Glycoconjugate Journal 21, 63–68, 2004

Approach for functional analysis of glycan using RNA interference

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The elucidation of the biological role of glycan is one of the most important issues to be resolved following the genome project. RNA interference is becoming an efficient reverse genetic tool for studying gene function in model organisms, including *C.elegans* and *Drosophila melanogaster.* Our molecular evolutionary study has shown that a prototype of glycosyltransferases, which synthesize a variety of glycan structures in the Golgi apparatus, was conserved between mammals and *Drosophila.* For analyses of the basic physiological functions of glycans, we established the *Drosophila* inducible RNAi knockdown system and applied it to one glycosyltransferase and one transporter, proteoglycan UDP-galactose: β-xylose β1,4galactosyltransferase I and the PAPS-transporter, respectively. If on the silencing of each gene induced ubiquitously under the control of a cytoplasmic actin promoter, the RNAi knockdown fly died, then the protein was indispensable for life. The expression of the target gene was disrupted specifically and the degree of interference was well correlated with the phenotype. The inducible RNAi knockdown fly obtained using the GAL4-UAS system will pave the way for the functional analysis of glycans.
Published in 2004.

Keywords: **RNA interference, *Drosophila*, glycosyltransferase, sugar-nucleotide transporter, carbohydrate**

Introduction

The genome project has been almost finished and proteome studies are now being carried out worldwide. One of the most important posttranslational modifications is glycosylation along with phosphorylation. Consequently, elucidation of the overall function of glycan at the whole body or cellular level is an important issue to be resolved. The glycosylation of proteins and lipids is performed in the Golgi apparatus by glycosyltransferases, which transfer sugars from sugar-nucleotides to acceptor substrates. Sugar-nucleotide transporters supply a variety of sugar-nucleotides, which are synthesized in the cytosol, as donor substrates of glycosyltransferases in the Golgi apparatus. Glycosyltransferases are responsible for synthesizing the huge diversity of complex oligosaccharides. By analyzing these glycosyltransferases in the cell, one can obtain information about the structure of glycan on the cells.

We have performed a molecular evolutionary study of 55 glycosyltransferase genes [1]. The divergence of gene families had finished before the branching of the deuterostome and proteostome, including *C.elegans* and *Drosophila melanogaster.* After that, early in the history of the vertebrate lineage, intrafamilial genes increased through gene duplication to obtain a variety of substrate specificities for synthesizing various glycan structures. The prototype of each glycosyltransferase was conserved in *Drosophila*, suggesting common roles for glycans in humans and *Drosophila*.

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RNA interference (RNAi) knockdown system in *Drosophila*

RNAi is an evolutionarily conserved event through which double-stranded RNA (dsRNA) induces gene silencing [2]. In the initiation step, a long dsRNA is processed by Dicer into 21–23 nt double stranded fragments, small interfering RNAs (siRNA). These siRNA duplexes are incorporated into a protein complex, an inactivated RNA-induced silencing complex (inactivated RISC). ATP-dependent unwinding of the siRNA duplex remodels the complex to generate an active RNA-induced silencing complex (RISC). Finally, the RISC can recognize and cleave specifically a target RNA complementary to the guide strand of the siRNA. The last step is so restricted that a single base mismatch between a target RNA and the guide strand of the siRNA is sufficient to prevent "target RNA destruction".

In many model organisms, including *Drosophila*, *C.elegans*, and plants, large dsRNAs efficiently induce gene-specific silencing [3,4], while only siRNA can suppress efficiently the expression of the corresponding gene in mammalian cells [5,6]. Moreover, *Drosophila* and humans do not have the pathway, "amplification of siRNA by RNA-dependent RNA polymerase", which reduces the specificity of RNAi, while *C.elegans* and plants have [2].

So we have established the *Drosophila* inducible RNAi knockdown system using the GAL4-upstream activating sequence system (GAL4-UAS system) for analyses of the basic physiological functions of glycans [7]. A scheme of the heritable and inducible RNAi system is shown in Figure 1. Two transgenic fly lines, *GAL4*-driver and *UAS-inverted repeat* (*UAS-IR*), were used in this system. The *GAL4*-driver fly has a transgene containing the yeast transcription factor *GAL4*, the expression of which is under the control of a tissue-specific promoter. The *UAS-IR* fly has a transgene containing an inverted repeat (IR) of the target gene ligated to the UAS promoter, a target of GAL4. In the F_1 generation of these flies, the dsRNA of the target gene is expressed in specific tissue under the control of the tissue-specific promoter to induce gene silencing. Then we applied this system to one glycosyltransferase and one transporter, as mentioned later.

Drosophila orthologs of a human glycosyltransferase and a transporter have similar substrate specificities to their human counterparts

The expression of glycosyltransferases and sugar nucleotide transporters in cells determines the glycan structures on the cells. We will show two examples of the conservation of the proteins between humans and *Drosophila*, the proteoglycan UDP-galactose: β-xylose β1,4galactosyltransferase I (β4GalTI) [7] and the 3′-phosphoadenosine 5′-phosphosulfate (PAPS) transporter [8] (Figure 2).

We carried out a TBLASTN search of the *Drosophila* genome database using all members of the human β1,4galactosyltransferase (hβ4GalT) family, hβ4GalT1∼6 and hβ4GalTI, whose activity had already been determined, and then found three *Drosophila* putative β4GalTs, dβ4GalTI, dβ4GalTA and dβ4GalTB (Figure 2A) [7]. A ClustalW alignment and phylogenetic tree of the *Drosophila* and human β4GalTs based on the amino acid sequences clearly showed that *dβ4GalTI* is the *Drosophila* ortholog of *hβ4GalTI*. Then we expressed a FLAG-tagged recombinant dβ4GalTI in insect cells and used the purified enzyme for a galactosyltransferase

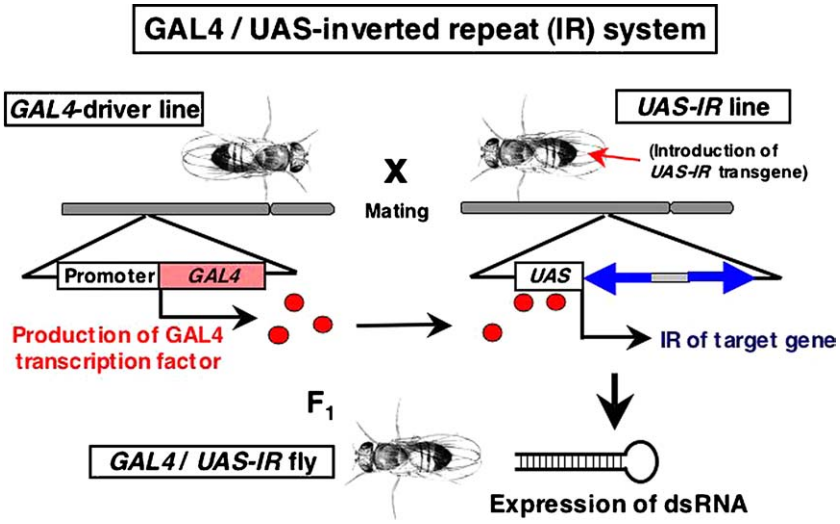

Figure 1. Scheme of the heritable and inducible RNAi system. Two transgenic fly stocks, *GAL4*-driver and *UAS-IR*, are used in this system. The *GAL4*-driver fly has a transgene containing the yeast transcription factor *GAL4*, the expression of which is under the control of a tissue-specific promoter. The *UAS-IR* fly has a transgene containing an IR of the target gene ligated to the UAS promoter, a target of GAL4. In the F_1 progeny of these flies, the dsRNA of the target gene is expressed in specific tissue to induce gene silencing.

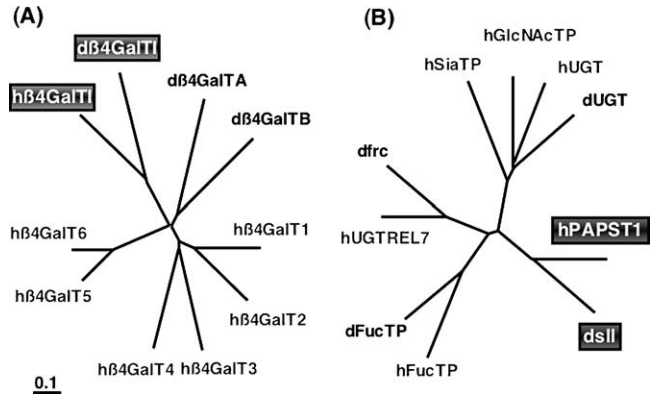

Figure 2. Phylogenetic tree of *Drosophila* and human proteins participating in the synthesis of glycan. The branch lengths indicate amino acid substitutions per site. (A) Phylogenetic tree of three *Drosophila* (dβ4GalTI, dβ4GalTA and dβ4GalTB) and seven human (hβ4GalT1$\sim$6 and hβ4GalTI) β4GalTs. (B) Phylogenetic tree of human and *Drosophila* sugar-nucleotide transporters and PAPS transporters. dsll, *Drosophila* PAPS transporter; hPAPST1, human PAPS transporter; dFucTP, *Drosophila* GDP-Fuc transporter; hFucTP, human GDP-Fuc transporter; dfc, *Drosophila* UDP-sugar nucleotide transporter; hUGTREL7, UDP-GlcA /UDP-GalNAc transporter; dUGT, *Drosophila* UDP-Gal/UDP-GalNAc transporter; hUGT, human UDP-Gal/UDP-GalNAc transporter; hGlcNAcTP, human UDP-GlcNAc transporter; hSiaTP, human CMP-Sia transporter.

assay (Figure 3A). Both dβ4GalTI and hβ4GalTI had strong activity toward the β-Xyl residue, but only slight activity toward α-Xyl and no activity toward β-GlcNAc, β-Glc, β-Gal, or β-GalNAc. It was demonstrated that dβ4GalTI was the *Drosophila* ortholog of hβ4GalTI in view of its activity, that is, it had similar substrate specificity to hβ4GalTI. But the β4GalT activity of dβ4GalTI toward the β-Xyl residue was almost half that of hβ4GalTI at both 25°C and 37°C. During evolution, β4GalTI might have acquired greater activity.

The other example is a PAPS transporter in the Glogi apparatus. PAPS is a sulfate-nucleotide, a universal sulfuryl donor for sulfation. We have identified novel *Drosophila* and human genes encoding putative PAPS transporters, *dsll* and *hPAPST1*, in a TBLASTN search of the *Drosophila* and human genome databases using sugar-nucleotide transporters as query sequences [8]. As shown in Figure 2B, a phylogenetic tree of the transporters clearly demonstrated that *dsll* was the *Drosophila* ortholog of putative *hPAPST1*. Then we expressed hPAPST1 and dSLL proteins in the yeast *Saccharomyces cerevisiae* and prepared Golgi membrane fractions. The expression of both hPAPST1 and dSLL in yeast significantly increased the transport of PAPS into the Golgi membrane fraction proving that both proteins were actual PAPS transporters (Figure 3B). The apparent *Km* values of hPAPST1 and dSLL for PAPS were estimated to be 0.8 μM and 1.2 μM, respectively. Therefore, it was also demonstrated that for PAPS dsll was the *Drosophila* ortholog of hPAPST1 in view of the similar substrate specificity. As mentioned above, β4GalTI and PAPST share a similar function between *Drosophila* and humans.

RNAi knockdown of *dβ4GalTI* and *dsll*, *dPAPS transporter*

We applied the *Drosophila* inducible RNAi knockdown system (Figure 1) to *dβ4GalTI* and *dsll*, which are orthologs of *hβ4GalTI* and *hPAPST1*, respectively. Proteoglycan β4GalTI contributes to the synthesis of the carbohydrate-protein linkage structure, GlcAβ1,3Galβ1,3Galβ1,4Xylβ1-*O*-Ser, of proteoglycans common to heparin/heparan sulfate and chondroitin sulfate/dermatan sulfate. If the proteoglycan β4GalTI is inactivated, all proteoglycans will lack glycosaminoglycan (GAG) chains and severe biological defects are expected. Proteoglycans are also sulfated at various positions along their GAG chains. Since PAPS is the sole donor substrate for sulfation, the down-regulation of PAPS transport into the Golgi lumen may result in undersulfation of all GAG chains. Both of these cases could be expected to cause severe defects.

Twenty-four *UAS-dβ4GalTI-IR* fly lines having a transgene containing two types of the IR of *dβ4GalTI*, were established [7]. No.C1$\sim$C11 and No.N1–N13 have a transgene containing the IR of the C-terminal and N-terminal regions of *dβ4GalTI*, respectively. *Act5C-GAL4* was used as a *GAL4*-driver. In the F_1 generations of the *Act5C-GAL4* fly and the *UAS-dβ4GalTI-IR* fly, dsRNA of *dβ4GalTI* is expressed ubiquitously under the control of the cytoplasmic actin promoter to induce *dβ4GalTI* gene silencing. Approximately 65% of these crosses caused lethality in the progeny, that is, the flies died in the late pupal stage. The severity of the phenotype differed between the lines. This was because the degree of expression of the transgene was known to depend on its sites of insertion in the chromosome.

Then the mRNA levels of all *Drosophila* *β4GalTs*, *dβ4GalTI*, *dβ4GalTA* and *dβ4GalTB*, were determined in each *dβ4GalTI* RNAi knockdown fly by competitive RT-PCR to estimate the efficiency and specificity of RNAi in this system. First, we determined the amounts of the three *dβ4GalT* mRNAs in the third instar larvae of the four RNAi flies, *Act5C-GAL4/N2*, *Act5C-GAL4/N4*, *Act5C-GAL4/N13* and *Act5C-GAL4/N6*, and the wild-type fly, *Actin5C-GAL4/+* (Figure 4B). N13 having 2 copies of the IR on chromosome 2 and 3 was made from the N2 and N4 lines. *Act5C-GAL4/N2*, *Act5C-GAL4/N4* and *Act5C-GAL4/N13* died as pupae, while *Act5C-GAL4/N6* was viable. The ratios of reduction in *dβ4GalTI* mRNA of *Act5C-GAL4/N13*, *Act5C-GAL4/N4*, *Act5C-GAL4/N2*, and *Act5C-GAL4/N6* were 0.26, 0.32, 0.36, and 0.76, respectively, demonstrating a correlation with the severity of the phenotype. F_1 progeny of the N13 line having two copies of the IR had less *dβ4GalTI* mRNA than those of the N2 line and N4 line which were crossed to make the N13 line. Reductions in *dβ4GalTA* mRNA and *dβ4GalTB* mRNA were not observed in any RNAi flies. It was clearly demonstrated that the *dβ4GalTI* mRNA was disrupted specifically and the ratio of degraded *dβ4GalTI* mRNA was well correlated with the severity of the phenotype.

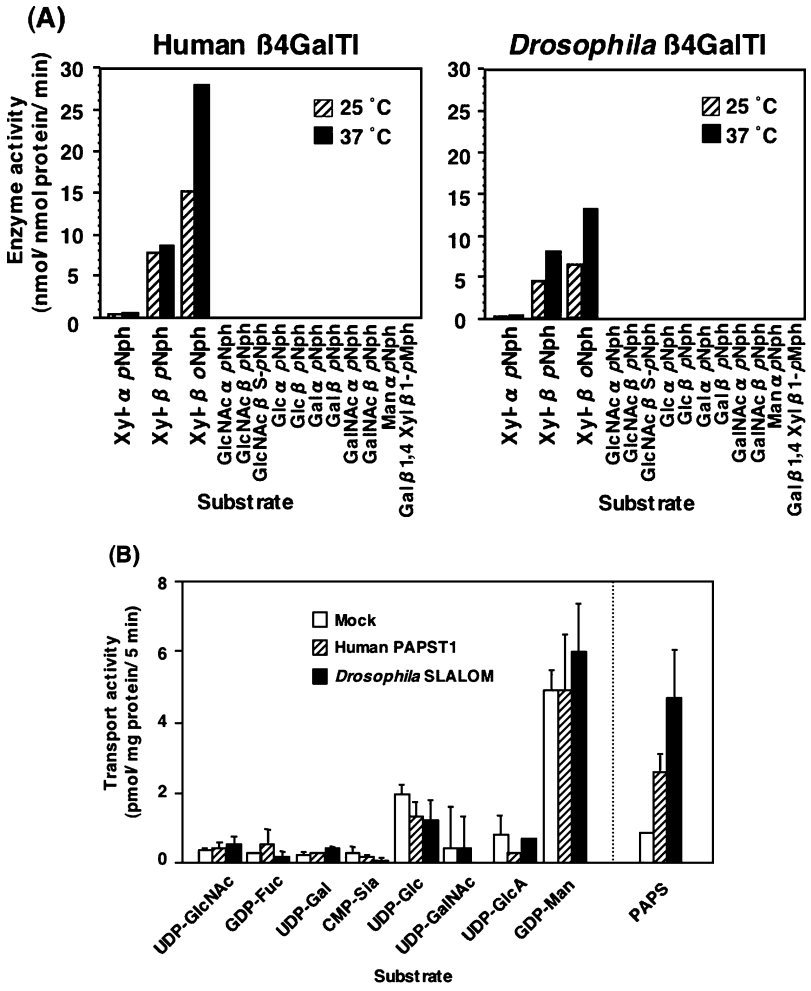

Figure 3. Substrate specificities of *Drosophila* and human proteins participating in the synthesis of glycan. (A) Acceptor substrate specificities of purified recombinant dß4GalTI and hß4GalTI expressed in the baculovirus system. The determined amounts of dβ4GalTI and hβ4GalTI and the same amount of each substrate were used for the enzyme reactions, so that we could determine relative activities that were comparable. (B) Substrate specificity of PAPST1 and SLL in the yeast Golgi membrane fraction (modified ref. [8]). The substrate specificities of PAPST1 and SLL were examined using radiolabeled substrates. The transport activity of PAPS into the Golgi membrane fraction prepared from yeast cells expressing hPAPST1 and dSLL was significantly higher than that for mock (2.5 and 4.9 times, respectively). No difference was observed among PAPST1, SLL, and mock in the transport of other sugar-nucleotides.

Similar analyses were performed for the F_1 progeny of C lines crossed with *Act5C-GAL4*, *Act5C-GAL4/C5* and *Act5C-GAL4/C6* (Figure 4C). Both the pupae died. The *dβ4GalTI* mRNAs in the third instar larvae were also interfered specifically and the ratios of degraded *dβ4GalTI* mRNA of *Act5C-GAL4/C5* and *Act5C-GAL4/C6*, 0.35 and 0.45, respectively, were almost the same as those of *Act5C-GAL4/N2* and *Act5C-GAL4/N4* (Figure 4B). The efficiency of RNAi did not depend greatly on the target sequences using the constructs of IR. We also determined the amount of *dβ4GalTI* mRNAs in prepupae of *Act5C-GAL4/N4* and *Act5C-GAL4/N13* (Figure 4D). The target efficiency in the prepupae was almost the same as that in the third instar larvae.

The second example was the RNAi knockdown of *dsll*, *dPAPST* [8]. We made four *UAS-sll-IR* fly lines, and then used *Act5C-GAL4* as a *GAL4*-driver to induce *sll* gene knockdown in all cells of the fly. All four lines of the F_1 progeny of the *UAS-sll-IR* flies crossed with *Act5C-GAL4* exhibited pupal lethality and no fly developed into an adult. The amounts of *dsll* mRNA in the third instar larvae of each F_1 progeny were analyzed by real-time PCR (Figure 4E). The ratios of reduction in *dsll* mRNA of *Act5C-GAL4/3* and *Act5C-GAL4/4* were 0.21 and 0.24, respectively. The above results clearly demonstrated that the expression of the target gene was interrupted specifically by RNAi in this *Drosophila* RNAi knockdown system and abnormal phenotypes were produced.

In this mini-review, we introduced our RNAi knockdown system and showed two examples, the *dβ4GalTI* [7] and *dsll* (*dPAPST*) RNAi knockdown flies [8]. These cases demonstrate that both genes play important roles in the viability of flies.

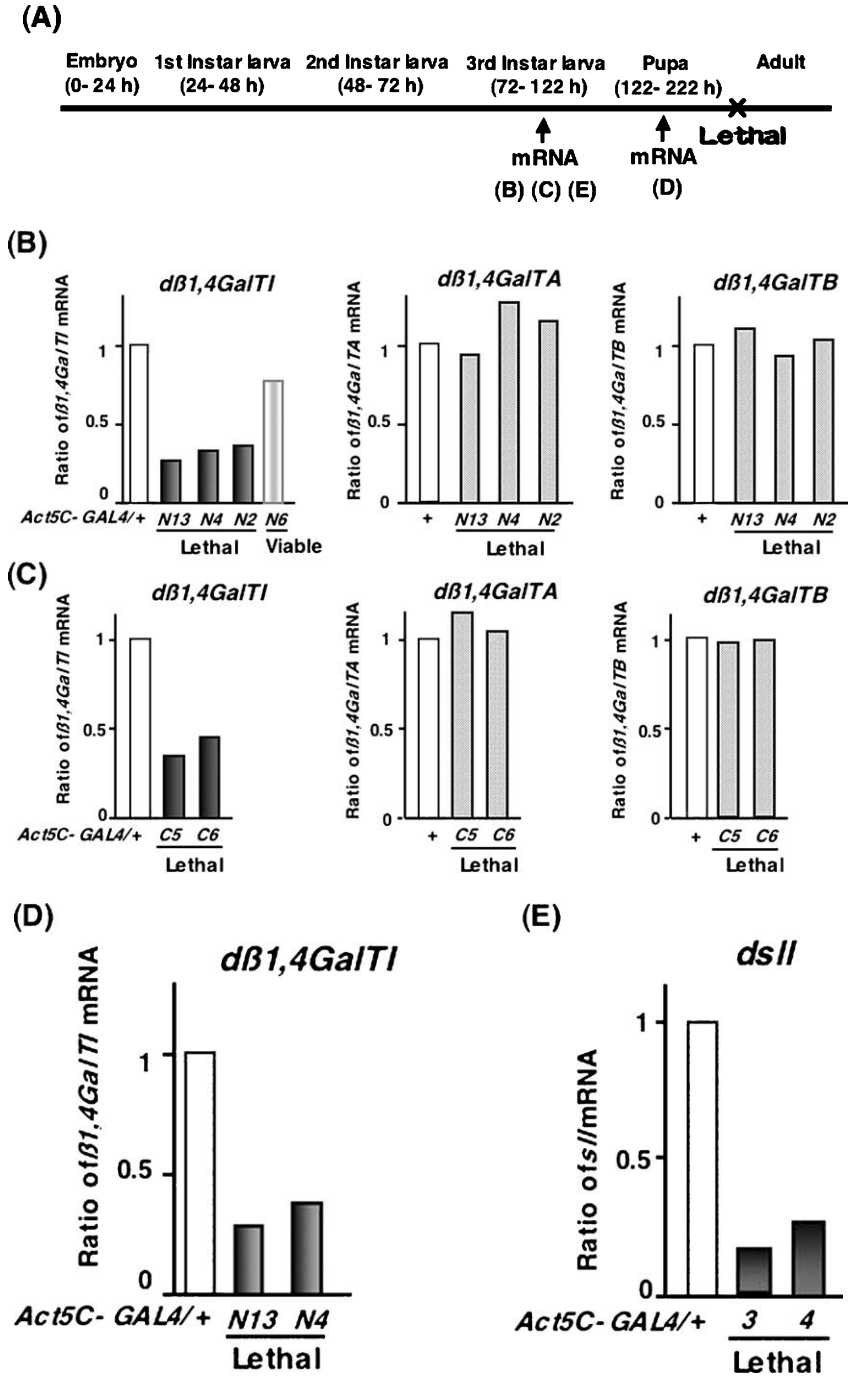

Figure 4. Quantitative analyses of *dβ4GalTI* mRNA and *sll* mRNA in each inducible RNAi knockdown fly: (A) Preparation of mRNA of RNAi knock down flies for quantitative analyses of mRNA. (B) The mRNA levels of three kinds of *dβ4GalTs* in the third instar larvae of the F_1 progeny of each N line of the *UAS-dβ4GalTI-IR* fly crossed with *Act5C-GAL4*, designated as *Act5C-GAL4/N* (modified ref. [7]). The actual amount of each *β4GalT* mRNA was divided by that of *RpL32* mRNA for normalization. The relative amount of each *β4GalT* mRNA to *RpL32* mRNA in F_1 progeny of the w^{1118} crossed with *Act5C-GAL4*, *Actin5C-GAL4/+*, which corresponds to the wild type, is presented as 1. Each N line has the IR corresponding to the N-terminal region of *dβ4GalTI*. *Act5C-GAL4/N2*, *Act5C-GAL4/N4* and *Act5C-GAL4/N13* died as pupae, while *Act5C-GAL4/N6* was viable and morphologically normal. (C) The mRNA levels of three kinds of *dβ4GalTs* in the third instar larvae of the F_1 progeny of each C line of the *UAS-dβ4GalTI-IR* fly crossed with *Act5C-GAL4*, designated as *Act5C-GAL4/C* (modified ref. [7]). Each C line has the IR corresponding to the C-terminal region of d*β4GalTI*. *Act5C-GAL4/C5* and *Act5C-GAL4/C6* died as pupae. (D) The mRNA level of *dβ4GalTI* in the prepupae of *Act5C-GAL4/N4* and *Act5C-GAL4/N13* (modified ref. [7]). (E) The mRNA level of *dsll* in the third instar larvae of the F_1 progeny of each line of the *UAS-dsll-IR* fly crossed with *Act5C-GAL4, Act5C-GAL4/3* and *Act5C-GAL4/4*. *Act5C-GAL4/3* and *Act5C-GAL4/4* died as pupae.

GAG chains and the sulfation of GAG chains were essential for maintaining life.

Recently, fly mutants with severe defects have begun to be used to study deficiencies of glycosyltransferases, including *Drosophila* fringe [9,10] protein *O*-fucosyltransferase [11], polypeptide *N*-acetylgalactosaminyltransferase [12–14], brainiac [15,16] and egghead [17]. Some flies with mutations related to proteoglycan, *dally* [18], *sugarless* [19], *tout-velu* [20], *brother of tout-velu* [20,21], *sister of tout-velu* [20] and s*ulfateless* [19], have demonstrated defects in the signaling of growth factors including Wingless, Decapentaplegic, Hedgehog and fibroblast growth factors.

We found almost seventy *Drosophila* glycosyltransferases by performing a TBLASTN search of the *Drosophila* databases using mammalian glycosyltransferases as the query sequence. *Drosophila melanogaster* is a well-established model system for genetic analysis. Combining our RNAi knockdown system with genetic analyses, *Drosophila melanogaster* will became a powerful tool for analysis of the essential biological roles of glycans. We are attempting to analyze fundamental glycan functions by using RNAi in a *Drosophila* model and in mammalian cultured cells. The results of RNAi technology should be applied to the analysis of the mechanisms and therapy of various diseases and cancer.

Acknowledgments

This work has been supported by the Core Research for Evolutional Science and Technology (CREST) program of the Japan Science and Technology Agency (JST).

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Received 12 March 2004; accepted 17 May 2004