Heparan sulfate proteoglycan plays an important role in developmental processes by modulating the distribution and stability of the morphogens Wingless, Hedgehog, and Decapentaplegic. Heparan and chondroitin sulfates share a common linkage tetrasaccharide structure, GlcA\(\beta\)1,3Gal\(\beta\)1,4Xyl\(\beta\)-Ser. In the present study, we identified Drosophila proteoglycan galactosyltransferase II (d\(\beta\)3GalTII), determined its substrate specificity, and performed its functional analysis by using RNA interference (RNAi) mutant flies. The enzyme transferred a galactose to Gal\(\beta\)1,4Xyl-pMph, confirming that it is the Drosophila ortholog of human proteoglycan galactosyltransferase II. Real-time PCR analyses revealed that d\(\beta\)3GalTII is expressed in various tissues and throughout development. The d\(\beta\)3GalTII RNAi mutant flies showed decreased amounts of heparan sulfate proteoglycans. A genetic interaction of d\(\beta\)3GalTII with Drosophila \(\beta\)1,4-galactosyltransferase 7 (d\(\beta\)4GalT7) or with six genes that encode enzymes contributing to the synthesis of glycosaminoglycans indicated that d\(\beta\)3GalTII is involved in heparan sulfate synthesis for wing and eye development. Moreover, d\(\beta\)3GalTII knockdown caused a decrease in extracellular Wingless in the wing imaginal disc of the third instar larvae. These results demonstrated that d\(\beta\)3GalTII contributes to heparan sulfate proteoglycan synthesis in vitro and in vivo and also modulates Wingless distribution.

Proteoglycans on the cell surface and in the extracellular matrix, which are distributed in various tissues, play important roles in the control of growth and pattern formation of tissues during developmental processes (1–5). They consist of a core protein and negatively charged glycosaminoglycans (GAGs)

that interact with various growth factors, components of the extracellular matrix, morphogens, and cytokines. GAGs are classified into two categories: heparin/heparan sulfate (HS) and chondroitin sulfate (CS)/dermatan sulfate. Particularly, HS proteoglycans (HSPG) have been demonstrated to regulate the signaling activities of the secreted morphogens Wingless (Wg), Hedgehog (Hh), and Decapentaplegic (Dpp) through their distribution (6–8). On the other hand, studies on the biological function of CS proteoglycans (CSPG) are underway; they cover points such as the factors responsible for pathogen infection (9, 10), embryonic cytokinesis and cell division (11, 12), and the inhibition of neurite outgrowth during neural development and regeneration (13–17).

The biosynthesis of GAG is initiated by the formation of the linkage tetrasaccharide structure, GlcA\(\beta\)1,3Gal\(\beta\)1,4Xyl\(\beta\)-Ser, which is common to heparin/HS and CS/dermatan sulfate. In human, genes encoding all four types of glycosyltransferases contribute to the synthesis of the linkage tetrasaccharide structure, and they have been cloned and identified. These include genes for two peptide O-xylitoltransferases (O-XylTs) (18, 19), one proteoglycan \(\beta\)1,4-galactosyltransferase I (\(\beta\)1,4-galactosyltransferase 7) (\(\beta\)4GalT7) (20, 21), one proteoglycan \(\beta\)1,3-galactosyltransferase II (\(\beta\)1,3-galactosyltransferase 6) (\(\beta\)3GalTII) (22), and one glucuronosyltransferase I (GlcATI) (23). In Caenorhabditis elegans, all four types of glucosyltransferases have been cloned and characterized as sgv genes having the squashed vulva phenotype (24–27). A biochemical analysis of GAGs has demonstrated that both C. elegans and Drosophila melanogaster have HS and CS (28–32). In Drosophila, three of the four types of glycosyltransferases, one peptide O-xylitoltransferase (dOXT), one \(\beta\)1,4-galactosyltransferase 7 (d\(\beta\)4GalT7), and three GlcATs, except for \(\beta\)3GalTII, have been identified and characterized for their activities (33–37). But a candidate Drosophila
β3GalTII (dβ3GalTII) has not yet been characterized for its activity (22, 27).

Previously, we reported on the Drosophila inducible RNA interference (RNAi) knockdown system for analyses of the basic physiological functions of glycans (38) and applied this system to two types of glycosyltransferases, namely, dβ4GalT7 and Drosophila protein O-mannosyltransferases, and two transporters, that is, the Drosophila PAPS-transporters slalom (slil) and dpAPST2 (34, 39–41). In the present study, we first performed the biochemical characterization of dβ3GalTII. We then generated inducible dβ3GalTII RNAi flies by using the GAL4–UAS system and subsequently performed the functional analysis.

**EXPERIMENTAL PROCEDURES**

**Materials**—UDP-Gal, p-nitrophenol-α-xlylopyranoside (Xyl-Nph), Xylβ-pNph, p-nitrophenyl-N-acetyl-1-thio-β-glucosaminide (GlcNAC–S-pNph), p-nitrophenyl-α-glucopyranoside (Glcα–pNph), Glcβ–pNph, p-nitrophenyl-α-galactopyranoside (Gal–pNph), Galβ–pNph, p-nitrophenyl-N-acetyl-α-galactosaminide (GlcNAc–pNph), and GalNACβ–pNph were purchased from Sigma; GlcNAc–pNph and GlcNACβ–pNph were purchased from Calbiochem (La Jolla, CA); Galβ1–4Xylβ1–p-methoxyphenyl (Galβ1,4Xylβ1–pMph) was provided by Seikagaku Corporation (Tokyo, Japan); and uridine diphosphate-[14C]galactose (UDP-[14C]Gal) (325 mCi/mmol) was supplied by PerkinElmer Life Sciences (Boston, MA).

**Vector Construction, Expression, and Purification of dβ3GalTII Proteins**—The putative catalytic domain of candidate dβ3GalTII (amino acids 39 to 382) (AAF59065, CG8734) (22) was cloned using the cDNA library of a Drosophila embryo and expressed as a secreted protein fused with a FLAG peptide (22) was cloned using the cDNA library of a

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**Assay for Galactosyltransferase Activity**—To determine the galactosyltransferase activity, Xylα–pNph, Xylβ–pNph, Glcα–pNph, Glcβ–pNph, GlcNACβ–pNph, GlcNACβ–pNph, Glcβ–pNph, Galβ–pNph, Galβ–pNph, Glcα–pNph, GlcNACβ–pNph, and Galβ1,4Xylβ1–pMph were used as the acceptor substrates. Using 10 nmol of each acceptor, the enzyme reaction was terminated by the addition of 400 μl of heparitinase buffer (pH 7.4), 0.5% Triton X-100, 11 mM MnCl₂, 1 mM UDP-[14C]Gal (325 mCi/mmol), 250 μM UDP-Gal, and 0.57 pmol of purified dβ1,3GalTII. The enzyme reaction was measured by a densitometer to determine the amount of the purified enzyme, using the FLAG-BAP control protein (Sigma).

For the Western blot analysis using the monoclonal antibody 3G10, the protein extract (see the subsection “sample preparation”) was subjected to 15% SDS-polyacrylamide gel electrophoresis. The separated protein was transferred to an Immobilon-P membrane (Millipore Corp., Bedford, MA). The membrane was blocked with 5% skim milk in TBST (TBS with 0.1% Tween 20) for 30 min at room temperature and washed with TBST. The blocked membrane was treated with 20 milli-units of heparitinase I (Seikagaku) for 1 h at 37°C and washed with TBST. After heparitinase treatment, the membrane was first probed with the monoclonal antibody 3G10 (1:2000, Seikagaku) and then with horseradish peroxidase-conjugated sheep anti-mouse secondary antibody (1:20000, GE Healthcare). The blots were visualized with the ECL plus Western blotting detection kit (GE Healthcare), according to the manufacturer’s instructions.

**Western Blot Analysis**—To determine the concentration of the purified enzyme, the enzyme purified above was subjected to 12.5% SDS-polyacrylamide gel electrophoresis followed by Western blot analysis. The separated protein was transferred to a Hybond-P membrane (GE Healthcare). The membrane was probed with the anti-FLAG M2-peroxidase conjugate (Sigma) and stained with Konica Immunostaining HRP-1000 (Konica, Tokyo, Japan). The intensity of a positive band obtained using Western blotting was measured by a densitometer to determine the amount of the purified enzyme, using the FLAG-BAP control protein (Sigma).

**Fly Stocks**—All stocks were raised at room temperature (23 to 25°C), using a standard medium. A9-GAL4, MS095-GAL4, hedgehog (hh)–GAL4, engrailed (en)–GAL4, scolloped (sd)–GAL4, patched (ptc)–GAL4, apterous (ap)–GAL4, and Actin5C (Act5C)–GAL4 were obtained from the Bloomington Stock Center, and GMR-GAL4 was obtained from Dr. M. Yamaguchi (Kyoto Institute of Technology). The descriptions of these
RNAi and GalTII Activity in GAG Synthesis

GAL4 lines are referred to as FlyBase. All fly lines except for the wild type have w (white) background mutations (hereafter, we have not described w in the genotype).

Sample Preparation for Western Blotting by the Monoclonal Antibody 3G10—Twenty to 30 third instar larvae were homogenized in 10 mM Hepes-Tris (pH 7.4) containing 0.25 M sucrose, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of leupeptin, apropiatin, and pepstatin A. The extract was centrifuged at 10000 × g for 10 min to remove the debris. The concentration of protein was assayed by the Bradford method.

dβ3GalTII, dβ4GalT7, ttv, sotv, dOXT, dHs3st-B, dHS6ST, and papss RNAi Flies—RNAi fly lines were obtained as described in previous reports (34, 39–41). The gene-specific primer and the ABI PRISM 7700 Sequence Detection System Mastermix QuickGoldStar (Eurogentec, Seraing, Belgium) kit (Invitrogen). Real-time PCR was performed using qPCR was synthesized using a Superscript II first-strand synthesis by using the TRIzol reagent (Invitrogen). First-strand cDNA for each expression sequence tag clone.

Each PCR fragment was inserted as an inverted repeat (IR) sequence into the pSC1 vector. The IR-containing fragments were then subcloned into the transformation vector pUAST, and these vectors were introduced into Drosophila embryos of the w1118 mutant stock that were used as hosts to construct UAS-IR flies according to the procedure reported by Spradling (42). Each line was mated with the appropriate driver fly lines and the F1 progeny were raised at 28°C to observe the phenotypes.

Quantitative Analysis of the dβ3GalTII transcript in tissues of the third instar larvae and genital organs of each expression sequence were determined using real-time PCR. The amount of transcript was normalized to that of the RpL32 transcript, which was measured in the same cDNA. A, distribution of the dβ3GalTII transcript in tissues of the third instar larvae and genital organs of the adult flies.

| Acceptor substrate | Enzyme activity (nmol/nmol protein/min) |
|--------------------|----------------------------------------|
| Xyla-pNph          | ND*                                   |
| Xylβ-pNph          | ND                                    |
| GlcNAc−pNph        | ND                                    |
| GlcNAcβ−pNph       | ND                                    |
| GlcNAcS−pNph       | ND                                    |
| Glcα−pNph          | ND                                    |
| Galα−pNph          | 0.42                                  |
| Galβ−pNph          | 1.4                                   |
| Galβ1,GalYβ1,pMph  | 5.5                                   |
| GalNAcα−pNph       | ND                                    |
| GalNAcβ−pNph       | ND                                    |

* ND, not detected.

FIGURE 1. Spatiotemporal expression pattern of dβ3GalTII. A, the levels of the dβ3GalTII transcript in each developmental stage were determined using real-time PCR. The amount of transcript was normalized to that of the RpL32 transcript, which was measured in the same cDNA. B, distribution of the dβ3GalTII transcript in tissues of the third instar larval and genital organs of the adult fly. CNS, central nervous system.

forward primer 5′-GCTTCAATGCTGCTTCTCT-3′, the reverse primer 5′-GCCAGTAGATTTGGTAATTTCT-3′, and the probe 5′-CCACGATCAGCTGGTAC-3′ were used. For the quantification of dβ3GalTII, the forward primer 5′-GCGTGGAGACCTCATGAG-3′, and the probe 5′-ATACGAGACCCAGATGCCAGTT-3′ were used. For the quantification of dβ3GalTII, the forward primer 5′-GCCAGTAGATTGCGTAAGTTGCT-3′, the reverse primer 5′-GCCGGACAGCAGCCA-3′ were used.
GATACTG-3’, and the probe 5’-AACAGAGTGCAGTCGCG-CCCTTCA-3’ were used. The probes were labeled with a reporter dye FAM and quencher dye TAMRA at the 5’- and 3’-ends, respectively. Relative amounts of the dβ3GalTII, sotv, doXT, dHs3st-B, or dHS6ST transcripts were normalized to those of the RpL32 transcripts in the same cDNA.

**Scanning Electron Microscopy**—Adult flies were separated in new food vials and allowed to age for several days. The preparation was subjected to triethylamine anesthesia prior to mounting on stubs covered with carbon tapes and viewed under a JEOL 5600LL scanning electron microscope (Japan Electron Optics Laboratory Co., Ltd., Tokyo, Japan) under low vacuum (30 Pa) conditions.

**Histochemistry**—Third instar larvae were dissected in ice-cold phosphate-buffered saline. Wing imaginal discs were fixed in phosphate-buffered saline with 4% paraformaldehyde (pH 7.0) for 15 min and rinsed three times with PBT (phosphate-buffered saline with 0.1% Triton X-100). After blocking with 10% goat serum in PBT, the samples were stained with primary antibodies. The primary antibodies were used in the following dilutions: mouse anti-Wg antibody 4D4 at 1:1000 (Developmental Studies Hybridoma Bank); rabbit anti-pMad (PS1) at 1:2000 (43); and rabbit anti-Hh antibody NHH1 at 1:1000 (6). The secondary antibodies were used: anti-mouse Alexa 488 and anti-rabbit Alexa 488 (Molecular Probes).

**Statistical Analysis**—t test was used for analysis of the data of Western blotting. Tukey tests were used for analysis of the eye data. All statistical analysis was performed using the public domain R program.

**RESULTS**

**Characterization of the Galactosyltransferase Activity of dβ3GalTII**—We prepared recombinant candidate dβ3GalTII (CG8734), which has been reported to be the *Drosophila* homolog of SQV-2 (24, 27), to identify proteoglycan galactosyltransferase II activity. The soluble form was prepared by replacing the N-terminal region, including the cytoplasmic and transmembrane domains (amino acids 1 to 38) with an Igκ signal sequence and FLAG peptide sequence. The secreted enzyme was purified using the anti-FLAG M1 gel and quantitated by Western blotting analysis using the anti-FLAG antibody.

The purified enzyme was used for galactosyltransferase assay with various acceptor substrates (Table 1). dβ3GalTII showed stronger activity toward Gal[1,4]Xylβ1-pMph than Galβ-pNph, only slight activity toward -α-Gal, and no activity toward -β-Xyl, -β-GlcNAc, -β-Glc, and -β-GalNAc. These results demonstrated that dβ3GalTII was the *Drosophila* ortholog of hβ3GalTII in view of their activities.
Spatiotemporal Expression Pattern of dβ3GalTII—The developmental expression profiles and tissue distribution of dβ3GalTII mRNA were investigated using a quantitative analysis by real-time PCR (Fig. 1, A and B). As shown in Fig. 1A, dβ3GalTII showed higher expression levels in the early embryonic stage (stages 0–2) than in the other stages. The tissue distribution of dβ3GalTII mRNA in the third instar larvae and adult flies is shown in Fig. 1B. The dβ3GalTII gene showed ubiquitous distribution.

Reduction of dβ3GalTII mRNA and Heparan Sulfate Proteoglycans in RNAi Mutant Flies—To generate a mutant of the dβ3GalTII gene, we performed inducible RNAi knockdown for this gene in the fly by using the GAL4-UAS system (34, 39–41). We constructed three UAS-dβ3GalTII-IR fly lines, namely, UAS-dβ3GalTII-IR(1), UAS-dβ3GalTII-IR(4), and UAS-dβ3GalTII-IR(5). All lines contained a UAS-controlled dβ3GalTII transgene in chromosome II. These lines were crossed with a GAL4 driver fly line, namely, Act5C-GAL4, to express the yeast transcription factor GAL4 under the control of the cytoplasmic actin promoter. The F1 fly lines Act5C-GAL4/UAS-dβ3GalTII-IR(1), Act5C-GAL4/UAS-dβ3GalTII-IR(4), and Act5C-GAL4/UAS-dβ3GalTII-IR(5) were expected to induce dβ3GalTII gene silencing in all cells at all developmental stages in the fly.

The efficiency of gene silencing in the third instar larvae was determined by real-time PCR. The level of the dβ3GalTII transcript in the three RNAi mutant fly lines Act5C-GAL4/UAS-dβ3GalTII-IR(1), Act5C-GAL4/UAS-dβ3GalTII-IR(4), and Act5C-GAL4/UAS-dβ3GalTII-IR(5) was 49.7, 32.6, and 32.1%, respectively, of that in the wild-type fly (Fig 2A).

Subsequently, we analyzed the amount of HSPG in these RNAi mutant flies by Western blotting. No signal was detected by 3G10 in the heparitinase-ununtreated membrane (data not shown). The signal was detected in the heparitinase-treated membrane (Fig. 2C). The ratios of total signal intensity in the Act5C-GAL4/UAS-dβ3GalTII-IR(1), Act5C-GAL4/UAS-dβ3GalTII-IR(4), and Act5C-GAL4/UAS-dβ3GalTII-IR(5) lines were 84, 41, and 49%, respectively, of that in the wild-type fly. Moreover, the signal intensity of the double RNAi mutant flies of dβ3GalTII and dβ4GalT7 was less than that in dβ4GalT7 single RNAi mutant flies (Fig. 2D). These results demonstrated that the knockdown of dβ3GalTII influenced HSPG production.

dβ3GalTII Genetically Interacts with the Drosophila dβ4GalT7 Gene in the Wing and Eyede We investigated the biological functions of dβ3GalTII in the fly. First, we crossed the UAS-dβ3GalTII-IR fly lines with seven GAL4 driver-lines: A9-GAL4, MS096-GAL4, hh-GAL4, en-GAL4, sd-GAL4, ptc-GAL4, and ap-GAL4. Each of the driver lines had a different promoter/enhancer that was expressed in a specific region of the wing. When ap-GAL4 was crossed with UAS-dβ3GalTII-IR, curly wings with concavity along the anteroposterior axis were observed (hereafter referred to as “A-P curly wing phenotype”) (Fig. 3).

Human and Drosophila β4GalT7 proteins are involved in the synthesis of GAGs by transferring a Gal to the Xyl-α-Ser in the tetrasaccharide structure of the protein-linkage region of GAGs. To investigate whether dβ3GalTII is involved in the synthesis of proteoglycans in vivo, we tested for a genetic interaction between dβ3GalTII and dβ4GalT7.

The double RNAi mutant flies have two UAS-IR transgenes. If the control flies have one UAS-IR transgene, theoretically, GAL4 binds the UAS sequence twice in these flies and the double strand RNA of the same gene is produced twice in the double RNAi mutant flies. Therefore, the decrease in the target mRNA is expected to be half of that in the control flies. To avoid this difference, we used the UAS-EGFP transgene. In our experiment, the control flies possessed one UAS-IR and one UAS-EGFP gene, and we obtained the same number of UAS sequences in both the control and double RNAi mutant flies.

First, we used ap-GAL4 for the genetic interaction analysis. However, we could not observe the genetic interaction because of the strong phenotype in the control flies (Table 2).

**TABLE 2**

| Genotype | Percentage of curly wings with concavity along the anteroposterior axis (number of observed wings) |
|----------|-------------------------------------------------------------------------------------------------|
| ap-GAL4/+;UAS-dβ3GalTII-IR(1);UAS-EGFP/+ | 100 (36) |
| ap-GAL4/+;UAS-dβ3GalTII-IR(4);UAS-EGFP/+ | 100 (89) |
| ap-GAL4/+;UAS-dβ3GalTII-IR(5);UAS-EGFP/+ | 100 (53) |
| A9-GAL4/+;UAS-dβ4GalT7-IR[N2]/+;UAS-EGFP/+ | 22 (148) |
| A9-GAL4/+;UAS-dβ3GalTII-IR(1);+;UAS-EGFP/+ | 0 (464) |
| A9-GAL4/+;UAS-dβ3GalTII-IR(4);+;UAS-EGFP/+ | 0 (220) |
| A9-GAL4/+;UAS-dβ3GalTII-IR(5);+;UAS-EGFP/+ | 0 (220) |
| A9-GAL4/+;UAS-dβ4GalT7-IR[N2]/+;UAS-dβ3GalTII-IR(1);+ | 49 (106) |
| A9-GAL4/+;UAS-dβ4GalT7-IR[N2]/+;UAS-dβ3GalTII-IR(4);+ | 54 (208) |
| A9-GAL4/+;UAS-dβ4GalT7-IR[N2]/+;UAS-dβ3GalTII-IR(5);+ | 44 (118) |
We then screened the GAL4 drivers to induce the weaker phenotype. The F1 fly line A9-GAL4/UAS-dβ3GalTII-IR[N2]/++;UAS-EGFP/+ showed a weak A-P curly wing phenotype (22% penetrance in this genotype, Table 2) and we used this driver as the test for the genetic interaction between dβ3GalTII and dβ4GalT7. Neither of the three, that is, A9-GAL4/++; UAS-dβ3GalTII-IR [1]/++;UAS-EGFP/+; A9-GAL4/++;UAS-dβ3GalTII-IR [4]/++;UAS-EGFP/+; and A9-GAL4/++;UAS-dβ3GalTII-IR [5]/++;UAS-EGFP/+, exhibited the A-P curly wing phenotype. On the other hand, 49, 54, and 44% of the double RNAi fly lines A9-GAL4/++;UAS-dβ4GalT7-IR[N2]/ UAS-dβ3GalTII-IR [1]/++; A9-GAL4/++;UAS-dβ3GalTII-IR [4]/++; and A9-GAL4/++;UAS-dβ3GalTII-IR [5]/++;, respectively, exhibited the A-P curly wing phenotype (Table 2). These results demonstrated that dβ3GalTII genetically interacted with dβ4GalT7 in wing development.

We also used a different GAL4 driver line-class multiple reporter (GMR)-GAL4 to express the GAL4 protein in the developing eye under the control of the GMR promoter. The compound eye of the wild type was normal (Fig. 5B). The eye of the dβ4GalT7 RNAi flies, GMR-GAL4/+;UAS-EGFP/++;UAS-dβ4GalT7-IR[R2]/+, had a weak rough-eye phenotype with disrupted ommatidial assembly, fused ommatidia, and duplication and/or loss of interommatidial bristles (Fig. 4, A and B). The eye of the dβ3GalTII RNAi flies, GMR-GAL4/++; UAS-dβ3GalTII-IR-[1]/++;;UAS-EGFP/+; and GMR-GAL4/++;UAS-dβ3GalTII-IR-[4]/++;UAS-EGFP/+; also exhibited the weak rough-eye phenotype (Fig. 4, C, E, and G). The percentages of abnormal interommatidial bristles in these flies were 20–30%. The double RNAi fly lines, namely, GMR-GAL4/+;UAS-dβ3GalTII-IR-[1]/++; UAS-dβ4GalT7-IR[R2]/+; GMR-GAL4/+;UAS-dβ3GalTII-IR-[4]/++;UAS-dβ4GalT7-IR[R2]/+; and GMR-GAL4/+;UAS-dβ3GalTII-IR-[5]/++;UAS-dβ4GalT7-IR[R2]/+, showed an enhanced rough-eye phenotype (Fig. 4, D, F, and H). The percentages of abnormal interommatidial bristles in these flies were 57, 71, and 39%, respectively (Fig. 4f). These results implied that dβ3GalTII knockdown enhanced the phenotype of dβ4GalT7 knockdown and indicated that dβ3GalTII genetically interacts with dβ4GalT7 during eye development. The above results clearly showed that dβ3GalTII genetically interacted with dβ4GalT7 in vivo.

dβ3GalTII Genetically Interacts with dOXt, ttk, sotv, dhs3st-B, dhs6ST, and papss in the Eye—To confirm the involvement of dβ3GalTII in the synthesis of HSPG, we also examined the genetic interaction between the dβ3GalTII gene and another gene involved in the synthesis of HS in the eye; first, we focused on the peptide O-xylosyltransferase gene dOXt. The dOXt protein catalyzes the first xylose of the linkage region of GAG in Drosophila (Fig. 5A). The RNAi fly line driven by GMR-GAL4, namely, GMR-GAL4/+;UAS-EGFP/+; UAS-dOXt-IR/+; showed a weak rough-eye phenotype with disrupted ommatidial assembly (Fig. 5E). The other control line, GMR-GAL4/++;UAS-dβ3GalTII-IR-[5]/++;UAS-EGFP/+, also showed the weak rough-eye phenotype (Fig. 5C). Here, the double-knockdown fly line GMR-GAL4/++;UAS-dβ3GalTII-IR-[5]/++;UAS-dOXt-IR/+ exhibited an enhanced rough-eye phenotype with disrupted ommatidial assembly and fused ommatidia (Fig. 5, C, K, and L), although one control fly showed a strong rough-eye phenotype and no significant difference in the
proportion of interommatidial bristles was observed (Fig. 5M).

To clarify the difference of eye phenotype of the single RNAi mutant flies, we examined the knockdown efficiency of dβ3GalTII RNAi fly line. The expression level of Hh and pMad, which is the activated form of Mad and activated by Dpp receptor Thickveins (Tkv) in response to Dpp signaling, did not change in the dβ3GalTII RNAi region in the wing disc of the third instar larvae (data not shown).

On the other hand, when we induced RNAi by en-GAL4, the amount of Wg in the dβ3GalTII RNAi region decreased. Wg is normally expressed at the wing margin, that is, the boundary between the dorsal and ventral sides. We used en-GAL4, which expresses the GAL4 protein, in the posterior region of the wing in the third instar larva (Fig. 6A). The dβ3GalTII RNAi fly line, namely, en-GAL4/ UAS-dβ3GalTII-IR(5), showed decreased amounts of extracellular Wg in the posterior region of the wing (Fig. 6, B–D). This result indicated that the decreased HSPG expression with a decrease in the linkage structure formation affected extracellular Wg distribution.

**DISCUSSION**

Human β3GalTII (hβ3GalTII) has been cloned as β3GalT6, the sixth member of the β1,3-galactosyltransf erase family, which transfers Gal to the GlcNAc or GalNAc residue (22). This dβ3GalTII shares a homology with Brainiac, that is, β1,3-N-acetylgalcosaminyltransferase, which transfers GlcNAc to β-linked mannose (Man), with a preference for the disaccharide Manβ1,4Glc, the core of arthro-series glycolipids (44, 45). In human, the β1,3-galactosyltransferase family shows a homology with the β1,3-N-acetylgalcosaminyltransferase family (46) but not with the β1,4-galactosyltransferase family, although both of the galactosyltransferases transfer a same Gal residue to β-linkage. This phenomenon is conserved between human and *Drosophila*.

dβ3GalTII transfers Gal to β-linked Gal, with a four times higher preference for the disaccharide Galβ1,4Xylβ- than for the monosaccharide Galβ- (Table 1). Its activity is completely consistent with hβ3GalTII (22), showing that dβ3GalTII is an ortholog of hβ3GalTII in terms of activity. *C. elegans* β3GalTIII showed no activity for the monosaccharide Galβ- (27). In a previous study, we cloned and characterized dβ4GalT7, which synthesizes the substrate of dβ3GalTII, the Galβ1,4Xylβ- structure (34). We assayed the

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**FIGURE 5. Genetic interaction between dβ3GalTII and dOXT, tvt, sotv, dhs3st-B, dhs6ST, and papp3 in the eye.** A shows the structure of HSPG and the genes that are involved in HSPG synthesis. B, C, and D show the representative eye of a fly in each genotype. B, wild type (Canton-S). C, GMER-GAL4/+; UAS-dβ3GalTII-IR(5); UAS-EGFP. D, GMER-GAL4/+; UAS-dβ3GalTII-IR(5); UAS-dOXT-IR/+; F, GMER-GAL4/++; UAS-dβ3GalTII-IR(5); UAS-dOXT-IR/+; H, GMER-GAL4/+; UAS-dβ3GalTII-IR(5); UAS-dOXT-IR/+; J, GMER-GAL4/++; UAS-dβ3GalTII-IR(5); UAS-dOXT-IR/+; K, GMER-GAL4/++; UAS-dβ3GalTII-IR(5); UAS-dOXT-IR/+; L, GMER-GAL4/++; UAS-dβ3GalTII-IR(5); UAS-dOXT-IR/+; M, GMER-GAL4/++; UAS-dβ3GalTII-IR(5); UAS-dOXT-IR/+; N, GMER-GAL4/++; UAS-dβ3GalTII-IR(5); UAS-dOXT-IR/+; O, GMER-GAL4/++; UAS-dβ3GalTII-IR(5); UAS-Hs3st-B; R, GMER-GAL4/++; UAS-dβ3GalTII-IR(5); UAS-Hs6ST; T, GMER-GAL4/++; UAS-dβ3GalTII-IR(5); UAS-Hs6ST; U, GMER-GAL4/++; UAS-papp3-IR; UAS-dβ3GalTII-IR(5); V, GMER-GAL4/++; UAS-papp3-IR; UAS-dβ3GalTII-IR(5); W, GMER-GAL4/++; UAS-papp3-IR; UAS-dβ3GalTII-IR(5); X, GMER-GAL4/++; UAS-papp3-IR; UAS-dβ3GalTII-IR(5); Y, GMER-GAL4/++; UAS-papp3-IR; UAS-dβ3GalTII-IR(5); Z, GMER-GAL4/++; UAS-papp3-IR; UAS-dβ3GalTII-IR(5). All lines were raised at 28°C. Eyes displayed in B had no aberrant phenotype. Eyes displayed in C, E, H, N, Q, and T exhibited a weak rough-eye phenotype. Eyes displayed in F, I, K, L, O, R, and U exhibited a strong rough-eye phenotype. D, G, J, M, P, S, and V show the proportion of abnormal interommatidial bristles in the eye of each genotype. The numbers on the Y axis of each graph indicate each of the genotypes described above. Values shown are the means ± S.E. All lines are n = 10. The proportion of abnormal interommatidial bristles in which there were significant differences between two control (single RNAi mutant) lines and the double RNAi mutant line have been indicated using three asterisks (Tukey test, p < 0.001).
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FIGURE 6. Decrease in extracellular Wingless in the wing. Panels A–C show wing imaginal discs in the third instar larvae. All discs are anterior left, dorsal up. A, en-GAL4/UAS-EGFP. The expression of en-GAL4 in the posterior region of the wing. B and C, en-GAL4/UAS-dβ3GalTII-IR(51). Both lines were raised at 28 °C. C shows a high magnification view of the white box in B, D, the staining intensity of Wg in the selected area (white box in C) was integrated along the anteroposterior axis and was plotted using the ImageJ program. The white and black arrowheads shows the anteroposterior boundary. Wg decreased in the dβ3GalTII knockdown region of the wing.

fβ4GalT7 activity using a protocol that was almost the same as that adopted for dβ3GalTII. The purified recombinant db4GalTII transferred Gal to Xylβ-, at a rate of 6.3 nmol/nmol of protein/min at 25 °C. This transfer rate is of the same order as that of dβ3GalTII, which is 5.5 nmol/nmol of protein/min at 25 °C. Thus, in the Golgi apparatus, both enzymes transfer Gal at almost equal rates for smooth synthesis of the linkage tetrasaccharide structure GlcAβ1,3Galβ1,3Galβ1,4Xylβ1-O-Ser.

HSPG are cell-surface, extracellular matrix molecules composed of HS and core proteins (47–49). In mammals, several core proteins in HSPG have been demonstrated. On the other hand, only four core proteins in HSPG are known in Drosophila; these include Syndecan, Dally, Dally-like, and Trol (50–53). In this study, we showed the possibility that there are several other core proteins. More than 10 signals were observed approximately between 30 and 80 kDa (Fig. 2C). Because the putative molecular masses of Syndecan, Dally, Dally-like, and Trol are 23 and 40, 63, 77, and 94 kDa, and, 320–460 kDa, respectively, novel core proteins in HSPG possibly exist in the larval Drosophila proteins. The RNAi knockdown flies of dβ3GalTII will be useful in analyzing these novel core proteins.

In addition to the biochemical function of dβ3GalTII in vitro, the present study showed its developmental function in vivo. The RNAi in the wing and eye caused the morphological changes (Figs. 3 and 4). Mutations in the genes involved in the synthesis of HSPG, including those encoding the core proteins (51, 54, 55), glycosyltransferases (6–8), sulfotransferases (56, 57), and nucleotide sugar transporter (58, 59), show several phenotypes in the wing and eye. For example, dHs3st-B RNAi in the eye resulted in the rough-eye phenotype (57). These similar phenotypes in comparison to the dβ3GalTII RNAi fly strongly indicate that dβ3GalTII is the gene involved in the synthesis of HSPG in vivo and, moreover, suggest that dβ3GalTII interacts with these genes. In fact, dβ3GalTII interacted with dβ4GalT7 in the wing (Table 2) and with dβ4GalT7, dOXt, ttv, sotv, dHs3st-B, dHS6ST, and paps in the eye (Figs. 4 and 5).

A number of reports have mentioned that HSPG is required in the signaling of growth factors such as Wg, Hh, and Dpp during development (6, 60–62). During photoreceptor differentiation in the eye of Drosophila, the Hh protein controls the progression of the morphogenetic furrow, and Dpp signaling follows the Hh signaling within and in front of the morphogenetic furrow (63, 64). Because dHs3st-B, dHS6ST; and dlp (dally-like) are expressed in the morphogenetic furrow (55, 57, 65), HSPG is also expected to be involved in the growth factor signaling in the developing eye. The genetic interactions between dβ3GalTII and dβ4GalT7, dOXt, ttv, sotv, dHs3st-B, dHS6ST, and paps in the eye (Figs. 4 and 5) suggest that HSPG also contributes to morphogenesis in the eye via Hh and/or Dpp signaling.

Baeg et al. (56) reported that sfl (sulfateless), one of the genes involved in HSPG synthesis, is involved in Wg signaling during development. The accumulation of extracellular Wg decreases in the sfl mutant clone in the wing discs. The same result was obtained from the mutant of the sfl (slalom) gene, which was involved in HSPG synthesis (66). In this study, we also observed a decrease in extracellular Wg expression in the dβ3GalTII RNAi flies (Fig. 6). The wing phenotypes of mutants in the genes involved in the synthesis of HSPG were also attributable to defective Hh and/or Dpp signaling (6–8, 61). However, the expression of Hh or pMad, a downstream molecule of Dpp, was not altered in dβ3GalTII RNAi flies (data not shown). dβ3GalTII was expressed in the wing and eye discs in the third instar larvae of Drosophila (Fig. 1A) and may be involved in Hh and Dpp signaling. Because the expression level of dβ3GalTII in dβ3GalTII RNAi flies was ~30–50% and that of the total HSPG was ~40–80% of that in wild type (Fig. 2, A and C), the Hh or Dpp signaling pathway might have been slightly altered; therefore, we could not detect changes in these pathways.

The linkage tetrasaccharide structure of GAGs, GlcAβ1,3Galβ1,3Galβ1,4Xylβ1-O-Ser, is common to HS and CS. dβ3GalTII is also possibly involved in CS synthesis because of the enzyme, which transfers a Gal to a -Gal residue in the linkage structure. Several studies have mentioned that CS is required in the regeneration of the central nervous system in mammal (15, 68–70) and binds to signaling molecules, which are involved in axon guidance in vitro (67). On the other hand, in Drosophila, although a few studies indicated the existence of CSPG (28, 31), the in vitro and in vivo functions of CSPG have yet been clarified. Further investigations will be necessary to clarify the functions of CSPG in Drosophila.

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