Genes to Cells

Phenotype-based clustering of glycosylation-related genes by RNAi-mediated gene silencing

Miki Yamamoto-Hino[1,2†], Hideki Yoshida[2,3,4†], Tomomi Ichimiya[3], Sho Sakamura[5], Megumi Maeda[5], Yoshinobu Kimura[5], Norihiko Sasaki[3,6], Kiyoko F. Aoki-Kinoshita[3], Akiko Kinoshita-Toyoda[2,7], Hidenao Toyoda[2,7], Ryu Ueda[2,8], Shoko Nishihara[2,3]* and Satoshi Goto[1,2]*

[1]*Department of Life Science, Rikkyo University, Toshima-ku, Tokyo, Japan*
[2]*Core Research for Evolutional Science and Technology (CREST) of Japan Science and Technology Agency (JST), Kawaguchi, Saitama, Japan*
[3]*Department of Bioinformatics, Faculty of Engineering, Soka University, Hachioji, Tokyo, Japan*
[4]*Department of Applied Biology, Insect Biomedical Research Center, Kyoto Institute of Technology, Sakyo-ku, Kyoto, Japan*
[5]*Department of Biofunctional Chemistry, Graduate School of Environmental and Life Science, Okayama University, Okayama, Japan*
[6]*Research Team for Geriatric Medicine (Vascular Medicine), Tokyo Metropolitan Institute of Gerontology, Itabashi-ku, Tokyo, Japan*
[7]*College of Pharmaceutical Sciences, Ritsumeikan University, Kusatsu, Shiga, Japan*
[8]*Invertebrate Genetics Laboratory, National Institute of Genetics, Mishima, Shizuoka, Japan*

Glycan structures are synthesized by a series of reactions conducted by glycosylation-related (GR) proteins such as glycosyltransferases, glycan-modifying enzymes, and nucleotide-sugar transporters. For example, the common core region of glycosaminoglycans (GAGs) is sequentially synthesized by peptide-*O*-xylosyltransferase, β1,4-galactosyltransferase I, β1,3-galactosyltransferase II, and β1,3-glucuronyltransferase. This raises the possibility that functional impairment of GR proteins involved in synthesis of the same glycan might result in the same phenotypic abnormality. To examine this possibility, comprehensive silencing of genes encoding GR and proteoglycan core proteins was conducted in *Drosophila*. *Drosophila* GR candidate genes (125) were classified into five functional groups for synthesis of GAGs, *N*-linked, *O*-linked, Notch-related, and unknown glycans. Spatiotemporally regulated silencing caused a range of malformed phenotypes that fell into three types: extra veins, thick veins, and depigmentation. The clustered phenotypes reflected the biosynthetic pathways of GAGs, Fringe-dependent glycan on Notch, and glycans placed at or near nonreducing ends (herein termed terminal domains of glycans). Based on the phenotypic clustering, CG33145 was predicted to be involved in formation of terminal domains. Our further analysis showed that CG33145 exhibited galactosyltransferase activity in synthesis of terminal *N*-linked glycans. Phenotypic clustering, therefore, has potential for the functional prediction of novel GR genes.

Introduction

A wide variety of glycans play important roles in a diverse range of biological processes, such as organ development (Haltiwanger & Lowe 2004), lymphocyte homing (Carlow *et al.* 2009), and cancer invasion (Isaji *et al.* 2010), by regulating protein–protein, lipid–protein, and cell–cell interactions. Glycans are synthesized by sequential reactions conducted by glycosylation-related (GR) proteins such as glycosyltransferases, glycan-modifying enzymes, and nucleotide-sugar transporters (Nishihara 2007; Yamamoto-Hino *et al.* 2012). Accordingly, different glycan structures are synthesized by different sets of GR proteins. Thus, it is likely that mutation of GR genes involved in synthesis of the same glycans will result in the same phenotype. For example, glycosaminoglycans (GAG) are

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**Correspondence:* stgoto@rikkyo.ac.jp or shoko@soka.ac.jp
†These authors contributed equally to this work.

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sequentially synthesized by peptide-*O*-xylosyltransferase, β1,4-galactosyltransferase I, β1,3-galactosyltransferase II, and β1,3-glucuronyltransferase (Nishihara 2010; Mikami & Kitagawa 2013). Mutations of these GAG synthesizing enzymes principally impair the same developmental pathways, namely those regulated by decapentaplegic, wingless, hedgehog, and fibroblast growth factor in *Drosophila* (Haltiwanger & Lowe 2004; Nishihara 2010; Yamamoto-Hino *et al.* 2012). However, because complete sets of GR gene mutants are not available in metazoa, no comprehensive examination has yet been undertaken to determine whether impairment of GR genes involved in synthesis of the same glycans results in the same phenotypes.

It is possible to silence almost all the genes in *Drosophila* and *Caenorhabditis elegans* by RNA interference (RNAi) (Yamamoto-Hino & Goto 2013). In particular, spatiotemporally regulated gene silencing is possible in *Drosophila* when it is implemented using the Gal4/upstream activation sequence (UAS) system (Brand & Perrimon 1993). In this system, the yeast Gal4 transcription factor binds to the UAS and activates expression of the downstream gene; theoretically, the gene downstream of the UAS is not expressed in the absence of Gal4. Consequently, a genetic cross between UAS- and Gal4-fly strains will induce expression of the gene downstream of the UAS. By placing genes expressing hairpin RNAs downstream of a UAS, RNAi is readily induced by genetic crossing. In addition, there are a large number of Gal4 strains in which the Gal4 gene is conditionally expressed, such as in a specific tissue, at a particular developmental stage, or under specific temperature conditions (Hayashi *et al.* 2002). Therefore, spatiotemporal patterns and levels of expression of hairpin RNAs can be controlled by the Gal4 strains and temperature conditions used.

In this study, we determined 120 *Drosophila* GR genes and five core proteins by sequence similarity searches and literature mining. Of these GR genes, 72 were silenced in the whole body. Silencing of 56 of these genes resulted in lethality before eclosion. Thus, it was not possible to assess phenotypic clustering of essential GR genes when genes were silenced in the whole organism. To overcome this difficulty, spatiotemporally regulated gene silencing was carried out using several Gal4 driver strains. The induced phenotypes were linked to the biosynthetic pathways of GAGs, Fringe-dependent glycan on Notch, and terminal domains of glycans. Based on this phenotypic clustering, the functionally unknown gene *CG33145* was predicted to be involved in the synthesis of terminal domains. Our biochemical analysis provided direct evidence that CG33145 functioned as a novel galactosyltransferase in terminal *N*-linked glycan synthesis. In summary, phenotypic clustering in this study proved useful for functional prediction of novel GR genes.

Results

Drosophila GR genes

Drosophila GR genes (67) were identified through similarity to human glycosylation genes using the human GlycoGene DataBase (http://jcggdb.jp/rcmg/ggdb/). The *Drosophila* GR gene set comprised 54 glycosyltransferases, seven glycan-modifying enzymes, and six nucleotide-sugar transporters. In addition, we manually identified *Drosophila* genes encoding 44 glycosyltransferases, eight glycan-modifying enzymes, one sugar-nucleotide transporter, and five core proteins from literature searches. In total, 98 glycosyltransferases, fifteen glycan-modifying enzymes, seven sugar-nucleotide transporters, and five core protein genes were identified (Table 1). Based on biochemical activities that were directly measured or predicted from homologous mammalian genes, 108 of these 125 GR proteins could be assigned to the following categories: formation of sugar linkages, modification of glycans, or core proteins (Fig. 1, Table 1).

There are structural variants of *N*-linked glycans. Aoki and colleagues determined the number of *N*-linked glycan variants in *Drosophila* embryo using mass spectrometry (Aoki *et al.* 2007). The authors detected GlcNAc structures that were synthesized by Mgat1, Mgat2, and Mgat4, and also observed extended forms such as Galβ-3GlcNAc and SAα2-6Galβ-3GlcNAc. However, no terminal GlcNAc structures synthesized by Mgat3, Mgat5, or Mgat6 were detected. Accordingly, sequence comparisons showed the absence of *Mgat5* and *Mgat6* in *Drosophila*, and expression of *Mgat3* was very low (Flybase). In addition, a small amount of *N*-linked glycans was capped by LacdiNAc (GalNAc-GlcNAc) or GlcA in the *Drosophila* embryo (Aoki & Tiemeyer 2010). LacdiNAc was also found in arthro-series glycosphingolipids in embryo. LacdiNAc structures on glycoproteins and glycosphingolipids were synthesized by *Drosophila* β4GalNAcTA (Sasaki *et al.* 2007).

Gene silencing in the whole *Drosophila* body

To examine the phenotypes caused by silencing of GR genes, we established RNAi-inducible fly strains for 72

Table 1 *Drosophila* GR genes

Family of proteins/protein name	Protein/gene name	CG No.	References	Glycan structure	Mammalian orthologue
Glycosyltransferase					
N-acetylgalactosaminyltransferase					
UDP-GalNAc:polypeptide	pgant1/GalNAc-T1	CG8182	Ten Hagen *et al.* 2003	Mucin-type *O*-glycan	
N-acetylgalactosaminyltransferase	pgant2	CG3254	Ten Hagen *et al.* 2003	Mucin-type *O*-glycan	GALNT2
	pgant3	CG4445	Ten Hagen *et al.* 2003	Mucin-type *O*-glycan	
	pgant4	CG31956	Ten Hagen *et al.* 2003	Mucin-type *O*-glycan	
	pgant5	CG31651	Ten Hagen *et al.* 2003	Mucin-type *O*-glycan	GALNT5
	pgant6	CG2103	Ten Hagen *et al.* 2003	Mucin-type *O*-glycan	GALNT1
	pgant7/GalNAc-T2	CG6394	Schwientek *et al.* 2002; Ten Hagen *et al.* 2003	Mucin-type *O*-glycan	GALNT7
	pgant8	CG7297	Ten Hagen *et al.* 2003	Mucin-type *O*-glycan	
	pgant35A	CG7480	Schwientek *et al.* 2002; Ten Hagen *et al.* 2003	Mucin-type *O*-glycan	GALNT11
	dppGalNAcT9	CG30463	ND	Mucin-type *O*-glycan	GALNT3
	dppGalNAcT10	CG10000	ND	Mucin-type *O*-glycan	
		CG31776	ND	Mucin-type *O*-glycan	
	dppGalNAcT11	CG7304	ND	Mucin-type *O*-glycan	
	dppGalNAcT12	CG7579	ND	Mucin-type *O*-glycan	
α1,4-*N*-acetylgalactosaminyltransferase	α4GT1	CG17223	Mucha *et al.* 2004	Glycolipid	A4GALT
	α4GT2	CG5878	Chen *et al.* 2007	Glycolipid	
β1,4-*N*-acetylgalactosaminyltransferase	β4GalNAcTA	CG8536	Haines & Irvine 2005; Chen *et al.* 2007; Sasaki *et al.* 2007	Glycolipid, *N*-glycan	B4GALT2
	β4GalNAcTB	CG14517	Haines & Irvine 2005; Chen *et al.* 2007	Glycolipid	B4GALT3
N-acetylglucosaminyltransferase					
UDP-GlcNAc:polypeptide *O*-β-*N*-acetylglucosaminyltransferase	dO-GnT/Sxc	CG10392	Sinclair *et al.* 2009★	*O*-GlcNAc	OGT
α3-*D*-mannoside-β1,2-*N*-acetylglucosaminyltransferase	dMGAT1/Mgat1	CG13431	Sarkar & Schachter 2001; Ichimiya *et al.* 2004	*N*-glycan	MGAT1
α6-*D*-mannoside-β1,2-*N*-acetylglucosaminyltransferase	dMGAT2/Mgat2	CG7921	Ichimiya *et al.* 2004	*N*-glycan	MGAT2
β4-*D*-mannoside-β1,4-*N*-acetylglucosaminyltransferase	dMGAT3	CG31849	ND	*N*-glycan	MGAT3
α3-*D*-mannoside-β1,4-*N*-acetylglucosaminyltransferase	dMGAT4-1	CG9384	ND	*N*-glycan	MGAT4A
	dMGAT4-2	CG17173	ND	*N*-glycan	MGAT4B

Table 1 (Continued)

Family of proteins/protein name	Protein/gene name	CG No.	References	Glycan structure	Mammalian orthologue
i-β1,3-*N*-acetylglucosaminyltransferase	diβ3GnT1	CG3253	ND	Unknown	
	diβ3GnT2	CG9171	ND	Unknown	
	diβ3GnT3	CG15483	ND	Unknown	
	diβ3GnT4	CG11149	ND	Unknown	
	diβ3GnT5	CG9996	ND	Unknown	
	diβ3GnT6	CG11388	ND	Unknown	
β1,3-*N*-acetylglucosaminyltransferase	Brn	CG4934	Muller *et al.* 2002	Glycolipid	
	Fng	CG10580	Bruckner *et al.* 2000; Moloney *et al.* 2000	Notch *O*-glycan	RFNG
β1,3-*N*-acetylglucosaminyltransferase or	dβ3GnT or GalT1	CG33145	this study	*N*-glycan	
β1,3-galactosyltransferase★	dβ3GnT or GalT2	CG11357	ND	Unknown	
	dβ3GnT or GalT3	CG3038	ND	Unknown	
	dβ3GnT or GalT4	CG8668	ND	Unknown	
	dβ3GnT or GalT5	CG8673	ND	Unknown	
Dolichyl phosphate *N*-acetylglucosaminyltransferase	dAlg14	CG6308	ND	*N*-glycan	ALG14
	dAlg7	CG5287	ND	*N*-glycan	DPAGT1/ALG7
	dAlg13	CG14512	ND	*N*-glycan	GLT28D1/ALG13
Chondroitin synthase					
Chondroitin synthase	dCHSY	CG9220	ND	GAG (CS)	CHSY1
Chondroitin polymerization factor	dCHPF	CG43313	ND	GAG (CS)	CHPF
Chondroitin *N*-acetylgalactosaminyltransferase	dCSGalNAcT1	CG12913	ND	GAG (CS)	ChGn
Chitin synthase	Chitin Syn1/Kkv	CG2666	ND	Chitin	
	Chitin Syn2	CG7464	ND	Chitin	
Fucosyltransferase					
α1,3/1,4-fucosyltransferase or	FucTA	CG6869	Fabini *et al.* 2001	*N*-glycan	
α1,3-fucosyltransferase★	FucTB	CG4435	ND	Unknown	FUT1
	FucTD	CG9169	ND	Unknown	
	FucTC	CG40305	ND	Unknown	
α1,6-fucosyltransferase	dα6Fut/FucT6	CG2448	Paschinger *et al.* 2005	*N*-glycan	FUT8
Protein *O*-fucosyltransferase	OFut1	CG12366	Okajima & Irvine 2002	Notch	POFUT1
	OFut2	CG14789	Luo *et al.* 2006	Thrombospondin	POFUT2
Galactosyltransferase					
GAGβ1,4-galactosy-ltransferase I	dGAGβ4GalTI/β4GalT7	CG11780		GAG (common)	B4GALT7

Table 1 (Continued)

Family of proteins/protein name	Protein/gene name	CG No.	References	Glycan structure	Mammalian orthologue
			Nakamura *et al.* 2002; Vadaie *et al.* 2002; Takemae *et al.* 2003		
GAGβ1,3-galactosyltransferase II	dGAGβ3GalTII	CG8734	Ueyama *et al.* 2008	GAG (common)	B3GALT6
core1β1,3-galactosyltransferase	dC1GalT1/C1GalTA	CG9520	Muller *et al.* 2005; Yoshida *et al.* 2008★	Mucin-type *O*-glycan	C1GALT1
	dC1GalT2	CG8708	Muller *et al.* 2005	Mucin-type *O*-glycan	
	dC1GalT3	CG18558	ND	Mucin-type *O*-glycan	
	dC1GalT4	CG2975	Muller *et al.* 2005	Mucin-type *O*-glycan	
	dC1GalT5/Tgy	CG7440	ND	Mucin-type *O*-glycan	
	dC1GalT6	CG34056	Muller *et al.* 2005	Mucin-type *O*-glycan	
		CG34057	Muller *et al.* 2005	Mucin-type *O*-glycan	
	dC1GalT7	CG3119	ND	Mucin-type *O*-glycan	
	dC1GalT8	CG2983	ND	Mucin-type *O*-glycan	
	dC1GalT9	CG9109	ND	Mucin-type *O*-glycan	
Glucosyltransferase					
Dolichyl phosphate glucosyltransferase	dAlg5/Wol	CG7870	ND	*N*-glycan	ALG5
Dolichyl pyrophosphate glucosyltransferase	dAlg6/Gny	CG5091	ND	*N*-glycan	ALG6
	dAlg8	CG4542	ND	*N*-glycan	ALG8
	dAlg10	CG32076	ND	*N*-glycan	ALG10
Glucosylceramide synthase	dGlcCerT/GlcT-1	CG6437	Kohyama-Koganeya *et al.* 2004	Glycolipid	UGCG
Protein *O*-glucosyltransferase	Rumi	CG31152	Acar *et al.* 2008	Notch	
	Ugt	CG6850	Parker *et al.* 1995	*N*-glycan	UGCGL1
Glucuronyltransferase					
GAG glucuronyltransferase I	dGlcAT-I	CG32775	Kim *et al.* 2003	GAG (common)	B3GAT1
β1,3-glucuronyltransferase	dGlcAT-BSI/GlcAT-S	CG3881	Kim *et al.* 2003	GAG (common), other glycan ?	
	dGlcAT-BSII/GlcAT-P	CG6207	Kim *et al.* 2003	GAG (common), other glycan ?	
		CG30438	ND	glucuronidation	CGT
Hereditary multiple exostoses (EXT) protein	dExt1/Ttv	CG10117	ND	GAG (HS)	EXT1
	dExt2/Sotv	CG8433	ND	GAG (HS)	
	dExt3/Botv	CG15110	Kim *et al.* 2002	GAG (HS)	EXTL3
Mannosyltransferase					
β1,4-mannosyltransferase	β1,4ManT/Egh	CG9659	Wandall *et al.* 2003	Glycolipid	

Table 1 (Continued)

Family of proteins/protein name	Protein/gene name	CG No.	References	Glycan structure	Mammalian orthologue
Dolichyl pyrophosphate mannosyltransferase	dAlg1	CG18012	ND	*N*-glycan	ALG1
	dAlg2	CG1291	ND	*N*-glycan	ALG2
	dAlg11	CG11306	ND	*N*-glycan	ALG11
	dAlg3/l(2)not	CG4084	ND	*N*-glycan	ALG3
	dAlg9	CG11851	ND	*N*-glycan	ALG9
	dAlg12	CG8412	ND	*N*-glycan	ALG12
	dDPM	CG10166	ND	*N*-glycan	DPM1
Protein *O*-mannosyltransferase	dPomt1/Rt	CG6097	Ichimiya *et al.* 2004	Dystroglycan	POMT1
	dPomt2/Tw	CG12311	Ichimiya *et al.* 2004	Dystroglycan	POMT2
Sialyltransferase					
Galactoside α2,6-sialyltransferase	dST6Gal I	CG4871	Koles *et al.* 2004	*N*-glycan	ST6GAL2
Xylosyltransferase					
Peptide-*O*-xylosyltransferase	dXylT/Oxt	CG32300	Wilson 2002	GAG (common)	XYLT1
Oligosaccharyltransferase					
Oligosaccharyltransferase	OST	CG33303		*N*-glycan	
		CG9022		*N*-glycan	
		CG7830		*N*-glycan	
		CG6370		*N*-glycan	
		CG13393		*N*-glycan	
	STT	CG1518		*N*-glycan	
	STT	CG7748		*N*-glycan	
Fukutin-related protein		CG15651	ND	Dystroglycan	FKRP
Sulfotransferase					
Chondroitin 4-*O*-sulfotransferase	dC4ST	CG31743	ND	GAG (CS)	CHST13
N-acetylgalactosamine-4-*O*-sulfotransferase	d4ST1	CG14024	ND	GAG (CS), *N*-glycan ?	CHST11
	d4ST2	CG13937	ND	GAG (CS), *N*-glycan ?	
N-acetylgalactosamine/*N*-acetylglucosamine/galactose	d6ST1	CG31637	ND	GAG (CS), *N*-glycan ?	
6-*O*-sulfotransferase	d6ST2	CG9550	ND	GAG (CS), *N*-glycan ?	
Heparan sufate sulfotransferase	Pipe	CG9614	Zhu *et al.* 2005★; Xu *et al.* 2007	GAG (HS)	
Heparan *N*-deacetylase/*N*-sulfotransferase	Sfl	CG8339	ND	GAG (HS)	NDST2
Heparan sulfate 2-*O*-sulfotransferase	HS2ST	CG10234	Kamimura *et al.* 2006★; Xu *et al.* 2007	GAG (HS)	HS2ST1
Heparan sulfate 6-*O*-sulfotransferase	dHS6ST	CG4451	Kamimura *et al.* 2001	GAG (HS)	HS6ST1

Table 1 (Continued)

Family of proteins/protein name	Protein/gene name	CG No.	References	Glycan structure	Mammalian orthologue
Heparan sulfate d-glucosaminyl 3-*O*-sulfotransferase	dHS3OSTA	CG33147	ND	GAG (HS)	HS3ST5
	dHS3OSTB	CG7890	Kamimura *et al.* 2004	GAG (HS)	HS3ST3A1
C5 epimerase	Heparan sulfate C5-epimerase	CG3194	ND	GAG (HS)	
Sugar-nucleotide transporter					
GDP-Fuc transporter (Golgi)	Gfr/Nac	CG9620	Luhn *et al.* 2004; Ishikawa *et al.* 2005; Geisler *et al.* 2012⋆		
GDP-Fuc/UDP-GlcNAc/UDP-Xyl transporter (ER)	Efr	CG3774	Ishikawa *et al.* 2010		SLC35B4
UDP-Gal/UDP-GalNAc transporter	Csat	CG2675	Segawa *et al.* 2002		SLC35A2
UDP-sugar transporter	Frc	CG3874	Goto *et al.* 2001; Selva *et al.* 2001	Notch, GAG	SLC35D1
Sugar-nucleotide transporter	Meigo	CG5802	ND		SLC35B1
PAPS transporter	Sll	CG7623	Kamiyama *et al.* 2003; Luders *et al.* 2003	GAG	SLC35B2
	dPAPST2	CG7853	Goda *et al.* 2006	GAG	SLC35B3
Core protein					
Glypican	Dally	CG4974		GAG (HS)	
Glypican	Dlp	CG32146		GAG (HS)	
Dystroglycan	αDystroglycan	CG18250		*O*-Man	
Syndecan	dSdc	CG10497		GAG (HS)	
Perlecan	dPerlecan/Trol	CG33950		GAG (HS)	
Glycosidase					
α-mannosidase I	α-Man-I	CG42275	ND	*N*-Glycan	
	α-Man-II	CG18802	Cao *et al.* 2011	*N*-Glycan	
β-*N*-acetylglucosaminidase	Fdl	CG8824	Leonard *et al.* 2006	*N*-Glycan	

⋆Determined by mutant phenotype.
ND: not determined.

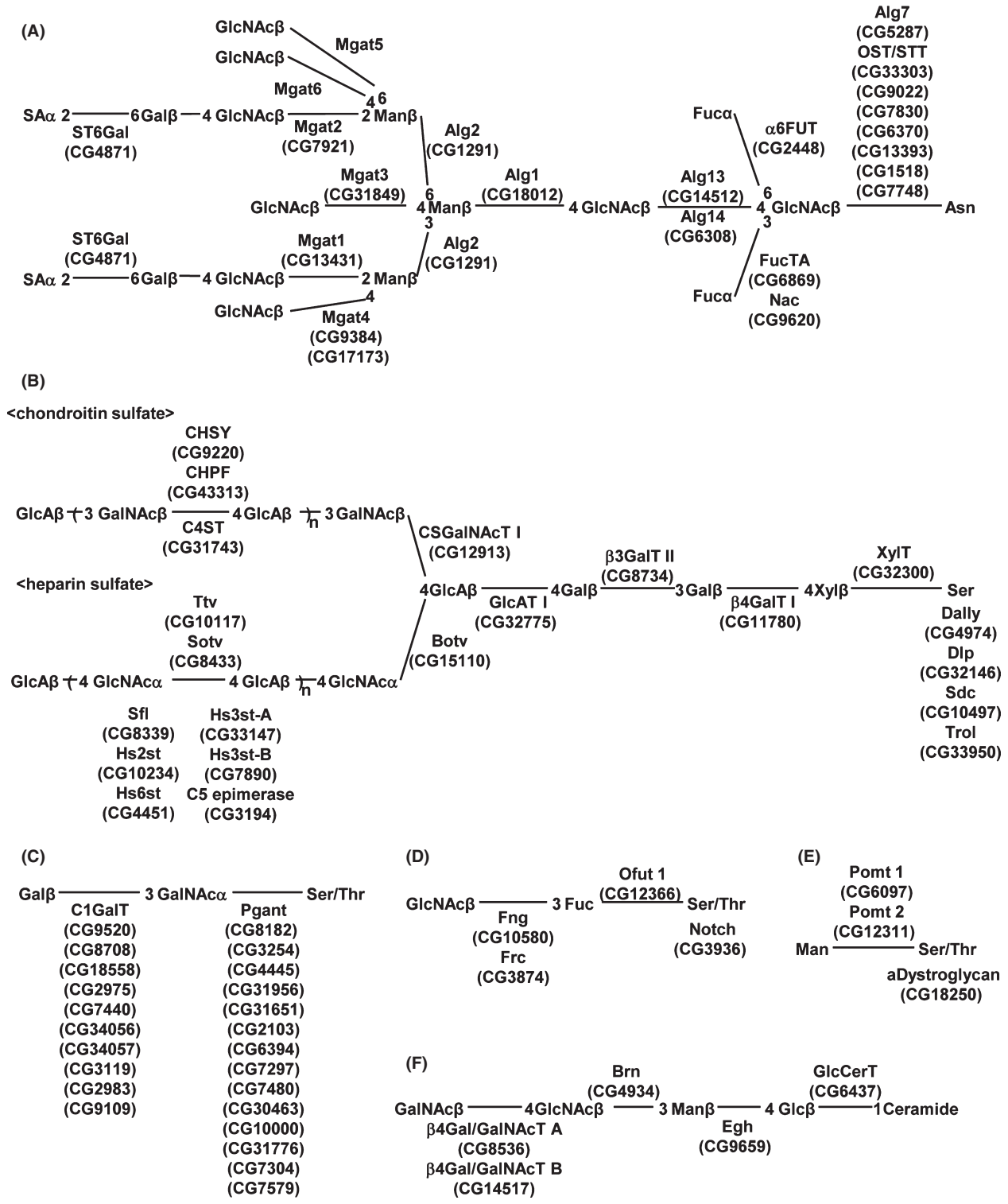

Figure 1 *Drosophila* GR genes assigned to linkage formation and modification of *N*-linked glycan (A), glycosaminoglycans (B), mucin-type glycans (C), Notch-related glycans (D), Dystroglycan-related glycan (E), and arthro-series of glycolipid (F). Core proteins are also assigned (B, D, and E).

Drosophila GR genes. RNAi could not be established for the remaining 53 genes. The established UAS-IR strains bore transgenes containing IR sequences of the target genes under the control of the UAS. First, we calculated off-target probability scores (OTPS) for each UAS-IR strain using the dsCheck website (http://dscheck.rnai.jp/, Table 2). Our previous research showed that UAS-IR strains with OTPS <3 were most likely to silence on-target genes (Yamamoto-Hino *et al.* 2010). Therefore, UAS-IR strains with OTPS >2 were not analyzed further.

Next, we examined whether RNAi-mediated gene silencing reduced the amounts of corresponding mRNA and glycan in *Drosophila*. Peptide-*O*-xylosyltransferase (XylT, CG17772) is required for the formation of the common core region of GAGs such as heparan sulfate (HS) GAG and chondroitin sulfate (CS) GAG, whereas hereditary multiple exostoses protein 3 (DExt3, CG15110) participates in the extension of HS but not CS. Expression of *XylT* and *DExt3* was silenced in whole larval bodies using Act5C-Gal4. *XylT* and *DExt3* mRNA in the silenced larvae were reduced to 15–30% of control levels (*Act5C-Gal4*) (Fig. 2). GAG fractions were extracted from the silenced larvae, treated with heparitinase, and subjected to HPLC for detailed analyses of GAGs. Silencing of *XylT* resulted in the reduction in both HS and CS, whereas *DExt3* silencing caused the specific reduction in HS (Fig. 2). These results clearly showed that the RNAi-mediated silencing in the present study resulted in specific reduction in GAGs as well as the mRNA expression levels of each glycosyltransferase.

The *Act5C-Gal4* driver strain was crossed to 72 UAS-IR strains to induce gene silencing in whole bodies during all developmental stages. Progeny from 56 of the crosses (78%) died before developing into third instar larvae, suggesting that these genes were essential for development (Table 2). As it was difficult to classify these GR genes from lethality alone, we next carried out spatiotemporally regulated gene silencing using several Gal4 driver strains.

Gene silencing in a spatiotemporally regulated manner

For spatiotemporal RNAi, *MS1096/A9-Gal4*, *scalloped (sd)-Gal4*, *patched* (*ptc*)*-Gal4*, and *engrailed* (*en*)*-Gal4* driver strains were used to induce gene silencing in wing disks, and *69B-Gal4* was used for expression in larval histoblasts and wing disks (Fig. 3). Of the 72 strains tested, 20 showed abnormalities in adult wings and abdomens. In wings, extra or thick veins were formed by gene silencing using *MS1096/A9-Gal4*, *scalloped* (*sd*)*-Gal4*, *patched* (*ptc*)*-Gal4*, and *engrailed* (*en*)*-Gal4* drivers (Figs 4,5, Table 3). By contrast, gene silencing using *69B-Gal4* caused abdominal depigmentation (Fig. 6, Table 3). Formation of extra and thick veins was mainly observed by silencing of genes involved in synthesis of GAGs and Fringe-dependent glycans on Notch, respectively (Figs 4,5, Table 3). These phenotypes corresponded with those observed for mutant strains (Panin *et al.* 1997; Goto *et al.* 2001; Selva *et al.* 2001; Nybakken & Perrimon 2002). By contrast, abdominal depigmentation has not been observed previously. Depigmentation was caused by silencing of *dα6fut/fucT6*, *gfr/nac*, *Csat*, and *CG33145* (Fig. 6, Table 3). Dα6Fut/FucT6 adds a fucose moiety to the core region of *N*-linked glycans via α1,6-linkage (Paschinger *et al.* 2005), whereas Gfr/Nac transports GDP-fucose to the Golgi lumen for fucose addition, including α1,3-fucosylation of the core *N*-linked glycans (Ishikawa *et al.* 2010; Geisler *et al.* 2012). As Gal and GalNAc are often added at or near nonreducing ends of glycans, Csat, a UDP-Gal/UDP-GalNAc transporter (Segawa *et al.* 2002), may be involved in terminal glycosylation. Therefore, the depigmentation group is possibly involved in synthesis of glycans at or near nonreducing ends, namely terminal domains. We therefore next examined whether CG33145 participated in terminal glycosylation.

CG33145 has β1,3-galactosyltransferase activity for terminal *N*-glycans

As CG33145 has high sequence similarity to the members of human β1,3-*N*-acetylglucosaminyltransferase family (60–64%) and those of human β1,3-galactosyltransferase family (61–68%), we searched for glycan structures, including GlcNAc or Gal moiety, via β1,3-linkage in insects including *Drosophila*. Galβ1,3GalNAc was found in a complex-type *N*-linked glycan on royal jelly glycoproteins of honeybee: Galβ1,3GalNAcβ1,4GlcNAcβ1,2Manα1,6(Galβ1,3GalNAcβ1,4GlcNAcβ1,2Manα1,3)Manβ1,4GlcNAcβ1,4GlcNAc (E5, Fig. 7A) (Kimura *et al.* 2006, 2007). Thus, we examined whether CG33145 added Gal to GalNAcβ1,4GlcNAcβ1,2Manα1,6(GalNAcβ1,4GlcNAcβ1,2Manα1,3)Manβ1,4GlcNAcβ1,4GlcNAc (E2, Fig. 7A) via β1,3-linkage. CG33145 protein was expressed in Sf9 cells, and the β1,3galactosyltransferase activity was assessed (Fig. 7B,C). An *in vitro* assay showed that CG33145 protein transferred the Gal moiety to E2 and produced the

Table 2 Off-target probability score (OTPS) and phenotypes caused by whole-body gene silencing

Family of proteins/protein name	Protein/gene name	CG No.	OTPS	Act5C
Glycosyltransferase				
N-acetylgalactosaminyltransferase				
UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase	pgant1/GalNAc-T1	CG8182	1	Lethal
	pgant2	CG3254	0	Lethal
	pgant3	CG4445	2	Viable
	pgant4	CG31956	0	Lethal
	pgant5	CG31651	0	Lethal
	pgant6	CG2103	0	Lethal
	pgant7/GalNAc-T2	CG6394	1	Lethal
	pgant8	CG7297	0	Lethal
	pgant35A	CG7480	0	Lethal
	dppGalNAcT9	CG30463	2	Lethal
	dppGalNAcT10	CG10000	1	N.T.
		CG31776	no line	N.T.
	dppGalNAcT11	CG7304	no line	N.T.
	dppGalNAcT12	CG7579	1	N.T.
α1,4-*N*-acetylgalactosaminyltransferase	α4GT1	CG17223	0	Viable
	α4GT2	CG5878	0	N.T.
β1,4-*N*-acetylgalactosaminyltransferase	β4GalNAcTA	CG8536	0	N.T.
	β4GalNAcTB	CG14517	1	Viable
N-acetylglucosaminyltransferase				
UDP-GlcNAc:polypeptide *O*-β-*N*-acetylglucosaminyltransferase	dO-GnT/Sxc	CG10392	0	Lethal
α3-*D*-mannoside-β1,2-*N*-acetylglucosaminyltransferase	dMGAT1/Mgat1	CG13431	2	Lethal
α6-*D*-mannoside-β1,2-*N*-acetylglucosaminyltransferase	dMGAT2/Mgat2	CG7921	1	Lethal
β4-*D*-mannoside-β1,4-*N*-acetylglucosaminyltransferase	dMGAT3	CG31849	1	Lethal
α3-*D*-mannoside-β1,4-*N*-acetylglucosaminyltransferase	dMGAT4-1	CG9384	2	Viable
	dMGAT4-2	CG17173	0	Lethal
i-β1,3-*N*-acetylglucosaminyltransferase	diβ3GnT1	CG3253	1	Viable
	diβ3GnT2	CG9171	38	N.T.
	diβ3GnT3	CG15483	0	Viable
	diβ3GnT4	CG11149	0	Lethal
	diβ3GnT5	CG9996	0	Lethal
	diβ3GnT6	CG11388	0	Lethal
β1,3-*N*-acetylglucosaminyltransferase	Brn	CG4934	0	Lethal
	Fng	CG10580	0	Lethal
β1,3-*N*-acetylglucosaminyltransferase or	dβ3GnT or GalT1	CG33145	0	Lethal
β1,3-galactosyltransferase	dβ3GnT or GalT2	CG11357	2	Lethal
	dβ3GnT or GalT3	CG3038	0	Viable
	dβ3GnT or GalT4	CG8668	0	Lethal
	dβ3GnT or GalT5	CG8673	11	N.T.
Dolichyl phosphate *N*-acetylglucosaminyltransferase	dAlg14	CG6308	no line	N.t.
	dAlg7	CG5287	0	Lethal
	dAlg13	CG14512	0	Lethal
Chondroitin synthase				
Chondroitin synthase	dCHSY	CG9220	2	n.t.
Chondroitin polymerization factor	dCHPF	CG43313	0	Lethal
Chondroitin *N*-acetylgalactosaminyltransferase	dCSGalNAcT1	CG12913	2	Viable
Chitin synthase	Chitin Syn1/Kkv	CG2666	0	Lethal
	Chitin Syn2	CG7464	0	Lethal
Fucosyltransferase				
α1,3/1,4-fucosyltransferase or	FucTA	CG6869	6	N.T.
α1,3-fucosyltransferase	FucTB	CG4435	0	Lethal

Table 2 (Continued)

Family of proteins/protein name	Protein/gene name	CG No.	OTPS	Act5C
	FucTD	CG9169	1	Lethal
	FucTC	CG40305	no line	N.T.
α1,6-fucosyltransferase	dα6Fut/FucT6	CG2448	1	Lethal
Protein *O*-fucosyltransferase	OFut1	CG12366	0	Lethal
	OFut2	CG14789	0	Viable
Galactosyltransferase				
GAGβ1,4-galactosyltransferase I	dGAGβ4GalTI/β4GalT7	CG11780	0	Lethal
GAGβ1,3-galactosyltransferase II	dGAGβ3GalTII	CG8734	1	Lethal
core1β1,3-galactosyltransferase	dC1GalT1/C1GalTA	CG9520	0	Viable
	dC1GalT2	CG8708	1	Lethal
	dC1GalT3	CG18558	0	N.T.
	dC1GalT4	CG2975	8	N.T.
	dC1GalT5/Tgy	CG7440	0	Lethal
	dC1GalT6	CG34056	8	N.T.
		CG34057	8	N.T.
	dC1GalT7	CG3119	2	N.T.
	dC1GalT8	CG2983	2	Viable
	dC1GalT9	CG9109	1	Lethal
Glucosyltransferase				
Dolichyl phosphate glucosyltransferase	dAlg5/Wol	CG7870	2	N.T.
Dolichyl pyrophosphate glucosyltransferase	dAlg6/Gny	CG5091	0	N.T.
	dAlg8	CG4542	2	N.T.
	dAlg10	CG32076	1	N.T.
Glucosylceramide synthase	dGlcCerT/GlcT-1	CG6437	1	Lethal
Protein *O*-glucosyltransferase	Rumi	CG31152	no line	N.T.
	Ugt	CG6850	no line	N.T.
Glucuronyltransferase				
GAG glucuronyltransferase I	dGlcAT-I	CG32775	0	Lethal
β1,3-glucuronyltransferase	dGlcAT-BSI/GlcAT-S	CG3881	0	Viable
	dGlcAT-BSII/GlcAT-P	CG6207	24	N.T.
		CG30438	0	Viable
Hereditary multiple exostoses (EXT) protein	dExt1/Ttv	CG10117	0	Lethal
	dExt2/Sotv	CG8433	0	Lethal
	dExt3/Botv	CG15110	?	Lethal
Mannosyltransferase				
β1,4-mannosyltransferase	β1,4ManT/Egh	CG9659	0	N.T.
Dolichyl pyrophosphate mannosyltransferase	dAlg1	CG18012	0	Lethal
	dAlg2	CG1291	2	N.T.
	dAlg11	CG11306	0	N.T.
	dAlg3/l(2)not	CG4084	0	N.T.
	dAlg9	CG11851	no line	N.T.
	dAlg12	CG8412	0	N.T.
	dDPM	CG10166	0	N.T.
Protein *O*-mannosyltransferase	dPomt1/Rt	CG6097	0	Lethal
	dPomt2/Tw	CG12311	0	Lethal
Sialyltransferase				
Galactoside α2,6-sialyltransferase	dST6Gal I	CG4871	3	N.T.
Xylosyltransferase				
Peptide-*O*-xylosyltransferase	dXylT/Oxt	CG32300	0	Lethal

Table 2 (Continued)

Family of proteins/protein name	Protein/gene name	CG No.	OTPS	Act5C
Oligosaccharyltransferase				
Oligosaccharyltransferase	OST	CG33303	0	N.T
		CG9022	no line	N.T
		CG7830	0	N.T
		CG6370	no line	N.T
		CG13393	no line	N.T
	STT	CG1518	0	N.T
	STT	CG7748	no line	N.T
Fukutin-related protein		CG15651	0	Lethal
Sulfotransferase				
Chondroitin 4-*O*-sulfotransferase	dC4ST	CG31743	0	Viable
N-acetylgalactosamine-4-*O*-sulfotransferase	d4ST1	CG14024	6	N.T.
	d4ST2	CG13937	0	Viable
N-acetylgalactosamine/*N*-acetylglucosamine/galactose	d6ST1	CG31637	0	N.T.
6-*O*-sulfotransferase	d6ST2	CG9550	0	Lethal
Heparan sufate sulfotransferase	Pipe	CG9614	1	Lethal
Heparan *N*-deacetylase/*N*-sulfotransferase	Sfl	CG8339	1	N.T.
Heparan sulfate 2-*O*-sulfotransferase	HS2ST	CG10234	0	Viable
Heparan sulfate 6-*O*-sulfotransferase	dHS6ST	CG4451	0	N.T.
Heparan sulfate d-glucosaminyl 3-*O*-sulfotransferase	dHS3OSTA	CG33147	2	N.T.
	dHS3OSTB	CG7890	3	N.T.
C5 epimerase	Heparan sulfate C5-epimerase	CG3194	0	lethal
Sugar-nucleotide transporter				
GDP-Fuc transporter (Golgi)	Gfr/Nac	CG9620	0	N.T.
GDP-Fuc/UDP-GlcNAc/UDP-Xyl transporter (ER)	Efr	CG3774	no line	N.T.
UDP-Gal/UDP-GalNAc transporter	Csat	CG2675	0	Lethal
UDP-sugar transporter	Frc	CG3874	2	Lethal
Sugar-nucleotide transporter	Meigo	CG5802	0	Lethal
PAPS transporter	Sll	CG7623	0	Lethal
	dPAPST2	CG7853	2	Lethal
Core protein				
Glypican	Dally	CG4974	4	N.T.
Glypican	Dlp	CG32146	2	Lethal
Dystroglycan	αDystroglycan	CG18250	1	N.T.
Syndecan	dSdc	CG10497	3	N.T.
Perlecan	dPerlecan/Trol	CG33950	0	N.T.
Glycosidase				
α-mannosidase I	α-Man-I	CG42275	no line	N.T.
	α-Man-II	CG18802	0	Lethal
β-*N*-acetylglucosaminidase	Fdl	CG8824	no line	N.T.

no line: no UAS-IR line; N.T.: not tested.

products Galβ1,3GalNAcβ1,4GlcNAcβ1,2Manα1,6(GalNAcβ1,4GlcNAcβ1,2Manα1,3)Manβ1,4GlcNAcβ1,4GlcNAc or GalNAcβ1,4GlcNAcβ1,2Manα1,6(Galβ1,3GalNAcβ1,4GlcNAcβ1,2Manα1,3)Manβ1,4GlcNAcβ1,4GlcNAc (E4), and Galβ1,3GalNAcβ1,4GlcNAcβ1,2Manα1,6(Galβ1,3GalNAcβ1,4GlcNAcβ1,2Manα1,3)Manβ1,4GlcNAcβ1,4GlcNAc (E5). Digestion of the fractionated E5 product by β1,3galactosidase produced E2 and E4 (Fig. 7D), confirming that the linkage between Gal and GalNAc was a β1,3-linkage. These data clearly show that CG33145 protein is a novel β1,3galactosyltransferase of *N*-glycan.

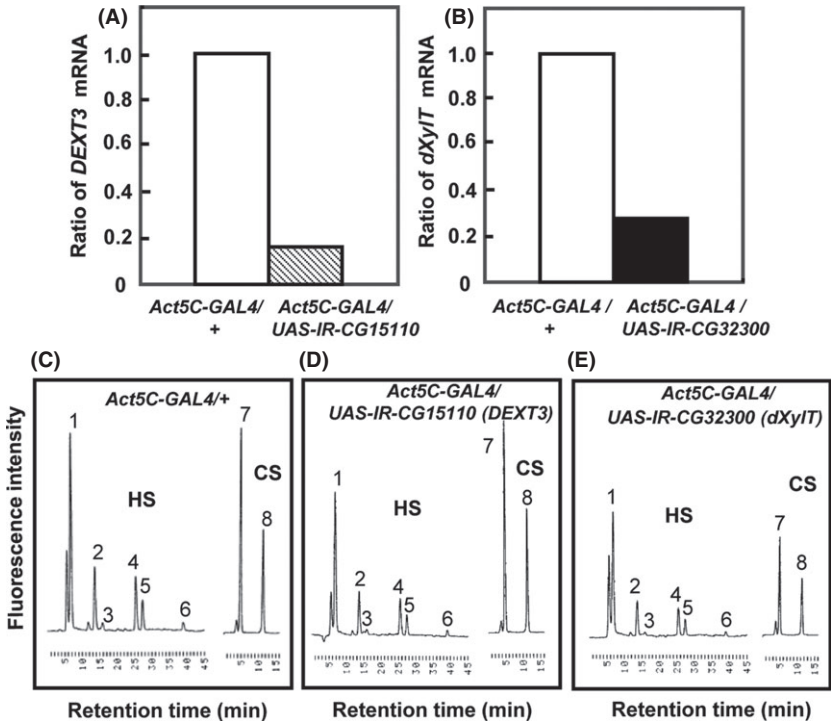

Figure 2 Reduction in mRNA and GAG levels by silencing of *CG15110* (*Dext3*) and *CG32300* (*dXylT*). (A, B) The mRNA levels of *CG15110* and *CG32300* in *Act5C-GAL4/UAS-IR-CG15110* (A), *Act5C-GAL4/UAS-IR-CG17772* (B), and *Actin5C-GAL4/+* (as control in A and B) were quantified by real-time PCR. (C–E) Typical chromatograms of GAG-derived oligosaccharides in the third instar larvae of *Actin5C-GAL4/+* (C), *Act5C-GAL4/UAS-IR-CG15110* (D), and *Act5C-GAL4/UAS-IR-CG17772* (E). HS, chromatograms of unsaturated disaccharides from heparan sulfate. CS, chromatograms of unsaturated disaccharides from low-sulfated chondroitin 4-sulfate. Peaks: 1, ΔUA-GlcNAc; 2, ΔUA-GlcNS; 3, ΔUA-GlcNAc6S; 4, ΔUA-GlcNS6S; 5, ΔUA2S-GlcNS; 6, ΔUA2S-GlcNS6S; 7, ΔDi-0S; and 8, ΔDi-4S. The peak heights in each chromatogram reflect the amount of oligosaccharides and can be compared between different genotypes.

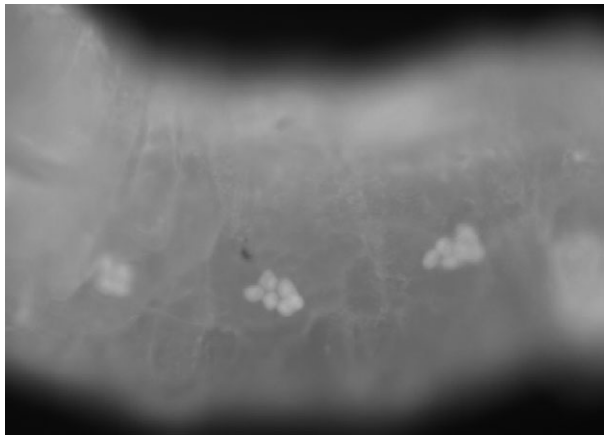

Figure 3 69B-Gal4 expression in larval histoblasts. The late third instar larva of *69B-Gal4/UAS-GFP* expressed GFP in histoblasts.

Discussion

A wide variety of glycans are involved in diverse biological processes. To date, more than 200 genes in the human genome have been identified as GR candidates. However, biological and biochemical functions of the gene products remain to be studied in detail. In this study, large-scale RNAi silencing was used with *Drosophila* GR genes. Silencing of genes involved in synthesis of the same glycan resulted in the same phenotypes. Phenotypic clustering was used to identify galactosyltransferase terminal *N*-glycosylation activity in the previously uncharacterized protein CG33145. This suggests that phenotypic clustering is potentially valuable for the identification of specific glycans synthesized by genes of interest.

Using sequence comparisons, we identified 132 GR gene candidates in the *Drosophila* genome. Of these, the biochemical and biological functions of 50

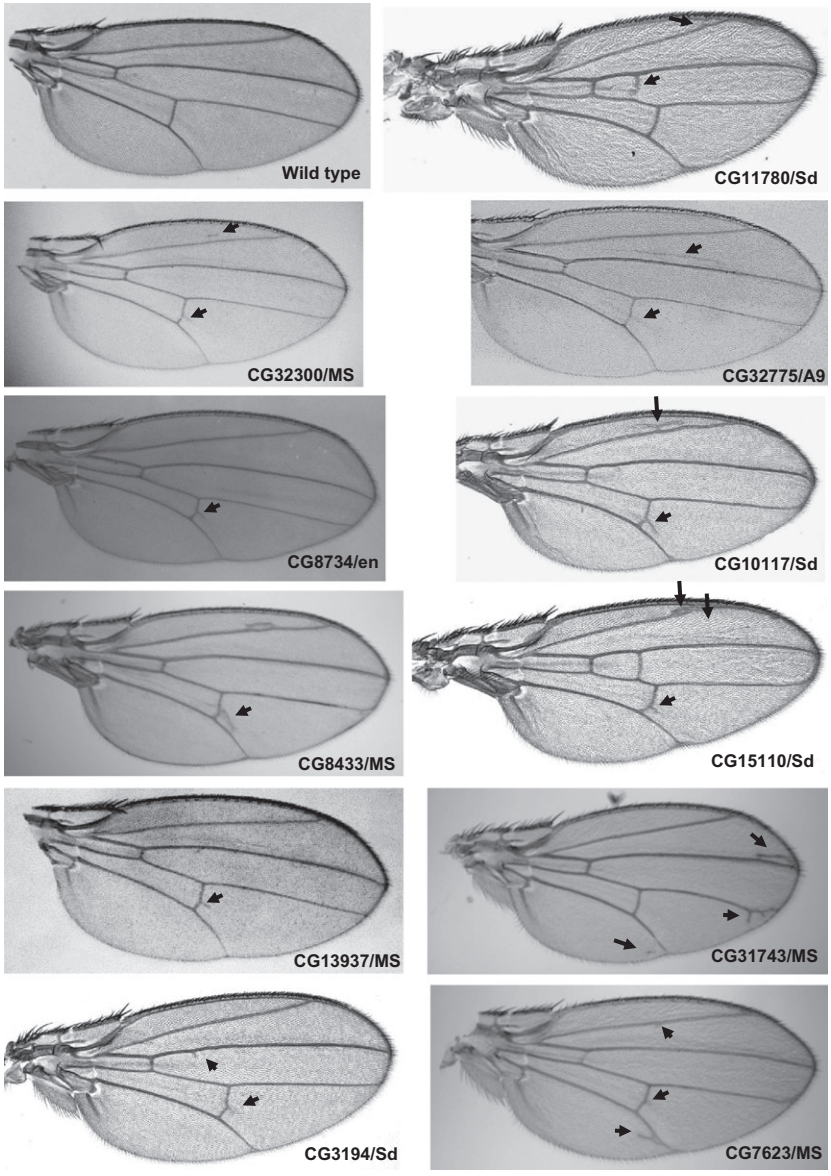

Figure 4 Adult wing phenotypes caused by silencing of GAG genes. Extra veins are indicated by arrows. The combination of UAS-IR and Gal4 strains is indicated under each panel.

genes remain to be studied in detail. However, it is difficult to determine the biochemical properties of GR proteins without predictive information because appropriate substrates and conditions are needed for biochemical assays. For example, a sialyltransferase that adds a sialic acid to a nonreducing end of *N*-glycans requires both CMP-sialic acid and a part of *N*-glycans for its biochemical assay. Pgant, a peptidyl-*N*-acetylgalactosaminyltransferase that transfers GalNAc to mucin-type proteins, needs both UDP-GalNAc and appropriate peptides. Therefore, to determine the biochemical property of a novel gene, it is advantageous to predict what type of glycosylation is involved. In the present study, we examined the utility of phenotypic clustering for glycosylation prediction.

Silencing of GR genes using several Gal4 drivers resulted in various phenotypes such as formation of extra and thick veins and abdominal depigmentation; however, RNAi abnormalities were less severe than those resulting from classical mutations such as deletion, point mutation, or transposon-insertion. For example, silencing of *fringe* (*fng*) and *fringe-connection*

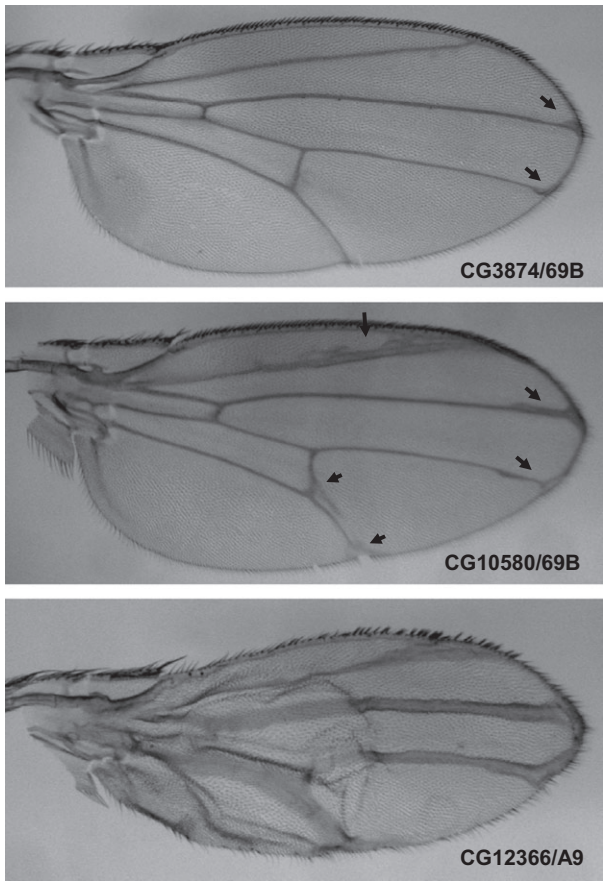

Figure 5 Adult wing phenotypes caused by silencing of Notch glycosylation genes. Thick veins are indicated by arrows. The combination of UAS-IR and Gal4 strains is indicated in each panel.

(*frc*), which play an essential role in Notch glycosylation, produced a thick vein phenotype that was milder than the deleted margin phenotype of their null mutations. This may be due to low efficiency of gene silencing by RNAi and/or unusual persistence of GR proteins. Maternally provided Frc protein and/or mRNA was sufficient for a strong *frc* mutant to survive to the late third larval stage (Goto *et al.* 2001).

Knockdown phenotypes also depend on the RNAi library. Mummery-Widmer *et al.* identified *CG12366* (*Ofut1*), but neither *CG10580* (*fng*) nor *CG3874* (*frc*), as a Notch regulator using Vienna RNAi library (Mummery-Widmer *et al.* 2009). The reason may be that the different lengths of dsRNAs between Vienna and NIG RNAi libraries. Long dsRNAs (500 bp) in NIG silence target gene expression more effectively than short ones (approximately 300 bp) in Vienna.

RNAi and conventional mutation phenotypes were similar, albeit with milder phenotypes observed with silencing. For example, knockdown of genes involved in GAG synthesis and Notch glycosylation resulted in formation of extra and thick veins, respectively. These phenotypes were also reported in strains with mutations in the corresponding genes. By contrast, the abdominal depigmentation phenotype produced upon knockdown of genes involved in synthesis of terminal domains of glycans has not been observed previously.

Sequence similarity and phenotype-based gene clustering in the present study suggested that CG33145 had a β1,3galactosyltransferase activity in *N*-glycan synthesis. Biochemical analysis confirmed that the CG33145 protein had β1,3galactosyltransferase activity for *N*-glycosylation. These results suggest that phenotype-based clustering can be indicative of molecular function. Similarly, *Csat* (*CG2675*), which also exhibited the abdominal pigmentation phenotype, may contribute to the synthesis of *N*-glycan.

The *N*-glycan gene cluster did not include glycosyltransferases involved in the production of core regions of *N*-linked glycans. It is possible that core regions of *N*-linked glycans are essential for protein folding and quality control and that deletion of whole *N*-linked glycan structures may cause lethal defects. By contrast, nonreducing ends of *N*-linked glycans play more specific roles such as regulation of ligand-receptor interactions, protein complex formation, and protein trafficking. Thus, defects of the nonreducing ends of *N*-linked glycans might result in specific, less severe phenotypes such as depigmentation.

In mice, branch positioning near nonreducing ends of *N*-glycans is required for proper trafficking of Glucose transporter 2, which is essential for glucose-stimulated insulin secretion (Ohtsubo *et al.* 2005). In *Drosophila*, the same branch structure and the insulin pathway were shown to be involved in cuticle pigmentation (Shakhmantsir *et al.* 2014). Therefore, abdominal depigmentation may be caused by impaired trafficking of membrane and/or secretory proteins in the insulin pathway.

Biological functions of some glycans are conserved between *Drosophila* and humans. For example, POMT1 and POMT2, which transfer a mannose to Dystroglycan via an *O*-type linkage, are mutated in Walker–Warburg syndrome, a type of muscular dystrophy (Akasaka-Manya *et al.* 2004; van Reeuwijk *et al.* 2005). *rotated abdomen* and *twisted*, *Drosophila* mutants of POMT1 (CG6097) and POMT2 (CG12311), respectively, which mediate *O*-linked mannosylation, also exhibit muscle defects in adults, suggesting a conserved biologi-

Table 3 Phenotypes caused by spatiotemporally regulated gene silencing

			Gal4 driver line					
Family of proteins/protein name	Protein/gene name	CG No.	MS1096/A9	SD	en	ptc	69B	Glycans
N-acetylgalactosamine-4-*O*-sulfotransferase	d4ST2	CG13937	Extra vein					GAGs
GAG glucuronyltransferase I	dGlcAT-I	CG32775	Extra vein[1]					
peptide-*O*-xylosyltransferase	dXylT/Oxt	CG32300	Extra vein	Extra vein				
GAGβ1,4-galactosyltransferase I	dGAGβ4GalTI/β4GalT7	CG11780		Extra vein	Extra vein			
C5 epimerase	Heparan sulfate C5-epimerase	CG3194	Extra vein	Extra vein	Extra vein[2]			
Hereditary multiple exostoses (EXT) protein	dExt1/Ttv	CG10117	Extra vein[3]	Extra vein				
Hereditary multiple exostoses (EXT) protein	dExt2/Stv	CG8433	Extra vein	Extra vein	Extra vein			
Hereditary multiple exostoses (EXT) protein	dExt3/Botv	CG15110		Extra vein		acv deletion		
β1,3-glucuronyltransferase	dGlcAT-BSII/GlcAT-P	CG6207		Extra vein				
GAGβ1,3-galactosyltransferase II	dGAGβ3GalTII	CG8734			Extra vein			
PAPS transporter	Sll	CG7623	Extra vein					
Chondroitin 4-*O*-sulfotransferase	dC4ST	CG31743	Extra vein					
Syndecan	dSdc	CG10497	Thick vein					
Protein *O*-fucosyltransferase	OFut1	CG12366	Thick vein[4]					Notch
β1,3-*N*-acetylglucosaminyltransferase	Fng	CG10580	Thick vein	Thick vein			Thick vein[5]	
UDP-sugar transporter	Frc	CG3874	Thick vein					
α1,6-fucosyltransferase	dα6Fut/FucT6	CG2448	Thick vein				Depigmentation[6]	Terminal
GDP-Fuc transporter (Golgi)	Gfr/Nac	CG9620					Depigmentation[7]	
UDP-Gal/UDP-GalNAc transporter	Csat	CG2675					Depigmentation[8]	
β1,3-*N*-acetylglucosaminyltransferase or β1,3-galactosyltransferase	dβ3GnT or GalT1	CG33145					Depigmentation[9]	
Glucosylceramide synthase	dGlcCerT/GlcT-1	CG6437	Thick vein					Glycolipid
UDP-GlcNAc:polypeptide *O*-β-*N*-acetylglucosaminyltransferase	d*O*-GnT/Sxc	CG10392	Thick vein	pcv deletion				*O*-GlcNAc

Number of abnormal wings/number of tested wings = 40/76 (1), 46/46 (2), 70/70 (3), 64/64 (4) and 8/8 (5).
Number of depigmented males/number of tested males = 66/79 (6), 5/15 (7), 45/175 (8) and 9/69 (9).

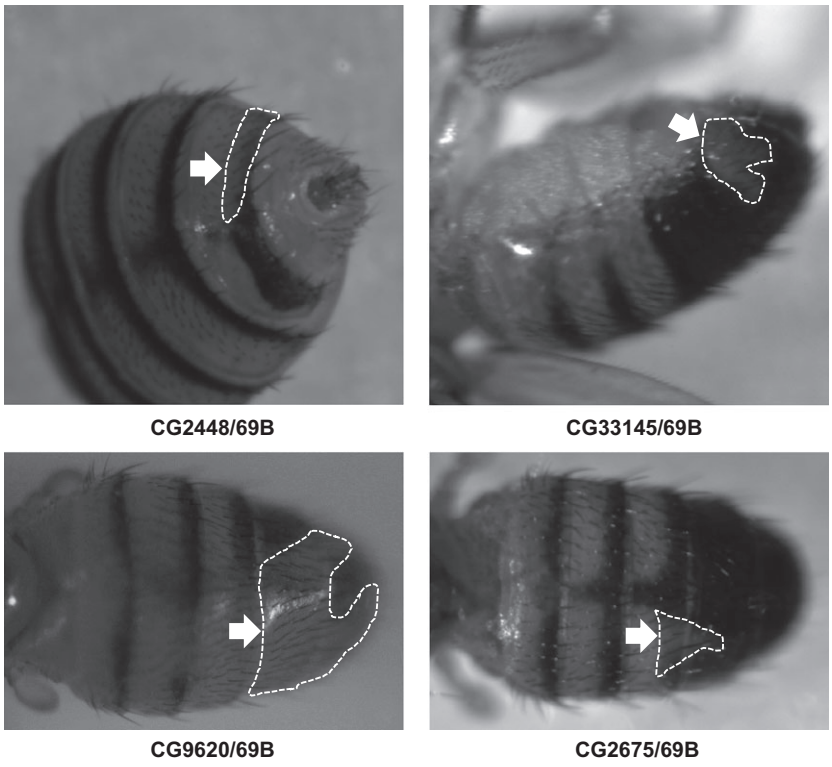

Figure 6 Adult abdominal phenotypes caused by silencing of *N*-glycan genes. Depigmented regions are indicated by arrows and surrounded by dotted lines. The combination of UAS-IR and Gal4 strains is indicated under each panel.

cal function of the *O*-mannosyl glycan (Martin-Blanco & Garcia-Bellido 1996; Ichimiya *et al.* 2004; Ueyama *et al.* 2010). These mutants exhibited the behavioral abnormalities, the shortened lifespan and ultrastructural defects of muscles, as seen in human patients, also indicating that *Drosophila POMT* mutants are models for human muscular dystrophy. Then enhanced apoptosis was found in muscle progenitor cells of these mutants and provided new insight into the mechanism of WWS development, namely increased numbers of apoptotic myoblasts causing muscle disorganization (Ueyama *et al.* 2010). Therefore, phenotypic information obtained in *Drosophila* may shed light on glycan functions in other organisms, including humans.

Experimental procedures

Generation of RNAi fly lines

A 500-bp-long cDNA fragment of the N-terminal region of the ORF of each target gene was amplified by PCR. The fragment was inserted as an inverted repeat (IR) into a modified pUAST transformation vector, pUAST-R57 (GenBank accession: AB233207), which possessed an IR formation site consisting of paired *Kpn*I-*Cpo*I and *Xba*I-*Sfi*I restriction sites. To enhance the RNAi effect (Kalidas & Smith 2002), pUAST-R57 carries a 282-bp-long genome fragment containing introns 5 and 6 of the *Drosophila Ret oncogene* between the two IR fragments. The IR was constructed in a head-to-head orientation using a combination of tag sequences in the PCR primers and restriction sites in the vector. Transformation of *Drosophila* embryos was carried out according to Spradling (Spradling 1986) in the w^{1118} fly backgrounds. Each line was mated with several of the GAL4 driver lines: *Act5C-GAL4* (Bloomington *Drosophila* Stock Center), *GMR-GAL4* (Freeman 1996), *eγ-GAL4* (Bloomington *Drosophila* Stock Center), *dpp-GAL4* (Staehling-Hampton *et al.* 1994), *en-GAL4* (Johnson *et al.* 1995), *pnr-GAL4* (Heitzler *et al.* 1996), *ptc-GAL4* (Speicher *et al.* 1994), *sd-GAL4* (Milan *et al.* 1997), *A9-GAL4* (Sun & Artavanis-Tsakonas 1997), *29BD-GAL4* (Nakayama *et al.* 1997), *69B-GAL4* (Brand & Perrimon 1993), and *MS1096-GAL4* (Capdevila & Guerrero 1994). F_1 progeny were raised at 28°C, and their phenotypes were analyzed. F_1 progeny of w^{1118} crossed with each of the GAL4 driver lines were used as a control, for example, *Act5C-GAL4/+*, *GMR-GAL4/+*, etc.

Quantitative analysis of mRNA by real-time PCR

Total RNA was extracted from *Act5C-GAL4/UAS-IR-CG4351*, *CG15110*, *CG17772*, and *Act5C-GAL4/+* third

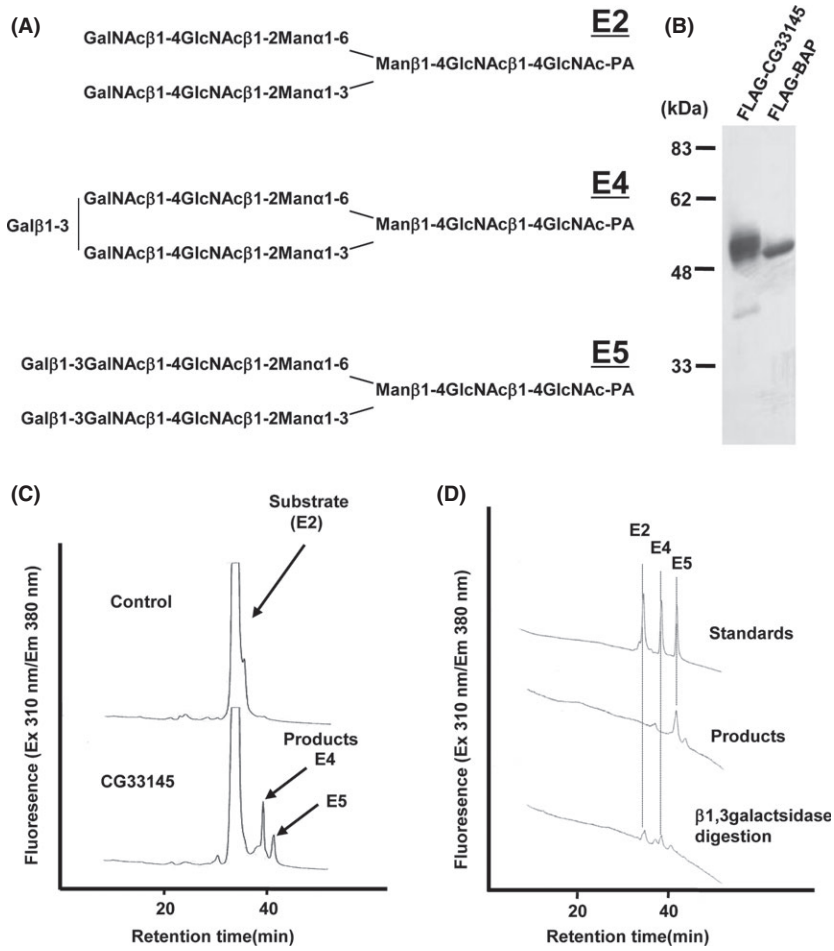

Figure 7 Identification of CG33145 as a novel β1,3galactosyltransferase of *N*-glycosylation**.** (A) The structures of an acceptor substrate E2 and its Gal extended forms, E4 and E5, which have one and two terminal Gal moieties, respectively. (B) FLAG-CG33145-PB and FLAG-BAP expressed in insect cells were purified and detected by anti-FLAG antibody. (C) Products of the CG33145-mediated reaction were analyzed by HPLC. CG33145 produced E4 and E5. (D) β1,3galactosidase treatment of reaction product E5. The E5 product peak shifted to peaks corresponding to E4 and E2 after β1,3galactosidase treatment.

instar larvae. First-strand cDNA was synthesized using a SuperScript II first-strand synthesis kit (Invitrogen) according to the manufacturer's instructions. Quantitation of *CG4351*, *CG15110*, and *CG17772* mRNA expression was carried out by real-time PCR using the following primers: forward, 5′-ccacgacgtgatcgctttct-3′ (*CG4351*), 5′-ggagtgcgcggaaatgg-3′ (*CG15110*), and 5′-gaaatctgcggcggattcta-3′ (*CG17772*); and reverse, 5′-cagtccctgcggatgtaagag-3′ (*CG4351*), 5′-tgtttgggcctcagttcacct t-3′ (*CG15110*), and 5′-agtggtggccgccagtt-3′ (*CG17772*). The probe, which consisted of 5′-tagtcgggattatgtccaggctcgca-3′ (*CG4351*), 5′-ccgcccgaaggaaatacctgcttaccta-3′ (*CG15110*), or 5′-ccatgaacatatacgagaccggaatagccaa-3′ (*CG17772*), was labeled at the 5′-end with the reporter dye 3FAM and at the 3′-end with the quencher dye TAMRA (Applied Biosystems, Foster City, CA). Real-time PCR was carried out using a TaqMan Universal PCR Master Mix (Applied Biosystems). Relative amounts of *CG4351*, *CG15110*, and *CG17772* mRNAs were normalized against *ribosomal protein L32* (*RpL32*) mRNA levels from the same cDNA.

Determination of the amount of chondroitin sulfate and heparan sulfate in *Drosophila*

GAGs were prepared from approximately 20 mg of lyophilized flies. Unsaturated disaccharides were produced by enzymatic digestion and analyzed by fluorometric postcolumn high-performance liquid chromatography, as described previously (Toyoda *et al.* 2000).

Expression and purification of CG33145 protein

The putative catalytic domain of candidate CG33145 protein (amino acids 92 to 466, CG33145-PB) was cloned using

DGC clone RE52041, expressed in insect cells as a secreted protein fused with a FLAG peptide, and purified using Anti-FLAG M1 Affinity gel (Sigma), as described previously (Ueyama *et al.* 2008).

Galactosyltransferase activity assay

β1,3galactosyltransferase activity was assessed. The acceptor substrate E2 and standards, E4 and E5, were prepared as described previously (Kimura *et al.* 2006, 2007). Enzymatic reactions, product detection, and product confirmation were also carried out as noted previously (Kimura *et al.* 2006, 2007).

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