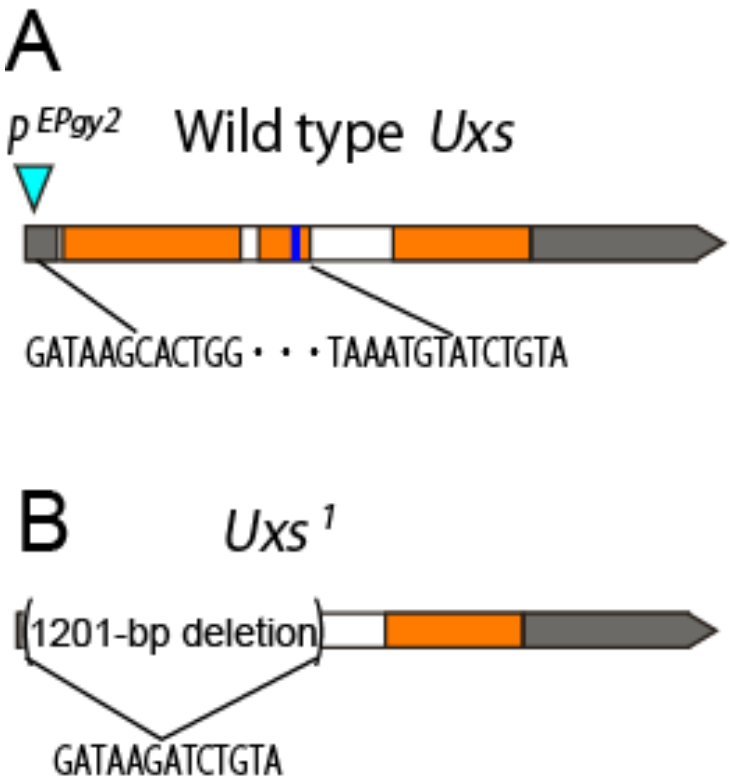

Fig. 11: *Uxs*^{1} is null allele mutant of *Uxs*

(A)Genomic organization of the *Drosophila Uxs* locus, showing the untranslated (gray) and protein-coding (orange) regions of the *Uxs* gene exon, introns (white), and the region encoding a nucleotide-binding site (blue). A P-element (EPgy2) insertion site is indicated by a blue triangle. (B) The 1201-bp deletion in *Uxs*^{1} is indicated by parentheses; the end-points are shown by the DNA sequence.

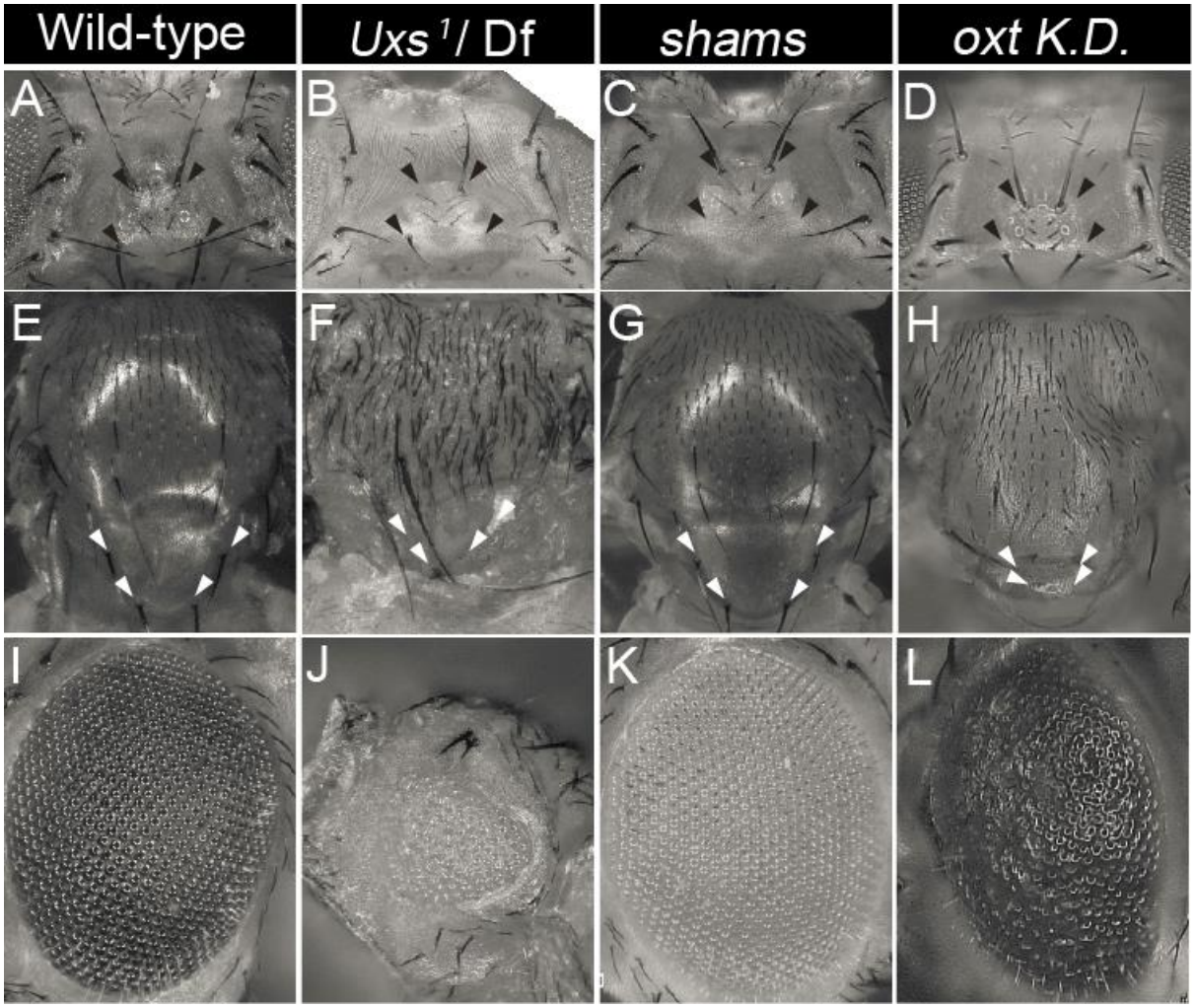

Fig. 12: Overlaps in the *Uxs*- and *shams*-mutant phenotypes

(A-L) Phenotypes of the head (A-D), notum (E-H), and eye (I-L) of wild-type (A, E, and I), and *Uxs*^{1}/*Df(3L)Exel6112* (B, F, and J) and *shams*^{34} homozygotes (C, G, and K), and *oxt* knock-down (D, H, and L) phenotypes. Ocellar and post-vertical bristles are indicated by black (A-D) and white (E-H) arrowheads, respectively. *sca-Gal4* (D), *ap-Gal4* (H), *GMR-Gal4* (L) was used for *oxt* knock-down.

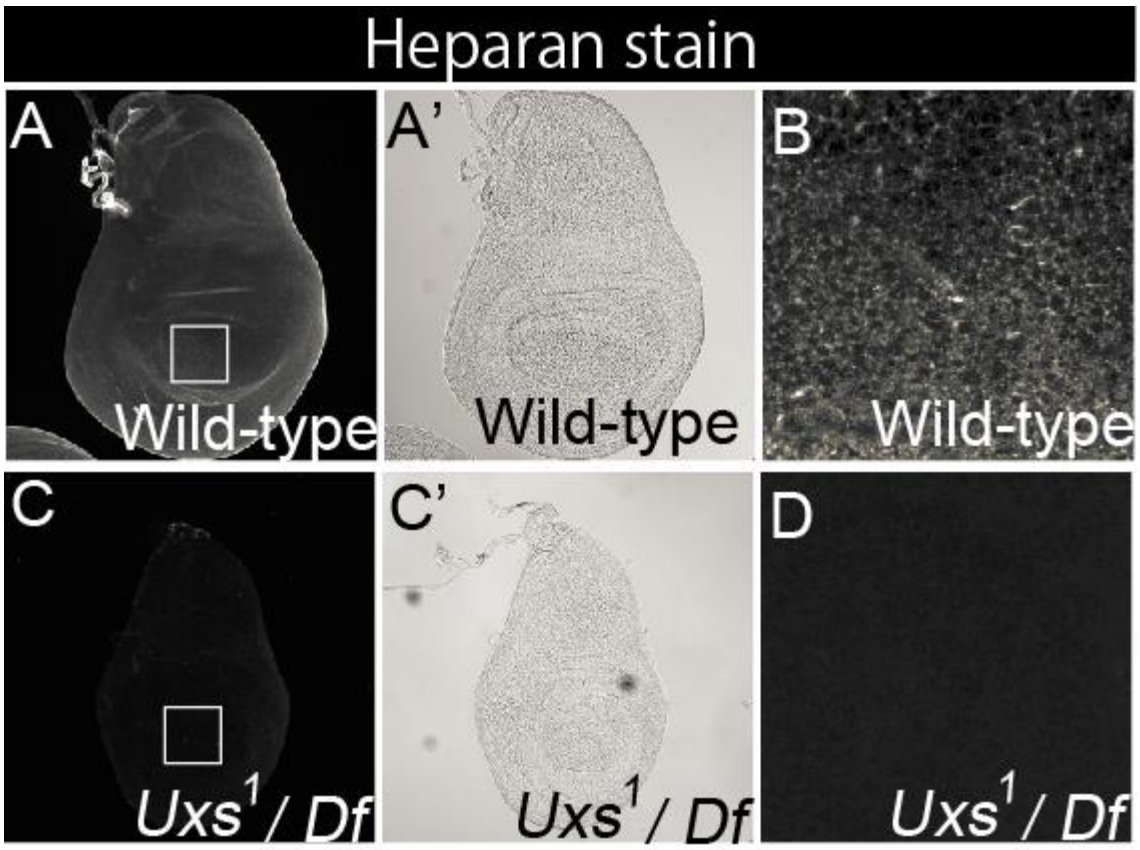

Fig. 13: Hepasan sulfate are missing in *Uxs* mutant

(A-D) Wild-type (A, A', and B) and *Uxs*^{1}/*Df(3L)Exel6112* (C, C', and D) wing discs stained with an anti-heparan sulfate antibody. A' and C' show optic-microscope images of A and C, respectively. B and D show magnified views of the white squares in A and C, respectively.

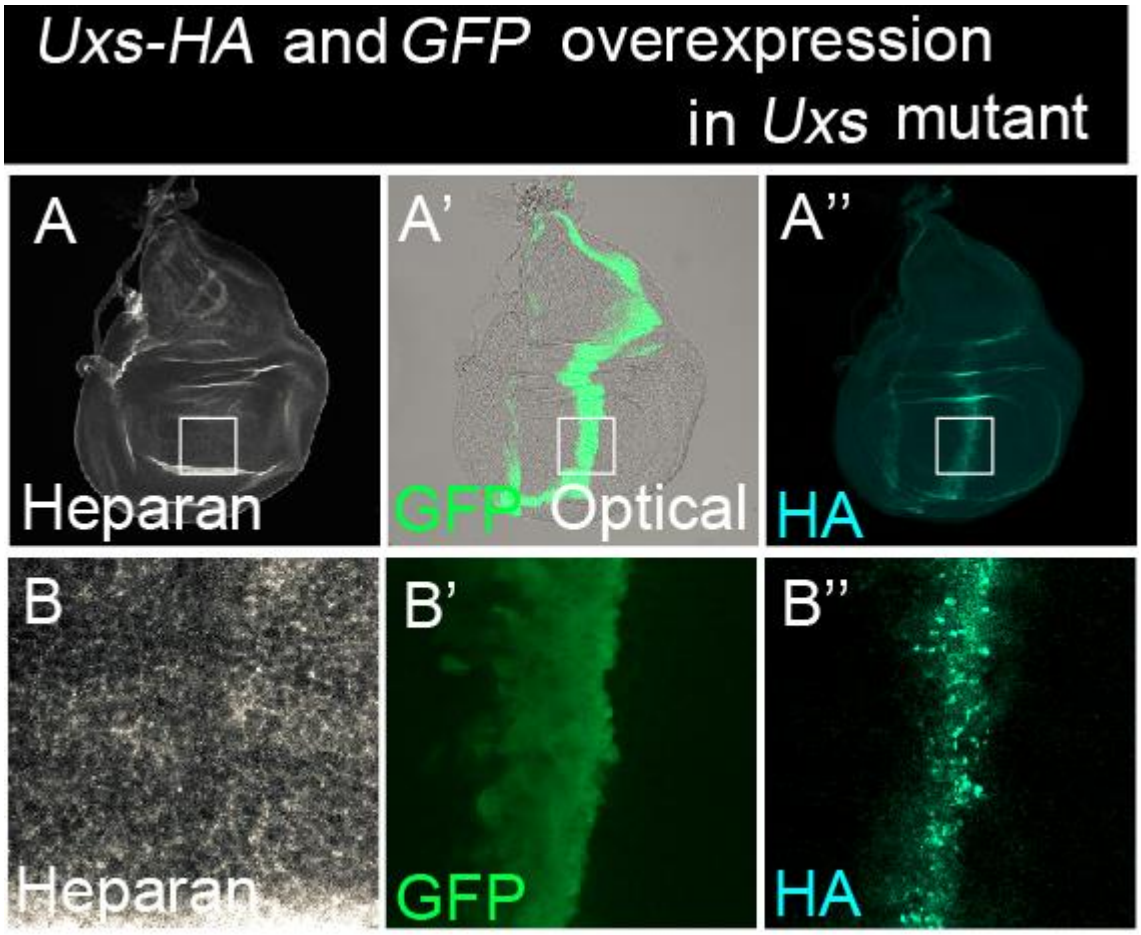

Fig. 14: *Uxs*^{1} phenotype are rescued by ectopic expression of Uxs

Third instar wing discs isolated from homozygote for *Uxs*^{1} were stained with anti-heparan sulfate antibody (A-B). Wing disc overexpressing *UAS-Uxs-HA* and *UAS-GFP* under the control of *ptc-Gal4* (A-B''). Anti-heparan sulfate antibody staining (A), anti-GFP antibody staining (A'), showing where *UAS-Uxs-HA* expression was driven in optical microscopic image, anti-HA antibody staining (A'') to detect the Uxs-HA protein. B, B' (fluorescence image), and B'' are higher magnification of the eras indicated by white squares in A, A', and A'', respectively.

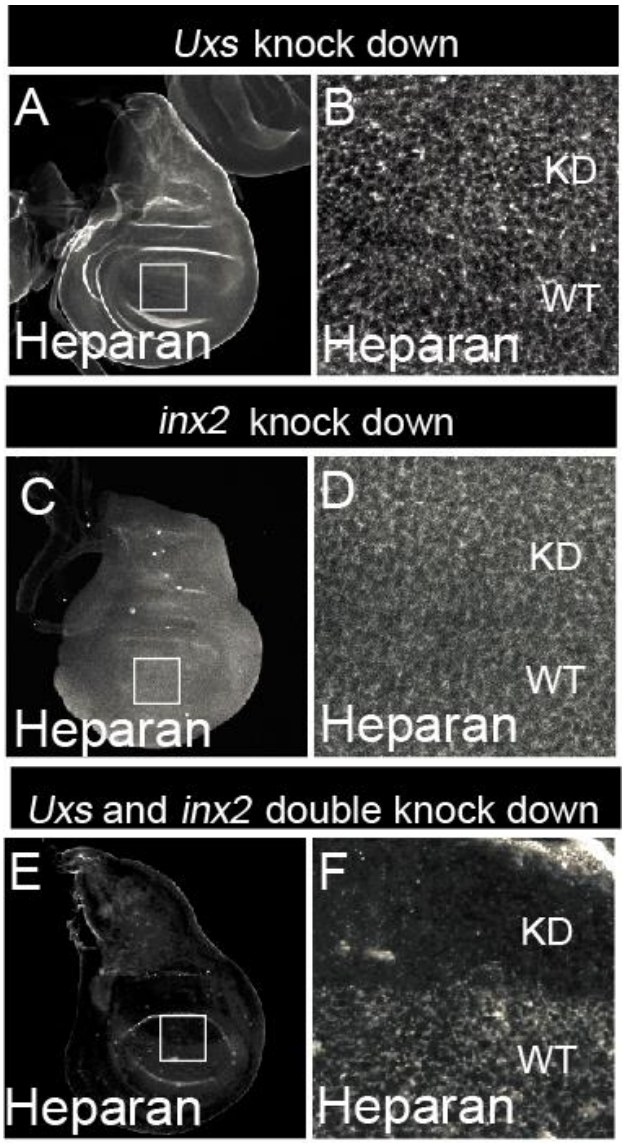

Fig. 15: UDP-xylose is diffused intercellularly through gap junction

Third instar wing discs isolated from *Uxs* (A and B), *inx2* (C and D), and *Uxs* and *inx2* (E and F) were knocked down in the notum and dorsal wing pouch region, under the control of *ap-Gal4*. B, D, and F are higher magnification of the eras indicated by white squares in A, C, and E, respectively. In A, C, and E, upper and lower parts of the images correspond to the arias where each gene was knocked down (indicated by KD) and to the eras of wild type (WT), respectively. All wing discs were dissected out from larvae raised at 25 °C.

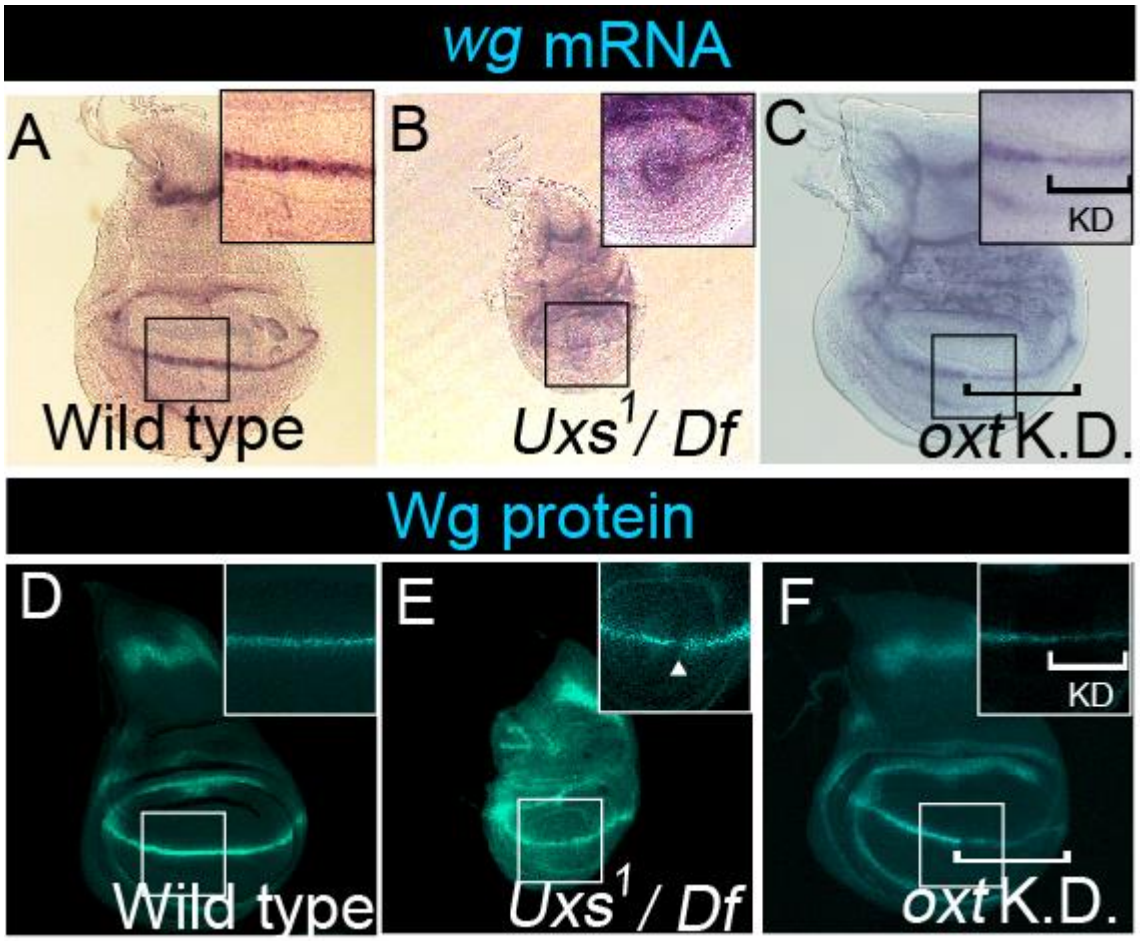

Fig. 16: Notch signaling is normal in D/V boundary when xylose modifications are missing

(A-F)Wing imaginal discs from Wild-type (A and D), *Uxs*^{1}*/Df(3L)Exel6112* (B and E), and and *oxt* knock-down (C and F). (A-C) Expression of *wg*, detected by *in situ* hybridization (Blue) in wild-type (A), *Uxs*^{1}*/Df(3L)Exel6112* (B), and *oxt* knock-down (C) wing discs. Insets show magnified views of the black squares. Black bucket in panel C indicate *oxt* knock-down region (C). (D-F) Distribution of Wg protein, detected by anti-Wg antibody staining (blue) in wild-type (C), *Uxs*^{1}*/Df(3L)Exel6112* (D), and and *oxt* knock-down (F) wing discs; insets show magnified views of the white squares. White arrowhead indicates a gap between Wg protein-expressing cells along the boundary of the dorsal and ventral compartments. White bucket in panel F indicate *oxt* knock-down region. All wing discs were isolated from third-

instar larvae at 25 °C except RNAi analysis. *hh-Gal4* (C and F) was used for *oxt* knock-down. All *oxt* knock-downed wing discs were isolated from third-instar larvae at 30 °C.

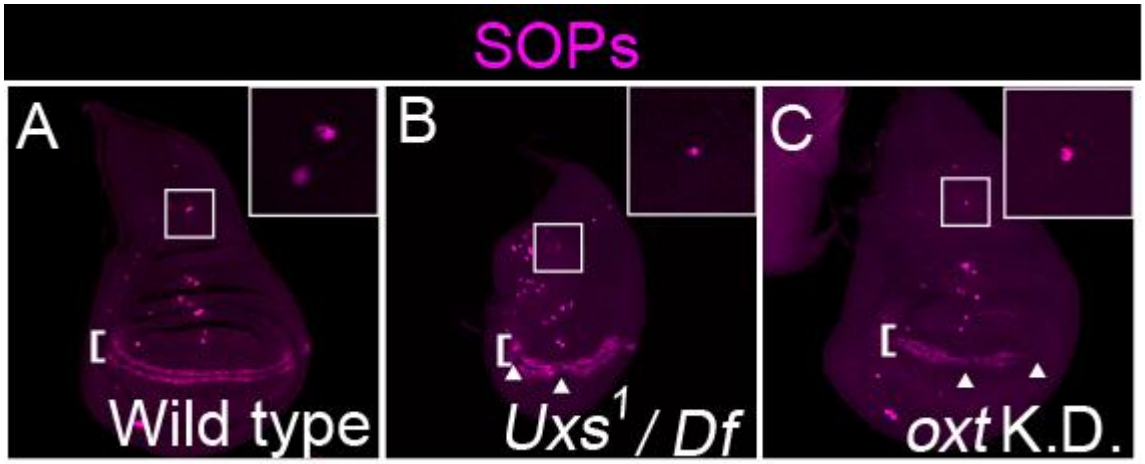

Fig. 17: The number of SA neurons are normal when xylose modifications are missing

(A-C) SOPs in wing discs were detected by anti-Sens antibody staining (magenta). Wing discs from wild type (A), *Uxs*^{1} homozygote (B), and *oxt* knock-down (C) were isolated from third-instar larvae. Insets show magnified views of the areas in white squares, which contained SA-neuron precursors. White arrowhead indicates a gap between Sens protein-expressing cells along the boundary of the dorsal and ventral compartments. All wing discs were isolated from third-instar larvae at 25 °C except RNAi analysis. *ap-Gal4* (C) was used for *oxt* knock-down. All *oxt* knock-downed wing discs were isolated from third-instar larvae at 30 °C.

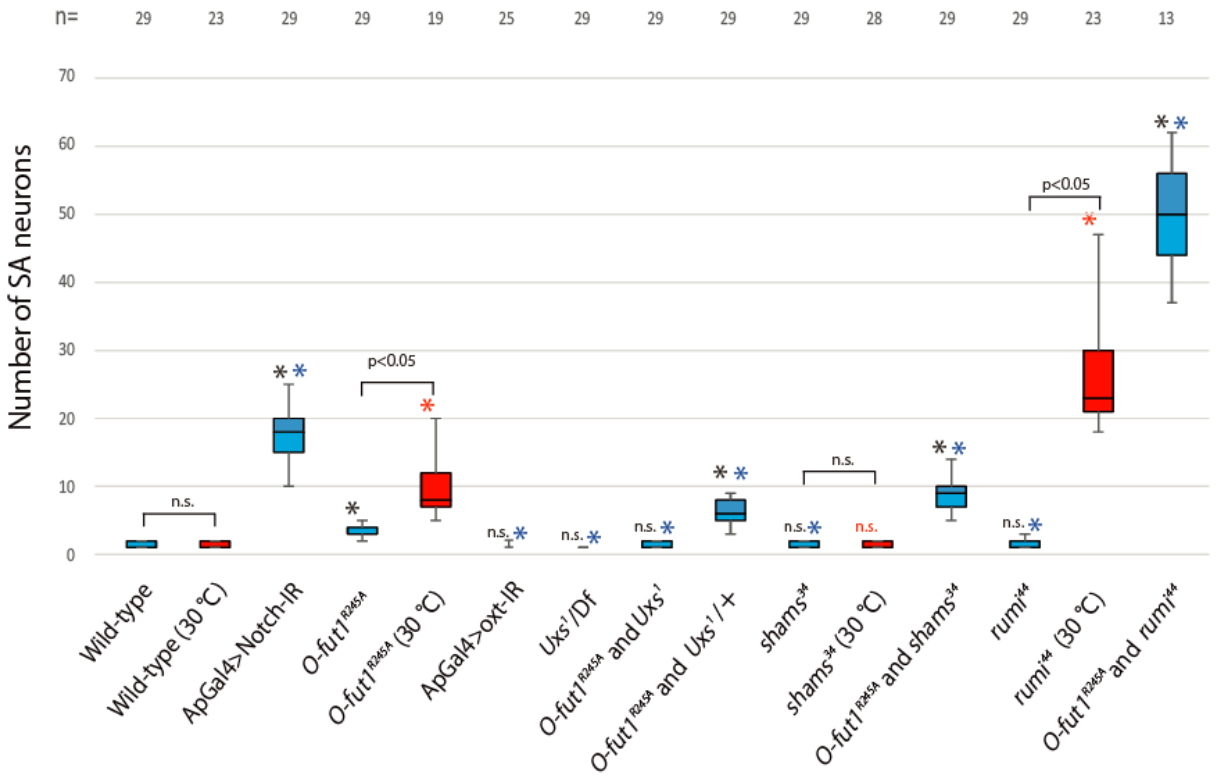

Fig. 18: Box plots of the number of SA neurons

(vertical axis) for each genotype (across the bottom). SA neurons were identified by anti-Sens antibody staining in wing discs isolated from third-instar larvae raised at 25 °C (blue boxes) or 30 °C (red boxes); black asterisks and n.s. indicate $*p < 0.05$ and no significant difference, respectively, compared with wild type at 25 °C. Blue asterisks indicate $*p < 0.05$ compared with *O-fut1*^{R245A} homozygotes at 25 °C. Red asterisks and n.s. indicate $*p < 0.05$ and no significant difference, respectively, compared with wild type at 30 °C.

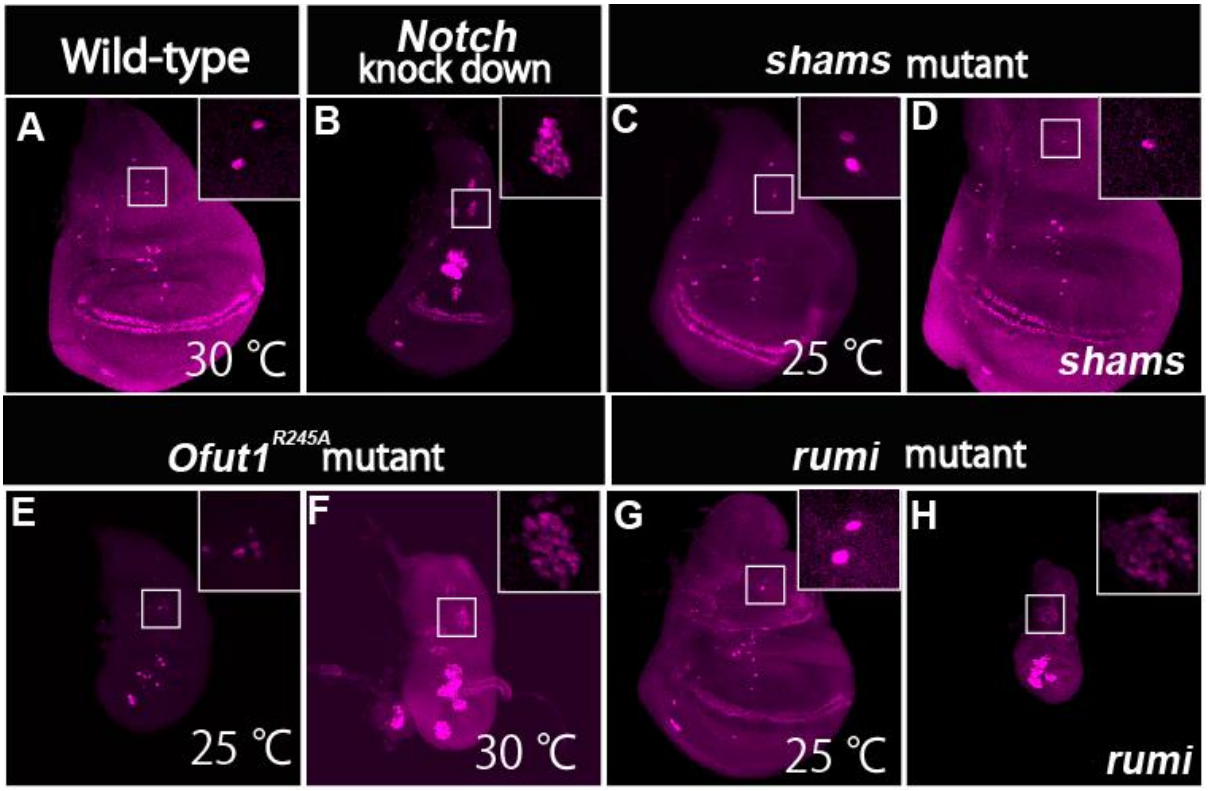

Fig. 19: The *O*-glucose glycan plays multiple roles in Notch-signaling activation and shows redundancy with monosaccharide *O*-fucose

(A-I) SOPs in wing discs were detected by anti-Sens antibody staining (magenta). (A-H) Wing discs from a Wildtype (A), *Notch* knock down (B), *shams*^{34}homozygote (C and D), *O-fut1*^{R245A} homozygote (E and F), , *rumi*^{44} homozygote (G and H); the wing discs were isolated from larvae raised at 25 °C (C, E, and G) and 30 °C (A, B, D, F, and H). Insets show magnified views of the areas in white squares, which contained SA-neuron precursors.

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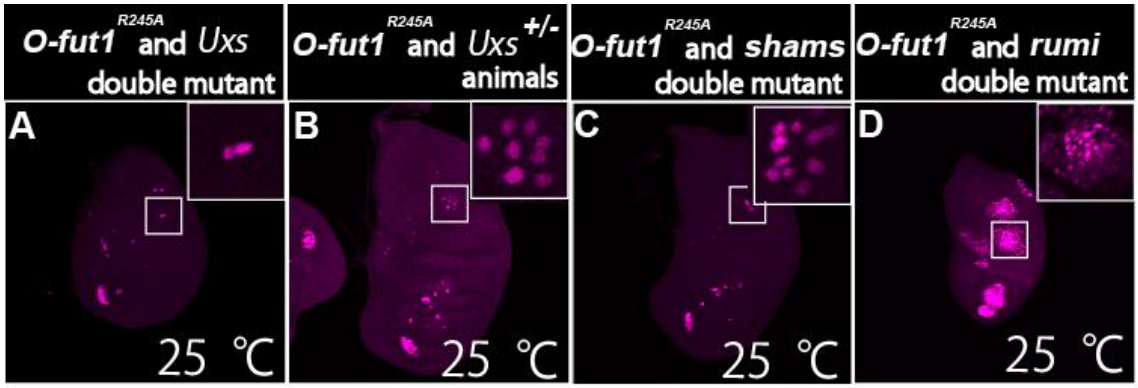

Fig. 20: Monosaccharide *O*-fucose have redundant role with monosaccaride *O*-glucose and terminal dixylose modification of Notch for Notch signaling activation

(A-D) SOPs in wing discs were detected by anti-Sens antibody staining (magenta). (A-D) Wing discs from *O-fut1*^{R245A} and *Uxs*^{1} double homozygote (A), *O-fut1* ^{R245A}/*O-fut1* ^{R245A}; *Uxs*/+ (B), *O-fut1*^{R245A} and *shams*^{34} double homozygote (C), and *O-fut1*^{R245A} and *rumi*^{44} double homozygote (D); the wing discs were isolated from larvae raised at 25 °C. Insets show magnified views of the areas in white squares, which contained SA-neuron precursors.

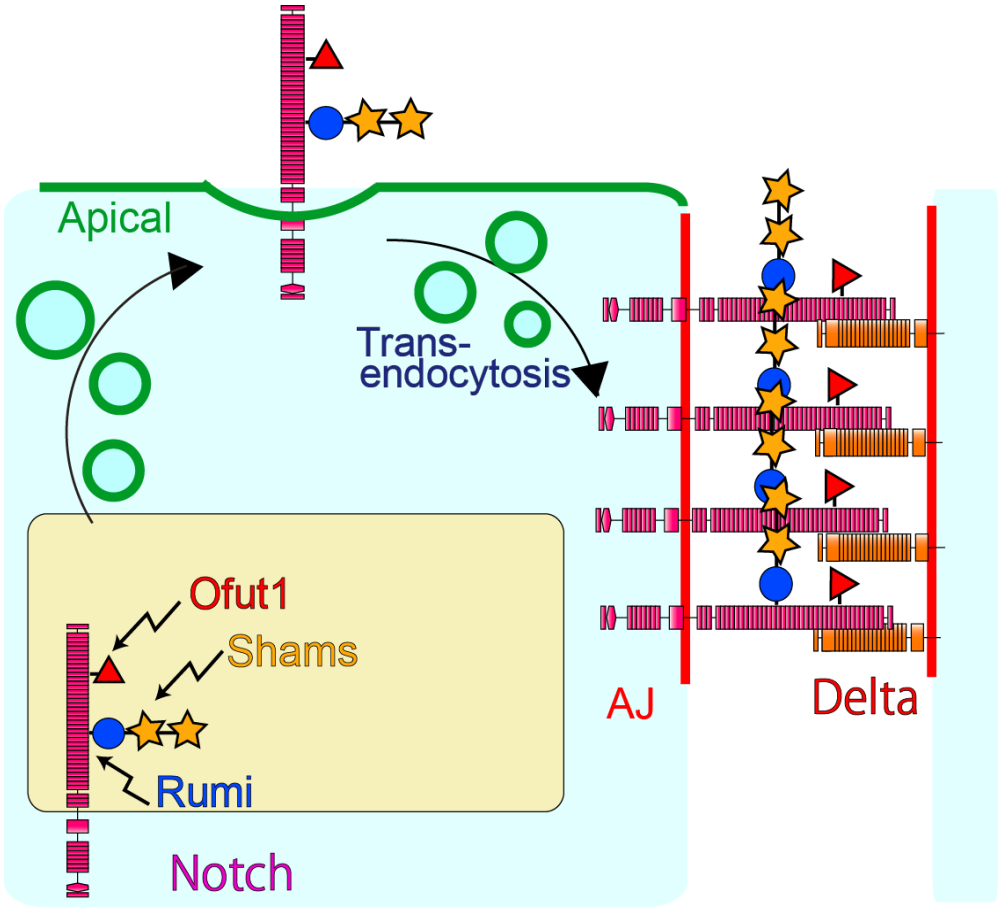

Fig. 21: Notch specifically localizes to AJs

Correct Notch transportation are essential for correct Notch signaling activation. First Notch are post-translational modification like glycan modification. Second, Notch is translocated to cell membrane. Last, Notch translocated apical cell membrane to Adherence junction by trans-endocytosis. And Notch bind its ligand Delta/ Serrate.

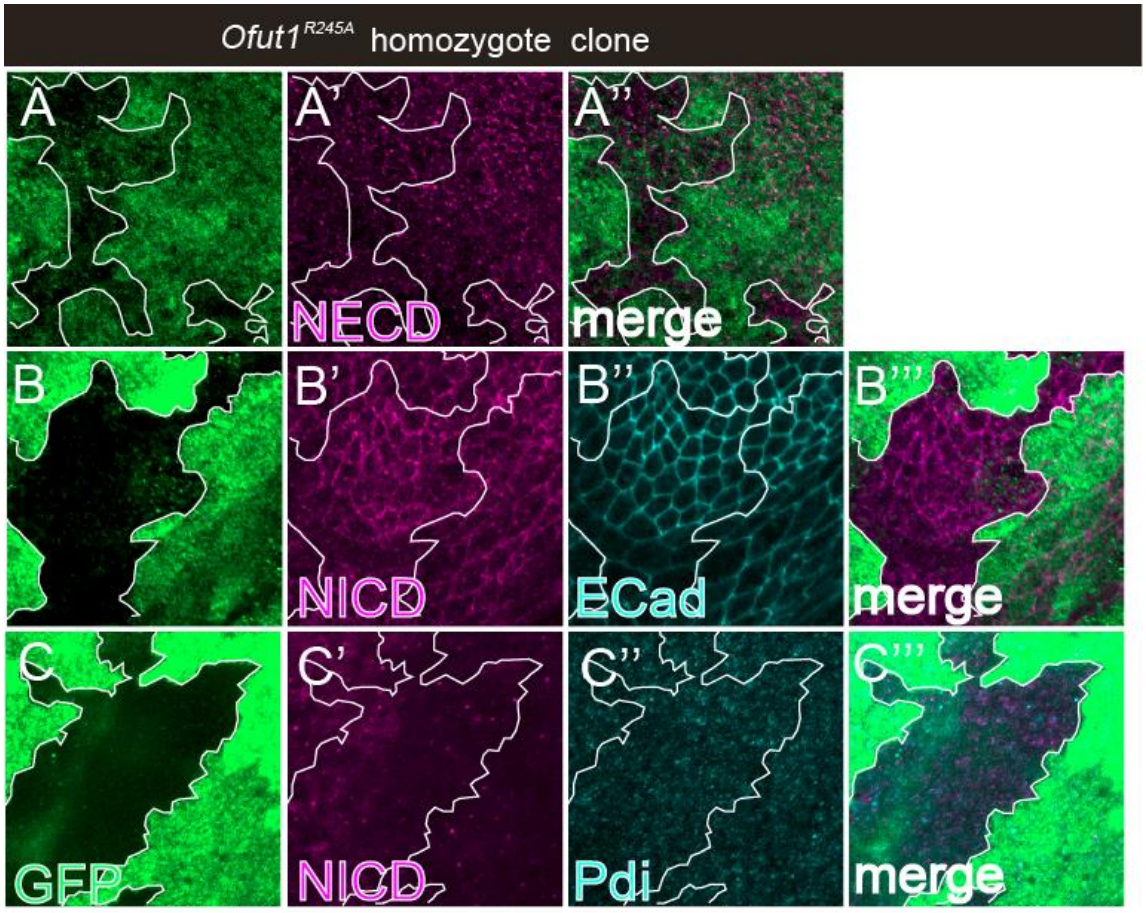

Fig. 22: Missing of monosaccharide *O*-fucose on Notch doesn't affect Notch trafficking at 25 °C

Wing discs with somatic clones homozygous for *O-fut1*^{R245A} (A-C''') were stained with antibodies against NECD (A' and A''), NICD (B', B''', C', and C'''), *D*E-cad (B''), and Pdi (C'', and C'''). Optical images show planes corresponding to the apical membrane (A-A''), AJs (B-B'''), and the medial region including the ER (C-C'''). Mosaic clones of mutant cells are indicated by the absence of GFP. Clone boundaries are indicated by white lines. Wing discs were isolated from larvae raised at 25 °C. A'', B''', and C''' show merged images from A and A', B and B', and C-C'' respectively.

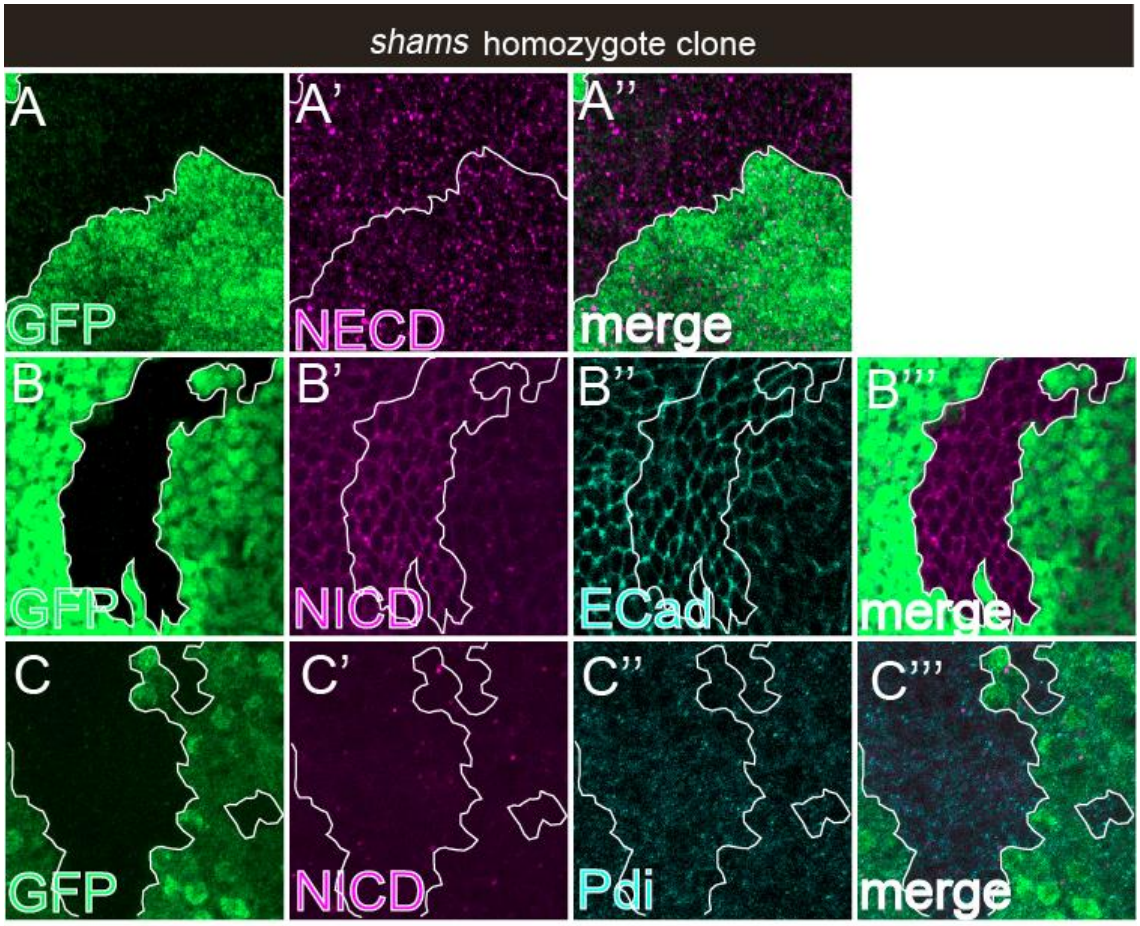

Fig. 23: Missing of terminal dixylose of *O*-glucose-linked saccharides on Notch doesn't affect Notch trafficking at 25 °C

Wing discs with somatic clones homozygous for *shams* (A-C''') were stained with antibodies against NECD (A' and A''), NICD (B', B''', C', and C'''), *D*E-cad (B''), and Pdi (C'', and C'''). Optical images show planes corresponding to the apical membrane (A-A''), AJs (B-B'''), and the medial region including the ER (C-C'''). Mosaic clones of mutant cells are indicated by the absence of GFP. Clone boundaries are indicated by white lines. Wing discs were isolated from larvae raised at 25 °C. A'', B''', and C''' show merged images from A and A', B and B', and C-C'' respectively.

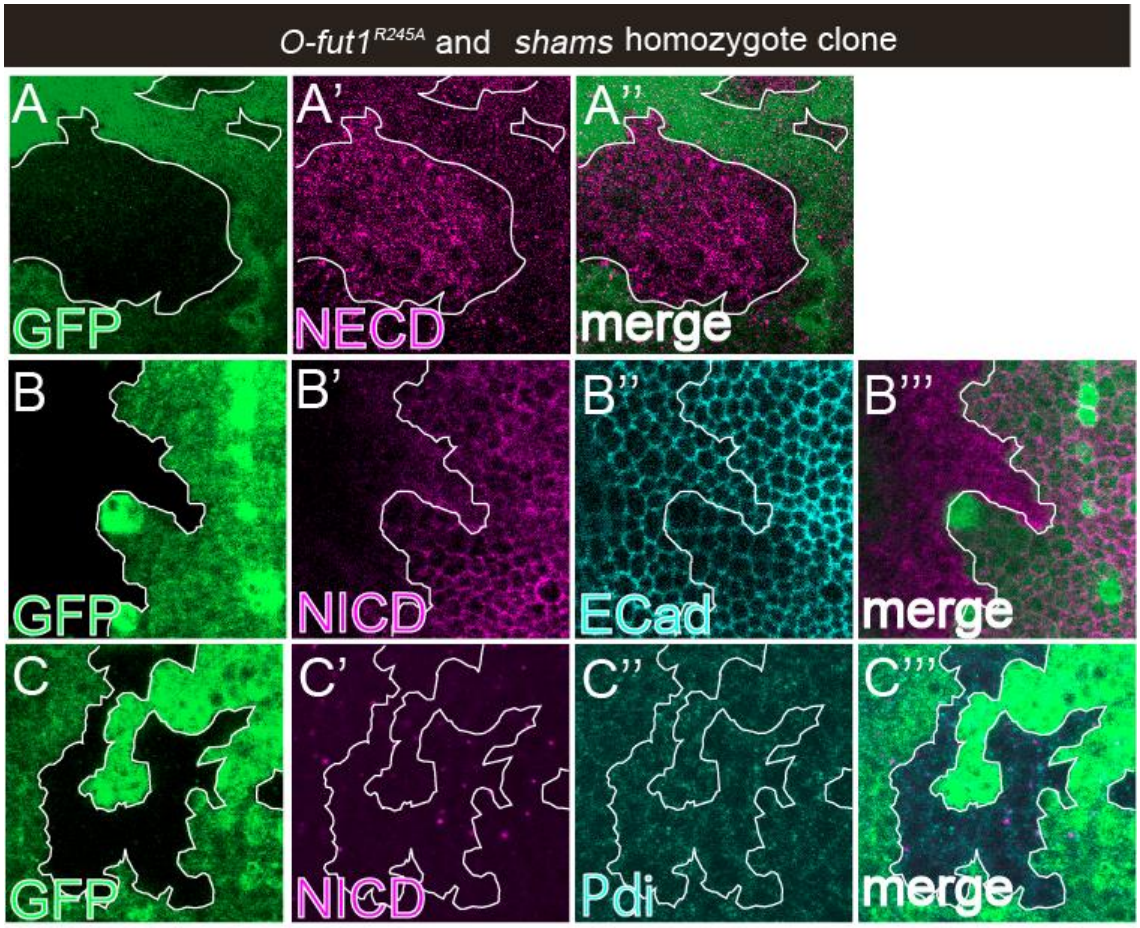

Fig. 24: Monosaccharide *O*-fucose and the terminal dixylose of *O*-glucose-linked saccharides **play redundant roles in relocating Notch from the apical plasma membrane to AJs at 25 °C**

Wing discs with somatic clones homozygous for double-homozygous for *O-fut1*^{R245A} and *shams* (A-C’’’) were stained with antibodies against NECD (A’ and A’’), NICD (B’, B’’’, C’, and C’’’), *D*E-cad (B’’), and Pdi (C’’, and C’’’). Optical images show planes corresponding to the apical membrane (A-A’’), AJs (B-B’’’), and the medial region including the ER (C-C’’’). Mosaic clones of mutant cells are indicated by the absence of GFP. Clone boundaries are indicated by white lines. Wing discs were isolated from larvae raised at 25 °C. A’’, B’’’, and C’’’ show merged images from A and A’, B and B’, and C-C’’ respectively.

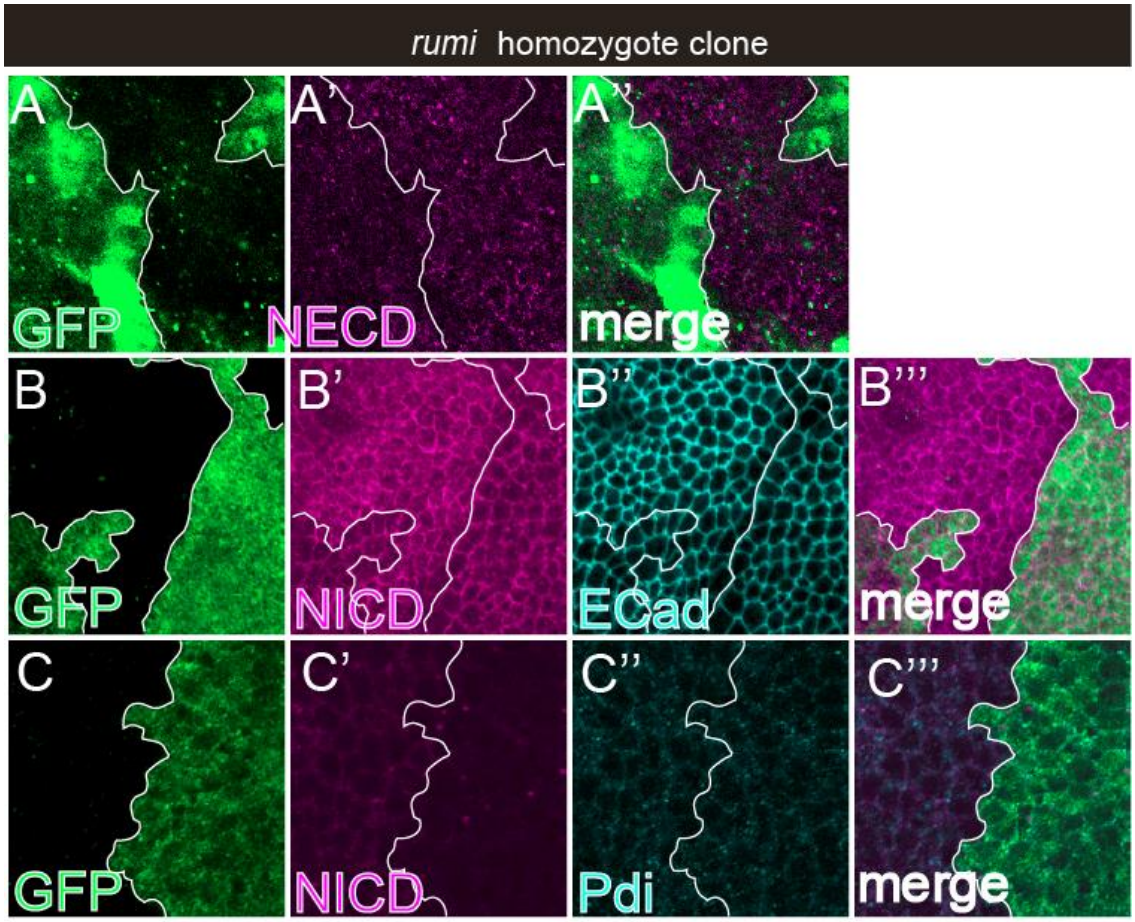

Fig. 25: Missing of monosaccharide *O*-glucose on Notch affect Notch trafficking at 25 °C

(A-F''') homozygous for *rumi*^{44} (A-C''') were stained with antibodies against NECD (A' and A''), NICD (B', B''', C', and C'''), *D*E-cad (B''), and Pdi (C'', and C'''). Optical images show planes corresponding to the apical membrane (A-A''), AJs (B-B'''), and the medial region including the ER (C-C'''). Mosaic clones of mutant cells are indicated by the absence of GFP. Clone boundaries are indicated by white lines. Wing discs were isolated from larvae raised at 25 °C. A'', B''', and C''' show merged images from A and A', B and B', and C-C'' respectively.

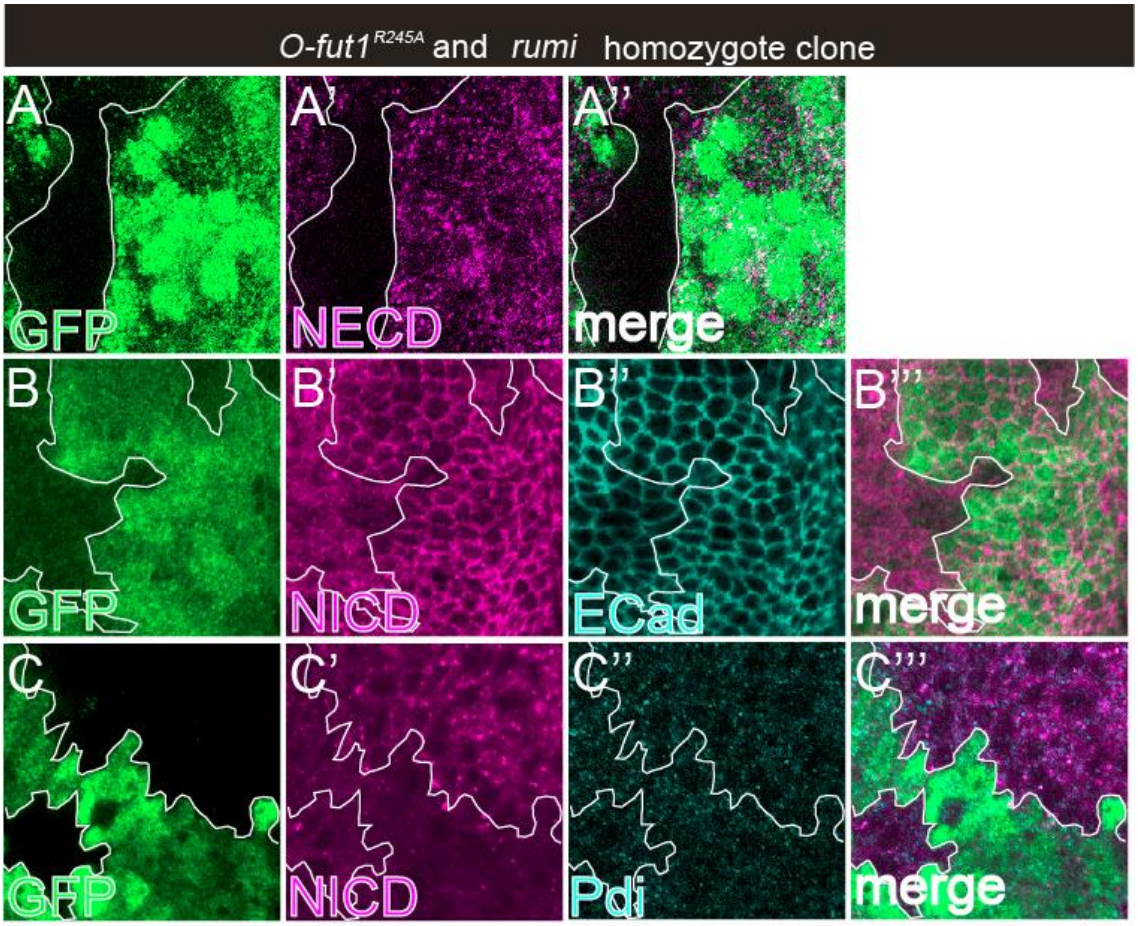

Fig. 26: Monosaccharide *O*-glucose and *O*-fucose play redundant roles in exporting Notch from the ER at 25 °C

(A-F’’’) double-homozygous for *O-fut1*^{R245A} and *rumi*^{44} (A-C’’’) were stained with antibodies against NECD (A’ and A’’), NICD (B’, B’’’, C’, and C’’’), *D*E-cad (B’’), and Pdi (C’’, and C’’’). Optical images show planes corresponding to the apical membrane (A-A’’), AJs (B-B’’’), and the medial region including the ER (C-C’’’). Mosaic clones of mutant cells are indicated by the absence of GFP. Clone boundaries are indicated by white lines. Wing discs were isolated from larvae raised at 25 °C. A’’, B’’’, and C’’’ show merged images from A and A’, B and B’, and C-C’’ respectively.

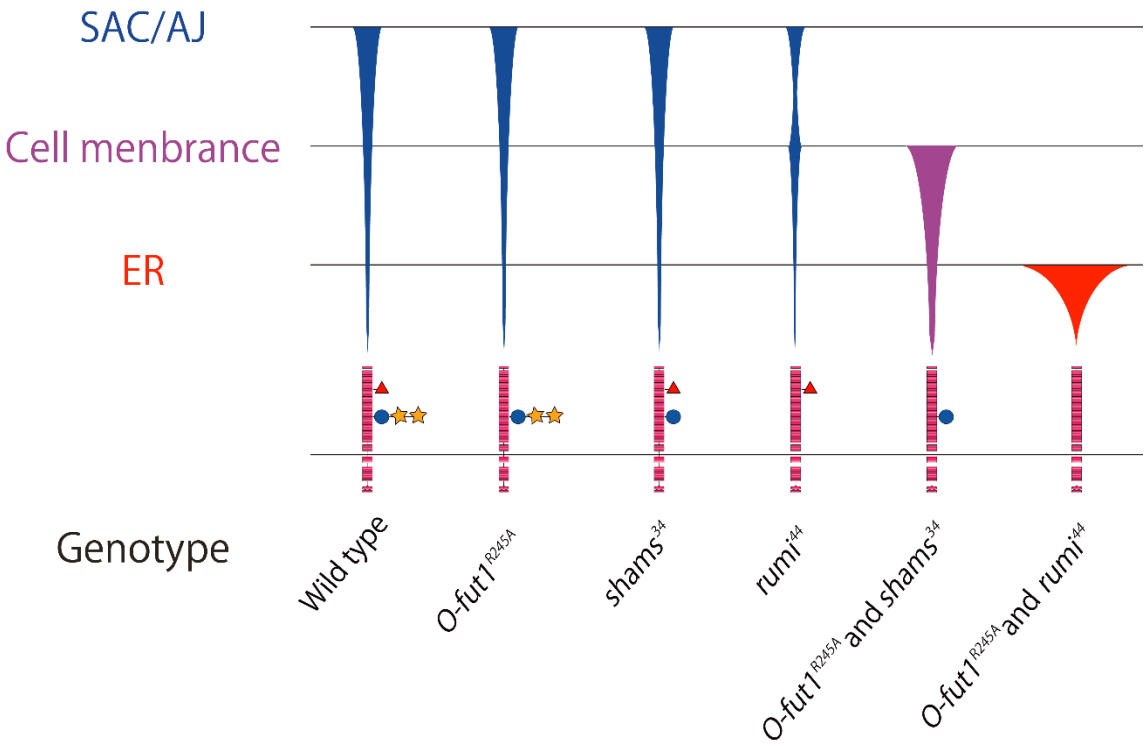

Fig. 27: Graphical representation of Notch distribution in each mutant

Schematic showing the final destinations of Notch transport at 25 °C in various genotypes (indicated at the bottom). SAC/AJ, sub-apical complex/adherens junction; ER, endoplasmic reticulum. Symbols: pink boxes, EGF-like repeats; red triangles, *O*-fucosylation; blue circles, *O*-glucosylation; and red triangles, *O*-fucose.

Table 1: Proteins containing EGF-like repeats with consensus sequence of *O*-fucose and *O*-glucose glycan modifications

Name	The number of EGF-like repeats	Structure or Function
Crumbs	29	Transmembrane protein
Cubilin	6	Transmembrane protein
Dumpy	12	Transmembrane protein
Eyeshut	13	Transmembrane protein
Delta	5	Delta/Serrate/lag-2 (DSL) protein-like
Serrate	7	Delta/Serrate/lag-2 (DSL) protein-like
Weary	6	Notch binding?
Fat	3	Cadherin-like
Kugelei (Fat2)	5	Cadherin-like
CG31999	7	Fibrillin-like
LDL receptor protein 1	6	LDL receptor
Arrow	2	LDL receptor-like
Lipophorin receptor 1	3	LDL receptor-like
Lipophorin receptor 2	3	LDL receptor-like
Slit	5	Laminin G-like
Faulty attraction	11	Matrix metalloprotease
Uninflatable	12	tenascin family protein
Tenascin accessory	6	tenascin family protein
Shifted	5	Wnt inhibitory factor-1like