Increased Apoptosis of Myoblasts in Drosophila Model for the Walker-Warburg Syndrome

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Abstract

Walker-Warburg syndrome, a progressive muscular dystrophy, is a severe disease with various kinds of symptoms such as muscle weakness and occasional seizures. The genes of protein O-mannosyltransferases 1 and 2 (POMT1 and POMT2), fukutin, and fukutin-related protein are responsible for this syndrome. In our previous study, we cloned Drosophila orthologs of human POMT1 and POMT2 and identified their activity. However, the mechanism of onset of this syndrome is not well understood. Furthermore, little is known about the behavioral properties of the Drosophila POMT1 and POMT2 mutants, which are called rotated abdomen (rt) and twisted (tw), respectively. First, we performed various kinds of behavioral tests and described in detail the muscle structures by using these mutants. The mutant flies exhibited abnormalities in heavy exercises such as climbing or flight but not in light movements such as locomotion. Defective motor function in mutants appeared immediately after eclosion and was exaggerated with aging. Along with motor function, muscle ultrastructure in the tw mutant was altered, as seen in human patients. We demonstrated that expression of RNA interference (RNAi) for the rt gene and the tw mutant was almost completely lethal and semi-lethal, respectively. Flies expressing RNAi had reduced lifespans. These findings clearly demonstrate that Drosophila POMT mutants are models for human muscular dystrophy. We then observed a high density of myoblasts with an enhanced degree of apoptosis in the tw mutant, which completely lost enzymatic activity. In this paper, we propose a novel mechanism for the development of muscular dystrophy: POMT mutation causes high myoblast density and position derangement, which result in apoptosis, muscle disorganization, and muscle cell defects.

Introduction

Congenital muscular dystrophies (CMDs) are genetic diseases that cause progressive muscle weakness and wasting [1,2]. CMDs result from dystrophin glycoprotein complex (DGC) dysfunction [3]. DGC, which connects the extracellular matrix to the intracellular cytoskeleton, comprises several kinds of proteins such as laminin 2, dystrophin, sarcoglycan, and dystroglycan [4].

Walker-Warburg Syndrome (WWS), the most severe CMD, is a rare recessive inherited disorder characterized by muscular dystrophy, severe brain malformations, and eye abnormalities [5–9]. Patients with WWS rarely survive to birth, and even if they do, the chances that they will survive to adulthood are low [10].

The genes of protein O-mannosyltransferase 1 and 2 (POMT1 and POMT2), fukutin (FCMD), and fukutin-related protein (FKRP) are responsible for WWS [11–17]. The POMT1/2 complex transfers mannose to the Ser/Thr residues of α-dystroglycan [18], one of the components of the DGC, and plays an important role in the first step of O-mannosylation. O-Mannosylation contributes to the stabilization of sarcolemma by binding to laminin, which attaches to the basal membrane [3,19–21].

Recently, several mutations were found in the POMT1 and POMT2 genes of WWS patients [11–15]. These mutations cause a reduction in O-mannosylation of α-dystroglycan, which results in WWS. In fact, recombinant mutant forms of POMT1 co-expressed with wild-type POMT2 did not show any O-mannosyltransferase activity [22].

Although a mouse model for WWS has been generated by targeted disruption of the Pomt1 gene, the mouse ortholog of POMT1, the adult phenotype is unknown because Pomt1 knockout mice are embryonic lethal [23].

In Drosophila, few studies on muscular dystrophy have been reported [24–26]. The Drosophila genome also has the components of DGC [27,28]. The Drosophila orthologs of human POMT1 and POMT2 are called rotated abdomen (rt) and twisted (tw), respectively [29,30]. In our previous study, we cloned genes of these orthologs and identified their activity. Their enzymatic activities are similar to those of the human enzymes; when both RT and TW were co-expressed in cultured insect cells, O-mannosyltransferase activity was observed [29]. Moreover, defective muscles and/or thin muscles with large sarcomeres were observed in larvae of rt or tw mutants [25,31], and the synaptic transmission in larvae was also...
abnormal with changes in the subunit composition of the postsynaptic glutamate receptors at the neuromuscular junction [26].

In the current paper, we first analyze the behavioral properties and ultrastructure of adult muscles in rt and/or tw mutants and then provide evidence that these mutants are highly useful for elucidating the mechanism of muscular dystrophy. Finally, we report that the number of apoptotic myoblasts increases in tw mutants and propose a new mechanism for the development of muscular dystrophy, which involves an increase in the number of apoptotic myoblasts, thereby causing muscle disorganization.

Results

Behavioral defects in rt and tw mutants

Patients with progressive muscular dystrophy show muscle weakness and motor dysfunction with age. Therefore, we evaluated the motor function in rt and tw mutant flies. We first examined age-related changes in climbing activities in rt mutants, which showed specific reduction of rt transcripts (Fig. S1), the climbing abilities of flies homozygous for rt were significantly decreased compared to those of flies heterozygous for rt at all ages (Fig. 1A, Table 1). In tw mutants, the climbing abilities of flies homozygous for tw were also significantly decreased compared to those of flies heterozygous for tw at all ages, except at the age of 41 days between Df[1]/+ and Df[1]/tw (Fig. 1B, Table 2). These data showed reduced climbing abilities in rt and tw mutants at almost all ages.

To understand the response of these mutants to milder exercises, we also evaluated the general locomotive activities of flies. The number of times a fly crossed the center of the glass tube was in the range of approximately 500–1000 in the rt group at all ages and was almost equal (Fig. 1C). Likewise, in the tw group, the number of times a fly crossed the center was in the range of approximately 1000–1800 at all ages, and we could not find any differences in the numbers between the tw mutant and the wild-type fly (Fig. 1D). These data showed that locomotive activities in rt and tw mutants did not decrease compared to those in wild-type flies.

Climbing ability and locomotive activities reflect leg muscle function. In order to determine the status of another behavioral function involved in other muscles, we evaluated flying ability, which reflects the function of flight muscles in the thorax as well as that of the relevant nervous system. The flying index of rtEP/rtKG flies was significantly lower than that of rtEP/+ and rtKG/+ flies (p<0.001, Tukey test) (Fig. 1E). In the case of tw mutants, the flying index of tw/tw flies was approximately half that of tw/+ flies, and the flying index of tw/Df[1] flies was approximately three-quarters of that of Df[1]/+ flies. There were significant differences between tw/tw and tw/+, and between tw/Df[1] and tw/+ or Df[1]/+ (each p<0.001, Tukey test) (Fig. 1F). The above results showed that flying ability was reduced in tw and rt mutants.

We further examined whether defects in the flying ability of tw mutants were rescued by overexpression of the tw gene. The flying index of tw/tw; Act5C-Gal4/+; UAS-tw/+ (rescued tw mutant) flies increased compared to that of tw/tw; Act5C-Gal4/+; tw/+ or tw/tw; tw/+; UAS-tw/+ flies (tw mutant) (each p<0.001, Tukey test) and did not differ from that of [+/+; Act5C-Gal4/+; +/+] or [+/+; +/+; UAS-tw/+] control flies (not significant, Tukey test) (Fig. 1G). These data showed that flying ability was completely rescued in tw mutants by overexpression of the tw gene. This clearly demonstrates that the tw gene plays an important role in motor function.

Here, we showed 3 behavioral features of rt and tw mutants: (1) mutant flies have impaired motor function; (2) defective motor function is observed from just after eclosion to death; and (3) mutant flies show abnormalities in heavy exercises such as climbing or flying, but not in light movements such as locomotion. These results were compatible with the idea that these mutant flies had abnormal muscles and/or motor neurons.

Behavioral defects of flies expressing RNAi for the rt gene

We examined in which tissue the expression of the rt and tw genes affects motor function. We used tissue-specific knockdown flies produced by the Gal4-UAS-IR technique [32], the tissue-specific gene knockdown technique that uses the Gal4-UAS system and RNA interference (RNAi) methods. Tissue-specific rt gene knockdown was induced when flies had both Gal4 driver and UAS-rt-IR, whereas gene knockdown was not induced when flies had only Gal4 driver or UAS-rt-IR [Fig. S2]. The climbing ability of flies with ubiquitous expression of RNAi for the rt gene as driven by Act5C-Gal4 was significantly lower than that of control flies at all ages (Fig. 2A, Table 3). We also examined the climbing ability of neuron- and glial cell-specific knockdown flies by using elav-Gal4 and repo-Gal4, respectively, since it has been reported that there are some defects in the efficacy of synaptic transmission and changes in the subunit composition of postsynaptic glutamate receptors at the larval neuromuscular junction of rt mutants [24].

Table 1. Statistical analysis of results of the climbing abilities of rt mutants.

| Control group | Experimental group | Days after eclosion |
|---------------|--------------------|---------------------|
|               | rtEP/rtKG          | 1 6 11 16 21 26 31 36 |
| rtEP/+       | rtEP/rtKG          | *** *** *** ** * * * * |
| rtEP/+       | rtEP/rtKG          | *** *** *** *** *** *** ** |
| rtKG/+       | rtKG/rtKG          | ns ns ns ns * * ns ns |

*p<0.05;  **p<0.01;  ***p<0.001 by Tukey test. ns, not significant.

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neuron- nor glia-specific knockdown of the rt gene resulted in a distinct reduction in climbing ability at any age, except at the age of 1 day after eclosion in neuron-specific flies (Figs. 2B and C, Table 3). It was noted that the presence of elav-Gal4 or repo-Gal4 has a deteriorative effect on climbing ability for unknown reasons. These data indicated that the climbing ability of adult flies is not mainly influenced by knockdown of the rt gene in neurons or glial cells, and that the expression of this gene in muscles should be relevant to climbing ability.

### Age-related abnormal patterning and ultrastructural abnormalities in muscles of tw and rt mutants

The behavioral data strongly suggest that Drosophila POMT mutants exhibit the muscle defect. Muscles of WWS patients also show structural abnormalities. Hence, we examined the effect of rt or tw on the patterning of muscles by checking the larval body wall muscles in mutants and in flies expressing RNAi for the rt gene. The normal structure of wild-type muscles is shown in Fig. 3A. Deficient or thin muscles were observed in the abdominal segment of mutant larvae (Figs. 3B and C) and in flies expressing RNAi for the rt gene (Fig. 3D).

The frequency of abnormal patterning of muscles detected by FITC-phalloidin staining in these mutants was approximately 10% (data not shown). In addition, we examined the frequency of abnormal patterning in larval muscles of tw mutants by using the MHC-tauGFP marker to observe live muscles. In MHC-tauGFP larvae, which did not show reduction of rt and tw transcripts (Fig. S3), the dorsal body wall muscles were structurally normal (Fig. 3E). A few muscles were absent in the mutant larvae (Figs. 3F and G), and many muscles were absent in rare cases of mutant larvae (Fig. 3H). Significantly higher frequencies of abnormal patterning were detected in the muscles of tw mutants (probabilities for all compared pairs were p<0.001, Fisher’s exact test) (Fig. 3I).

We further examined the effect of the tw mutation on sarcomeric structure by performing detailed electron microscopic analysis on the leg and thoracic muscles from wild-type, tw mutant and the rescue flies. Similar changes in ultrastructure were observed in the leg and thoracic muscles of both male and female tw mutant flies (Table 4). The normal sarcomeric structure of the muscles of wild-type flies is shown in Figs. 4A, 4C, and 5A. Sarcomeric disarray was frequently observed in the muscles of tw mutant flies (Figs. 4B, 4D, 5B, and 5C). In the mutant muscles, Z-lines were irregular and often streaming (Figs. 4D, 5B, and 5C), nemaline bodies were observed in the muscle fiber (Fig. 4D), actin and myosin filaments were disorganized (Figs. 4E and 5B), and accumulated glycogen granules were seen (Fig. 4F). Enlarged mitochondria (Fig. 5F) and swollen sarcoplasmic reticulum (SR) were seen in the tw mutant muscles (Figs. 4H and 5D), while normal mitochondria and SR were observed between the muscle fibers (Figs. 4A, 4C, 4G, 5A, and 5E). The basement membrane was duplicated and multilayered in tw mutant muscles (Figs. 4J and 5H), while normal basement membrane was observed continuously along the sarcolemma in wild-type muscles (Figs. 4I and 5G). The abovementioned defective muscle phenotypes were observed both in 15- and 35-day-old tw mutants but were hardly detected in wild-type flies. The number of mutants with defective phenotypes was higher in 35-day-old tw mutants than in 15-day-old tw mutants. Moreover, these defective phenotypes could not be found among the rescue flies (Table 4), indicating that the defective phenotypes in the mutants were fully rescued. These results demonstrated that tw contributed to the maintenance of muscle ultrastructure.

### Myoblasts in the wing discs of tw mutants

In Drosophila, flight muscles in the thorax develop from myoblasts in the wing imaginal discs of larvae. The climbing ability of the tw mutant at 1 day of age was less than that of the wild-type fly (Fig. 1B). These facts suggest that something happens to the myoblasts of tw mutants. We determined whether the number of myoblasts in the tw mutant changed by observing myoblasts in third instar larvae. Myoblasts were present in the nodal plane of the third instar larva (Figs. 7A–D). The wing disc arc occupied by myoblasts in tw mutant was less than that in wild-type flies (Fig. 7E). The number of myoblasts per unit area of mutant tissue was higher than that of wild-type tissue (Fig. 7F). As a result, the total number of

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**Table 2. Statistical analysis of results of the climbing abilities of tw mutants.**

| Control group | Experimental group | Days after eclosion |
|---------------|--------------------|---------------------|
| tw/+          | tw/tw              | 1 6 11 16 21 26 31 36 41 46 51 |
| tw/+          | tw/Df(1)           | *** *** *** *** *** *** *** *** *** *** |
| Df(1)/+       | tw/Df(1)           | *** *** *** *** *** *** *** *** *** *** |
| tw/+          | Df(1)/+            | ns * ns * ns *** *** *** *** *** *** |

*p<0.05; **p<0.01; ***p<0.001 by Tukey test. ns, not significant. doi:10.1371/journal.pone.0011557.t002
flies at all ages. Neither neuron- nor glial cell-specific knockdown of the gene resulted in a distinct reduction in climbing ability at any age except the (C) are shown in Table 3. The climbing ability of flies expressing RNAi for the gene. The climbing ability of whole-body knockdown flies using Act5C-Gal4 (A), that of neuron-specific knockdown flies using elav-Gal4 (B), and that of glial cell-specific knockdown flies using repo-Gal4 (C). Error bars indicate standard error. Results of the statistical analyses in (A), (B), and (C) are shown in Table 3. The climbing ability of flies expressing RNAi for the rt gene driven by Act5C-Gal4 was significantly less than that of control flies at all ages. Neither neuron- nor glial cell-specific knockdown of the rt gene resulted in a distinct reduction in climbing ability at any age except at 1 day after eclosion in neuron-specific knockdown flies.

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Figure 2. Climbing ability in flies expressing RNAi for the rt gene. The climbing ability of whole-body knockdown flies using Act5C-Gal4 (A), that of neuron-specific knockdown flies using elav-Gal4 (B), and that of glial cell-specific knockdown flies using repo-Gal4 (C). Error bars indicate standard error. Results of the statistical analyses in (A), (B), and (C) are shown in Table 3. The climbing ability of flies expressing RNAi for the rt gene driven by Act5C-Gal4 was significantly less than that of control flies at all ages. Neither neuron- nor glial cell-specific knockdown of the rt gene resulted in a distinct reduction in climbing ability at any age except at 1 day after eclosion in neuron-specific knockdown flies.

Increased α-spectrin in myoblasts and βPS-integrin around myoblasts of the tw mutant

Cytoskeletal and cell adhesion molecules, such as spectrins, cadherins, and integrins, proteolyze during apoptosis [33–35]. Thus, we examined the expression of α-spectrin, DE-cadherin, and βPS-integrin in myoblasts of the tw mutant. Surprisingly, the signals of α-spectrin and βPS-integrin excessively increased in the region of myoblasts compared to the lateral region of myoblasts in the tw mutant (Figs. 9E and M) although apoptosis increased in the myoblasts of tw mutant. These signals in wild-type flies did not change between the 2 regions (Figs. 9A and I). We could not find any difference in the DE-cadherin signal (Figs. 9B, F, J, and N). These data suggest that α-spectrin and βPS-integrin might be induced to protect myoblasts of the tw mutant from apoptosis.

Lethality in tw mutants and flies expressing RNAi for the rt gene

It is known that WWS has high lethality rates during early development. Therefore, we determined whether Drosophila POMT genes play an important role in viability. We crossed females heterozygous for tw with male hemizygous for tw and checked the number of F1 progenies (Fig. 10A). If all the eggs of F1 progenies hatched and developed normally, the ratio of the number of individuals having a twisted abdomen, the tw phenotype, to those having a normal abdomen is expected to be 1. The ratio was found to be 0.2, significantly lower than the expected ratio \(p<0.001\), chi-square test) (Table 6). In addition, in order to determine the necessity of the tw gene for viability, we performed a rescue experiment by expressing the tw gene in tw mutants (Fig. 10B). The ratio was found to be 1.25 and was significantly increased \(p<0.001\), chi-square test) (Table 7). These data indicated that the tw gene plays an important role in viability and normal development. We also examined fly viability after knockdown of the rt gene under 3 different temperature conditions. Knockdown at high temperature is more efficient than that at low temperature because yeast transcriptional factor GAL4 binds strongly to UAS sequences. Ratios of the number of knockdown flies to the number of non-knockdown flies were 0.26, 0.12, and 0.00 at 18, 25, and 28°C, respectively (Fig. 10C, Table 8). Growing flies at a higher temperature resulted in higher lethality, indicating that the rt gene also contributed to normal development. The abovementioned results showed that the tw and rt genes were essential for the viability of the embryo, larva, and/or pupa. Elucidation of the reason for the lethal phenotype in these mutants could clarify the mechanism of low viability in human patients.
Drosophila cells did not influence lifespan. Together with the results of the tissues reduced the lifespan, while knockdown in neurons or glial respectively, were not affected (Figs. 11B and C, Tables 9 and hand, the lifespans of flies expressing RNAi for the gene in all epidermal cells or gastrointestinal tract may lead to early death in flies. TWMut was expressed. POMT-specific activity was detected when RT WT, TW WT, and mutant we used was an almost null or a strong hypomorphic mutant for POMT activity.

Table 3. Statistical analysis of results of the climbing abilities of flies expressing RNAi for the rt gene.

| Control group | Experimental group | Days after eclosion |
|---------------|--------------------|---------------------|
| Act5C-Gal4/+  | Act5C-Gal4/UAS-rt-IR | *** *** *** *** *** *** *** |
| +/UAS-rt-IR   | Act5C-Gal4/UAS-rt-IR | *** *** *** *** *** *** *** |
| +/UAS-rt-IR   | +/UAS-rt-IR         | ns ns ** * ns ** *** |
| elav-Gal4/+; +/+ | elav-Gal4/+; +/UAS-rt-IR | ** ns ns ns ns ns ns |
| +/+; +/UAS-rt-IR | elav-Gal4/+; +/UAS-rt-IR | *** *** *** *** *** *** *** |
| elav-Gal4/+; +/+ | +/+; +/UAS-rt-IR     | *** *** *** *** *** *** *** |
| +/+; +/repo-Gal4 | +/UAS-rt-IR; repo-Gal4/+ | ns ns ns ns *** * |
| +/+; +/repo-Gal4 | +/UAS-rt-IR; repo-Gal4/+ | *** *** *** *** *** *** *** |
| +/+; +/repo-Gal4 | +/UAS-rt-IR; +/+     | *** *** *** *** *** *** *** |

*p<0.05; **p<0.01; ***p<0.001 by Tukey test. ns, not significant.

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Shortened lifespan of flies expressing RNAi for the rt gene

Patients with WWS rarely survive to adulthood [10]. Therefore, we investigated whether rt knockdown mutants had shortened lifespans. Flies with ubiquitous expression of RNAi for the rt gene driven by Act5C-Gal4 had shorter lifespans than those of the control groups (both p<0.001, log-rank test, Table 9) (Fig. 11A). The median lifespan of Act5C-Gal4/rt-IR flies was 23 days, which was ~51.1% of that of UAS-rt-IR/+ flies (47 days) and ~66.7% of that of Act5C-Gal4/+ flies (69 days) (Tables 9 and 10). On the other hand, the lifespans of flies expressing RNAi for the rt gene in neurons and glial cells driven by elav-Gal4 and repo-Gal4, respectively, were not affected (Figs. 11B and C, Tables 9 and 10). These results indicated that knockdown of the rt gene in all tissues reduced the lifespan, while knockdown in neurons or glial cells did not influence lifespan. Together with the results of the muscle phenotype in Drosophila POMT mutants, these results suggest that age-related weakness of the muscles in the heart and/or gastrointestinal tract may lead to early death in flies.

No enzymatic activity in the protein of the tw mutant form or in the larval extracts of rt and tw mutants

Since there is no evidence that POMT2 mutation in WWS patients influences POMT activity, we examined the POMT activity in the Drosophila POMT2 mutant. We prepared recombinant wild-type RT (RTWT), wild-type TW (TWWT), and mutant TW (TWTmut) in order to examine POMT activity in the tw mutant. After pVL1393-rtWT-HA and pVL1393-twWTmut or pVL1393-twWTmut was co-transfected into Sf21 insect cells, microsomal membrane fractions were collected from each infected cell. The specific expression of recombinant proteins was confirmed by western blot analysis using anti-HA monoclonal antibody and anti-dPOMT2 antibody (Figs. 12A and B). POMT activity toward GST-α-DG was measured using each microsomal membrane fraction as described under “Materials and Methods.” We could not detect enzymatic activity when only RTWT-HA, TWTWT, or TWTmut was expressed. POMT-specific activity was detected when RTWT-HA and TWTWT were co-expressed (p<0.001, Tukey test). However, no POMT-specific activity was detected when RTWT-HA and TWTmut were co-expressed (n.s., Tukey test) (Fig. 12C). These data clearly demonstrated that the mutation involved in the tw mutant was a null mutation for POMT activity. Moreover, we examined the POMT activity of larval extracts of rt and tw mutants. POMT-specific activities of the rt and tw mutants were significantly reduced compared to those of wild-type flies (Fig. 12D). Taken together, the tw mutant was a null mutant and the rt mutant we used was an almost null or a strong hypomorphic mutant for POMT activity.

Genetic interaction between rt or tw and Dg in the wing

Dg is one of the putative core proteins that are O-mannosylated by RT and TW. Wild-type flies had normal-shaped wings (Fig. 13A); however, knockdown of Dg in the posterior region of the wing disc resulted in a blistered phenotype (Fig. 13B). The blistered phenotype results from cell adhesion failure in the 2 cell layers during wing development. Actually, Dg expression was dramatically decreased in the posterior region (Fig. S5), indicating that Dg contributed the attachment of the 2 cell layers during wing development. We examined the genetic interaction between rt or tw and Dg by using this phenotype. At 25°C, the penetrances of the blistered phenotype in wings of single knockdown flies of rt, tw, and Dg were 0, 0, and 0.06, respectively. The penetrances of the phenotype in double knockdown flies of rt-Dg and tw-Dg were 0.14 and 0.08, respectively, but they were not significantly higher than those of the single knockdown flies (Fisher’s exact test). As mentioned in the above section, “Lethality in tw mutants and flies expressing RNAi for the rt gene,” knockdown at 28°C is more efficient than that at 25°C. Thus, we performed the knockdown at 28°C. At 28°C, the penetrances of the blistered phenotype in wings of single knockdown flies of rt, tw, and Dg were 0, 0, and 0.34, respectively. The penetrances of double knockdown flies of rt-Dg and tw-Dg were 0.86 and 0.48, and they were significantly higher than those of single knockdown flies (p<0.001 and p<0.05, Fisher’s exact test) (Fig. 13C). These data showed that rt or tw genetically interact with Dg to contribute to cell adhesion in the wings. Together with the high density of myoblasts observed in the tw mutant (Fig. 7F), these results suggest that both epidermal cells and muscle progenitor cells of Drosophila POMT mutants give rise to cell adhesion derangement.

Discussion

In this study, we first presented not only the behavioral abnormalities but also the shortened lifespan and ultrastructural...
defects of muscles in flies with mutations in \textit{rt} and/or \textit{tw}, the \textit{Drosophila} orthologs of human \textit{POMT1} and \textit{POMT2}, respectively. Our data strongly indicate that these mutants are a \textit{Drosophila} model for WWS. We then discovered that apoptosis is enhanced in muscle progenitor cells of these mutants and provided new insight into the mechanism of WWS development, namely increased numbers of apoptotic myoblasts causing muscle disorganization.

\textbf{Figure 3. Larval body wall muscles in \textit{rt} and \textit{tw} mutant flies.} Larval body wall muscles in abdominal segments 2–4 of wild-type flies (A), \textit{rt}^P/\textit{rt}^P flies (B), \textit{rt}^P/\textit{rt}^P flies (C), and flies expressing RNAi for the \textit{tw} gene (\textit{Act5C}::\textit{tw}-IR) (D). These muscles were stained by FITC-phalloidin. The arrowhead in (A) shows muscle 5. Deficient or thin muscle 5 were observed in \textit{rt} mutants and in flies with ubiquitous expression of RNAi for the \textit{tw} gene. We visualized live muscles by using a \textit{MHC}-\textit{tauGFP} marker in wild-type (\textit{MHC}-\textit{tauGFP}/Y) (E) and \textit{tw} mutant (F–H) (\textit{tw}, \textit{MHC}-\textit{tauGFP}/Y) larvae. The arrows indicate the absent muscles. A few muscles were absent in the mutant larvae (F and G), and many muscles were absent in rare cases of mutant larvae (H). The frequencies of abnormal patterning of larval body wall muscles in \textit{tw} mutants (I). Error bars indicate standard error. The frequencies of abnormal patterning of the muscles were significantly higher in \textit{tw} mutants. ***p<0.001 by Fisher’s exact test.

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Behavioral and developmental similarities between the phenotypes of rt and tw mutant flies and the symptoms of WWS patients

The climbing abilities of rt and tw mutant flies were reduced compared to those of flies that were heterozygous for rt and tw, respectively (Figs. 1A and B, Tables 1 and 2). Reduced climbing ability was also observed in flies with ubiquitous expression of RNAi for the rt gene driven by Act5C-Gal4 (Fig. 2, Table 3). In addition, Dg and Dys (Dystrophin) mutant flies lack climbing ability [24]. These data indicate that the O-mannosyl glycans on Dg contributes to motor functions such as climbing. The mutation of rt or tw in Drosophila causes the behavioral defect like WWS, since the defect of O-mannosyl glycans on Dg leads to WWS [14,22]. Furthermore, the climbing abilities of mutants rapidly decreased with age (Figs. 1A and B). These changes are similar to the behaviors seen in patients with WWS, such as difficulty in walking with age.

Both our study (Fig. 3) and other studies [25,31] have revealed structural defects in the larval body wall muscles in Dg, rt, and/or tw mutants. Moreover, we observed various kinds of defective ultrastructural phenotypes in the thoracic and leg muscles of adult tw mutant flies (Figs. 4 and 5, and Table 4) that have been reported in muscle biopsies of WWS patients [36]. A recent study revealed that expression of GluRIIB, a subunit of the postsynaptic glutamate receptor, and the efficacy of synaptic transmission decreases at the neuromuscular junctions of larval Dg and tw mutant flies [26]. Muscle contraction and membrane resistance in larval body wall muscles changed in flies expressing RNAi for the Dg gene [25]. These changes might cause decreased motor function in adult rt and tw mutant flies. On the other hand, we observed no difference in locomotive activity between mutant and control flies (Figs. 1C and D). The mutant flies showed abnormalities in heavy exercises, such as climbing or flying, but not in light movements, such as locomotion, probably because some muscles and/or neuromuscular junctions had normal functioning while other muscles had defective functioning.

The growing of flies with ubiquitous expression RNAi for the rt gene at a higher temperature resulted in higher lethality rates, and knockdown at 28°C was almost entirely lethal (Table 8). Some rt alleles that were hemizygous for the deficiency and entirely lacked the genomic region of rt showed partial lethality [31]. Thus, a null mutation in rt appears to be lethal. On the other hand, the tw allele is semi-lethal (Table 6). Davis [37] also reported that flies carrying a tw mutation showed reduced viability. Furthermore, we rescued the lethality of tw mutant flies by ubiquitous expression of the tw gene (Table 7). The apparent incidence rate of WWS is very low [10] because of its high lethality rate in the embryonic stage. Indeed, defective development in the early embryonic stages causes embryonic lethality in Pom1 mutant mice [23]. However, the mechanism underlying this lethality is unknown. Thus, the mechanism of the high lethality rate in the embryonic stage in mammals may be understood by elucidating the mechanisms of the high lethality rates in rt and tw mutant flies. Lethality in rt mutant flies is not associated with any particular developmental stage [31]. Several studies have revealed that Dg is involved in epithelial and oocyte polarity determination [38–40], so the O-mannosyl glycans on Dg at least contribute to the viability through fly oocyte formation and motor function.

Even though WWS patients may survive to birth, their lifespan is generally short and they typically die before reaching adulthood. Flies with ubiquitous expression of RNAi for the rt gene have shortened lifespans as well, but those with neuron- or glial cell-specific expression of RNAi do not have shortened lifespans (Fig. 11, Tables 9 and 10). These results suggest that expression of POMT1 and POMT2 in the tissues other than neurons and glial cells plays a crucial role in longevity in humans.

Anatomical similarities between the muscles of tw mutant flies and WWS patients

In the present study, we showed that the larval body wall muscles (Fig. 3A, muscle 5) were thin or missing in flies expressing RNAi for the rt and tw genes as in flies carrying other mutant alleles (Figs. 3B–D). Furthermore, we observed larval body wall muscles in tw mutant flies by using the MHC-tauGFP reporter, a muscle marker (Figs. 3F–H). Studies have shown that larval body wall muscles of rt and tw mutant flies are sometimes thin and missing [25,31]. In our analysis, the frequency of abnormal patterning in the body wall muscles is approximately 50% in female and 40% in male tw mutant flies, both of which are higher than that reported in one study (10%) [25]. The discrepancies between the data seem to result from differences in the methods used: our methods did not involve dissection of larvae but, rather, observation of all muscles in living larvae using MHC-tauGFP reporter for muscle visualization; on the other hand, methods used

### Table 4. Ultrastructure defects of thoracic and leg muscles from 15- and 35-day-old tw mutants.

| Abnormal phenotype       | 15-day-old fly muscles | 35-day-old fly muscles |
|--------------------------|------------------------|------------------------|
|                          | Thorax | Leg | Thorax | Leg |
|                          | +/Y | tw/Y | +/Y | tw/Y | rescue | +/Y | tw/Y | rescue |
| sarcomeric disarray *     | 0/6 | 0/6 | 0/6 | 6/6 | 0/6 | 6/6 | 0/6 | 1/6 | 6/6 | 0/6 |
| irregular Z-lines *       | 0/6 | 0/6 | 2/6 | 6/6 | 0/6 | 6/6 | 0/6 | 0/6 | 6/6 | 0/6 |
| filament disorganization *| 0/6 | 0/6 | 0/6 | 1/6 | 0/6 | 0/6 | 0/6 | 0/6 | 6/6 | 0/6 |
| swollen SR                | 0/6 | 6/6 | 0/6 | 6/6 | 0/6 | 6/6 | 0/6 | 0/6 | 6/6 | 0/6 |
| accumulation of glycogen  | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 6/6 | 0/6 |
| mitochondrial enlargement | 0/6 | 6/6 | 0/6 | 6/6 | 0/6 | 6/6 | 0/6 | 0/6 | 0/6 | 6/6 |
| basement membrane dup.    | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 | 0/6 |

(Number of individuals that have abnormal phenotype)/(Number of individuals observed) is shown. The “rescue” genotype was [tw/Y; Act5C-Gal4/+; UAS-tauGFP]. A muscle area of 590 μm² per individual was observed.

*: The number of abnormal phenotypes in the muscle area of 590 μm² per individual was counted and the percentage of abnormal structures (Number of abnormal structures/Number of structures observed) was calculated and is shown in Figure 6. SR, sarcoplasmic reticulum.

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Figure 4. Representative electron micrographs of thoracic muscles in aged wild-type and tw mutant flies. (A, C, G, and I) Thirty-five-day-old wild-type fly muscles. (B, D, E, F, H, and J) Thirty-five-day-old tw mutant fly muscles. (A and B) Low-magnification images of muscles. (C and D) High-magnification view of the area bordered by the rectangle in Figs. 4A and B. (C) Normal sarcomere with regular Z-lines (arrowheads). (D) Z-lines (arrowheads) are irregular and often streaming. Nemaline bodies (arrows) in the muscle fiber. (E) Actin and myosin filaments are disorganized. (F) Glycogen granules (arrows) are accumulated. (G) Normal sarcoplasmic reticulum (SR). (H) SR is swollen. (I) Normal basement membrane. (J) The basement membrane (arrowheads) is duplicated and multilayered. MT: mitochondria. Bars: (A and B) 2 μm, (C, D, F, G, and H) 1 μm, and (E, I, and J) 500 nm.

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Figure 5. Representative electron micrographs of leg muscles in aged wild-type and tw mutant flies. (A, E, and G) Muscles of 35-day-old wild-type flies. (B, C, D, F, and H) Muscles of 35-day-old tw mutant flies. (A) Normal sarcomere in a wild-type fly with regular Z-lines. (B) In the tw mutant, numbers of actin and myosin filaments are decreased and disorganized. (C) Z-lines are irregular and often incomplete. (D) Sarcoplasmic reticulum is swollen. Normal mitochondria are observed in wild-type fly muscles (E) while enlarged mitochondria are accumulated in tw mutant fly muscles (F). The normal basement membrane (arrowhead) runs continuously along the sarcolemma in wild-type fly muscles (G), while the basement membrane (arrowheads) is duplicated and multilayered in the tw mutant fly muscles (H). SR: sarcoplasmic reticulum. Bars: (A–D) 1 μm and (E–H) 500 nm.

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to obtain the previous data included dissection, fixation, FITC-phalloidin staining for muscle visualization, and observation of particular muscles.

The ultrastructure of muscles in adult mutant flies has not yet been reported, although age-dependent muscle degeneration and large sarcomeres in the larval body wall muscles of the flies in mutants have been reported based on light microscopic observations [24,25]. Here, we observed ultrastructural characteristics of tw mutant flies and discovered various phenotypes: sarcomeric disarray, irregular Z-lines, filament disorganization, swollen SR, accumulation of glycogen granules, enlargement of mitochondria, and duplication of basement membranes (Figs. 4 and 5). The ultrastructural defects observed in the thorax and leg muscles of tw mutant flies are consistent with muscle characteristics of patients with Duchenne muscular dystrophy, including WWS. Myofibril shearing, myofilament loss, wavy or disrupted Z-lines, changes in

Figure 6. Frequency of abnormal structures in the muscles of tw mutant flies. (A) Thoracic muscles. (B) Leg muscles. The percentages of sarcomeric disarray, irregular Z-line, and filament disorganization noted in the muscle area of 590 μm² per individual in 15- and 35-day-old wild-type and tw mutant flies are shown. In thoracic muscles, these abnormal structures were observed in 35-day-old mutant flies but not in wild-type flies. In leg muscles, these abnormal structures were observed in both 15- and 35-day-old mutant flies but were hardly detected in wild-type flies. We compared data of each abnormal phenotype in 15- and 35-day-old wild-type, tw mutant, and rescued flies. Each bar represents the mean of 6 individuals. Error bars indicate standard error. **p<0.01, ***p<0.001 by Welch's two sample t test. n.s., not significant.
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the muscle structure are conserved in flies and humans. POMTs were found to be coexpressed in muscles of flies and humans, similar to what was previously reported by others [29]. In humans, mutations in the POMT1 and POMT2 genes have been identified in patients with WWS, indicating that the first transmembrane region of TW may play an important role in the development of WWS.

In flies, the Drosophila orthologs of POMTs have been shown to be important for cell adhesion [26] and basal membrane [43]. The basal membrane is duplicated in muscles of WWS patients, suggesting that cell adhesion in both muscle and epithelial cells, such as wing cells, is involved in the WWS phenotype.

**Table 5. Numbers of abnormal muscle ultrastructures increasing with age in tw mutant flies.**

| Muscle | Abnormal phenotype | +/-Y | tw/Y |
|--------|--------------------|------|------|
| Thorax | sarcomeric disarray | ns   | ***  |
|        | irregular Z-lines  | ns   | **   |
|        | filament disorganization | ns | ***  |
| Leg    | sarcomeric disarray | *    | ***  |
|        | irregular Z-lines  | *    | ***  |
|        | filament disorganization | ns | ***  |

The percentage of abnormalities observed in 35-day-old flies compared to that in 15-day-old flies of the same genotype. The percentage of abnormalities was significantly exaggerated with age in tw mutant flies but not significantly exaggerated with age in wild-type flies except for sarcomeric disarray in the legs of muscles of 15-day-old flies.

**p<0.05; **p<0.01; ***p<0.001 by Welch’s two sample t test. ns, not significant. doi:10.1371/journal.pone.0011557.t005

Interestingly, we showed that apoptosis was increased in myoblasts of wing imaginal discs in tw mutant larvae, cells that otherwise differentiate into indirect flight muscles (Fig. 8G). Myoblast density was observed to be high (Fig. 7F), although no mitotic abnormality was observed (Fig. S4). Moreover, expression of α-spectrin and βPS-integrin in the myoblasts was increased (Fig. 9). These results suggest that myoblasts of the tw mutant might avoid enhanced apoptosis or compensate for a DGC defect by overexpressing cytoskeletal or cell adhesion molecules such as α-spectrin and βPS-integrin. Mutations in the tw gene will lead to a high density of myoblasts, causing disruption of myoblast intercellular interactions, and result in enhanced myoblast apoptosis. Thus, the tw gene also controls myoblast density or intercellular interaction.

Here, we propose a novel mechanism for the development of WWS. At first, POMT mutation causes cell adhesion abnormalities, myoblast positioning, and high density of myoblasts because O-mannosyl glycans do not form on core proteins, such as Dg. Since high densities of myoblasts do not develop into normal muscles, some of them are excluded by apoptosis. As a result, muscles in the POMT mutant have duplicated basal membranes and decreased motor function compared to those of wild-type flies. Apoptosis occurs not only in myogenesis but also in cell differentiation events such as neurogenesis. Thus, severe phenotypes will appear in several organs of patients with WWS.

**Genetic interaction between Dg and rt and/or tw in the wing.**

Several studies have revealed the genetic interaction between Dg and rt and/or tw. One study demonstrated that Dg and rt contribute to the promotion of synaptic vesicle release and regulate glutamate receptor subunit composition at the neuromuscular junction [26]; moreover, it showed that muscle attachment formation and sarcomere size determination in third instar larvae require functional Dg and rt or tw [26].

In the present study, we showed that basement membranes are duplicated and multilayered in tw mutant muscles (Figs. 4J, 5H). This change in the basement membrane is due to the muscle attachment failure that results from mediation of Dg and laminin by O-mannosyl glycan [43]. In addition, knockdown of Dg in the wing resulted in the blistered phenotype (Fig. 13B), which was caused by cell adhesion failure of the 2 cell layers during wing development. Moreover, double knockdown of Dg and rt or tw enhanced this phenotype (Fig. 13C). Consequently, we demonstrated that the effect of O-mannosyl glycan on Dg contributes to epidermal cell attachment in the wing. Incidentally, a mutant of ieb (wing blister), a Drosophila laminin, also showed blistered wings [44]. Thus, it is suggested that O-mannosyl glycan mediates the binding
Figure 7. Myoblasts in the wing imaginal disc of tw mutant larvae. (A) Myoblasts in the wing imaginal disc of wild-type larvae. Myoblasts were visualized by nuclear localization of GFP (GFPnls), which was driven by 1151-Gal4. (B) Wing imaginal disc of wild-type (1151-GFPnls) larvae. (C)
of Dg with molecules such as laminin in the extracellular matrix, and that failure of this binding leads to the blistered wing phenotype observed in mutants. Laminin and adhesion molecules play important roles in muscle attachment [45–47]. Thus, the binding of Dg to laminin mediated by O-mannosyl glycan might contribute to adhesion between the 2 epithelial cell layers of the wing as well as muscle attachment.

Conclusion

Analyses of rt and tw mutants, the Drosophila models for WWS, help in understanding not only the symptoms of this human disease but also the mechanisms of muscular dystrophy. We proposed a new mechanism for the development of muscular dystrophy involving increased apoptosis in developing muscles that causes muscle disorganization. Further studies with these mutants will provide additional insight into the mechanisms of muscular dystrophies and will help in the development of useful drugs for WWS patients.

Materials and Methods

Fly stocks

All stocks were raised at room temperature (23–25°C) using a cornmeal-yeast-glucose medium. All stocks except those for rtEP571, MHC-tauGFP, 1151-Gal4, and Canton-S were obtained from Bloomington Stock Center (http://flystocks.bio.indiana.edu/). We refer to the rtEP571 line (stock number: 14434) as rtKG. rtEP571 was obtained from Szeged Stock Center. In this paper, we refer to this line as rtKG. Df(1)Exel6223, which disrupts the tw gene, is referred to as Df(1). The tw stock was backcrossed onto the Canton-S background for 20 generations. MHC-tauGFP [48], a muscle marker, and 1151-Gal4 [49], a driver for marking the myoblasts in wing imaginal discs, were supplied by Dr. T. Maqbool. Canton-S was a gift from Dr. D. Yamamoto. UAS-tw-IR and UAS-rt-IR have been referred to by Ichimiya et al. [29] as UAS-dPOMT2-IR and UAS-dPOMT1-IR, respectively. UAS-Dg-IR and UAS-tw were generated by the following methods.

Figure 8. Excessive apoptosis of myoblasts in the wing imaginal disc of tw mutant larva. (A, C, and E) The wild-type fly (1151->GFPnls). (B, D, and F) The tw mutant fly (tw, 1151->GFPnls). (A) and (B) Myoblasts in the wing imaginal disc of larvae. The GFP localize in myoblast nuclei. (C) and (D) Myoblasts stained by caspase-3 antibody, a marker of apoptotic cells. (E) and (F) Merged images of (A) and (C) and of (B) and (D), respectively. The arrowheads and arrows show the co-localization of GFP and caspase-3. The arrowheads show the nuclei just before breakdown. The arrows show degraded nuclei in more of a progressive apoptotic stage than the nuclei shown by arrowheads. (G) The number of myoblasts positive for caspase-3 in the wing imaginal disc of wild-type and tw mutant larvae. Error bars indicate standard error. The number of apoptotic myoblasts in tw mutant larvae was significantly lower than that in wild-type larvae. ***p<0.001 by t test.

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The UAS-IR fly line was obtained as described in previous reports [29,32]. The cDNA fragment of Dg (nucleotide positions 327–826 of the coding sequence of Dg-RA) was amplified by PCR using a cDNA library derived from Drosophila melanogaster. The amplified fragment was inserted as an inverted repeat (IR) sequence into the pSC1 vector. The IR-containing fragment was then subcloned into the transformation vector pUAST, and this vector was introduced into Drosophila embryos of the w1118 mutant stock, which was used as the host to construct the UAS-IR fly line according to the procedure reported by Spradling and Rubin [50].

**UAS-Dg-IR flies**

The UAS-IR fly line was obtained as described in previous reports [29,32]. The cDNA fragment of Dg [nucleotide positions 327–826 of the coding sequence of Dg-RA] was amplified by PCR using a cDNA library derived from Drosophila melanogaster.

**Figure 9. Increased α-spectrin and βPS-integrin in the myoblasts of tw mutant.** (A–D and I–L) wild type (1151>GFPnls). (E–H and M–P) tw mutant (tw, 1151>GFPnls). (A) and (E) Images stained by anti-α-spectrin antibody. α-spectrin is a component of cytoskeleton inside of cell membrane and bind to actin. (B), (F), (J), and (N) Images stained by anti-DE-cadherin antibody. DE-cadherin is a cell adhesion molecule located in cell surface. (C), (G), (K), and (O) Nuclei of myoblasts. (D), (H), (L), and (P) Merged images of (A–C), (E–G), (I–K), and (M–O), respectively. (I) and (M) Images stained by anti-βPS-integrin antibody. βPS-integrin is a cell adhesion molecule located in cell surface and bind to extracellular matrix. TB, arrowheads, and arrows in (A), (E), (I), and (M) show tracheoblast, the region of myoblasts, and lateral region of myoblasts, respectively. The signals of α-spectrin and βPS-integrin excessively increased in the region of myoblasts compared to the lateral region of myoblasts in tw mutant although apoptosis increased in the myoblasts of tw mutant. These signals in wild type did not change between two regions. But we could not find any difference in the signal of DE-cadherin.

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**UAS-tw flies**

The DNA fragment containing the *tw* gene was amplified by two-step PCR. For the first PCR, we used the plasmid DNA from an EST clone (LP01681) as a template and the following primer set for the amplification of the *tw* coding region: forward primer 5′-AAAAAGCAGGCTTGGCAGCAAGTGTTGTTA-3′; and reverse primer 5′-AGAAAAGCTGGGTCTAGAAACTCCGAGG-TAGAAAAG-3′. For the second PCR, we used the first PCR product as a template; a forward primer that included the attB1 site, 5′-GGGGAGCATTTTGTACAAAAAAGCAGGCT-

| Phenotype  | [tw∗] | Ratio* ([tw∗]/[tw+]) | p (x²) |
|------------|-------|----------------------|--------|
| [tw+], [tw∗] | 912   | 0.28                 | <0.001 (1359) |

Number of adult *tw*/tw and *tw*/Y flies divided by number of *tw+* and *+*/Y flies. If all flies hatch and develop normally, the ratio is expected to be 1.

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**Figure 10. Crosses for examining *tw* and *rt* mutant fly viability.** (A) The cross scheme for examining *tw* mutant fly viability. Female *tw/+* flies were crossed with male *tw* mutants (*tw/Y*). Genotypes of F₁ progeny are *tw/tw*, *tw/Y*, *tw/+*, and *+*/Y. Individually with genotype *tw/tw* or *tw/Y* have the twisted abdominal phenotype, which is represented as [tw∗]. On the other hand, individuals with genotype *tw/+* or *+*/Y have the normal abdominal phenotype, which is represented as [tw]. The number of F₁ progeny with phenotype [tw∗] or [tw] is shown in Table 6. (B) The cross scheme for the rescue experiment of the *tw* mutant. Female *ActSC-Gal4* driver flies with *tw* mutation were crossed with male *UAS-tw* flies with *tw* mutation. In the F₁ progeny, rescued and non-rescued individuals are born and are described as [Cy−, GFP+] and [Cy+, GFP+], respectively. The number of F₁ progeny with phenotypes [Cy−, GFP+] or [Cy+, GFP+] is shown in Table 7. (C) The cross scheme for examining the viability of flies expressing RNAi for the *rt* gene. Female *ActSC-Gal4* driver flies were crossed with male *UAS-rt-IR* flies. In the F₁ progeny, flies expressing RNAi for the *rt* gene, *ActSC-Gal4* driver, and *UAS-rt-IR* individuals were born. Flies expressing RNAi for the *rt* gene do not have CyO GFP balance; thus, the phenotype is described as [Cy−, GFP+]. *ActSC-Gal4* driver and *UAS-rt-IR* flies have CyO GFP balance; thus, the phenotype is described as [Cy+, GFP+]. The number of F₁ progeny with ([Cy−, GFP+]) or without ([Cy+, GFP+]) ubiquitous expression of RNAi for the *rt* gene is shown in Table 8.

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**Table 7. Viability of rescued *tw* mutant flies.**

| Phenotype | [Cy−, GFP+] | [Cy+, GFP+] | Ratio* ([Cy−, GFP+]/[Cy+, GFP+]) | p (x²) |
|-----------|-------------|-------------|----------------------------------|--------|
| 948       | 757         | 1.25        | <0.001 (21.4)                    |

*Number of adult *tw/tw; ActSC-Gal4/+; UAS-tw/+* and *tw/Y; ActSC-Gal4/+; UAS-tw/+* flies divided by number of *tw/tw; CyO GFP+*; *UAS-tw/+* and *tw/Y; CyO GFP+*; *UAS-tw/+* flies. If the lethal phenotype of *tw* mutant flies is rescued, the ratio is expected to be more than 1.

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Flight assay

We used a modified version of the method by Stockinger et al. to assess flying ability [53]. Twenty individual flies were gently introduced into a glass vial height 240 mm tall and 25 mm in diameter. After a 5-minute rest, the bottom of the vial was gently tapped and the maximum height reached in 10 seconds was recorded by a digital camera. Five trials were performed in each experiment. Climbing ability was then calculated from the 5 trials.

Climbing assay

We used a modified version of a previously described assay to assess climbing ability [51]. Twenty individual flies were gently introduced into a glass vial height 240 mm tall and 25 mm in diameter. After a 5-minute rest, the bottom of the vial was gently tapped and the maximum height reached in 10 seconds was recorded by a digital camera. Five trials were performed in each experiment. Climbing ability was then calculated from the 5 trials.

Locomotion assay

We used the procedure described in Kaneuchi et al. to assess locomotive ability [52]. One day after eclosion, flies were individually introduced into a glass tube 65 mm long and 3 mm in diameter. The media was changed every week until the C-50 (weight/volume), 10% glucose, 0.6% propionic acid, 2% yeast extract, and 1.2% agar. After the tubes were placed in the Drosophila Activity Monitor (TriKinetics Inc., http://www.trikinetics.com/), the medium was changed every 30 minutes. At least 16 fly died. The number of times a fly crossed the center of the glass tube was automatically recorded every 30 minutes. At least 16 individuals were tested in each group and data were pooled for statistical analysis.

Longevity assay

About 30 test flies were maintained in the standard medium after eclosion for assessment of longevity. Every 2 or 3 days, flies were transferred to a fresh food vial and the number of dead flies was determined until all flies died. More than 4 replicate vials were tested in each group and data were pooled for statistical analysis.

Staining of muscles and examination of the frequencies of abnormal muscle patterning

Third instar larvae were dissected along the dorsal midline in ice-cold phosphate-buffered saline (PBS: 130 mM NaCl, 7 mM Na2HPO4, and 3 mM NaH2PO4). After removal of the digestive organs, fat bodies, and main trachea, the preparations were fixed with 4% formaldehyde in PBS for 30 minutes at room temperature. The tissue was then washed in PBS with 0.1% Triton X-100 (PBT) and stained with phalloidin conjugated with FITC. Stained samples were mounted in 90% glycerol in PBS and observed under a Zeiss LSM5 Pascal confocal microscope. We counted the number of individuals that had more than 1 abnormal muscle and calculated the frequencies of abnormal muscle patterning.

Immunohistochemistry

Third instar larvae were dissected in ice-cold PBS. Wing imaginal discs were fixed with 4% paraformaldehyde (pH 7.0) in PBS for 15 minutes and washed 3 times with PBT. After being blocked with 10% goat serum in PBT, the samples were stained with primary antibodies. The primary antibodies were used in the following dilutions: anti-cleaved caspase-3 (Asp175) rabbit polyclonal antibody, 1:300 (Cell Signaling, http://www.cellsignal.com); anti-phospho-histone H3 (Ser10) rabbit polyclonal antibody, 1:100 (Millipore, http://www.millipore.com); anti-PS-integrin mouse monoclonal antibody (3A9), 1:25 (Developmental Studies Hybridoma Bank, http://dshb.biology.uiowa.edu/); anti-β-FBS-integrin mouse monoclonal antibody (CF.6G1), 1:200 (Developmental Studies Hybridoma Bank); anti-DE-cadherin rat monoclonal antibody (DCAD2), 1:25 (Developmental Studies Hybridoma Bank); and anti-Dg rabbit polyclonal antibody, 1:100 (Invitrogen). Stained samples were mounted in FluoroGuard™ Antifade Reagent (BIO-RAD, http://www.bio-rad.com) and observed under a Zeiss LSM5 Pascal confocal microscope.

Electron microscopy and analysis of ultrastructural phenotypes

Legs and thoraces were isolated from adult flies (15 and 35 days old, males and females, n = 6) and fixed with 2.5% glutaraldehyde

| Table 8. Effect of temperature on lethality in flies expressing RNAi for the rt gene. |
|---------------------------------|-----------------|-----------------|-----------------|
| Phenotype                        | Temperature (°C) | [Cy5, GFP+]     | [Cy5, GFP-]     | Ratio* ([Cy5, GFP+] / [Cy5, GFP-]) |
|---------------------------------|-----------------|-----------------|-----------------|-----------------------------------|
|                                | 18              | 206             | 781             | 0.26                              |
|                                | 25              | 182             | 1541            | 0.12                              |
|                                | 28              | 1               | 405             | 0.00                              |

*Number of adult ActSC-Gal4/t-IR flies divided by number of ActSC-Gal4/+ and +/t-IR flies. If all flies hatch and develop normally, the ratio is expected to be 0.33.

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| Table 9. Statistical analysis of lifespans of flies expressing RNAi for the rt gene. |
|---------------------------------|-----------------|-----------------|-----------------|
| Control group                   | Experimental group | % Difference in median lifespan | p value         |
|                                 |                  |                            |                  |
| +/-                             | +/+; ActSC-Gal4/UAS-t-IR; +/- | -81.3                | <0.001           |
| ¥/¥                            | +/+; ActSC-Gal4/UAS-t-IR; +/- | -66.7                | <0.001           |
| ¥/¥                            | +/+; UAS-t-IR; +/- | 21.3                  | <0.001           |
| ¥/¥                            | elav-Gal4/+; UAS-t-IR; +/- | -12.3                | <0.001           |
| ¥/¥                            | +/+; UAS-t-IR; repo-Gal4/+ | 25.5                  | <0.001           |
| ¥/¥                            | +/+; UAS-t-IR; repo-Gal4/+ | -11.9                | <0.001           |

C, control group; E, experimental group. Percent difference (calculated as [E-C]/C×100) and log-rank test p values are given.

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in PBS overnight at room temperature. They were then postfixed with 1% OsO4 in 100 mM phosphate buffer (pH 7.3) for 1 hr at 4°C and dehydrated in a graded series of alcohol. After passage through propylene oxide, the specimens were embedded in Epon 812. Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and observed with a JEM-1010C transmission electron microscope (JEOL, http://www.jeol.co.jp).

Quantitative analysis of the \( rt \) and \( tw \) transcripts in flies by real-time PCR

Total RNA was extracted from third instar larvae of each fly by using TRIzol® Reagent (Invitrogen). First-strand cDNA was synthesized using a SuperScript® II First-Strand Synthesis Kit (Invitrogen). Real-time PCR was performed using QuickGoldStar qPCR MasterMix (Eurogentec, http://www.eurogentec.com/) and the ABI PRISM® 7700 Sequence Detection System (Applied Biosystems, http://www3.appliedbiosystems.com/). The gene-specific primer pairs and TaqMan probes were used for each gene as follows. For \( rt \) quantification, the forward primer 5'-ACACCTGTGGCACTGCTTAGC-3', reverse primer 5'-AC-TTTAGGATCCATCAGATG-3', and probe 5'-ACGCC-GGCTCTACCCGATCCG-3' were used. For \( tw \) quantification, the forward primer 5'-TTTCCGGCTTGATCTTCAA-3', reverse primer 5'-GGGACGAAACCCTACTAAGTGAAC-3', and probe 5'-TTCGATCTACCCGCTGGT-3' were used. For Ribosomal protein L32 (Rpl32) quantification, the forward primer 5'-GGGAAGCAGAGGATACGTCA-3', reverse primer 5'-CGATGGTGCGACAGATCTAG-3', and probe 5'-ACAGAG-GTGCGACAGATCTAG-3' were used. The probes were labeled with reporter dye FAM and quencher dye TAMRA at the 5'-3' ends, respectively. The relative amounts of the \( rt \) and \( tw \) transcripts were normalized to those of the RpL32 transcripts in the same cDNA.

Vector construction and expression of recombinant h-Dg proteins for the assay of POMT activity

Human \( \alpha \)-dystroglycan (\( \alpha \)-hDG), which was used as the substrate for the \( \Omega \)-mannosylation reaction, was amplified using the forward primer that included the EcoRI site, 5'-GAATTCC-CATCCAGGATCGTGCCA-3', and the reverse primer that included the Nod site, 5'-GGCGGGCGCTTAGGTAGCAA-CTGCGAGGCG-3'. The amplified fragment up to the region of amino acids 335–421 was subcloned into pGEX-6P-1 (GE Healthcare, http://www.gehealthcare.com/), the \( \Omega \)-terminal glutathione-S-transferase (GST) fusion vector. The pGEX-6P-1-\( \alpha \)-hDG transformant of *Escherichia coli* BL21 (DE3) was cultured until the OD600 reached 1.2 at 20°C and then incubated with 0.1 mM IPTG at 20°C for 6 hr. The cells were sonicated with a PBS solution containing 0.5% n-octyl-\( \beta \)-D-thioglucoide (DOGG) from the cDNA of wild-type *Homo sapiens* (DNAX Laboratories, http://www.dnax.com/), 1 mM diithiothreitol (DTT), and protease inhibitors (5 μg/ml pepstatin A, 2 μg/ml leupentin, 2 μg/ml aprotin, 1 mM benzamidine-HCl, and 1 mM phenylmethylsulfonyl fluoride (PMSF)) and centrifuged; the supernatant was then applied to Glutathione-Sepharose™ 4B beads (GE Healthcare). The recombinant GST-fused \( \alpha \)-hDG protein was eluted with a solution containing 50 mM reduced glutathione, 20 mM Tris-HCl (pH 8.0), 10 mM ethylenediaminetetraacetic acid (EDTA), and 0.5% n-octyl-\( \beta \)-D-thioglucoide. To remove the glutathione, the eluate solution was changed to a solution containing 20 mM Tris-HCl (pH 8.0), 10 mM EDTA, 2 mM 2-mercaptoethanol, and 0.5% n-octyl-\( \beta \)-D-thioglucoide using a PD-10 column (GE Healthcare).

Vector construction and expression of mutant form of \( TW \)

The full-length ORFs of the wild-type \( rt \) form, wild-type \( tw \) form, and mutant \( tw \) form were expressed in insect cells as described previously [29]. One mutant in \( tw \), \( tw^\text{ mutant} \), has been reported. Sequencing of the \( tw \) gene in \( tw^\text{ mutant} \) flies revealed 3 alterations. Two of these alterations do not cause amino acid substitutions. The third alteration, a 2-base substitution and 3-base insertion, is predicted to affect the translated protein sequence: the 59th threonine residue from the initiating methionine changes to glycine and serine residues [30]. Hereafter, we represent the wild-type \( rt \) form, wild-type \( tw \) form, and mutant \( tw \) form as \( rt^\text{WT}, tw^\text{WT} \), and \( tw^\text{mut} \), respectively. The coding region of \( tw^\text{mut} \) was amplified from the cDNA of \( tw \) mutant flies using the same primer sets as that for \( tw^\text{WT} \) [29], and the amplified fragment was inserted into vector pVL1393-g, pVL1393-nI1-tw-HA, pVL1393-tw3, and pVL1393-tw3-tw, were co-transfected with BD BaculoGold Linearized Baculovirus DNA (BD Biosciences, http://www.bdbiosciences.com) into Sf21 insect cells, and the cells were incubated for 7 days at 25°C to produce recombinant viruses. Sf21

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**Table 10. Median lifespan of flies expressing RNAi for the rt gene.**

| Genotype | Number of animals | Median lifespan (days) |
|----------|-------------------|------------------------|
| +/+      | 163               | 23                     |
| +/+; Act5C-Gal4+actRT-IR; +/+ | 112 | 47 |
cells were infected with each recombinant virus and incubated to express RTWT-HA, TWWT, and TWMut proteins.

Preparation of cellular microsomal membrane fraction and assay of POMT activity

The microsomal membrane fraction was prepared and the POMT activity was assayed as described previously [29,55] with some modification. The infected cells were first collected and washed twice in PBS. They were then suspended in a solution containing 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 250 mM sucrose, 1 mM DTT, and protease inhibitors (5 μg/ml pepstatin A, 2 μg/ml leupeptin, 2 μg/ml aproptin, 1 mM benzamidine-HCl, and 1 mM PMSF) and homogenized using a 1-ml Dounce homogenizer. After centrifugation at 900 × g for 10 minutes, the supernatant was subjected to ultracentrifugation at 100,000 × g for 1 hr. The pellet was suspended in 20 mM Tris-HCl (pH 8.0), 10 mM EDTA, 2 mM 2-mercaptoethanol, and 0.5% n-octyl-β-D-

Figure 12. POMT activity of recombinant wild-type RT and wild-type or mutant TW flies. Western blot analysis of HA-tagged wild-type RT using anti-HA monoclonal antibody (A) and of TW using anti-TW antibody (B). The prepared microsomal membrane fractions of infected cells were applied to 7% SDS-PAGE at 20 μg for western blot analysis with anti-HA monoclonal antibody and 5 μg for that with anti-TW antibody. The arrowhead and arrow indicate HA-tagged RT and TW, respectively. (C) POMT activity for GST-α-DG of recombinant mutant TW (TWMut). POMT-specific activity was detected when RTWT-HA and TWWT were co-expressed as we reported. However, POMT-specific activity was not detected when RTWT-HA and TWMut were co-expressed. Each bar represents the mean of 8 replicates. Error bars indicate standard error. ***p<0.001 by the Tukey test. n.s. not significant. (D) POMT activity for GST-α-DG of larval extract of rt and tw mutant. POMT-specific activities of rt and tw mutant were significantly reduced. Each bar represents the mean of 6 replicates. Error bars indicate standard error. ***p<0.001 by t test.

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Figure 13. Genetic interaction between rt or tw and Dg in the wing. (A) Normal wing shape in wild-type flies. (B) Blistered phenotype in the wings of knockdown flies. (C) Penetrances of the blistered phenotype in knockdown flies. At least 30 individuals were observed in each knockdown group. At 28°C, the penetrances of the blistered phenotype with the double knockdowns rt-Dg and tw-Dg were significantly higher than those with single knockdown. *p<0.05; ***p<0.001 by Fisher’s exact test. n.s., not significant.

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thiogluicoside, and this suspension was used as the microsomal membrane fraction. The reaction mixture contained 20 mM Tris-HCl (pH 9.0), 10 mM EDTA, 2 mM 2-mercaptoethanol, 0.5% n-octyl-β-D-thiogluicoside, 100 nM Dol-P-[3H]Man (153,200 dpm/μmol) (American Radiolabeled Chemicals, http://www.arcincusa.com/), 2.5 μg GST-α-hDG, and 80 μg of microsomal membrane fraction as the enzyme source in a total volume of 20 μl. After 1-hr incubation at 18°C, the reaction was stopped by addition of 100 μl of PBS containing 1% Triton X-100, and the reaction mixture was centrifuged at 10,000×g for 5 minutes. The supernatant was transferred, mixed with 400 μl of PBS and 20 μl of Ghtathione-Sepharose™ 4B beads, rotated for 1 hr, and washed 3 times with PBS containing 0.2% Triton X-100. The radioactivity of the beads was measured using a liquid scintillation counter.

Western blot analysis

Each microsomal membrane fraction was subjected to 7% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The membrane to which the separated proteins were transferred was probed with anti-HA mouse monoclonal antibody (1:2000 dilution) (Santa Cruz Biotechnology, Inc., http://www.scbt.com/) or anti-iPOMT2 rabbit polyclonal antibody (1:400 dilution) [29]. Each membrane was then reacted with HRP-conjugated secondary antibody and stained with Amersham™ ECL™ Plus (GE Healthcare).

Preparation of larval extract and assay of POMT activity

Fourth instar larval larvae of nortf, tw/Y, and wild-type (Canton-S) flies were homogenized in 20 mM Tris-HCl (pH 8.0), 10 mM EDTA, 2 mM 2-mercaptoethanol, and 0.5% n-octyl-β-D-thiogluicoside with protease inhibitors (5 μg/ml pepstatin A, 2 μg/ml leupeptin, 2 μg/ml aproptin, 1 mM benzamidine-HCl, and 1 mM PMSE) (300 μl for every 40 larvae). The supernatant was obtained by centrifugation at 9,000×g for 10 minutes and used as larval extract. The same reaction mixture and conditions described in the “Preparation of cellular microsomal membrane fraction and assay of POMT activity” section above were used except for the following 2 modifications: (1) the amount of GST-α-hDG in the reaction mixture was 10 μg; and (2) the incubation time was 2 hr.

Statistical analyses

All statistical analyses were performed using the public domain R program (http://www.r-project.org/).

Supporting Information

Figure S1 Quantitative analysis of rt and tw mRNAs in rt mutant fly. rt (A) and tw (B) transcript levels of rtP1/P2 and twP1/P2 mutant flies of third instar larva were determined by real-time PCR. Error bars indicate standard error. Lines above the bars show compared groups by one-way ANOVA. *p<0.05 by the one-way ANOVA. n.s., not significant. * above the bar of rtP1/P2 means p<0.05 by Tukey test.

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Figure S2 Quantitative analysis of rt and tw mRNAs in the flies with the expression of RNAi for rt gene. rt (A) and tw (B) transcript levels of the flies with the expression of RNAi for rt gene of third instar larva were determined by real-time PCR. The expression level of rt in Act5C>rt-IR was significantly reduced, while that in other lines was not significantly reduced. The expression level of tw was no different among all lines. Error bars indicate standard error. Lines above the bars show compared groups by one-way ANOVA. ***p<0.001 by the one-way ANOVA. n.s., not significant. * above the bar of Act5C>rt-IR means p<0.05 by Tukey test.

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Figure S3 Quantitative analysis of rt and tw mRNAs in HIG-hDG flies. rt (A) and tw (B) transcript levels of HIG-tauGFP flies of third instar larva were determined by real-time PCR. The expression levels of rt and tw did not differ between in HIG-tauGFP and wild type. Error bars indicate standard error. n.s., not significant by one-way ANOVA.

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Figure S4 Dividing myoblasts in the wing imaginal disc of tw mutant larva. The number of myoblasts positive for phosphohistone H3 in the wing imaginal discs of wild-type and tw mutant larvae. The phosho-histone H3 is marker of dividing cells. The number of dividing myoblasts did not differ between tw mutant and wild-type larva. n.s., not significant by t test.

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Figure S5 Reduced expression of Dg in the posterior region of the wing. (A–D) Wing imaginal discs in the third instar larvae of en-Gal4>UAS-EGFP, UAS-Dg-IR flies. All discs are anterior left, dorsal up. (A) Differential interference contrast (DIC) image of the wing disc. (B) The knockdown region of Dg, which is visualized by EGFP (green). The expression of en-Gal4 is the posterior region of the wing. (C) The expression of Dg (red) decreases in the posterior region of the wing. (D) Marged image of (B) and (C). EGFP and Dg do not co-localize in the wing. Dg dramatically decreases in the knockdown region of Dg.

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Author Contributions

Conceived and designed the experiments: MU SN. Performed the experiments: MU YA TI. Analyzed the data: MU YA. Contributed reagents/materials/analysis tools: RU HK TA. Wrote the paper: MU YA SN.

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