The *twisted* Gene Encodes Drosophila Protein O-Mannosyltransferase 2 and Genetically Interacts With the *rotated abdomen* Gene Encoding Drosophila Protein O-Mannosyltransferase 1

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ABSTRACT

The family of mammalian O-mannosyltransferases includes two enzymes, POMT1 and POMT2, which are thought to be essential for muscle and neural development. Similar to mammalian organisms, *Drosophila* has two O-mannosyltransferase genes, *rotated abdomen* (*rt*) and *DmPOMT2*, encoding proteins with high homology to their mammalian counterparts. The previously reported mutant phenotype of the *rt* gene includes a clockwise rotation of the abdomen and defects in embryonic muscle development. No mutants have been described so far for the *DmPOMT2* locus. In this study, we determined that the mutation in the *twisted* (*tw*) locus, *tw*¹, corresponds to a *DmPOMT2* mutant. The *twisted* alleles represent a complementation group of recessive mutations that, similar to the *rt* mutants, exhibit a clockwise abdomen rotation phenotype. Several *tw* alleles were isolated in the past; however, none of them was molecularly characterized. We used an expression rescue approach to confirm that *tw* locus represents *DmPOMT2* gene. We found that the *tw*¹ allele represents an amino acid substitution within the conserved PMT domain of DmPOMT2 (TW) protein. Immunostaining experiments revealed that the protein products of both *rt* and *tw* genes colocalize within *Drosophila* cells where they reside in the ER subcellular compartment. *In situ* hybridization analysis showed that both genes have essentially overlapping patterns of expression throughout most of embryogenesis (stages 8–17), while only the *rt* transcript is present at early embryonic stages (5 and 6), suggesting its maternal origin. Finally, we analyzed the genetic interactions between *rt* and *tw* using several mutant alleles, RNAi, and ectopic expression approaches. Our data suggest that the two *Drosophila* O-mannosyltransferase genes, *rt* and *tw*, have nonredundant functions within the same developmental cascade and that their activities are required simultaneously for possibly the same biochemical process. Our results establish the possibility of using *Drosophila* as a model system for studying molecular and genetic mechanisms of protein O-mannosylation during development.

Linked carbohydrate modifications of proteins can play important roles during animal development. O-mannosylation represents a prominent example of such a post-translational protein modification that has recently been linked to mammalian muscular and neural development (reviewed in MICHELE and CAMPBELL 2003; MARTIN 2003). Abnormal O-mannosylation has been implicated in several congenital neuromuscular disorders (VAN REEUWIJK *et al.* 2005; reviewed in ENDO and TODA 2003; MARTIN and FREEZE 2003); while the targeted disruption of the *Pomt1* gene encoding O-mannosyltransferase, an enzyme initiating the biosynthesis of O-mannosyl glycans, results in early embryonic lethality in mice (WILLER *et al.* 2004).

In mammalian organisms, one of the main targets of O-mannosylation is thought to be α -dystroglycan (CHIBA *et al.* 1997), a cell adhesion glycoprotein important for the integrity of the muscle cells and neuronal migration during development (MARTIN 2003). Dystroglycan plays a central role in the dystrophin-associated glycoprotein complex (DGC) that provides a crucial link between the extracellular basal lamina and the cytoskeletal proteins (MICHELE and CAMPBELL 2003). The O-mannosyl glycans of α -dystroglycan have been found to affect its binding to the extracellular matrix-associated ligand laminin, which indicates the importance of O-mannosylation for the functional integrity of DGC and its interaction with extracellular matrix (CHIBA *et al.* 1997; MICHELE *et al.* 2002).

Intriguingly, the *Drosophila* genome encodes homologs of all essential components necessary for the formation of DGC but with substantially less diversity (GREENER and ROBERTS 2000; DEKKERS *et al.* 2004), which presents an attractive possibility for using fruit flies as a model system for studying the biology of DGC and several human congenital neuromuscular diseases.

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Protein *O*-mannosyltransferase enzymes are best characterized in yeast. A total of seven *O*-mannosyltransferases have been identified in *Saccharomyces cerevisiae* (GENTZSCH and TANNER 1996). They comprise three evolutionary conserved subfamilies of proteins, PMT1, PMT2, and PMT4 (WILLER *et al.* 2003). On the other hand, the family of mammalian *O*-mannosyltransferases includes two enzymes, POMT1 and POMT2 that belong to PMT4 and PMT2 subfamilies, respectively (JURADO *et al.* 1999; WILLER *et al.* 2002). These enzymes are thought to modify numerous serine and threonine residues of α -dystroglycan with *O*-linked mannose, which initiates the biosynthesis of *O*-mannosyl glycans of dystroglycan (WILLER *et al.* 2003). Recently, it was demonstrated that elevated *O*-mannosyltransferase activity could be detected in extracts from cultured cells only if POMT1 and POMT2 were coexpressed in these cells (MANYA *et al.* 2004). Although this result indicated the possibility that these enzymes function as a heterocomplex, no evidence for physical interaction between POMT1 and POMT2 proteins have been obtained so far. Thus, molecular and genetic mechanisms governing the function of these proteins remain elusive.

Similar to higher animals, *Drosophila* has two *O*-mannosyltransferases, RT (encoded by the *rotated abdomen* gene) and DmPOMT2, which are evolutionary related to mammalian POMT1 and POMT2, respectively (MARTIN-BLANCO and GARCIA-BELLIDO 1996; WILLER *et al.* 2002; ICHIMIYA *et al.* 2004). Several mutations in the *rotated abdomen* gene (*rt*) were previously isolated and phenotypically characterized (BRIDGES and MORGAN 1923; LINDSLEY and ZIMM 1992; MARTIN-BLANCO and GARCIA-BELLIDO 1996). Two molecularly characterized mutants, *rt*² and *rt*^p, are semiviable recessive alleles associated with disruptions of the gene coding region by *P*-element insertion; they possibly represent null mutations (COOLEY *et al.* 1988; MARTIN-BLANCO and GARCIA-BELLIDO 1996). The phenotypes of these mutations include some larval and adult muscle abnormalities, as well as a prominent, up to 90° clockwise rotation of abdominal segments in adult flies (MARTIN-BLANCO and GARCIA-BELLIDO 1996). No mutations in *DmPOMT2* gene have been reported so far, although the possibility that mutations in the *twisted* locus might represent *DmPOMT2* mutants has been suggested (J. CRUCES, cited in WILLER *et al.* 2002). This hypothesis was further supported by “twisted abdomen” phenotype recently obtained in RNAi-mediated *DmPOMT* knockdown experiments (ICHIMIYA *et al.* 2004). Similar to their mammalian counterparts, RT and DmPOMT2 proteins have to be coexpressed to produce *O*-mannosyltransferase activity (ICHIMIYA *et al.* 2004).

The *twisted* alleles represent a complementation group of viable and semiviable recessive mutations on the X chromosome that also exhibit clockwise rotated abdominal segments in the adult (LINDSLEY and ZIMM 1992). Several *tw* mutant alleles had been isolated

(DAVIS 1980; LINDSLEY and ZIMM 1992); however, none of them was characterized in detail and no molecular data are currently available for the *tw* locus. Most of these *tw* mutants have been lost and there is only one mutant allele, *tw*^l, available from public collections (FlyBase information, DRYSDALE *et al.* 2005).

In this study, we have tested the hypothesis that *tw* corresponds to *DmPOMT2*. By using an expression rescue approach, we established that the *tw* locus represents the *DmPOMT2* gene. We also characterized the molecular nature of *tw*^l mutation. Our immunostaining analysis of subcellular localization of tagged RT and DmPOMT2 (TW) proteins revealed their colocalization in the endoplasmic reticulum (ER) of *Drosophila* cells. The pattern of embryonic *tw* expression was analyzed by *in situ* hybridization and compared to the expression of *rt*. Our data showed striking overlap of *tw* and *rt* expression at different stages of embryogenesis. Moreover, we found a genetic interaction between *tw* and *rt* mutant alleles. All these results are consistent with the hypothesis that RT and TW, the two *Drosophila* protein *O*-mannosyltransferases, participate in the same developmental cascade and may collaborate at the molecular level, potentially functioning as an enzymatic heterocomplex.

MATERIALS AND METHODS

Mutant and transgenic *Drosophila* stocks: The following *Drosophila* mutant alleles, chromosomal aberrations, and transgenic insertions used in the study were obtained from the Bloomington *Drosophila* Stock Center, Indiana University: *tw*^l, *rt*^p, *rt*², *Df(1)su(s)83*, *Dp(1;Y)y²sc*, *Act5C-GAL4-25* (second chromosome insertion), *Act5C-GAL4-17* (third chromosome insertion), *tubP-GAL4 (LL7)*, and *C155-GAL4*. The *rt*⁵⁷¹ allele (also designated as EP(3)0571) was obtained from the Exelixis EP collection (Exelixis; San Francisco). In our experiments, we used three different *rt* alleles to exclude potential influence of genetic background and possible peculiarities of some alleles. All three alleles of *rt* include an independent *P*-element insertion in the first exon of the gene and likely represent null or strong hypomorphic mutations (see MARTIN-BLANCO and GARCIA-BELLIDO 1996; DRYSDALE *et al.* 2005; and Figure 6). PDI::GFP transgenic flies were kindly provided by Alain Debec (BOBINNEC *et al.* 2003). The following transgenic insertions were generated by *P*-element mediated transformation: *UAS-DmPOMT2* and *UAS-twRNAi-39* (on the second chromosome) and *UAS-twRNAi-77* (on the third chromosome).

Sequencing of the *DmPOMT2* gene in *tw*^l mutant flies: The DNA fragment of *DmPOMT2* locus was PCR amplified from the genomic DNA of *tw*^l homozygous flies using primers 5' CGTGGCCAGGATAACAACACTGGC 3' and 5' AACGTTGACAGGGTTGTGGGTGTGGT 3'. The amplified genomic region included the transcribed region [determined by the alignment of the *DmPOMT2* cDNA (clone LP01681) with *Drosophila* genomic sequences (ADAMS *et al.* 2000)], along with the 2-kb upstream and 500-bp downstream fragments. The PCR product was sequenced and several deviations of the *DmPOMT2* locus sequence from the corresponding wild-type genomic sequence determined by the *Drosophila* genome project (ADAMS *et al.* 2000) were uncovered. The alterations

include: CA(249,250) → AGGAT; C(472) → T; T(2545) → C (nucleotide numbers are relative to the beginning of coding region). The first alteration is predicted to affect the translated protein sequence [T(59) → GS], while the two other mutations are silent.

DNA constructs for cell-culture expression and fly transformation The cDNA clones were obtained from the *Drosophila* Genomics Resource Center at Indiana University. For cell culture expression experiments, the coding region of *DmPOMT2* was PCR amplified from the *DmPOMT2* cDNA (LP01681) using PCR primers containing *Bgl*II and *Xba*I restriction sites. Using these sites, the PCR product was inserted into the *pMK33* vector (KOELLE *et al.* 1991). The final construct, *pMK-DmPOMT2*, also included a short DNA fragment encoding the HA tag (NIMAN *et al.* 1983); it was obtained by annealing two synthesized oligonucleotides and then introduced into *DmPOMT2* cDNA using *San*DI restriction site. The resulting construct encoded a DmPOMT2 (TW) protein with HA-tag inserted into nonconserved region immediately after G(650). The same construct was subcloned into the *pUAST* vector (BRAND *et al.* 1994) to obtain *pUAST-DmPOMT2* for fly transformation and *in vivo* expression. Functionality of the construct was confirmed by Western-blot analysis (data not shown) and immunostaining of *pMK-DmPOMT2*-transfected S2 cells (Figure 4), as well as by an *in vivo* rescue assay (Figure 2). For the expression of *rt* in *Drosophila* cell culture, the coding region of *rt* was PCR amplified from the *rt* cDNA (clone RE30211) and inserted in the *pRMHA3* vector (BUNCH *et al.* 1988). In the final expression construct, the *rt* coding region was modified by the addition of a short DNA fragment encoding two MYC tags (EVAN *et al.* 1985) immediately following the last amino acid-coding triplet of *rt*. As revealed by sequencing, the RE30211 clone includes a short unspliced intron preceding the last coding exon of the gene. Efficient expression of the construct and removal of the intron by in-cell splicing was confirmed using anti-MYC immunostaining of *pRMHA3-rt-MYC*-transfected cells (Figure 4) and Western-blot analysis of RT-MYC protein expressed in *Drosophila* S2 cells (data not shown). The functionality of the MYC-tagged RT protein was also confirmed *in vivo* by its ability to rescue the *rt* mutant phenotype (data not shown). The *UAS-twRNAi* construct was produced essentially according to the published strategy (LEE and CARTHEW 2003). Briefly, the two PCR-amplified DNA fragments of *DmPOMT2* gene, both including the 6th exon (~700 bp) and one also including the following intronal sequence, were ligated together in the opposite orientation. The resulting inverted repeat was cloned into the *pUAST* vector using PCR-introduced *Eco*RI and *Xba*I restriction sites. The *UAS-twRNAi* construct is predicted to produce ~700 bp hairpin dsRNA with 12 unpaired bases of loop region.

Cell culture: *Drosophila* S2 cells were maintained and transfected using the protocols described earlier (KOLES *et al.* 2004).

Immunostaining and epifluorescent microscopy: Expression of the *UAST-DmPOMT2* construct was induced in salivary glands using the *C155-GAL4* driver. Third instar larvae were dissected, fixed, and stained as described earlier (PANIN *et al.* 1997). The following primary antibodies and corresponding dilutions were used for immunostaining: rabbit anti-LVA (1:2000) (a gift from John Sisson, University of Texas, Austin, TX); mouse and rabbit anti-HA (1:1000); mouse anti-MYC (1:1000) (BabCo, Berkeley, CA). We used the following fluorescent secondary donkey antibodies: anti-mouse-Cy3 (1:250); anti-rabbit-FITC (1:150); and anti-mouse-Cy5 (1:150) (Jackson Laboratories). Digital images were obtained using Zeiss Axioplan 2 fluorescent microscope with the ApoTome module for optical sectioning.

In situ hybridization was performed as described earlier (KOLES *et al.* 2004) using the *tw* and *rt* cDNA clones LP01681 and RE30211, respectively, as templates for the synthesis of DIG-labeled probes. Every *in situ* hybridization experiment included a negative control staining with a probe transcribed from the corresponding antiparallel cDNA sequence (data not shown).

Phenotype analysis: All flies were grown at 25°. The rotation of abdomen was scored in CO₂-anesthetized flies 1 day after eclosion (to eliminate possible influence of aging or altered morphology in very young flies). The rotation angle was measured from posterior viewpoint using Nikon SMZ microscope with a protractor reticle objective.

RESULTS

The *tw*¹ mutant allele contains a mutation within the *DmPOMT2* gene region encoding the conserved PMT domain: The novel *Drosophila* O-mannosyltransferase 2 gene was initially found by BLAST (ALTSCHUL *et al.* 1990) searches of *Drosophila* genomic sequences on the basis of the homology of its conceptual translation to the sequences of RT, yeast, and mammalian O-mannosyltransferases (WILLER *et al.* 2002; FlyBase information, DRYSDALE *et al.* 2005). The software prediction was further confirmed by the identification and sequencing of the corresponding full-length cDNA clone LP01681 from the *Drosophila* EST collection (WILLER *et al.* 2002; ICHIMIYA *et al.* 2004). We also identified a mosquito EST sequence (GenBank accession no. XM 312249) encoding a protein with high homology to DmPOMT2, which suggested that this sequence corresponds to the mosquito ortholog of *DmPOMT2*, *AgPOMT2* (Figure 1). The similarity between the products of *rt* and *DmPOMT2* (Figure 1), along with functional data on the homologous yeast PMT family members, allowed us to suggest that both *Drosophila* genes might function within the same biochemical pathway. This scenario would predict similar phenotypes for *rt* and *DmPOMT2* mutations. Interestingly, we noticed that the chromosomal position of *DmPOMT2* (1D4) maps to the cytogenetic region that also includes *tw* (1C3-D4), the complementation group of mutations causing a clockwise rotation of the adult abdomen (LINDSLEY and ZIMM 1992). This phenotype is strikingly similar to that of *rt* mutants (Figure 2, A–C). These data indicated the possibility that *tw* corresponds to *DmPOMT2*. This hypothesis has also been mentioned by other researchers (J. CRUCES, cited in WILLER *et al.* 2002) and supported by *DmPOMT2* RNAi experiments that generated *rt*-like abdomen rotation phenotype (ICHIMIYA *et al.* 2004). Thus, we decided to sequence the genomic region of *DmPOMT2* in *tw*¹ mutant flies carrying a semiviable recessive *twisted* allele. The *tw*¹ homozygous female and hemizygous male flies have a fully penetrant phenotype that manifests in the clockwise rotation of the abdomen (20°–40°) along the anteroposterior axis, when scored from a posterior viewpoint (DAVIS 1975; Figure 2C). Genetically, the *tw*¹ mutation behaves as a hypomorphic allele since its phenotype is

FIGURE 1.—Protein sequence alignment of several protein *O*-mannosyltransferases from different species. DmPOMT2, fruit fly (*Drosophila melanogaster*) POMT2; AgPOMT2, mosquito (*Anopheles gambiae*) POMT2; HsPOMT2, human POMT2; ScPmt2p, yeast (*S. cerevisiae*) protein *O*-mannosyltransferase 2; RT, *D. melanogaster* POMT1 (GenBank accession nos. Q9W5D4, XM_312249, NP_037514, NP_009379, and Q9VTK2, respectively). The aligned conserved PMT domain (solid line) and MIR domains (dashed lines) are indicated. Asterisk indicates the position of T(59) → GS mutation identified in the *tw1* allele. Alignment was performed using CLUSTAL_W algorithm (THOMPSON *et al.* 1994) at BCM server (<http://searchlauncher.bcm.tmc.edu/multi-align>).

significantly enhanced in *tw1*-hemizygous females carrying a deficiency that includes the *tw* locus [*tw1*/*Df(1)su(s)83*], while the phenotype is completely rescued in males carrying a duplication of the *tw* locus on the Y chromosome [*tw1*/*Dp(1;Y)y2sc*] (Figure 2C and Figure 3). We sequenced the *DmPOMT2* locus including the transcribed region together with 2-kb upstream and 500-bp downstream fragments. The sequencing revealed several deviations of the *DmPOMT2* genomic sequence from the corresponding wild-type sequence determined by the *Drosophila* genome project (ADAMS *et al.* 2000). All but one of the sequence alterations likely represent polymorphisms or mutations neutral for the gene's function (see MATERIALS AND METHODS). Notably, one of the identified mutations maps to the coding region and results in the amino acid substitution/insertion [T(59) → GS] within the conserved PMT domain of DmPOMT2 (Figure 1), which suggests an altered functionality of the DmPOMT2 protein in the *tw1* mutants. This finding further supported the hypothesis that the phenotype of *tw* mutants results from mutations in *DmPOMT2*.

The phenotype of *tw1* mutation can be rescued by *DmPOMT2* expression: To confirm that the *DmPOMT2* mutation(s) causes the abdominal rotation phenotype in *tw1* mutants, we carried out a rescue assay. To this end, we induced *DmPOMT2* expression in the *tw1* mutant background using the UAS/GAL *in vivo* expression system (BRAND *et al.* 1994).

FIGURE 2.—Abdomen rotation phenotype of *rt* and *tw* mutants. (A) Wild type; (B) *rt^p/rt²*; (C) *tw1/tw1*; and (D) *tw1/Df(1)su(s)83*. Only females are shown; corresponding males have similar phenotypes (see also Figure 3). Ventral view, anterior is up.

FIGURE 3.—The *tw*¹ allele is a hypomorphic mutation in the *DmPOMT2* gene. The following numbers of flies were analyzed for corresponding genotypes: *tw*¹/*Y*, 102 flies; *tw*¹/*Y*; UAS-*DmPOMT2*/+, 40 flies; *tw*¹/*Y*; *Act5C-GAL4-17*/+, 51 flies; *tw*¹/*Y*; UAS-*DmPOMT2*/+; *Act5C-GAL4-17*/+, 90 flies; *tw*¹/*tw*¹, 72 flies; *tw*¹/*tw*¹; UAS-*DmPOMT2*/+, 39 flies; *tw*¹/*tw*¹; *Act5C-GAL4-17*/+, 84 flies; *tw*¹/*tw*¹; UAS-*DmPOMT2*/+; *Act5C-GAL4-17*/+, 73 flies; *tw*¹/*Df*(1)*su*(s)83, 65 flies; *tw*¹/*Dp*(1;Y)*y2sc*, 89 flies. Error bars indicate standard deviations.

We produced UAS-*DmPOMT2* transgenic flies and crossed them to flies carrying a ubiquitous *Act5C-GAL4-17* driver (see MATERIALS AND METHODS). There were no visible defects in abdominal morphology of the flies from either parental stocks or UAS-*DmPOMT2*/+; *Act5C-GAL4-17*/+ progeny of the cross (data not shown). The presence of the UAS-*DmPOMT2* or *Act5C-GAL4* transgene alone did not modify the phenotype of *tw*¹ mutants (Figure 3). At the same time, the complete rescue of the abdomen rotation phenotype was observed in *tw*¹ hemizygous male and homozygous female flies carrying both UAS-*DmPOMT2* and *Act5C-GAL4-17* together (Figure 3). Similar results were obtained when *DmPOMT2* was expressed using *Act5C-GAL4-25* and *tubP-GAL4* drivers (data not shown). These rescue experiments proved that *tw* indeed represents the *DmPOMT2* gene. Thus, we suggest changing the designation of the *DmPOMT2* (CG12311) gene to “*twisted*” (*tw*), the name of the originally discovered mutants that is in an agreement with the traditional nomenclature of *Drosophila* genetics (LINDSLEY and ZIMM 1992). Below, we use “*tw*” and “TW” to designate the *DmPOMT2* gene and its protein product, respectively.

Subcellular localization of RT and TW proteins: Glycosyltransferases that modify secreted glycoproteins commonly function in the Golgi apparatus. Interestingly, yeast PMT family members localize to the ER subcellular compartment (WILLER *et al.* 2003). The localization of O-mannosyltransferase proteins in animal cells has been reported only for human POMT2 protein expressed in human culture cells (WILLER *et al.* 2002). In that study, the C-terminally tagged POMT2 protein was detected in the ER by immunostaining; however, the functionality of this fusion protein remains undetermined. Thus, we decided to investigate the subcellular localization of the TW protein, the *Drosophila* ortholog of mammalian POMT2, using the UAS-*DmPOMT2* transgenic construct that was functional in our rescue assay (Figure 2). Immunostaining

for TW protein expressed *in vivo* in the salivary gland cells of *Drosophila* larvae revealed its colocalization with an ER marker, PDI-GFP (BOBINNEC *et al.* 2003). At the same time, the localization of TW showed minimal overlap with a Golgi marker, the LVA protein (Sisson *et al.* 2000), when TW was expressed in *Drosophila* S2 cells (Figure 4, D–F). Double immunostaining of S2 cells expressing both RT and TW proteins demonstrated their colocalization inside the cell (Figure 4, G–I). Thus, we concluded that both RT and TW proteins reside in the ER subcellular compartment.

Analysis of *tw* and *rt* expression during *Drosophila* embryogenesis by *in situ* hybridization: Detailed analysis of the spatial and temporal patterns of gene expression by *in situ* hybridization can provide important information about the functioning and regulation of the gene. The pattern of embryonic *rt* expression was previously analyzed by *in situ* hybridization (MARTIN-BLANCO and GARCIA-BELLIDO 1996). The pattern of *tw* (*DmPOMT2*) expression has been reported only for embryonic stage 10, while the expression at other stages was estimated only using a real-time PCR assay (ICHIMIYA *et al.* 2004). Thus, we decided to perform *in situ* hybridization analysis of *tw* expression and compare it with the pattern of *rt* expression at different embryonic stages. In agreement with previous reports (MARTIN-BLANCO and GARCIA-BELLIDO 1996; ICHIMIYA *et al.* 2004), we found no significant expression of *tw* at early embryonic stages, while *rt* mRNA was detected at stages 5 and 6, which probably indicates the presence of maternally provided transcript (Figure 5, A and D). This early *rt* expression decays quickly and it is not readily detectable after stage 7. Although we did detect some weak staining for *tw* and *rt* expression during the stages of germband extension (stages 10 and 11), this staining was diffuse and barely detectable above the background (data not shown). Thus, we decided to concentrate our analysis on *tw* and *rt* expression at later stages, when the expression of these

FIGURE 4.—Subcellular localization of the TW and RT proteins. (A–C) Immunofluorescent staining for HA-tagged TW protein expressed in the third larva instar salivary gland cells of PDI::GFP transgenic flies (BOBINNEC *et al.* 2003). (A) TW (red, Cy5); (B) PDI-GFP (green); (C) overlay of the red A and green B channels. The staining reveals TW localization in the ER compartment. (D–F) Double-immunofluorescent staining for HA-tagged TW and LVA proteins expressed in the *Drosophila* S2 cell culture. (D) LVA (red, Cy3); (E) TW (green, FITC); (F) overlay of the red D and green E channels. The staining indicates TW exclusion from the Golgi compartment. (G–I) Double-immunofluorescent staining for TW (HA-tagged) and RT (MYC-tagged) coexpressed in *Drosophila* culture cells. (G) TW (red, Cy3); (H) RT (green, FITC); (I) overlay of the red (G) and green (H) channels. The double immunostaining shows colocalization of TW and RT within *Drosophila* cells. Circular staining around nuclei of S2 cells represents perinuclear ER (OKAJIMA *et al.* 2005). Bars, 20 μ m in C and 6 μ m in F and I.

genes is readily detectable. The prominent expression of both genes, *tw* and *rt*, appears at early stage 14 that corresponds to the period of active muscle differentiation (Figure 5, B and E). However, we have not detected

significant expression of *tw* or *rt* in the developing somatic muscle cells. Instead, the expression of these genes appeared to be pronounced in other tissues, including certain developing epidermal cells, as well as hindgut and foregut regions. At that stage, the expression of *tw* is also present in the developing trachea, while *rt* expression appears in the tracheal cells slightly later, during stage 15. Thus, remarkably overlapping patterns of *rt* and *tw* expression are established by stages 14 and 15, and this expression appears to persist through the late embryogenesis (Figure 5, C and F).

Genetic interaction between *tw* and *rt* genes: The similarity of mutant phenotypes (Figure 2) strongly suggests that both genes function in the same developmental cascade. To test this possibility, we assayed the genetic interaction between *tw*¹ and three *rt* alleles, *rt*², *rt*^p, and *rt*⁵⁷¹. Somewhat surprisingly, we found that the presence of *tw*¹ significantly suppressed the abdomen rotation phenotype of *rt* mutants (Figure 6A). Notably, this effect is dominant since even one copy of *tw*¹ mutation in females is sufficient for the suppression. In fact, the suppression of *rt* phenotype in *tw*¹ homozygous or hemizygous flies appears to be nearly complete since the phenotype of *tw*¹/*tw*¹ (or *tw*¹/Y); *rt*/*rt* double mutants is virtually indistinguishable from the phenotype of *tw*¹-homo-/hemizygous mutants alone (Figure 6B).

We considered two competing explanations of these results. First, the relative excess of TW as compared to RT in the *rt* mutants might have a negative effect on the *O*-mannosylation pathway. In this case, a potential decrease of TW might explain the suppression of *rt* phenotype in *tw*¹ mutant background. Alternatively, the suppression of *rt* phenotype by *tw*¹ might be explained by a special feature of the *tw*¹ allele, which would not implicate the dependence of phenotype on relative concentrations of RT and TW. To discriminate between these two possibilities, we analyzed further the genetic interaction between *rt* and *tw*.

The first possibility would predict that an increase of *tw* activity would increase the severity of *rt* mutant

FIGURE 5.—The pattern of *rt* and *tw* expression at different embryonic stages as revealed by *in situ* hybridization. (A–C) *tw* expression: A, stage 5; B, stage 14; C, stage 15. (D–F) *rt* expression: D, stage 5; E, stage 14; F, stage 16. At stage 14, the expression of the genes is elevated in the epidermis (arrows), foregut (open triangles), hindgut (solid triangles), and trachea (asterisk). The presence of the *rt* mRNA at stage 5 in D suggests maternal contribution of the transcript. Anterior is to the left. B, C, E, and F are dorsolateral views.

phenotype, while a decrease of *tw* would result in *rt* mutant phenotype suppression. To test this prediction, we varied the level of *tw* in *rt* mutants by several alternative ways: (i) by adding an extra copy of the *tw* locus [using duplication *Dp(1;Y)y^{2sc}*], (ii) by overexpressing TW using UAS–GAL4 system, and (iii) by decreasing the *tw* activity via UAS–*tw*RNAi construct expression. Neither the increase of *tw* expression (Figure 7A) nor the decrease of *tw* activity (Figure 7B) revealed the predicted sensitivity of *rt* mutant phenotype to varied concentrations of TW. Hence, we interpret the dominant suppression of *rt* phenotype by the *tw¹* mutation as a special feature of the *tw¹* allele that somehow bypasses the requirement for *rt* activity in the genetic pathway. This interpretation is further supported by a synergistic

effect of *tw* RNAi and heterozygous *rt* mutant background (Figure 8), which revealed a positive interaction between *rt* and *tw*, indicating their close collaboration within the pathway. These data also support the hypothesis that the O-mannosylation pathway requires the simultaneous activities of both *rt* and *tw* genes (ICHIMIYA *et al.* 2004). This simultaneity requirement can explain the absence of any significant effect of varied TW levels on the phenotype of tested *rt* mutants that represent very strong hypomorphic or amorphic mutations and presumably lack the RT protein (see MARTIN-BLANCO and GARCIA-BELLIDO 1996; *rt* phenotypes in Figure 6A).

DISCUSSION

The family of protein O-mannosyltransferases in *Drosophila* includes two members, RT and DmPOMT2, and exhibits obvious evolutionary relation to the mammalian POMT protein family (WILLER *et al.* 2003). While several mutations in the *rt* locus have been previously isolated and the *rt* gene has been molecularly characterized (LINDSLEY and ZIMM 1992; MARTIN-BLANCO and GARCIA-BELLIDO 1996), the novel *DmPOMT2* gene (*CG12311*) has been described only recently (FlyBase; ICHIMIYA *et al.* 2004), and no mutations in *DmPOMT2* have been reported so far. In this study, we have established the relationship between *DmPOMT2* and *twisted*, the previously isolated complementation group of recessive mutations with the characteristic phenotype of a clockwise twisted abdomen. Our results demonstrate that the *tw¹* semiviable recessive allele is associated with a mutation in the coding region of the *DmPOMT2* gene, which alters the amino acid sequence of the conserved PMT domain of DmPOMT2 protein. We have also found that this mutation is associated with the decrease of *tw* activity, since the *tw¹* phenotype is enhanced over deficiency for *tw* locus, and it can be completely rescued by genetic duplication including the *tw* gene or by

FIGURE 6.—Genetic interactions between *tw¹* and *rt* mutant alleles. (A) The suppression of *rt* mutant phenotype by the *tw¹* allele. The following numbers of flies were analyzed for shown genotypes: males *rt^P/rt^P*, 73 flies; males *tw¹/Y; rt^P/rt^P*, 35 flies; females *rt^P/rt^P*, 30 flies; females *tw¹/+; rt^P/rt^P*, 83 flies; males *rt⁵⁷¹/rt⁵⁷¹*, 139 flies; males *tw¹/Y; rt⁵⁷¹/rt⁵⁷¹*, 143 flies; females *rt⁵⁷¹/rt⁵⁷¹*, 62 flies; females *tw¹/+; rt⁵⁷¹/rt⁵⁷¹*, 157 flies; males *rt^P/rt⁵⁷¹*, 131 flies; males *tw¹/Y; rt^P/rt⁵⁷¹*, 156 flies; females *rt^P/rt⁵⁷¹*, 45 flies; females *tw¹/+; rt^P/rt⁵⁷¹*, 73 flies; males *rt^P/rt²*, 59 flies; males *tw¹/Y; rt^P/rt²*, 67 flies; females *rt^P/rt²*, 50 flies; females *tw¹/+; rt^P/rt²*, 124 flies. Error bars indicate standard deviations. (B) The phenotype of *tw¹* homo/hemizygous mutants is not significantly influenced by the level of *rt* activity. The following numbers of flies were analyzed for shown genotypes: males *–tw¹/Y*, 99 flies; *tw¹/Y; rt⁵⁷¹/+*, 116 flies; *tw¹/Y; rt⁵⁷¹/rt⁵⁷¹*, 70 flies; females *–tw¹/tw¹*, 90 flies; *tw¹/tw¹; rt⁵⁷¹/+*, 102 flies; *tw¹/tw¹; rt⁵⁷¹/rt⁵⁷¹*, 63 flies. To minimize potential influence of different genetic backgrounds, all these flies were obtained from the same cross: *tw¹/Y; rt⁵⁷¹/+ × tw¹/tw¹; rt⁵⁷¹/+.* Error bars indicate standard deviations.

FIGURE 7.—Genetic interactions between *tw* and *rt*. (A) The increased level of *tw* does not have a significant effect on the phenotype of *rt* mutants. The following numbers of flies were analyzed for indicated genotypes: males *rt^P/rt⁵⁷¹*, 58 flies; males *+ / Dp(1;Y)y²sc; rt^P/rt⁵⁷¹*, 58 flies; males *Act5C-GAL4-25/+; rt^P/rt²*, 55 flies; males *Act5C-GAL4-25/UAS-DmPOMT2; rt^P/rt²*, 43 flies; females *Act5C-GAL4-25/+; rt^P/rt²*, 78 flies; females *Act5C-GAL4-25/UAS-DmPOMT2; rt^P/rt²*, 28 flies. Error bars indicate standard errors of the mean. The difference between phenotypes of compared male genotypes is small but statistically significant (*t*-test, *P* < 0.05). The difference between corresponding females is not statistically significant (*P* > 0.3). (B) The phenotype of *rt* mutants is insensitive to the decreased level of *tw*. The following numbers of flies were analyzed for indicated genotypes: males *Act5C-GAL4-25/UAS-twRNAi-39*, 138 flies; females *Act5C-GAL4-25/UAS-twRNAi-39*, 111 flies; males *Act5C-GAL4-25/+; rt^P/rt²*, 38 flies; males *Act5C-GAL4-25/UAS-twRNAi-39; rt^P/rt²*, 48 flies; females *Act5C-GAL4-25/+; rt^P/rt²*, 49 flies; females *Act5C-GAL4-25/UAS-twRNAi-39; rt^P/rt²*, 60 flies. The difference between phenotypes of compared genotypes is not statistically significant (*t*-test, *P* > 0.07). Error bars indicate standard errors of the mean.

ubiquitous ectopic expression of the *UAS-DmPOMT2* construct. Thus, *tw^l* represents the first molecularly and genetically characterized mutant allele of the *DmPOMT2* gene.

Interestingly, we found that *tw^l* could efficiently suppress the phenotype of three tested *rt* alleles (Figure 6). This finding was unexpected, since previously it was reported that RT and DmPOMT2 collaborate biochemically *in vitro*, and *DmPOMT2* RNAi phenotype is enhanced in the *rt^P* heterozygous background (ICHIMIYA *et al.* 2004). Yet the possibility existed that the suppression is the result of a decreased activity of *tw* in *tw^l* mutants. In this case, the suppression would suggest that an unbalanced relative increase of TW in *rt* mutants had a negative effect on the pathway [which, for instance, might result from a competition between nonproduc-

tive homomeric TW complexes and active RT-TW heterocomplexes (GIRRBACH and STRAHL 2003; ICHIMIYA *et al.* 2004)]. To test this possibility, we further analyzed the genetic interaction between *tw* and *rt* by varying the level of *tw* in the *rt* mutant background. We found that the phenotype of *rt* homozygous or heteroallelic mutants was neither significantly sensitive to an increase of *tw* expression by introducing a duplication of the *tw* locus or by ectopic expression of *UAS-DmPOMT2* construct nor significantly sensitive to a decrease of *tw* activity by *UAS-twRNAi* expression (Figure 7 A and B). At the same time, we also confirmed the synergistic effect of the partial reduction of *tw* and *rt* activities on the mutant phenotype using *UAS-twRNAi* construct expression in *rt* heterozygotes (Figure 8). Thus, we ruled out the possibility of negative effect of *tw* in *rt* mutants and

FIGURE 8.—Synergistic genetic interactions between *rt* and *tw*. The following numbers of flies were analyzed for indicated genotypes: females *Act5C-GAL4-25/+; UAS-twRNAi-77/+*, 33 flies; females *Act5C-GAL4-25/+; UAS-twRNAi-77/rt^P*, 42 flies; males *UAS-twRNAi-39/+; tubP-GAL4/+*, 198 flies; males *UAS-twRNAi-39/+; tubP-GAL4/rt^P*, 110 flies; females *UAS-twRNAi-39/+; tubP-GAL4/+*, 192 flies; females *UAS-twRNAi-39/+; tubP-GAL4/rt^P*, 84 flies. The difference between compared phenotypes is statistically significant (*t*-test, $P \leq 0.001$). Error bars indicate standard errors of the mean.

concluded that the suppression of *rt* phenotype is a special feature of *tw^l* allele.

Taken together, our results indicate that both *tw* and *rt* are involved in the same developmental pathway, where they execute nonredundant functions. Insensitivity of *rt* mutant phenotype to the varied level of *tw* expression formally characterizes *rt* as epistatic to *tw*. At the same time, the *tw^l* mutation could dominantly suppress the phenotype of strong (probably amorphic) *rt* mutations, thus revealing its epistatic position relative to *rt* (Figure 6). We interpret these mutually epistatic relationships between *rt* and *tw* as the evidence for possible functioning of their protein products within the same molecular complex or being involved in a regulatory interaction within the same biochemical pathway. This conclusion is in agreement with previously reported data on simultaneous requirement of RT and DmpPOMT2 for their biochemical activity *in vitro* (ICHIMIYA *et al.* 2004). The conclusion is also consistent with our other observations presented here, including (i) essentially identical phenotypes of clockwise abdomen rotation in both *rt* and *tw* mutants (Figure 2), (ii) the subcellular colocalization of RT and TW proteins within the ER compartment in *Drosophila* cells (Figure 4), and (iii) the overlapping pattern of *rt* and *tw* expression during different stages of embryogenesis (Figure 5). Although the close relationship between RT and TW functioning is obvious from all these data, molecular events underlying this relationship remain to be elucidated. Further biochemical and cell biological experiments are necessary to discriminate between different possible molecular mechanisms, including stable physical interaction between RT and TW, their enzymatic modifications of one another, chaperone activity of these proteins, or yet other possibilities.

Our combined genetic and molecular characterization of *tw^l* mutant highlighted the functional importance of the conserved PMT domain of TW protein. We found that *tw^l* mutation should result in the expression of the TW protein with just a subtle alteration of amino acid sequence [T(59) → GS] of the PMT

domain (Figure 1). Despite the apparent subtleness of this mutation and the fact that T(59) is not well conserved between different species, this mutation causes decreased *tw* function and a pronounced rotated abdomen phenotype in *tw^l* homozygotes (Figures 2 and 3). In addition, the *tw^l* phenotype is insensitive to the decreased level of *rt* activity, thus indicating that TW^l mutant protein can bypass the requirement for RT activity that is obligatory for wild-type TW (Figures 6–8). On the basis of protein sequence alignment of TW with other members of the POMT family (Figure 1 and data not shown) and the predicted topology of HsPOMT1 and ScPmt1p proteins (GIRRBACH *et al.* 2000; WILLER *et al.* 2003), the alteration in TW^l protein sequence maps to the luminal terminus of the first transmembrane domain of TW protein. It is tempting to speculate that this protein region might be involved in TW–RT regulatory interactions; however, other possible mechanisms could also explain the properties of the *tw^l* mutation. Further biochemical and genetic experiments are necessary to clarify the properties of TW^l protein.

Several features of *rt* expression detected in our experiments are new and different from the previously reported expression of *rt* (MARTIN-BLANCO and GARCIA-BELLIDO 1996). In contrast to the previous report, we could not detect significant expression in the somatic muscle precursors and the midgut at stages 13 and 14. At the same time, we found the presence of the *rt* transcript in the developing epidermis at stages 14–16 (Figure 5), which was not reported earlier (MARTIN-BLANCO and GARCIA-BELLIDO 1996). A possible explanation for these differences arises from the difference between the *rt* cDNAs used in these studies. The sequence of cDNA clone used in our *in situ* hybridization experiments matches precisely the predicted sequence of the *rt* transcript reported in the FlyBase. We have also used this clone for *in vivo* expression experiments and found that it was able to rescue *rt* mutant phenotype (data not shown), which confirmed its functionality. On the other hand, the cDNA used in the previous study deviates

from our sequence by ~90 nucleotides (MARTIN-BLANCO and GARCIA-BELLIDO 1996), thus possibly representing a fusion with an irrelevant DNA fragment, which may explain the different *in situ* hybridization pattern reported earlier.

In vertebrates, *O*-mannosylation of α -dystroglycan plays an important role in muscle and neural development (MARTIN 2003; MICHELE and CAMPBELL 2003). *Drosophila Dystroglycan (Dg)* is a fly homolog of the vertebrate *Dystroglycan* gene (GREENER and ROBERTS 2000; DENG *et al.* 2003). The predicted product of this gene, *Drosophila DG* protein, is structurally related to its vertebrate counterpart (DENG *et al.* 2003), thus representing a potential molecular target of RT/TW *O*-mannosyltransferase activity. Embryonic expression of *Dg* was detected in a dynamic fashion in many tissues, including visceral and somatic muscles, epidermis, nervous system, gut, and tracheal pits (DEKKERS *et al.* 2004). This expression has only partial overlap with the expression of *rt* and *tw* determined in our experiments, indicating that putative *O*-mannose modification of *Drosophila DG* protein may be limited to just a subset of DG-expressing cells. Interestingly, the expression of *rt* and *tw* in the developing epidermis revealed in our study corresponds to the region of epidermal segment border cells that are known to participate in the development of muscle attachment sites and to influence patterning of larval somatic muscles (VOLK and VIJAYRAGHAVAN 1994). These results indicate the intriguing possibility that these genes function in the epidermal muscle attachment cells, which would provide a novel mechanism for the involvement of *O*-mannosylation in muscle development. Further detailed characterization of *rt* and *tw* mutant phenotypes should shed light on this possibility and help elucidate the functions of these genes in *Drosophila* development.

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