4.05 *Drosophila* Development, RNAi, and Glycobiology

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4.05.1 Introduction

The fruit fly *Drosophila melanogaster* is a cosmopolitan, holometabolous insect that lives in all warm countries. *Drosophila melanogaster* was first used for genetic studies by Thomas Hunt Morgan in 1908. Since then, a very large number of studies have exploited this organism. *Drosophila* has many advantages for genetic studies, such as short life

cycle, large number of offspring, polytene chromosomes, and low cost. For these and other reasons, *Drosophila* has become one of the most important model organisms in the fields of classical and molecular genetics as well as developmental biology. Recently, effective analyses of glycan functions *in vivo* have been achieved using a genetic approach in *Drosophila*. In contrast, the structural complexity and functional redundancy of glycans has proved to be particularly problematic for biochemical studies. Induced mutations in some glycosyltransferases are associated with distinct phenotypes in *Drosophila*, and these mutations have been invaluable in clarifying the role of glycosylation in Wg/Wnt, Hh, FGF, and Notch signaling *in vivo*. The next section provides a brief, general introduction to *Drosophila melanogaster*, and the subsequent sections provide an overview of *Drosophila* glycobiology.
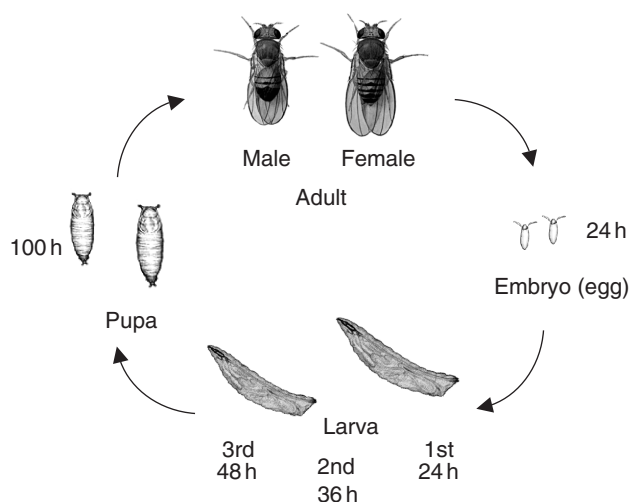
4.05.1.1   Normal Development in *Drosophila*

The *Drosophila* life cycle[1] consists of six stages: embryo, first-instar larva, second-instar larva, third-instar larva, pupa, and adult (summarized in **Figure 1**). A *Drosophila* egg is about a half millimeter in length. The time taken for embryogenesis and subsequent development varies with temperature. At 25°C, an embryo takes about 1 day after fertilization to develop and hatch into a worm-like larva. The larva grows continuously and undergoes three molts: the first after 1 day, the second after 2 days, and the third after 4 days. The final molt produces an immobile pupa. The pupal stage lasts about 4 days, during which the body is completely remodeled to give the adult winged form. The adult hatches from the pupal case and is fertile within about 12h. Overall, it takes *Drosophila* approximately 10 days, at 25°C, to develop from an egg to an adult fly.

   Drosophila melanogaster is a molting insect and has two distinct stages in its life cycle with totally different body plans, the larva and the adult. During larval development, cells that will make adult structures are put aside as imaginal discs, such as wing discs, eye discs, leg discs, and so on. Metamorphosis occurs during the pupal stage; the larval body breaks down and the adult body is reconstructed from the imaginal discs.

   Oocytes have anterior–posterior and dorsal–ventral axes determined by maternal factors, for example, *bicoid* and *nanos* mRNAs, which are distributed during oogenesis. During embryogenesis, cellular membranes do not form until after the 13th nuclear division. Prior to this stage, all nuclei share the same cytoplasm (a syncitium), and materials can diffuse throughout the embryo. After the 13th nuclear division, when about 5000 nuclei are present in the syncitium, the nuclei migrate to the surface of the embryo and are then separated by plasma membranes, forming cells that surround the yolk sac. This cleavage process is unique to insects. The germ line segregates from the somatic cells through the formation of pole cells at the posterior end of the embryo.

   As in other metazoa, gastrulation leads to the formation of three germ layers – the endoderm, the mesoderm, and the ectoderm. The mesoderm invaginates from a ventral furrow that consists of prospective mesodermal cells.

Figure 1   The life cycle of *Drosophila melanogaster*. The life cycle consists of six parts, embryo, first-instar larva, second-instar larva, third-instar larva, pupa and adult. *Drosophila melanogaster* takes about 9–10 days to develop from the fertilized egg to the adult fly at 25°C.

The internalization of cells from the surface of the blastoderm leads to the separation of the mesodermal and endodermal primordium from the ectodermal primordium. (Pole cells are internalized by a different route.) After this stage, cellular behavior in the three germ layers is conspicuously different. The ectoderm gives rise to the midgut.

Germ band elongation involves many rearrangements of the cells belonging to the three germ bands. The posterior region, including the hindgut, expands and extends toward the anterior pole along the dorsal side of the embryo. Segmentation begins to appear with the formation of parasegmental furrows. At this stage, the tracheal pits also form.

Germ band retraction returns the hindgut to the dorsal side of the posterior pole and coincides with clear segmentation, which is determined by expression of segmentation genes. After that, the nervous system internalizes and the internal organs form.

4.05.1.2 *Drosophila* Mutagenesis

4.05.1.2.1 Overview of *Drosophila* mutagenesis

In *Drosophila*, genetic screens for mutations have enabled the identification and characterization of many genes (and the molecules they encode) involved in growth factor signaling. The most direct strategy for identification of gene function is inactivation of individual genes by either random or site-directed mutagenesis. In *Drosophila*, mutagenesis has classically been based on chemically induced random mutagenesis using ethyl methanesulfonate[1] or on transposon-mediated insertional mutagenesis using *P* transposable elements.[2,3] However, both methods have their limitations for performing genome-wide gene function analyses. Chemically induced mutations take a significant amount of time for mapping *de novo* mutations to chromosomes. In contrast, *P* element-induced mutations can be made and identified easily on a large scale. Mutagenesis is induced by crossing two strains, one of which contains *P* element transposase. The insertion of a *P* element causes a new mutation. However, *P* elements integrate preferentially into certain hot spots.[4] This makes saturation mutagenesis difficult. Both chemically induced mutagenesis and transposon-mediated insertional mutagenesis are suitable for forward genetics.

Following completion of the genome project in *Drosophila melanogaster*, a large number of genes with unknown functions were identified. Other methods of mutagenesis are required for the analysis of the functions of these genes. That is, we need methods that allow a reverse genetics approach. Recently, two novel mutagenesis methods have been developed: gene targeting by homologous recombination[5,6] and gene silencing by RNA interference (the latter is described in Section 4.05.1.2.2).[7–9]

A method for targeted gene replacement using homologous recombination in *Drosophila* was developed in 2000.[5] Prior to this, research into gene function in *Drosophila* was hindered by the inability of performing homologous recombination between introduced DNA and the corresponding chromosomal locus, unlike the situation in other model organisms such as yeast and mouse. In organisms in which gene targeting was successful, cut or broken DNA molecules proved to be more recombinogenic than covalently closed circular DNAs. In *Drosophila*, double-strand breaks in DNA are also recombinogenic. There are two strategies for gene targeting by homologous recombination, and these are termed ends-in targeting[5] and ends-out targeting[6] (**Figure 2**). In ends-in targeting, a DNA double-strand break is made within the region of DNA that is homologous to the target locus. As a result of recombination, a marker gene, such as the *mini-white* marker, is inserted into and interrupts the target gene, thereby knocking out the function of the target. In contrast, in ends-out targeting, DNA double-strand breaks are made at both ends of the homologous DNA region. Recombination induces a simple replacement of the genomic sequence with the homologous sequence.

4.05.1.2.2 RNA interference

4.05.1.2.2.1 Mechanism of RNA interference

RNA interference (RNAi) is an evolutionarily conserved event in which double-stranded RNA (dsRNA) induces gene silencing (**Figure 3**).[10–12] RNAi is considered to originate from an ancient endogenous defense mechanism against viral and other heterologous dsRNA.[13,14] RNAi-mediated gene silencing is widely used as a tool for studying gene function in higher eukaryotes including humans and *Drosophila*. In the initiation step, a long dsRNA is processed by the RNase III enzyme Dicer into 21–23 nt small RNA duplexes containing 2-nt 3′ overhangs, which are called small interfering RNAs (siRNAs). These siRNAs are incorporated into a multimeric protein complex termed the RNA-induced silencing complex (RISC). The evolutionarily conserved family of Argonaute proteins is an essential component of the RISC. The RISC can recognize and specifically cleave a target RNA complementary to the guide strand of the siRNA. This last step is so sensitive that a single base mismatch between the target RNA and the guide strand of the siRNA is sufficient to stop destruction of the target RNA. This process, involving degradation of mRNA, is a type of post-transcriptional gene silencing (PTGS). In many model organisms, including *Drosophila*, *Caenorhabditis*
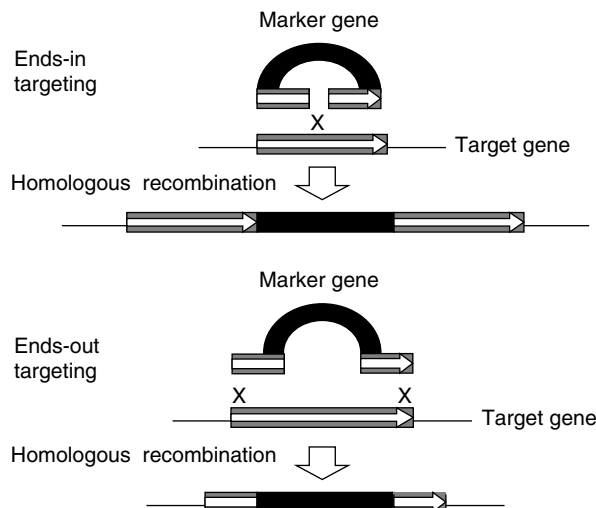
Figure 2   Two typical gene targeting strategies in *Drosophila*. In ends-in targeting, a DNA double-strand break is made within the region of DNA that is homologous to the target locus. In ends-out targeting, DNA double-strand breaks are made at the both ends of the region of homology.
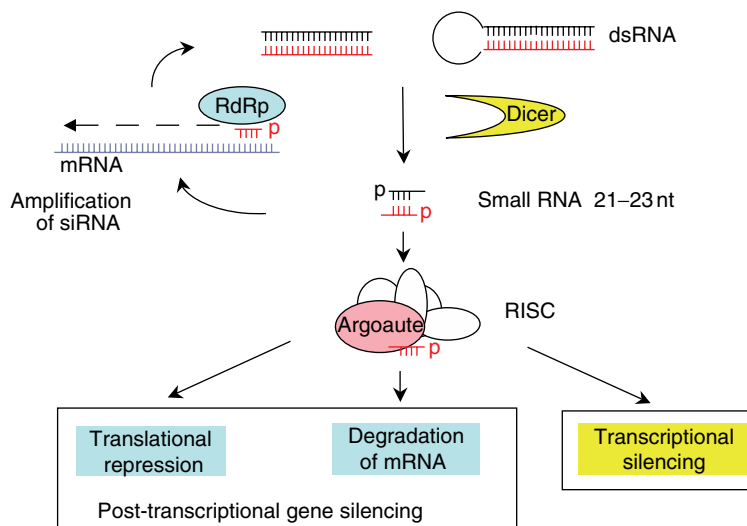

Figure 3   Three types of RNA silencing. Long dsRNAs are processed by the RNase III enzyme Dicer into 21–23nt small RNA duplexes containing 2-nt 3′ overhangs. These small RNA duplexes are then incorporated into a multimeric protein complex, called the RNA-induced silencing complex (RISC). Subsequently, the RISC, or a RISC-like complex, induces three different types of RNA silencing – 'translational repression', 'degradation of mRNA', and 'transcriptional silencing'. During 'degradation of mRNA', the conventional RNAi pathway, the RISC can recognize and cleave specifically a target RNA complementary to the guide strand of the small interfering RNA (siRNA). Usually, micro RNAs (miRNAs) are incorporated into a RISC-like micro ribonucleoprotein particle, miRNP, and induce 'translational repression'. 'Degradation of mRNA' and 'translational repression' are types of post-transcriptional gene silencing. RISC can also induce transcriptional gene silencing.

elegans (*C. elegance*) and plants, large dsRNAs efficiently induce gene-specific silencing,[8,15,16] whereas only siRNAs can efficiently suppress the expression of the corresponding gene in mammalian cells.[17,18] Moreover, *C. elegans* and plants contain a metabolic pathway that enables amplification of siRNAs by an RNA-dependent RNA polymerase (RdRp) (**Figure 3**). *Drosophila* and humans do not have this pathway, which reduces the specificity of RNAi in these species.[11]

Gene silencing by micro RNA (miRNA) is another PTGS pathway that differs from the above-mentioned conventional RNAi pathway (**Figure 3**).[19–22] More details of each pathway are shown in **Figure 4**. miRNAs comprise a large family of small regulatory RNAs expressed in animals and plants. These RNAs are derived from long primary transcripts, termed pri-miRNAs, that are cleaved by the RNase III enzyme Drosha into precursor miRNAs hairpins of about 70nt RNA duplexes with 2-nt 3′ overhangs (pre-miRNAs). After export from the nucleus to the cytoplasm, pre-miRNAs are cleaved into double-stranded miRNAs with 2-nt 3′ overhangs (similar to siRNAs) by Dicer 1 and are then incorporated into a RISC-like micro ribonucleoprotein particle, miRNP. The miRNAP contains Argonaute 1 and Dicer 1, while the RISC contains Argonaute 2 and Dicer 2. In contrast to siRNA, which induces mRNA cleavage, the single-stranded miRNA guide shows imperfect sequence complementarity to a target mRNA and induces repression of translation.

The same RNAi machinery that acts to repress genes post-transcriptionally can cause chromatin modification at homologous loci and induce transcriptional gene silencing (TGS) (**Figure 3**). TGS was initially observed in plants and, more recently, was also found in *Drosophila* and mammals.[11,12] TGS is triggered by double-stranded RNAs, induces transcriptional shutdown of the corresponding genes, and is associated with *de novo* DNA methylation on DNA sequences homologous to double-stranded RNAs.
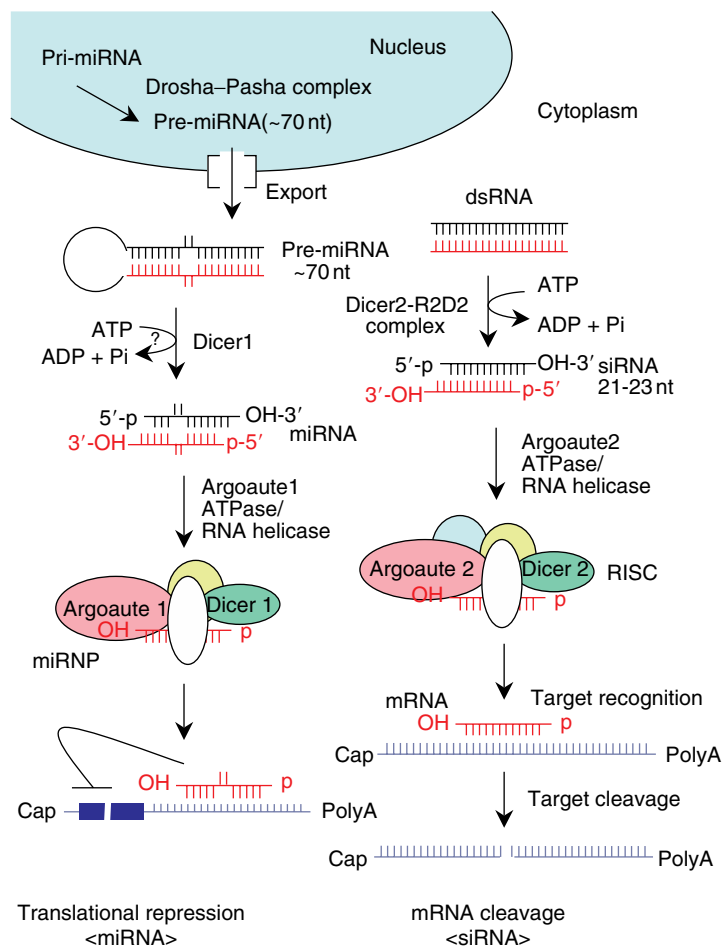

Figure 4   Post-transcriptional gene silencing by miRNA and siRNA. There are two kinds of post-transcriptional gene silencing – 'translational repression' by micro RNA (miRNA) and 'mRNA cleavage' by small interfering RNA (siRNA). The miRNAs are derived from long primary transcripts (pri-miRNAs), which are cleaved by the RNase III enzyme Drosha into pre-miRNA hairpins. After export from the nucleus to the cytoplasm, the pre-miRNA is cleaved into double-stranded miRNA with a 2-nt 3′ overhang by Dicer 1 and incorporated into miRNP. miRNAP contains Argonaute 1 and Dicer 1, while RISC contains Argonaute 2 and Dicer 2. In contrast to siRNA, which induces conventional 'mRNA cleavage', the single-stranded miRNA guide shows imperfect sequence complementarity to the target mRNA and induces 'translational repression'.

4.05.1.2.2.2   *Drosophila* RNAi system for the functional analysis of genes

Analysis of genes that have no mutant alleles requires the use of reverse genetic approaches such as homologous recombination-mediated gene replacement or RNAi-mediated gene silencing. It takes a considerable time to carry out targeted gene replacement by homologous recombination. Consequently, this approach is not suitable for identifying the functions of large numbers of genes. In contrast, RNAi is a powerful reverse genetics tool for studying gene function in many model organisms, including plants, *C. elegans*, and *Drosophila melanogaster*.[8,16] The large dsRNAs efficiently induce gene-specific silencing without interferon responses. Higher organisms, however, have complex antiviral defense mechanisms that induce interferon secretion in response to long dsRNAs. Interferon secretion activates a cascade of interferon-stimulated genes. Therefore, long dsRNAs are unsuitable for use as an experimental tool in RNAi knockdown analyses of gene expression in mammalian cells.

A *Drosophila* inducible RNAi knockdown system for the functional analyses of glycans was established using the GAL4-upstream activating sequence system (GAL4-UAS system).[8,9] An outline of this approach is shown in **Figure 5**. Two transgenic fly lines, *GAL4*-driver and *UAS-inverted repeat* (*UAS-IR*), are used. 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. To date, we can get 4300 *GAL4*-driver fly lines from the *Drosophila* stock centers. 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 under the control of a tissue-specific promoter to induce gene silencing. Using this system, we can induce tissue-specific gene silencing and analyze gene function, even for lethal null mutations.

The glycosaminoglycan (GAG) β-1,4-galactosyltransferase I (β4GalTI) was used to validate the above system as a practical approach for inducible RNAi knockdown in *Drosophila*.[9] A phylogenetic tree based on the amino acid sequences of *Drosophila* and human β-1,4-galactosyltransferases (β4GalTs) is shown in **Figure 6a**. The *Drosophila* β4GalT family consists of one β4GalT (dβ4GalTI) and two β-1,4-*N*-acetylgalactosaminyltransferase (β4GalNAcTs) (dβ4GalNAcTA and dβ4GalNAcTB). In comparison, the human β4GalT family consists of seven β4GalTs (hβ4GalTI and hβ4GalT1–6). dβ4GalTI is the *Drosophila* ortholog of hβ4GalTI and both enzymes show the same acceptor substrate specificity and both contribute to the synthesis of the GAG–protein linkage region.[9,23] Using the knockdown system described above (**Figure 5**), we established 24 *UAS-dβ4GalTI-IR* fly lines carrying a transgene containing two types of inverted repeat of *dβ4GalTI*.[9] Two groups of fly lines, labeled C1–C11 and N1–N13, were distinguished by the presence of a transgene containing an IR of the C-terminal or N-terminal region of *dβ4GalTI*, respectively. The efficiency of RNAi is not affected by either the constructs used or the target sequences of the constructs (**Figure 6c**). *Act5C-GAL4* was used as the GAL4 driver and *dβ4GalTI* gene silencing was induced ubiquitously under the control of
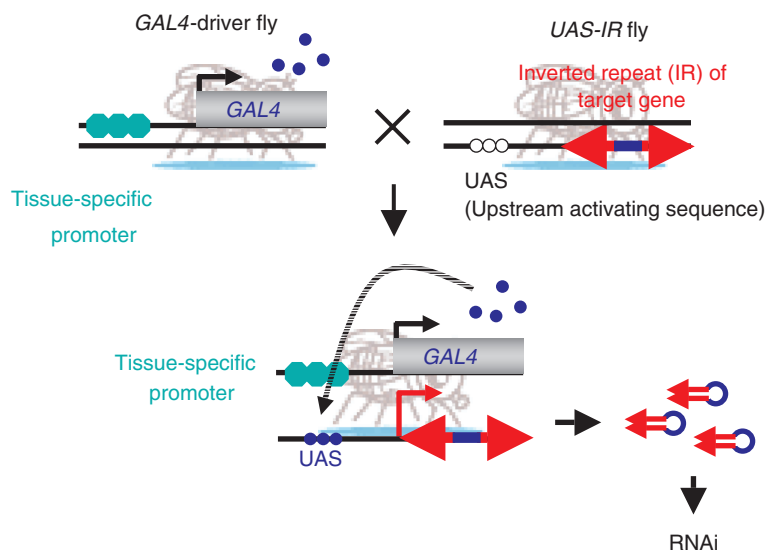
Figure 5 Outline of the GAL4-UAS system for producing heritable and inducible RNAi mutants in *Drosophila*. Two transgenic fly stocks, *GAL4*-driver and *UAS-IR*, are used. The *GAL4*-driver fly has a transgene containing the yeast transcription factor *GAL4*, the expression of which is under the control 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 tissues to induce gene silencing.
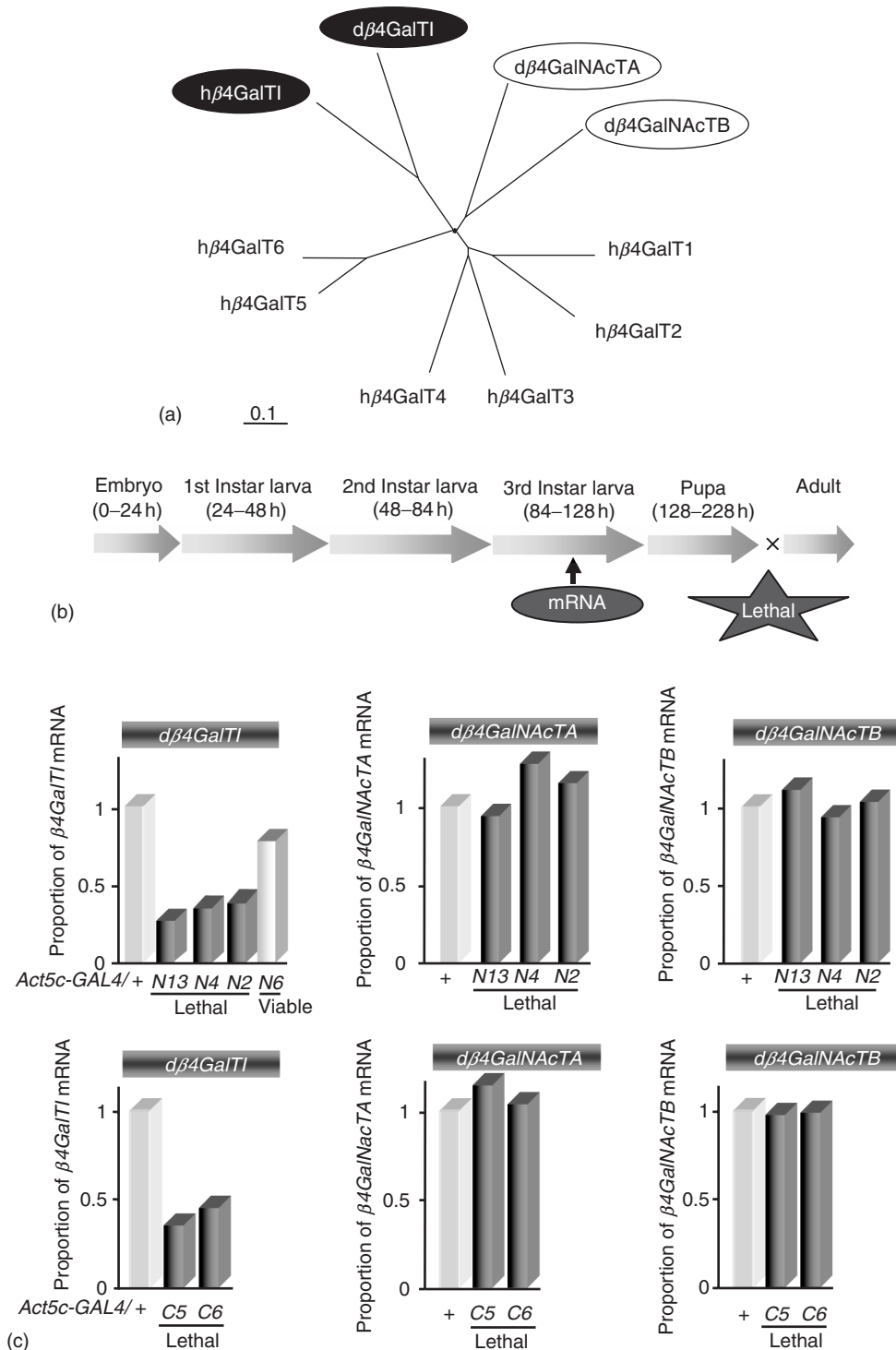
Figure 6 Validation of the *Drosophila* inducible RNAi knockdown system. a, Phylogenetic tree of *Drosophila* and human β4GalT families. *Drosophila* β4GalT family: dβ4GalTI, dβ4GalNAcTA, and dβ4GalNAcTB. Human β4GalT family: hβ4GalTI and hβ4GalT1–6. b, Preparation of mRNA from RNAi knockdown flies for quantitative analyses of mRNA. c, The mRNA levels of dβ4GalT family members in the third-instar larvae of dβ4GalTI knockdown flies. Two kinds of transgenic lines were established: one with an IR at the N-terminal region of dβ4GalTI (N), the other with the IR at the C-terminal region (C). The F_1 progeny of each N or C line of the *UAS-dβ4GalTI-IR* fly crossed with *Act5C-GAL4* was designated as *Act5C-GAL4/N* or *Act5C-GAL4/C*, respectively. 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.
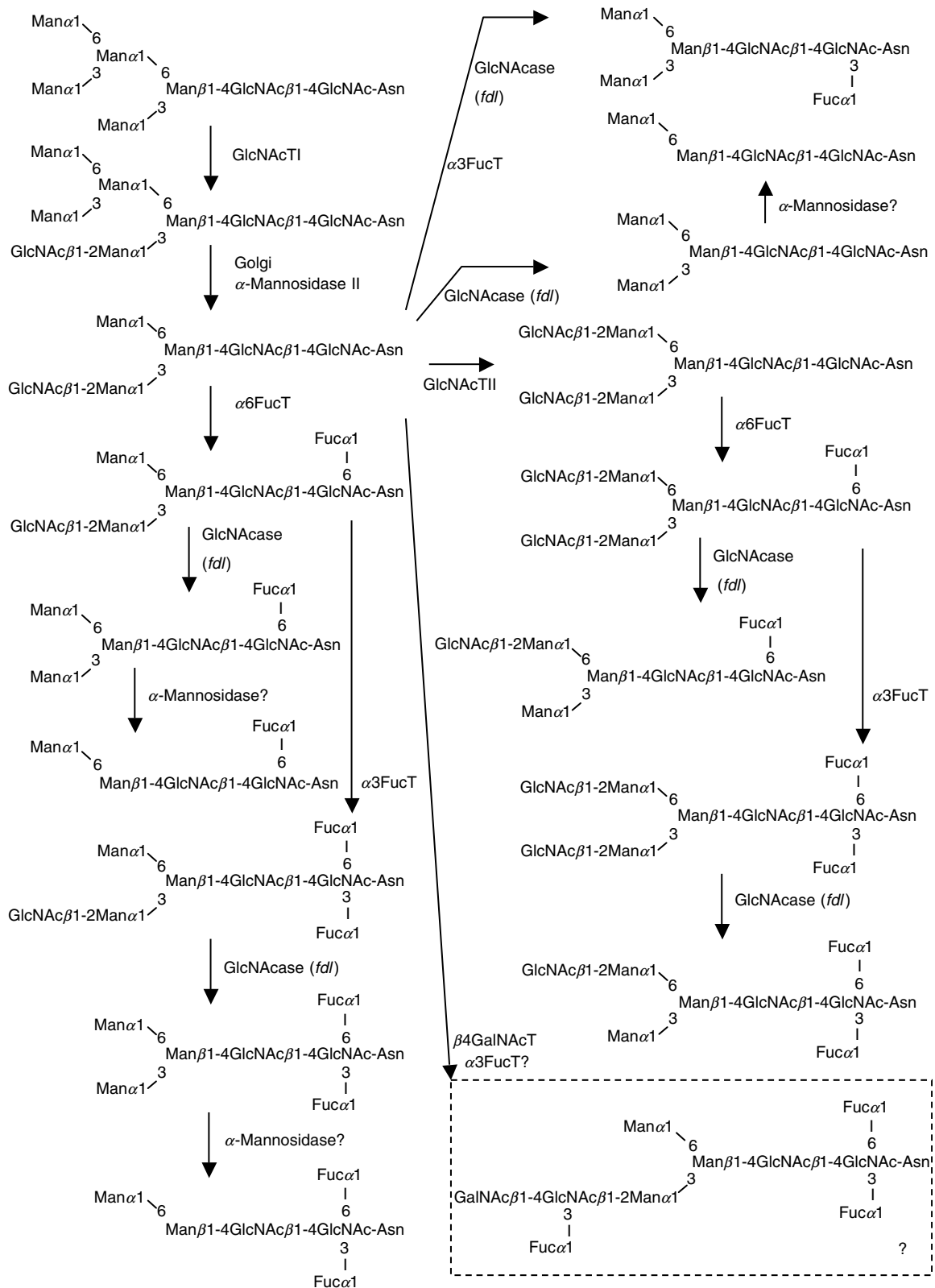
Figure 7   (continued)

the cytoplasmic actin promoter. In approximately 65% of the crosses, lethality occurred with the progeny dying at the late pupal stage (**Figure 6b**). The severity of the phenotype displayed by the progeny differs between the lines because the degree of expression of the transgene depends on the sites of insertion in the target chromosome. The severity of the phenotype in third instar larvae shows a correlation with the level of reduction of *dβ4GalTI* mRNA (**Figure 6c**). Reduction of *dβ4GalNAcTA* mRNA or *dβ4GalNAcTB* mRNA does not occur in any of the RNAi flies. The experiment clearly demonstrates that RNAi can specifically disrupt the expression of the target gene in this *Drosophila* RNAi knockdown system and that this disruption produces flies with abnormal phenotypes.

Injection of dsRNA corresponding to a single gene into an embryo can induce rapid degradation of mRNA. This may prove to be useful for the functional analyses of genes in embryogenesis.[24,25] However, in this case, the RNAi effect is transient and not stably inherited. So use of this method to study gene function in the later stages of development is limited.

4.05.2   N-Glycans in *Drosophila melanogaster*

4.05.2.1   Structure and Biosynthetic Pathway of N-Glycans

Structural analyses of *Drosophila* N-glycans have recently been performed using HPLC and mass spectroscopy.[26–32] The biosynthetic pathway of N-glycans after the Man5 structure in *Drosophila melanogaster* is summarized in **Figure 7**; this pathway is based on reported glycan structures. The synthetic process before Man5 bears a close resemblance to the mammalian N-glycan synthetic pathway. Oligomannosidic glycans including Man9, Man8, Man7, Man6, and Man5 structures have been found in adult flies. These glycans account for almost 40% of the total glycans found.[27,31,32] The other main N-glycan structures are paucimannosidic glycans, for example, Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-Asn and Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc-Asn, which arise from insect-specific β-*N*-acetylglucosaminidase (GlcNAcase);[27] these glycans account for almost 60% of the total glycans present. Moreover, in adult flies, the core α1-6 fucosylated paucimannosidic glycans predominate with Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc-Asn accounting for almost 40% of the total glycans present.[27,31,32] Similar results have been reported from N-glycan structural analysis of purified glycoproteins from Schneider 2 (S2) cells.[33] However, in the neuronal cell line, BG2-c6, core α1-3 and core α1-6 fucosylated paucimannosidic glycans are present as the principal types of glycan as these cells express core α-1,3-fucosyltransferase, FucTA.[28] In contrast to oligomannosidic glycans and paucimannosidic glycans, hybrid and complex glycans are minor components in *Drosophila*. Indeed, glycans with extended LacdiNAc structures have not yet been detected, although fucosylated LacdiNAc structures have been reported on bee venom glycoproteins.[34]

4.05.2.2   Glycosyltransferases and Glycosidases in N-Glycan Synthesis

The activities of seven enzymes, including glycosyltransferases and glycosidases, that are members of the biosynthetic pathway of N-glycans in **Figure 7** have been identified (**Table 1**). Of these, β-*N*-acetylglucosaminidase (GlcNAcase, *fdl*, CG8824)[27] and α-1,3-fucosyltransferase A (FucTA, CG6869)[31] contribute to the synthesis of paucimannosidic and core α1-3 fucosylated N-glycans, respectively, that are characteristic of insect N-glycan structures (**Figure 7**). The other five enzymes, UDP-glucose:glycoprotein glucosyltransferase (DUGT, CG6850),[35] α3-D-mannoside-β-1,2-*N*-acetylglucosaminyltransferase (dGlcNAcTI, dMGAT1, CG13431)[36] Golgi α-mannosidase II (dGMII, CG18802),[37] α6-D-mannoside-β-1,2-*N*-acetylglucosaminyltransferase (dGlcNAcTII, CG7921)[38] and α-1,6-fucosyltransferase (dα6FUT, CG2448),[29] have similar substrate specificities to the corresponding human enzymes and their product sugar structures have been detected in *Drosophila* (see **Figure 7**).

The enzymes β-1,4-*N*-acetylgalactosaminyltransferase A (β4GalNAcTA, CG8536), β-1,4-*N*-acetylgalactosaminyl-transferase B (β4GalNAcTB, CG14517)[39] and galactoside α-2,6-sialyltransferase (dST6GalI, CG4871)[40] have

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Figure 7   Biosynthetic pathway of N-glycans. A summary of the biosynthetic pathway of N-glycans in *Drosophila mela-nogaster*, based on reported glycan structures, after the Man5 structure. The pathway before Man5 bears a close resemblance to the mammalian N-glycan synthetic pathway. Oligomannosidic glycans and paucimannosidic glycan are the main components of *Drosophila* N-glycans. α-Mannosidase?: enzyme has not yet been cloned nor its activity identified. α3FucT?: FucTA has not yet been reported to transfer fucose to the LacdiNAc structure. ?: Fucosylated LacdiNAc structures have yet not been found in *Drosophila*.

Table 1 Glycosyltransferases and glycosidases related to the N-glycan biosynthetic pathway

Enzyme	Abbreviated name	CG no.	Conventional mutants and RNAi	Identified activity
Dolichyl phosphate mannosyltransferase	*dARG3*	CG4084	l(2)not	
UDP-glucose:glycoprotein glucosyltransferase	*DUGT*	CG6850		Glycoprotein GlcT activity [35]
Golgi α-mannosidase I	*dGMI*	CG18799	*P* element insertion[41,42]	
α3-D-mannoside-β-1,2-*N*-acetylglucosaminyltransferase	*dMGAT1, dGlcNAcTI*	CG13431	Deletion [32]	β2GlcNAcT activity[36]
Golgi α-mannosidase II	*dGMII*	CG18802	*P* element insertion[43,44]	α-Mannosidase activity[37]
α6-D-mannoside-β-1,2-*N*-acetylglucosaminyltransferase	*dMGAT2, dGlcNAcTII*	CG7921		β2GlcNAcT activity[38]
β-*N*-acetylglucosaminidase	*GlcNAcase, fdl*	CG8824	*P* element insertion[45]	GlcNAcase activity[27]
α-1,6-Fucosyltransferase	*dα6FUT*	CG2448		α6FucT activity[29]
α-1,3-Fucosyltransferase A	*FucTA*	CG6869		α3FucT activity[31]
β-1,4-*N*-acetylgalactosaminyltransferase A	*β4GalNAcTA*	CG8536	Deletion[39]	GalNAcT activity[39]
β-1,4-*N*-acetylgalactosaminyltransferase B	*β4GalNAcTB*	CG14517	Gene targeted[39]	GalNAcT activity[39]
Galactoside α-2,6-sialyltransferase	*dST6Gal I*	CG4871		α6ST activity[40]

N-acetylgalactosamine (GalNAc) transferase and sialyltransferase activities, respectively, but their product sugar structures have not been detected in *Drosophila*. As shown in **Figure 6a**, β4GalNAcTA and β4GalNAcTB are members of the β-1,4-galactosyltransferase family. During evolution, they are believed to have changed their donor substrates from galactose (Gal) to GalNAc. Thus the LacdiNAc structure, GalNAcβ1-4GlcNAc, of insects corresponds to the mammalian LacNAc structure, Galβ1-4GlcNAc. *Drosophila* galactoside α-2,6-sialyltransferase (dST6GalI) shows sialyltransferase activity to LacdiNAc structure.[40] However, neither the LacdiNAc nor sialylated LacdiNAc structures have been reported in *Drosophila*. In addition, putative *N*-acetylglucosaminyltransferases, which show high homology to human α3-D-mannoside-β-1,4-*N*-acetylglucosaminyltransferases (GlcNAcT-IVs, MGAT4s), are present in *Drosophila* databases. The product glycan structures expected of these enzymes have not yet been reported in *Drosophila*. One reason for this may be the fact that hybrid and complex glycans are minor components in *Drosophila*.

4.05.2.3 Function of N-Glycans

Mutations of several enzymes in the biosynthetic pathway of N-glycans have been reported (**Table 1**).[32,41–45] The *P* element insertion lines of *Golgi α-mannosidase I* (*dGMI, CG18799*) cause mutation of the enzyme that contributes to the earliest step of the biosynthetic pathway, that from the Man8 to Man5 structure. The null mutant of *dGMI* shows defects in embryonic peripheral nervous system (PNS), and in the wings and eyes of adults. However, the presence of Man5 structures in this null mutant suggests either redundancy in the biosynthetic pathway or the presence of an alternative pathway.[41,42] The *dGlcNAcTI* null mutant has been reported to have defects in locomotory activity, a reduced life span, and fused β-lobes in the mushroom bodies,[32] similar to those found in the *fused lobes* (*fdl*) mutant.[45] Recently, the *fdl* gene was found to code for the GlcNAcase responsible for the paucimannosidic glycan structure that is characteristic of insect glycans.[27] With respect to *dGMII*, two types of mutant have been reported: one shows a rough eye phenotype[43] while the other is lethal.[44] In addition to the above mutants, mutations of *β4GalNAcTA* and *β4GalNAcTB* have been isolated; the *β4GalNAcTA* mutant shows behavioral defects.[39] However, the expected product oligosaccharide structure of *β4GalNAcTA* (the LacdiNAc structure) has not been reported yet in *Drosophila*.

Antigenic epitopes that are recognized by anti-horseradish peroxidase (HRP) can be used as neuronal cell markers in *Drosophila* (**Figure 8a**).[46] The epitope includes the core α1-3 fucosylated N-glycan structure.[47] This structure is synthesized by FucTA[28] and is present on many types of neuronal glycoprotein, such as Fasciclin I–III, neurotactin, receptor-like protein tyrosine phosphatase, Nervana, and others.[48,49] No mutants of FucTA have been reported to date. However, two mutants, *neuronally altered carbohydrate* (*nac*)[50,51] and *tollo/toll-8*,[52] are known to affect the expression of HRP epitopes. The *nac* mutant exhibits altered axonal trajectories in the central nervous system
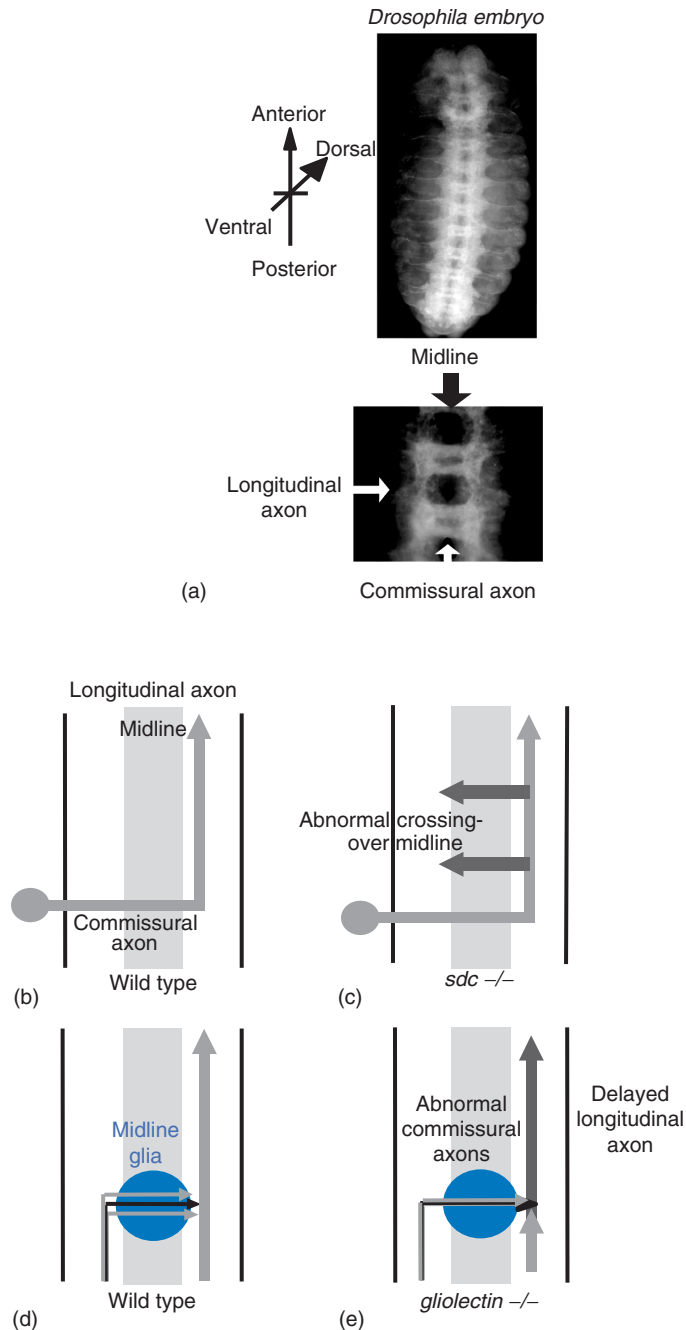
Figure 8 Axon guidance defects in *Drosophila* embryos. a, Immunostaining of a *Drosophila* wild-type embryo using an anti-HRP antibody. The HRP epitope is expressed specifically on neuronal cells and the anti-HRP antibody can be used as a neuronal marker. b–e, Pattern diagrams of the formation of commissural and longitudinal axons. b and d, Wild type. c, *sdc* null embryos show defects in midline axon guidance, abnormal crossing over in the midline, and reduced Slit distribution. Sdc is believed to have a role in Slit–Roundabout (Robo) signaling through its effect on either the stability or transport of Slit. d, Gliolectin is expressed on midline glial cells concurrently with early pathfinding of commissural axons across the midline. e, Loss of Gliolectin results in defective commissure formation accompanied by reduced axon-glial contact and delay in the completion of longitudinal pathfinding. Early commissural axons grow upon other axons rather than in close association with the midline glia. Black narrow arrows, pioneer commissural axons; gray narrow arrows, follower axons; blue circles, midline glial cells.