Title
Phosphoinositide regulation of integrin trafficking required for muscle attachment and maintenance.

Permalink
https://escholarship.org/uc/item/764072jq

Journal
PLoS genetics, 7(2)

ISSN
1553-7390

Authors
Ribeiro, Inês
Yuan, Lin
Tanentzapf, Guy
et al.

Publication Date
2011-02-10

DOI
10.1371/journal.pgen.1001295

Peer reviewed
Abstract

Muscles must maintain cell compartmentalization when remodeled during development and use. How spatially restricted adhesions are regulated with muscle remodeling is largely unexplored. We show that the myotubularin (mtm) phosphoinositide phosphatase is required for integrin-mediated myofiber attachments in Drosophila melanogaster, and that mtm-depleted myofibers exhibit hallmarks of human XLMTM myopathy. Depletion of mtm leads to increased integrin turnover at the sarcolemma and an accumulation of integrin with PI(3)P on endosomal-related membrane inclusions, indicating a role for Mtm phosphatase activity in endocytic trafficking. The depletion of Class II, but not Class III, PI3-kinase rescued mtm-dependent defects, identifying an important pathway that regulates integrin recycling. Importantly, similar integrin localization defects found in human XLMTM myofibers signify conserved MTM1 function in muscle membrane trafficking. Our results indicate that regulation of distinct phosphoinositide pools plays a central role in maintaining cell compartmentalization and attachments during muscle remodeling, and they suggest involvement of Class II PI3-kinase in MTM-related disease.

Introduction

Myofibers are large, highly differentiated contractile cells that rely on strong extracellular attachments to preserve their integrity during force-generating muscle contractions. Myofiber attachments are mediated by integrin adhesion complexes (IACs) composed of α- and β-transmembrane heterodimers that associate with cytoskeletal bridging factors, similar to those found in non-muscle cells [1]. IACs are crucial at myotendinous junctions (MTJs), attaching the ends of myofibers to tendons. In addition, IACs concentrated at costameres associated with repeating sarcomeric Z-lines attach peripheral myofibrils to the extracellular matrix. IACs are known to be essential for invertebrate and vertebrate muscle cell attachments and organization [2,3], but it is unclear how the critical pattern of spatially restricted adhesions is continuously maintained.

In non-muscle cells, integrin turnover through endocytic recycling has clear roles in localization of dynamic adhesion complexes that mediate cell migration and membrane remodeling in cytokinesis. Trafficking pathways that engage specific endocytic adaptors, protein kinases and Rab GTPases for internalization and recycling of specific integrins are emerging, as primarily understood in isolated cells [4]. In contrast, it is not clear how important regulated integrin turnover is in differentiated muscle, or how this turnover is regulated. In isolated myofibers, uptake of markers for endocytic recycling occurred in the vicinity of adhesion sites and trafficked to perinuclear compartments, distinct from a degradative pathway [5], suggesting common trafficking themes shared with non-muscle cells. Experiments using fluorescence recovery after photobleaching (FRAP) in intact flies recently provided the first observation of endocytosis-dependent, growth-regulated mobility of IAC proteins at MTJs [6], underscoring the significance of regulated endosomal integrin trafficking in muscles, as well.

Dynamic membrane compartment identity and functions are in part conveyed through phosphoinositides. Phosphoinositides exist as seven phosphorylated phosphatidylinositol forms interconverted by dedicated lipid kinases and phosphatases [7]. Different phosphoinositide forms can recruit specific binding proteins to distinct membranes in order to elicit spatiotemporal responses that include localized signaling, cytoskeletal reorganization, membrane deformation and trafficking. However, the complex cellular relationships in vivo are less defined.

The control of phosphoinositide balance by the Myotubularin (MTM) phosphoinositide phosphatases is both elaborate and crucial in metazoans. MTMs are encoded as a large family of genes (15 humans, 7 flies, 1 yeast) associated with human disease [8]. Mutations in human MTM1 lead to human X-linked myotubular (centronuclear) myopathy (XLMTM, OMIM #310400) characterized by centrally misplaced nuclei in hypertrophic myofibers [9,10]. Disruption of related MTMR2 leads to Charcot-Marie-Tooth neuropathy (CMT4B1, OMIM #601382) with abnormal morphology and plasma membrane outfalls in

Affiliations

1 Section of Cell and Developmental Biology, University of California San Diego, La Jolla, California, United States of America, 2 Department of Cellular and Physiological Sciences, University of British Columbia, Vancouver, Canada, 3 Department of Pediatrics, University of Michigan Medical Center, Ann Arbor, Michigan, United States of America.
Mtm and PI3-Kinase Control of Muscle Remodeling

Author Summary

Muscles require strong extracellular attachments to preserve cellular integrity during force-generating contractions. Integrin transmembrane receptors mediate muscle attachments at highly localized sites, but how this pattern of attachments is continuously maintained with muscle use is not understood. Human X-linked myotubular myopathy (XLMTM), a frequently fatal muscle disease, is associated with mutations in the MTM1 lipid regulator. Myotubulinarin (MTM) lipid phosphatases are implicated in endocytosis, a process of cellular uptake that can traffic transmembrane receptors for redelivery to the plasma membrane or to protein destruction. Here, we address MTM roles in muscle, using the genetically tractable fruit fly for detailed investigation of muscle cellular organization and functions. We show that fly muscle cells depleted for mtm function exhibit hallmarks of human XLMTM. We found that mtm regulates integrin localization through endocytosis and, in this role, is needed to maintain muscle attachments. Co-depletion of Class II PI3-kinase with mtm restores normal integrin localization at muscle attachment sites and fly survival, identifying a potential therapy target in MTM-related disease. Importantly, we show that integrin localization is also disrupted in human XLMTM. Our work shows conservation of MTM function in integrin trafficking and reveals insights into regulation of muscle cell maintenance and human disease.

Results

Mtm is required to maintain myofibers during remodeling and for adult muscle function

Given the role for myotubularin in human myopathy, and our discovery of an mtm requirement in muscle essential for fly viability [18], we investigated the contribution of mtm-dependent phosphoinositide regulation to muscle cell function and compartmentalization. Loss of mtm function using either null alleles or muscle-directed RNAi had no visible effects on muscle in larva, which remained mobile and exhibited normal body wall muscle formation, attachments and growth (Figure 1A and 1B–1D, Figure S1A–S1C). However, targeted RNAi depletion revealed muscle requirements for mtm at later developmental stages. Muscle-specific mtm knockdown, as indicated by protein depletion, showed either animal lethality (24B-GAL4) or developmental delay (Dve2-GAL4) around the stage of adult eclosion that was rescued by co-expression of either wildtype mtm or human MTMR2 (Figure 1A and 1C; Figure S1B).

Metamorphosis occurs inside a rigid pupal case that adult flies escape at eclosion with the help of muscle contractions, including supporting contractions from a subset of abdominal persistent larval muscles (PLMs) [24,25]. The PLMs called dorsal temporary internal oblique muscles (IOMs) are large, individual, multinucleated myofibers that span abdominal segments (Figure 1D; Figure S1D). Consistent with defects in eclosion, there was a decrease in the number of both dorsal IOMs and ventral PLMs in mtm-depleted abdomens (Figure 1D–1F; Figure S1D–S1E). The remaining mtm-depleted myofibers were frequently detached and seen as rounded-up balls or as elongated fibers with one completely detached end (Figure 1D, 1F; Figure S1D’, S1F), never observed in controls.

To explore the developmental requirement for an mtm muscle function, we first characterized myofibers using timelapse microscopy in intact animals. With mtm depletion, GFP-labeled IOMs were properly maintained during early pupal stages, when other larval muscles undergo developmentally regulated cell death (Figure 1A,1G–1G, 2 days after pupal formation, APF). The mtm-depleted IOMs subsequently underwent normal myofiber thinning (3d APF) and rereckening (4d APF), indicative of developmental turnover and rebuilding of the contractile myofibrils [25]. While no detachment was observed in control animals (n = 19), IOM detachment occurred during remodeling in late pupal stages with mtm knockdown (n = 15) (Figure 1G, 4d APF). Thus, mtm is not essential for IOM formation or survival, but is important for muscle attachments and maintenance upon remodeling.

To address whether mtm plays a role in other muscles, we examined different developmental stages and myofiber types. Although adult somatic muscles appeared to form normally with muscle-specific mtm knockdown (Figure 1D and not shown), 100.0±0.0% of the viable adult flies were flightless (versus 22.2±5.4% control; n = 10, ≥124 flies). Visceral muscles that normally migrate to ensheath the testis [26] were present but also disrupted with mtm muscle-depletion (Figure S1G–S1G’, 24B-GAL4). Taken together, mtm function appears dispensable for myogenesis, but is broadly required in both somatic and visceral muscles for myofiber remodeling, maintenance and function.

Mtm disruption models centronuclear myopathy, including T-tubule disorganization

Pathological hallmarks of XLMTM are small, rounded myofibers with nuclei displacement and disorganization of the perinuclear compartment [8]. In wildtype IOMs, myofibrils are normally tightly packed around centrally aligned nuclei following myofiber remodeling [25] (Figure 1H). In contrast, in mtm-depleted IOMs, central myofibrils were misaligned or absent around a normal number of centrally-displaced nuclei (2.1-fold increased nuclei distance from midline; Figure 1H–1J). The nuclei were otherwise normal in size and morphology (Figure S2A–S2A) and pharate adult IOMs were impermeable to propidium iodide staining (Figure S2B–S2C), while ultrastructural analysis confirmed normal mitochondrial integrity (Figure S2D–S2D), all indicating viability of mtm-deleted IOM cells. The peripheral...
Figure 1. Mtm depletion leads to myofiber detachment and morphological defects common with human myotubular myopathy. (A) Timeline of fly and muscle development, indicating stages of mtm requirements; days after puparium formation (APF). (B–B’) Normal body wall muscles in OreR and mtm±/mtm± third instar larvae. F-actin. (C) Percent viable and lethal progeny 13 days after egg lays with 24B-GAL4 and DMe2-GAL4 muscle-targeted mtm RNAi. (D–D’) Pharate dorsal abdominal muscles F-actin. Large IOMs (arrows) and smaller adult muscles (open.
myofibrils appeared normal (Figure S2E–S2H), suggesting that  

mtm is unlikely to function directly in sarcomere assembly.

We also found that transverse (T)-tubules were disrupted in mtm-depleted myofibers, consistent with defects recently described in vertebrate XLMTM [14,27]. T-tubules are an extensive membrane network, continuous with the sarcolemma, which mediates excitation-contraction coupling throughout the myofiber interior. Although critical for force-generating contractions, there is little understanding of T-tubule biogenesis and structural regulation.

We confirmed that both the Amphiplysin (Amp) BAR-domain protein and Dlg1 membrane-associated guanylate kinase scaffold protein localize to T-tubules in wildtype abdominal myofibers (Figure S3A, S3B, S3C), as in flight muscles [28]. In mtm-depleted IOMs, although longitudinal elements of T-tubule membranes were present, lack of Amp and Dlg1 indicated that transversal membranes were specifically disorganized or absent (Figure S3A’, S3B’; 9.5% control versus 96.3% mtm RNAi with transversal membranes in ˂half of IOM; n=21). These conclusions were confirmed by transmission electron microscopy (Figure S3D–S3D’). Altogether, the conserved mutant phenotypes and timing of onset suggests that mtm-depleted muscles in flies model hallmarks of XLMTM.

**Mtm is required for βPS-integrin trafficking for adhesions in muscle and wing**

Given the muscle detachment and myofibril misalignment observed in mtm mutant myofibers, we considered a possible defect in IACs at MTJs and costameres (Figure 2A–2C). We found that βPS-integrin, the single D. melanogaster β-integrin subunit encoded by nys (GenBank NM_080054), was dramatically mislocalized in mtm-depleted muscles (Figure 2B’). In contrast to wildtype muscle, βPS-integrin was absent at the ends of detached myofibers (Figure 2B’) and from costameres (Figure 2C’), consistent with detachment due to disruption of integrin adhesions. Although an intracellular pool of βPS-integrin protein was detected as small punctae within wildtype myofibers (Figure 2D), upon mtm knockdown, βPS-integrin became enriched along abnormal vacuolar inclusions within the myofiber center (Figure 2D’). Ultrastructural analyses revealed large, lucent membrane-bound compartments within the central regions of the mtm-depleted but not control myofibers (Figure 2E–2E’). Other proteins of the integrin adhesion complex, αPS2-integrin and Talin, were also both detected at MTJs but not at the inclusions, suggesting βPS-integrin as a primary target of mtm function (Figure S4A–S4B’).

To address the possible relationship between the appearance of membrane inclusions and muscle detachment, we examined βPS-integrin localization at earlier developmental stages. In mtm null or RNAi depleted larval muscles, normal integrin localization was detected at myofiber attachments, without any βPS-integrin-containing central inclusions (Figure S4C, S4C’). This indicates that appearance of inclusions coincides with detachment, and that mtm function is not needed for initial IAC formation. Although detected at the larval myofiber surface, βPS-integrin was not organized into uniform striations in either wildtype or mutant myofibers, as compared to the costameres observed in wildtype pharate adult IOMs. To address whether mtm function affects integrin trafficking prior to myofiber remodeling and detachment, we performed FRAP analysis of βPS-integrin:YFP along MTJs in intact larvae [6]. The mobile fraction of βPS-integrin:YFP was significantly increased in mtm mutant larval muscles (Figure 2F), indicating that mtm is required to stabilize sarcolemmal βPS-integrin localization, preceding myofiber remodeling.

To explore a basis for the sensitivity to mtm loss of function in pupal stages, we investigated integrin localization during IOM remodeling in metamorphosis. In wildtype myofibers at 2–3 days after puparal formation (APF), we discovered that there was a normal loss of integrin from the cell surface, along with detectable presence of integrin-marked inclusions (Figure 2G–2H). By 4 days APF, integrin was again predominantly absent in the myofiber center, with reappearance at costameres. This result reveals a normal redistribution of integrin that occurs with IOM remodeling, and suggests a distinct requirement for myofiber integrin regulation in pupal stages. To test a temporal requirement for mtm function specifically in pupal stages, we performed temperature shift experiments to induce conditional mtm knockdown. Due to the temperature sensitivity of the GAL4 transcription factor, flies with muscle-targeted mtm hairpin expression maintained normal myofiber attachments when raised continuously at 18°C with low GAL4 activity (Figure S4D). However, when flies were shifted during metamorphosis to 29°C for 1–2 days with increased GAL4 activity, the pharate adults then exhibited myofiber detachment (Figure S4D’–S4E, 0% versus 71% mtm pharates, respectively). Similarly, flies also carrying the temperature sensitive GAL80Δ, an inhibitor of GAL4, raised continuously at 18°C did not exhibit integrin-containing inclusions (Figure S4F, 0%). In contrast, flies shifted to 29°C for 3 days (with shorter metamorphosis at higher temperatures), exhibited myofibers with integrin-containing inclusions (Figure S4F’–S4G, 58%). These results indicate a requirement for mtm function in pupal stages that is important for integrin localization at the cell surface following myofiber remodeling, and further supports a primary role for mtm in integrin trafficking.

The requirement for mtm function in myofiber remodeling during development raised the question whether there is a similar mtm requirement during cellular remodeling that may occur with ongoing adult muscle use, repair or ageing. We investigated integrin localization in adult abdominal myofibers, which are derived from a different developmental program from the persistent larval muscles. The long, thin adult ventral abdominal muscles, called lateral transversal muscles, normally exhibit a striated pattern of intense integrin localization at repeating costameres (Figure 2I). In contrast, integrin deviated from this pattern with a diffuse distribution in portions of mtm-depleted myofibers in both six and ten day old adult flies (Figure 2I’). This result points to an important role for mtm in the maintenance of integrin adhesions with ongoing muscle use in adult flies.

To test whether mtm function has a specific role for integrin localization in broader developmental contexts, we assayed function of integrin-mediated adhesions in the epithelial bilayer of the developing fly wing [1]. The low frequency of wing blisters...
Figure 2. Mtm is required for βPS-integrin flux from intracellular compartments and localization at sarcolemmal adhesions. (A) Schematic of individual pharate IOM and regions imaged. MTJ, myotendinous junction. (B–B') βPS-integrin in IOM z-projections. (B) βPS-integrin at MTJs (arrow) and costameres (open arrowheads) in control. (B'–B'') With mtm RNAi, βPS-integrin was absent from detached ends (B'', arrow) and...
resulting from a hypomorphic allele of zPS2-integrin, $i^{f2}$ [29], was dominantly enhanced when in combination with heterozygous mtm null alleles with reduced function (Figure 2J), similar to interactions seen with components known to be required for integrin adhesions [30]. The interaction in two different tissues suggests a specific and fundamental role for mtm in maintenance of integrin-mediated attachments.

**Integrin adhesions and T-tubules have independent requirements for Mtm function**

The disruption in mtm mutants of both IACs and T-tubules, and their normal proximity along the sarcolemma, raised the question whether a structural or functional relationship between the two compartments normally exists or is relevant to the abnormal membrane inclusions (Figure 3A). Moreover, in mtm-depleted myofibers, we noted that Dlg, similar to BPS-integrin, appeared along abnormal central inclusions (Figure 3B, 3B'). To characterize the membrane identity of the inclusions, we first tested whether BPS-integrin and Dlg or Amph co-localized, either in normal or mtm mutant muscles. In wildtype myofibers, Dlg and Amph were not detected at IACs. However, internal BPS-integrin frequently co-localized with Dlg (Figure 3C) and occasionally with Amph (Figure S5A, S5B, S5C) along apparent longitudinal elements of T-tubules. Upon mtm depletion, BPS-integrin extensively co-localized with Dlg and Amph on longitudinal T-tubules (Figure S5A, S5B') and along the central inclusions (Figure 3C', Figure S5B'), suggesting accumulation of a possible common precursor membrane or trafficking compartment in mtm-depleted muscles.

**Figure 3. Integrin adhesions are independent of T-tubules, but share an mtm function for maintained organization.** (A) IOM longitudinal section schematic of alternating sarcolemmal structures: T-tubule membranes (green) marked by Amph and Dlg; costameres (red) with IACs linked to Z-lines of peripheral myofibrils; z-section images from central, central periphery or central perinuclear regions, as shown. (B–B') Dlg, green; Zormin, red. Dlg detected continuously on longitudinal and transversal T-tubules in control (B), but only on longitudinal tubules and on central inclusions with mtm RNAi (B', arrowheads). (C–C') BPS-integrin (red) and dlg1:GFP (green) partially co-localized at longitudinal T-tubules in control (C, open arrowheads), and on abnormal central inclusions with mtm RNAi (C', arrowheads). Single channels, right. (D) Normal BPS-integrin localization at costameres and (D') internal punctae in amph$^{26}$ mutants that lack T-tubules. (E) Persistent BPS-integrin-inclusions with mtm RNAi in amph$^{26}$ mutant. amph$^{26}$, UAS-IR-mtm$^{97}$/amph$^{26}$; DMel2-GAL4/+. (F) Normal transverse tubule formation and Amph localization in mys$^{1}$/mys$^{51}$ mutants reared at non-permissive temperature, with reduced BPS-integrin function. Scale bars 10 µm.

doi:10.1371/journal.pgen.1001295.g003
We next considered whether there is functional co-dependence between integrin adhesions and T-tubules. In amph26 null mutants that lack T-tubules in IOMs (Figure S5D), as in adult flight muscles [29], we observed normal muscle attachments, normal βPS-integrin localization to costameres, and no βPS-integrin- or Dlg-inclusions (Figure 3D–3D'). This suggests that T-tubules are not required for βPS-integrin trafficking in the formation or maintenance of IACs, and that the inclusion defects in mtm mutants do not reflect a general consequence of failed T-tubule formation. Furthermore, we found βPS-integrin mislocalized to internal membrane inclusions upon mtm depletion in amph26 null mutants (Figure 3E), signifying that the abnormal inclusions are independent of transverse tubule membrane and possible misregulation of amph function. Conversely, transverse tubules were present normally in abdominal muscles with hypomorphic mys conditions that were pharate lethal (Figure 3F), suggesting that T-tubule organization does not require normal levels of βPS-integrin protein or IACs. The dramatic defects in both IACs and T-tubule organization upon mtm depletion therefore appear to reflect a requirement for two independent mtm functions.

**PI(3)P role in Mtm-dependent muscle compartmentalization**

The disrupted βPS-integrin localization together with the enlarged membrane inclusions suggested defective membrane trafficking in mtm mutant myofibers. Characterization of the central inclusions could point to a specific compartment or trafficking step that normally requires Mtm phosphatase activity in muscle remodeling. The inclusions did not noticeably contain markers of endoplasmic reticulum, the trans-Golgi network or autophagosomes (Figure S5E–S5E'), KDEL; Figure S5F–S5F' PH-FAPP1; Figure S5G–S5G' Atg8). In contrast, the majority of inclusions were decorated by the endosome-lysosomal marker, GFP:LAMP (Figure 4A–4A'). The inclusions were frequently colocalized with an indicator of early

---

**Figure 4. Mtm depletion disrupts integrin trafficking at endosomal compartments.** (A–A') GFP:LAMP (green, and single channels) found as punctae throughout IOM controls (A) and localized to inclusions with mtm RNAi (A', arrowheads; A'). F-actin, red; DNA, blue. (B) GFP:Rab5 (green, and single channels) found as punctae with normally little overlap with βPS-integrin (red) throughout IOM controls. (B'–B') Rab5 partially co-localized with βPS-integrin on inclusions and accumulated at the plasma membrane and perinuclear with mtm RNAi, DNA, blue. (C–C') PI(3)P detected by GFP:2xFYVE (green, and single channels) and βPS-integrin (red) exhibited little overlap in control (C), but co-localized on inclusions with mtm RNAi (C', arrowheads). (D–D') PI(3)P detected by GFP:2xFYVE (green) and Dlg (red) exhibited little overlap in control (D), but co-localized on inclusions with mtm RNAi (D', arrowheads). Scale bars 10 μm, except zooms A', C', D' 2.5 μm.

doi:10.1371/journal.pgen.1001295.g004
endosomes, GFP:Rab5 (Figure 4B–4H), and infrequently by the Rab5 effector, Rho5 (Figure S5H–S5I), but not an indicator of late endosome identity, GFP:Rab7 (Figure S3J–S3L). Together, these results suggest a relationship between the inclusions and early endocytic traffic, and that mtm depletion disrupts endocytic traffic.

PI(3)P is normally enriched at endosomal membranes. We have recently shown that the normal Mtm PI(3)P phosphatase activity promotes membrane efflux, effecting both endosomal homoeostasis and cortical remodeling in macrophages [18]. We therefore explored PI(3)P distribution in muscle with respect to integrin adhesion and T-tubule compartments. In wildtype animals, muscle expression of the PI(3)P biosensor, GFP:2xFYVE, was detected along the sarcolemma and localized to punctae distributed throughout abdominal myofibers, with the greatest concentration in the perinuclear area without obvious overlap with βPS-integrin (Figure 4C) or Dlg (Figure 4D). Upon mtm-depletion, enlarged and more eccentrically positioned PI(3)P-containing compartments were detected (Figure 4C’, 4D’). In addition, GFP:2xFYVE co-localized with βPS-integrin (Figure 4C”) and with Dlg (Figure 4D”) along the abnormal inclusions in mtm-depleted myofibers, suggesting a possible role for Mtm phosphatase activity in PI(3)P turnover involved in integrin trafficking.

**Pi3Kc2 suppresses Mtm-related defects in βPS-integrin localization and muscle maintenance**

To test if PI(3)P regulation is involved in mtm muscle functions, we investigated the contribution of Class II and III PI3-kinases (Pik3c2d and Vps34, respectively), known to synthesize PI(3)P, to abdominal muscle maintenance. Muscle-targeted knockdown of P3Kc2d or expression of dominant negative kinase-dead Vps34- KD did not individually disrupt eclosion or animal viability. However, P3Kc2d depletion in combination with mtm RNAi was able to rescue the lethality and delayed development; in contrast, Vps34-4KD expression enhanced lethality in combination with mtm depletion (Figure S6A, S6B). Neither P3Kc2d nor Vps34 knockdown rescued the loss of T-tubules with mtm-depletion (Figure S6C), and accordingly adult flies remained flightless (Figure S6D). These results indicate separable P3Kc2d-independent and dependent mtm muscle functions required for normal T-tubules and viability, respectively.

A similar functional relationship was seen between P3Kc2d and mtm for roles related to integrin adhesions, as with viability. Importantly, P3Kc2d, but not Vps34, depletion rescued muscle detachment (Figure 5A, 5B) and loss of βPS-integrin localization at costameres (Figure 5C, 5C”, 5D) that occurs with loss of mtm function. Consistent with rescue of the IACs, co-depletion of mtm and P3Kc2d, and not Vps34, also eliminated the βPS-integrin-and Dlg-containing membrane inclusions (Figure 5E, 5E’, 5F, Figure S6E), indicating a functional relationship between the abnormal central inclusions and IACs at the sarcolemma. The tests visceral muscle function was also restored to normal with P3Kc2d and mtm co-depletion, implicating turnover of integrin-mediated adhesions in the gonadal muscle. Altogether, these results suggest that P3Kc2d function mediates mtm RNAi mutant defects in maintenance of IACs, and suggest that P3Kc2d may synthesize a PI(3)P subpool co-regulated by Mtm important for integrin trafficking and localization.

Interestingly, muscle-targeted disruption of Vps34 shared with mtm depletion a similar staged semi-lethality (Figure S6A), IOM detachment (Figure 5A’, 5B) and loss of βPS-integrin at costameres (Figure 5C’, 5D), however, without inducing abnormal inclusions (Figure 5E’, 5F, Figure S6E). This points to possible shared or sequential roles for Vps34 and Mtm in phosphoinositol-mediated steps in IAC maintenance, distinct from trafficking points that involve antagonistic P3Kc2d and Mtm co-regulation. Vps34 is broadly attributed with roles in PI(3)P-regulated endocytosis and autophagy. We found that inhibition of autophagy upon depletion of the central regulator, Atg1, phenocopied the Vps34 integrin defects (Figure S6F, S6G, S6H), indicating an important role for autophagy in IOM remodeling. We tested whether the phenotypes associated with mtm phosphatase depletion are a result of increased PI(3)P-mediated autophagy. The myofiber detachment and integrin localization defects, including integrin-containing inclusions, persisted with co-depletion of Atg1 and mtm (Figure S6F’, S6G’, S6H’), indicating that autophagy is not responsible for the integrin-related defects upon mtm depletion.

Integrin is mislocalized in human myofibers with XLMTM myopathy

Given shared defects observed in mtm- and MTM1-disrupted myofibers in flies and human XLMTM, respectively (Figure 1H, Figure S3A’–S3D’), we asked whether an mtm function required for integrin adhesions is also shared with MTM1 in human muscle. β1-integrin, the major β-integrin isoform found in vertebrate muscle, was detected along the myofiber sarcolemma in cross-sections of skeletal muscle from control subjects, as expected (Figure 6A). In contrast, β1-integrin localized throughout the perinuclear compartment of centronucleated myofibers in muscle from neonates with XLMTM (Figure 6B). The Dystroglycan adhesion complex (DAC) is a second complex localized to MTJs and costameres with key roles in muscle attachments, and mutations of DAC components are frequently associated with muscular dystrophy. Unlike integrin, the dystroglycan transmembrane protein exhibited only the expected peripheral staining along the sarcolemma in both control and XLMTM myofibers, without any abnormal centronuclear localization or inclusion (Figure 6C–6D). These results show that MTM1 is specifically required for normal β1-integrin localization in human myofibers, and suggests that disruption of integrin trafficking and adhesion complex function is important in XLMTM.

**Discussion**

We found that mtm regulates integrin adhesions in muscle and in the developing wing, and that integrin localization was disrupted in human XLMTM, pointing to a central role for Mtm/MTM1 in a trafficking pathway important for localization of β-integrin at the plasma membrane. It is well-established that integrin turnover contributes to cell motility, whereby targeted integrin recycling and reassembly of localized adhesions mediate polarized matrix attachments and signaling responses [4]. Our results reveal that regulated integrin turnover is also important for integrin adhesions in non-motile myofibers, after the establishment of attachments. Importantly, mtm disruption uncovered a demand for βPS-integrin trafficking in the maintenance of adhesions both at MTJs as well as at costameres, a less-understood adhesion site with putative roles in muscle integrity, mechanotransduction, and myofibril assembly [31,32]. Although integrin was destabilized at larval MTJs in mtm mutants, the most severe consequences occurred later with specific loss of pupal or adult mtm function during developmental myofiber remodeling or adult muscle use, respectively. This is consistent with costamere sensitivity to integrin depletion in adult muscle [33] and the possibility that mtm similarly regulates integrin turnover with myofiber remodeling that occurs both in development and with demands in adult muscle growth, repair and aging.

In fly macrophages, Class II P3Kc2d and mtm co-depletion could revert both an imbalance in PI(3)P and defects in cortical...
remodeling that impaired macrophage shape and in vivo immune cell distribution [18]. Here, we found Pi3K68D disruption is also a specific and potent suppressor of integrin adhesion defects in mtm-depleted muscle. Despite distinct macrophage and myofiber morphology and function, a shared requirement for a PI3KC2/Mtm pathway highlights common functions during cellular remodeling.

Figure 5. Class II and Class III PI3-kinases affect mtm-dependent integrin adhesions differently. (A–A’) Pharate abdominal muscles, F-actin. (A) IR-Pi3K68D and (A’) IR-Vps34 single RNAi (top) and mtm co-RNAi (bottom). Arrowheads, detached IOMs. (B) Number of visibly detached IOMs. (C, C’, C”) Sarcolemmal β1-integrin detected at costameres; (C) control, (C’) IR-Pi3K68D and (C”) IR-Vps34 in single RNAi (top) and mtm co-RNAi (bottom). Only Pi3K68D, mtm co-RNAi restored β1-integrin at costameres. (D) Percentage IOMs that lack costameres ≈ half of myofiber surface. (E, E’, E”) βPS-integrin central z-sections; (E) control, (E’) IR-Pi3K68D and (E”) IR-Vps34 single RNAi (top) and mtm co-RNAi (bottom). Only Pi3K68D, mtm co-RNAi reverted abnormal βPS-integrin-inclusions. (F) Percentage IOMs with βPS-integrin on inclusions. (B,D,F) IOMs in single RNAi (light bars) and mtm co-RNAi (dark bars) conditions. Scale bars 10 μm, except A–A' 200 μm. doi:10.1371/journal.pgen.1001295.g005

Figure 6. β1-integrin is mislocalized to perinuclear inclusions in XLMTM myopathy. Cryosections from XLMTM (n = 3) and age-matched control human muscle biopsies were immunostained either with anti-β-dystroglycan or with anti-β1D-integrin. (A–B) β1D-integrin was found only along the sarcolemma in control muscle (A), but was mislocalized to the perinuclear compartment in XLMTM fibers (B, arrows). (C–D) In contrast, Dystroglycan was found normally distributed along the sarcolemma membrane in both control (C) and XLMTM (D) muscle. Scale bar, 25 μm. doi:10.1371/journal.pgen.1001295.g006
Loss of Mtm phosphatase activity could be considered a gain of function condition, analogous to ectopic kinase activity, leading to inappropriate phosphoinositide accumulation. In line with this, either mtm deletion (this study) or P3K68D overexpression [34] disrupted integrin adhesion in the fly wing, presumably through imbalanced responses to an accumulation of the same phosphoinositide pool. PI3KC2 and Mtm family members in vertebrates have been associated with antagonistic functions related to regulation of traffic to the plasma membrane. PI3KC2 isoforms are required to promote while overexpression of MTM1 impairs GLUT4 trafficking [20,35] and integrin-mediated cell motility [21,36]. Together, the observations point to a broad and conserved relationship for PI3KC2/Mtm co-regulation at the plasma membrane.

How might PI3KC2 and Mtm co-regulate integrin trafficking? One possibility is that the cycle of phosphoinositides co-regulated by PI3KC2/Mtm tunes the balance between endocytic-exocytic flux. The strong genetic interaction between mtm and P3K68D, in conjunction with PI3KC2 ability to create PI(3)P in vivo [18,20–25], supports the possibility that P3K68D could generate a PI(3)P substrate pool acted on by Mtm phosphatase. Alternatively, P3K68D could act more distantly on an interrelated phosphoinositide pool. We envision that P3K68D mediates early endocytic trafficking, tethering or sorting of integrin-containing vesicles. The integrin detected on large inclusions in mtm-depleted and XLMTM muscles in flies and humans, respectively, and evidence that mtm promotes membrane tubulation from PI(3)P compartments [18,37], point to an Mtm/MTM1 role in membrane efflux for delivery of integrin to the plasma membrane. Mtm phosphatase could act to promote recycling or to negatively regulate retention, for example, through a PI(3)P-mediated fusion of integrin-containing vesicles with endosomes-lysosomes. An accumulation of β1-integrin on enlarged, perinuclear compartments has been observed with certain genetic manipulations in non-muscle cells. These results raise the possibility that normal Mtm phosphatase activity functions antagonistically to Rab21 GTPase or in concert with PKCδ kinase, Rab11 and/or Arf6 GTPase, respectively, to control redelivery of β-integrin to the plasma membrane [38–40]. We found that class III PI3K, Vps34, also contributes to integrin localization upon myofiber remodeling, but with no effect on integrin-containing inclusions. A requirement for class III PI3K could be at a shared step with the early endosomal Rab5 GTPase shown to be involved in integrin turnover at larval MTJs [6]. Thus, regulation of distinct PI(3)P pools is important for differential regulation of integrin endosomal trafficking, whereby PI3KC2 and Mtm are dedicated to specific paired antagonistic functions.

We discovered that mtm is required in muscle for both integrin-mediated adhesions and T-tubule organization. The T-tubule requirement for mtm was similar to but not as severe as for amph, the sole homolog of human AMPH2 that is also associated with centronuclear myopathy [41]. However, unlike mtm, null alleles of amph did not share a defect of myofiber detachment. Despite localization of βPS-integrin at T-tubules, and the dual requirements for mtm, we found that normal integrin adhesions and abnormal βPS-integrin localization on inclusions are independent of T-tubule organization. This suggests that mtm may serve a common function for integrin turnover and T-tubule formation at a shared precursor compartment, for example, at recycling endosomes, or alternatively, act independently at two distinct sites. β-integrin, Dlg and Amph are known to functionally interact at postsynaptic junctions [42–44], and MTMR2 has been shown to interact withDlg1/PSD-95 and Dlg1/PSD-95 to promote postsynaptic function [45,46]. Thus, the shared accumulation of βPS-integrin, Dlg and Amph on central membrane inclusions in mtm-depleted myofibers, and their elimination with P3K68D co-deletion, points to a possible role for PI3KC2/Mtm pathway in endocytic recycling at neuromuscular junctions, as well as at MTJs.

Many of the defects observed in mtm mutant muscle parallel those associated with the human disease, XLMTM, demonstrating that the fly offers a tractable model for the cellular basis of centronuclear myopathy. Importantly, the discovery that mtm broadly regulates βPS-integrin turnover through endocytic trafficking led us to uncover a previously untested defect in β1-integrin localization in human XLMTM myofibers. Normal myofiber organization and function rely on integrin adhesions in vertebrate muscle [2,3,31,47]. Thus, disruption of integrin regulation provides a basis for aspects of the severity of myofiber disorganization and dysfunction observed in XLMTM. The conservation between fly mtm and human Mtm1 functions brings further significance to the potent interaction demonstrated between mtm and class II P3K68D for integrin regulation in flies. Whereas class I and III PI3-kinases have been the focus of intense study as potential therapeutic targets of specific inhibitory compounds, the Class II PI3-kinases have received little attention. The knowledge of PI3KC2 contributions to specific MTM pathways is significant towards motivating similar studies for potential strategies addressing MTM-related disease.

Mtm is the single fly homolog related to both human MTM1 and MTMR2, and human MTMR2 expression was able to rescue integrin-related defects in mtm-depleted fly myofibers. An mtm pathway function in endocytic trafficking is therefore relevant to a more general understanding of the cell biological functions employed by Mtm subfamily members. Mutations in MTMR2 associated with CMT1B neuropathy affect the morphology and function of myelinating Schwann cells [11], which like myofibers, share features of having an extensive plasma membrane and a reliance on integrin adhesions [48]. The regulation of integrin trafficking under the control of a conserved PI3KC2/Mtm pathway may be an important mechanism for controlling cell compartmentalization more broadly in different contexts, and relevant to different MTM-related human disease.

Materials and Methods

Ethics statement

Human samples were obtained and used as per institutional IRB accepted protocol.

Fly genetics

Flies were reared at 25°C, unless stated. Stocks used include: mtmN77, mtmN2–44 [17] and RNAi hairpins w; UAS-IR-mtmN1 and w; UAS-IR-mtmN1,5 interchangeably or in combination when “2x IR-mtm” noted, w; UAS-mtm:GFP and w; UAS-GFP-MTMR2 [18]; UAS-IR-P3K68D [12–14] and UAS-IR-Vps34 [100296 (VDRC); UAS-Vps34-KD [49]; Hasp24GAL4, Dmef2-GAL4, UAS-De2; Dmef2-GAL4, UAS-2xGFP [42], myD [42], and myD [Bloomington]; if [Schöck]; Ubi-βPS-integrin:YFP [6]; amph[52] [28]; w, d1-GFP:GAL80 (FlyTrap, [50]; UAS-GFP:LAMP [51]; UAS-GFP;Rob5 and UAS-GFP;myc:2xMYC [52]; UAS-GFP;Rab7 [53]; UAS-mRFP-PH-FAPP1 (G. Polevoy and J. Brill); UAS-GFP-2Aglu [54] and Ubi-Talin-GFP [55]. To reduce βPS-integrin function, myD [myD] flies raised at permissive 22°C were shifted to 29°C at pupation until lethality at pharate stage. To reduce mtm function specifically during pupation, UAS-EGFP+/+; Dmef2-GAL4, tubulin-Gal80°/+ and UAS-EGFP, UAS-IR-mtmN1,5/+; Dmef2-GAL4, tubulin-Gal80°/+ flies raised at permissive 18°C were shifted to 29°C at 2 days after pupation until pharate stage. To reduce mtm function in adults,
Muscle preparations and immunofluorescence

Staged pharate adults were removed from pupal case fastened to double-sided tape and pinned on a silgroid covered petri dish in dissecting buffer (5 mM HEPES, 128 mM NaCl, 2 mM KCl, 4 mM MgCl₂, 36 mM sucrose, pH 7.2). Abdomens were opened with longitudinal and two lateral incisions, pinned flat, washed and fixed 30 min. (3.7% formaldehyde, 50 mM EGTA, PBS); washed, unpinned and blocked (0.3% bovine serum albumin, 2% goat serum, 0.1% Triton, PBS), incubated with primary antibody overnight 4°C, washed (0.1% Triton PBS), reblocked and incubated overnight 4°C with Alexa-secondary antibodies (Molecular Probes), counterstained with phalloidin for F-actin and DNA as needed, and mounted in Fluorosave. Antibodies included Amphi Ra29 (C. O’Kane), Dlg 4F3 and βPS-integrin CG.6G11 (Developmental Studies Hybridoma Bank), α-tubulin (Sigma-Aldrich), Zormin B1 (B. Ballard), muscle myosin (D. Kiehart), βPS2-integrin 7A10 [56], anti-Rabenosyn-5 [57] and KDEL (Babraham Institute). Propidium iodide (PI) staining was done on dissection buffer as above, washed once with PBS 0.2% BSA and maintained in 0.3 mg/ml PI final concentration in PBS 0.2% BSA. Anesthesia, by releasing 12 females in a 2 L cylinder (50.8 cm diameter) for 6 days old were tested for flight at least 24 hours after CO₂ inhalation. Flies were marked inclusions, and number of nuclei per IOM. ImageJ software was used to segment and quantify nuclei morphology. Statistical analysis in Prism software used to determine mean, standard error and Student’s t-test, where possible.

Statistical analyses

Visual quantification was made for number of IOMs in tergites 3 and 4, number of detached IOMs per abdomen, number of IOMs displaying βPS-integrin costameres or βPS-integrin- or Dlg-marked inclusions, and number of nuclei per IOM. ImageJ software was used to draw and measure nuclei distance to IOM midline and sarcomere length. CellProfiler was used to segment and quantify nuclei morphology. Statistical analysis in Prism software used to determine mean, standard error and Student’s t-test, where possible.

Genotypes

Full genotypes used are as shown in Figure Legends and as follows:

**Figure 1.** (A) Control: UAS-EGFP/+; DMef2-GALA/+; UAS-EGFP, UAS-IR-mtm3.1/+. (B) Control: UAS-EGFP, UAS-IR-mtm3.1/+. (C) 24B-GAL4. Control: UAS-EGFP/24B-GALA. RNAi: UAS-EGFP, UAS-IR-mtm3.1/24B-GALA. RNAi with mtm cDNA: UAS-mtm:EGFP, UAS-IR-mtm3.1/24B-GALA. RNAi with human MTM12 cDNA: UAS-EGFPHumanMTM12, UAS-IR-mtm3.1/24B-GALA. DMef2-GALA. Control: UAS-EGFP/+; DMef2-GALA, UAS-lacZ/+. RNAi: UAS-EGFP/-; DMef2-GALA, UAS-IR-mtm3.1/+. (E, F) 24B-GAL4: Control: UAS-EGFP/24B-GALA. 1x RNAi: UAS-IR-mtm3.1/24B-GALA. 2x RNAi: UAS-IR-mtm3.1/24B-GALA, UAS-lacZ/+. DMef2-GALA. Control: UAS-EGFP/+; DMef2-GALA/+; UAS-lacZ/+. 2x RNAi: UAS-IR-mtm3.1/24B-GALA, UAS-lacZ/+. (G, H) UAS-IR-mtm3.1/+. 2x RNAi: UAS-IR-mtm3.1/24B-GALA, UAS-lacZ/+. (I) Control: UAS-De2; Mef2-GALA/UAS-EGFP, UAS-Rtmn1/+. 1x RNAi: UAS-IR-mtm3.1/+. 2x RNAi: UAS-IR-mtm3.1/24B-GALA, UAS-lacZ/+. (J) Control: UAS-De2; Mef2-GALA/UAS-EGFP, UAS-Rtmn1/+. 1x RNAi: UAS-IR-mtm3.1/+. 2x RNAi: UAS-IR-mtm3.1/24B-GALA, UAS-lacZ/+. (K) Control: UAS-De2; Mef2-GALA/UAS-EGFP, UAS-Rtmn1/+. 1x RNAi: UAS-IR-mtm3.1/+. 2x RNAi: UAS-IR-mtm3.1/24B-GALA, UAS-lacZ/+. (L) Control: UAS-De2; Mef2-GALA/UAS-EGFP, UAS-Rtmn1/+. 1x RNAi: UAS-IR-mtm3.1/+. 2x RNAi: UAS-IR-mtm3.1/24B-GALA, UAS-lacZ/+. (M) Control: UAS-De2; Mef2-GALA/UAS-EGFP, UAS-Rtmn1/+. 1x RNAi: UAS-IR-mtm3.1/+. 2x RNAi: UAS-IR-mtm3.1/24B-GALA, UAS-lacZ/+. (N) Control: UAS-De2; Mef2-GALA/UAS-EGFP, UAS-Rtmn1/+. 1x RNAi: UAS-IR-mtm3.1/+. 2x RNAi: UAS-IR-mtm3.1/24B-GALA, UAS-lacZ/+. (O) Control: UAS-De2; Mef2-GALA/UAS-EGFP, UAS-Rtmn1/+. 1x RNAi: UAS-IR-mtm3.1/+. 2x RNAi: UAS-IR-mtm3.1/24B-GALA, UAS-lacZ/+.
Supporting Information

Figure S1 Mtm maintains myofiber attachments and visceral muscle function. (A) RT-PCR of mtm RNAi depletion detected in whole adult thorax. Rp42L control RT-PCR from same tissue. (B) Western Blot of Mtm:GFP (anti-GFP) knockdown with muscle-targeted mtm RNAi and control protein (anti-α-tubulin) detected in whole pharate adults. (C) Normal third instar larval body wall muscle in control and (C') mtm knockdown animals, with two copies of RNAi hairpins. Muscle myosin. (D–D') Pharate ventral abdominal muscles. Arrowheads, detached PLMs. F-actin. (A,B,C) Control: UAS-EGFP+/+; DmF2-GAL4, UAS-lacZ+/+. (A',B,C') mtm RNAi: UAS-EGFP, UAS-IR-mtm1.1+/+. (E–F) Ventral PLMs quantified in filleted abdomen with 24B-GAL4 or DmMef2-GAL4 expression of 1 or 2 copies of mtm RNAi hairpins. (E) Number of ventral PLMs present, including detached PLMs. (F) Number of present but detached ventral PLMs. (G) Normal tubular shape of testis enshathed by visceral muscle (green). Asterisk, testis tip. 24B-GAL4/UAS-EGFP, green; F-actin, red; DNA, blue. (G') Abnormally round, short testis indicative of visceral muscle defect. Arrowhead, testis base attached to seminal vesicle. 24B-GAL4/UAS-EGFP, UAS-IR-mtm1.1. Scale bar 200 μm, except F–F' 50 μm.

Found at: doi:10.1371/journal.pgen.1001295.s001 (3.43 MB TIF)

Figure S2 Mtm is unessential for myofiber cell viability and normal sarcomere assembly. (A) Normal nuclei morphology in (A') control and (A') mtm RNAi IOMs. Myofiber nuclei analyzed for condensation (DAPI intensity) and nuclei size (perimeter) were similarly normal in control and mtm RNAi IOMs. Mean, s.d. for nuclei measured from 3–7 experiments. (B–B') Nuclei (blue; white arrowhead positions) in pharate IOMs do not stain with propidium iodide (red, PI) in either control (B) or mtm RNAi (B') conditions. GFP, green; DNA, blue. (C) Nuclei (blue) in IOMs of 1 day old adults were permeable to propidium iodide (red, PI; yellow arrowheads) in both control (C) and mtm RNAi (C') conditions. GFP, green; DNA, blue. (D–D') Transmission electron microscopy of IOM cross-sections, showing mitochondria with intact membranes and normal size and morphology. (E–F') Normal microtubule organization between myofibrils and in perinuclear space. α-tubulin, green; DNA, blue. (F–F') Normal Zormin sarcomere localization at repeating Z-lines. (G–G') Normal Z-line and sarcomere ultrastructure. (H) Normal sarcomere length (μm), as measured in Zormin-stained myofibers. IOMs, control: UAS-EGFP+/+; DmF2-GAL4+/+. RNAi: UAS-EGFP, UAS-IR-mtm1.1+/+. Scale bars 10 μm, except D–D', G–G' 1 μm.

Found at: doi:10.1371/journal.pgen.1001295.s002 (4.96 MB TIF)

Figure S3 Mtm is required for transverse tubule formation and organization. (A–A') Amph protein (green, top; single channel, bottom) localized at T-tubules. (A') Higher density of longitudinal and lack of transversal membrane elements in mtm-depleted muscle. F-actin, red (top). (B–B') Dig protein (green, top; single channel, bottom) showed similar localization to Amph at T-tubules. Zormin, red (top). (C) Schematic of dorsal IOM. Cell surface (top; integrin adhesions shown in grey) and cut-away central section of myofiber (bottom; nuclei shown in center), indicating the orientation of longitudinal and transversal T-tubules (green) in the central periphery regions in images shown (blue box). (D–D') Transmission electron microscopy shows radial spokes of transverse-tubule membranes (arrowheads) in myofiber cross-sections of control muscles (D), undetected in mtm-depleted IOMs (D'). IOMs, control: UAS-EGFP+/+; DmF2-GAL4+/+. RNAi: UAS-EGFP, UAS-IR-mtm1.1+/+. Scale bar 10 μm, except D–D', G–G' 1 μm.

Found at: doi:10.1371/journal.pgen.1001295.s003 (4.47 MB TIF)

Figure S4 βPS-integrin inclusions specifically accumulate due to a requirement for an mtm function during metamorphosis. (A–A') Normal βPS2-integrin localization at IOM MTJs (arrows; green and single channels) and undetected on Dlg-containing inclusions (red) with mtm RNAi. Control: UAS-EGFP+/+, DmF2-GAL4, UAS-lacZ+/+. RNAi: UAS-EGFP+/+, DmF2-GAL4, UAS-IR-mtm1.1+/+. (B–B') Normal GFP: Talin localization at IOM MTJs (arrows; green and single channels) and undetected on βPS-integrin inclusions (red) with mtm RNAi. DNA, blue. Control: Ubi-GFP: Talin+/+, DmF2-GAL4, UAS-lacZ+/+. RNAi: Ubi-GFP: Talin+/+, DmF2-GAL4, UAS-IR-mtm1.1+/+. (C–C') Normal βPS-integrin localization at larval MTJs (arrows; green and single channels), along peripheral cell surface and in small internal punctae in OreR and mtm+/+; mtm2–4747 third instar larvae. F-actin, red; DNA, blue. (D–D') UAS-EGFP, UAS-IR-mtm1.1+/+; DmF2-GAL4, tub>Gal80ts/+ pharates. (E) Percent pharate adults with one or more detached IOMS. (F–F') UAS-EGFP, UAS-IR-mtm1.1+/+; DmF2-GAL4, tub>Gal80ts/+ pharates. (F) Normal βPS-integrin localization (red, and single channel) in pharate adult IOM in flies raised continuously at 18°C, due to GAL80ts-inhibited GAL4 activity. (F') Abnormal integrin inclusions (arrowheads) in IOMs of flies raised at 18°C until pupation, then upshifted to 29°C for 3 days, due to mtm RNAi during pupal stages. GFP, green; DNA, blue. (G) Percent of IOMs with integrin-containing inclusions. Scale bars 10 μm, except D–D' 200 μm.

Found at: doi:10.1371/journal.pgen.1001295.s004 (5.75 MB TIF)

Figure S5 Membrane inclusions in mtm-depleted myofibers are an endosomal-related compartment. (A) Co-localization of βPS-integrin and Amph increased along longitudinal T-tubules in IOM central periphery regions with mtm knockdown, as determined by Pearson correlation. (B–B') βPS-integrin (red) and Amph (green) infrequently co-localized (arrowheads) in control IOMs (B). With mtm RNAi, βPS-integrin and Amph became continuously co-localized along longitudinal T-tubules in central periphery (B', βPS-integrin single channel at right) and showed punctuated co-localization along inclusions (B", arrowheads). Control: dlg1:GFP; DmF2-GAL4, UAS-lacZ+/+. RNAi: dlg1:GFP; DmF2-GAL4, UAS-IR-mtm1.1+/+. (C) Schematic of dorsal IOM. Cell surface (top; integrin adhesions shown in grey) and cut-away central section of myofiber (bottom; nuclei shown in center), indicating the orientation of longitudinal and transversal T-tubules (green) in the central periphery regions in images shown (blue box). (D) amph26 null mutant. Dlg (white) detected in discontinuous longitudinal patches between myofibrils and missing from transversal elements of T-tubules, and normal aligned nuclei (blue) in myofiber perinuclear area. (E–F') Normal KDEL (green) distribution in IOMs localized to sarcoplasmic reticulum between myofibrils and perinuclear endoplasmic reticulum, but undetected on abnormal inclusions with mtm RNAi. Control: UAS-EGFP+/+, DmF2-GAL4+/+. RNAi: UAS-EGFP, UAS-IR-mtm1.1+/+. Scale bar 10 μm. (F–F') Normal mRFP:PH-FAPP1 (green) distribution indicating trans-Golgi network in IOMs localized between but not on abnormal inclusions with mtm RNAi. Control: UAS-EGFP/ UAS-mRFP:PH-FAPP1; DmF2-GAL4+/+. RNAi: UAS-EGFP, UAS-IR-mtm1.1+/ UAS-mRFP:PH-FAPP1; DmF2-GAL4+/+. (F–F') F-actin and cytoplasmic GFP, co-imaged and pseudocolored red. (G–G')
Few small GFP:Atg8a punctae (green), indicative of autophagosomes, with similar distribution in control and mtm RNAi IOMs, and not detected on inclusions. Control: UAS-GFP:Atg8a/+; DMel2-GAL4, UAS-laCZ/+; RNAi: UAS-GFP:Atg8a/+; DMel2-GAL4, UAS-IR-mtm3.5/b+; (H–H) DMel2-GAL4/+; RNAi: UAS-GFP:Rab7 is absent from periphery and does not colocalize with integrin (red) on inclusions with mtm RNAi (H). Control: UAS-EGFP/+; DMel2-GAL4/+; RNAi: UAS-EGFP, UAS-IR-mtm3.5/b+; DMel2-GAL4/+; EGFP not detected. In control IOMs, GFP:Rab7 (green) detected as small particles throughout myofiber periphery and in larger clusters in perinuclear region. In mtm RNAI IOMs, GFP:Rab7 is absent from periphery and does not colocalize on inclusions. Control: UAS-GFP:Rab7/+; DMel2-GAL4, UAS-laCZ/+; RNAi: UAS-GFP:Rab7/+; DMel2-GAL4, UAS-IR-mtm3.5/b+; DMel2-GAL4/+; RB5-integrin, red; DNA, blue. Scale bars 10 μm. 

Figure 56  Lethality and Dlg inclusions with muscle-specific mtm RNAi are rescued by P3K68D co-depletion. (A) Percent viable and lethal staged progeny at 17 days after staged egg lays for Dcr2; DMel2-GAL4 single and double RNAi hairpin conditions, as shown; ≥3 experiments. Disruption of class II or III P3-kinases alone (IR-P3K68D hairpin and Vps34-KD, kinase dead) did not alter viability. Co-disruption of P3K68D or Vps34 with mtm co-RNAi, respectively, restored or enhanced lethality and delayed development. (B) RT-PCR of P3K68D depletion and GAPDH control detected in adult abdomens with DMel2-GAL4 muscle-driven P3K68D RNAi. (C) Percentage of IOMs with severity of T-tubule defects, as detected by Dlg White, transverse elements detected throughout >50% of each myofiber; grey, transverse elements detected in 10–50% of each myofiber; dark grey, transverse elements detected in <10% of each myofiber. Pooled from 2 experiments. (D) Percent of flightless adults, aged 1–6 days old, from ≥5 experiments (semi-lethality prevented equivalent numbers from IRmtm, Vps34-KD). (E) Percentages of IOMs that contain Dlg detected along abnormal inclusions, for single (light grey, 0%) and mtm double (dark grey) RNAi conditions as shown. Genotypes as in Figure 5, in addition to UAS-Vps34-KD. (F–G) Pharate abdominal muscles, F-actin. (F) IR-Atg1 alone and (F) with mtm co-RNAi. Arrowheads, detached IOMs. (G–G) Sarcolemmal βPS-integrin no longer detected at costameres; (G) IR-Atg1 alone and (G) with mtm co-RNAi. (H–H) βPS-integrin central z-sections; (H) no inclusions detected with IR-Atg1 alone and (H) inclusions present with mtm co-RNAi. 

Acknowledgments

We thank D. Bäder, J. Brill, B. Bullard, T. Bunch, L. Cooley, M. Gonzalez-Gaitán, D. Kiehart, H. Krämer, T. Neufeld, C. O’Kane, F. Schöck, Developmental Studies Hybridoma Bank, and the stock centers Bloomington, Fly Trap, and VDRC for reagents. We thank members of the Kiger lab, R. Firtel, and C. Weaver for comments. We are grateful to T. Meerlo for technical assistance with electron microscopy preparation and to Stu Sharqur for use of the CMU Electron Microscope Facility. Imaging was conducted in part in the UCSD Neuroscience Microscopy Shared Facility.

Author Contributions

Conceived and designed the experiments: IR GT JJD AK. Performed the experiments: IR LY JJD AK. Analyzed the data: IR LY JJD AK. Contributed reagents/materials/analysis tools: IR. Wrote the paper: IR.

References

1. Brower DL (2003) Platelets with wings: the maturation of Drosophila integrin biology. Curr Opin Cell Biol 15: 607–613.
2. Mayer U, Saher G, Fesler R, Beeram A, Echtremer F, et al. (1997) Absence of integrin alpha 7 causes a novel form of muscular dystrophy. Nat Genet 17: 318–323.
3. Volk T, Fesler LI, Fesler JH (1990) A role for integrin in the formation of sarcomere cytoarchitecture. Cell 63: 525–536.
4. Caswell PT, Vadhura S, Norman JC (2009) Integrins: masters and slaves of endocytic transport. Nat Rev Mol Cell Biol 10: 453–453.
5. Kaisto T, Rahikila P, Mäyniemi V, Paron RC, Metsävel K (1999) Endocytosis in skeletal muscle fibers. Exp Cell Res 253: 551–560.
6. Yuan L, Fairchild MJ, Perkins AD, Tanentzapf G (2010) Analysis of integrin biology. Curr Opin Cell Biol 15: 607–613.
7. Di Paolo G, De Camilli P (2006) Phosphoinositides in cell regulation and membrane dynamics. Nature 443: 651–657.
8. Nicot A, Laspeyres J (2008) Endosomal phosphoinositides and human diseases. Traffic 9: 1240–1249.
9. Buja-Bello A, Laugel V, Messaddeq N, Zahreddine H, Laporte J, et al. (2002) Loss of phosphoinositide 3-kinase C2alpha in insulin signaling. J Biol Chem 282: 28226–28236.
10. Rider DL, (2003) Platelets with wings: the maturation of Drosophila integrin biology. Curr Opin Cell Biol 15: 607–613.
11. Altmann A, Mischler M, Conforti FL, Le Guern E, Salih MA, et al. (2000) Charcot-Marie-Tooth disease type 4B1. Hum Mol Genet 11: 1569–1579.
12. Bolino A, Muglia M, Conforti FL, Le Guern E, Salih MA, et al. (2000) Charcot-Marie-Tooth disease type 4B1 is caused by mutations in the gene encoding the myotubulin-related protein-2. Nat Genet 25: 17–19.
13. Taylor GS, Maehama T, Dixon JE (2000) Inaugural article: myotubularin, a protein tyrosine phosphatase mutated in myotubular myopathy, dephosphorylates the lipid second messenger, phosphatidylinositol 3-phosphate. Proc Natl Acad Sci U S A 97: 8910–8915.
14. Taylor GS, Maehama T, Dixon JE (2000) Inaugural article: myotubularin, a protein tyrosine phosphatase mutated in myotubular myopathy, dephosphorylates the lipid second messenger, phosphatidylinositol 3-phosphate. Proc Natl Acad Sci U S A 97: 8910–8915.
15. Tsujita K, Itoh T, Ijuin T, Yamamoto A, Shisheva A, et al. (2004) Myotubularin regulates the function of the late endosome through the gram domain-phosphatidylinositol 3,5-bisphosphate interaction. J Biol Chem 279: 13091–13094.
fibers lacking myotubularin lipid phosphatase. Proc Natl Acad Sci U S A 106: 18763–18768.

28. Razzak A, Robinson IM, McMahon HT, Sleper JN, Su Y, et al. (2001) Amphiphysin is necessary for organization of the excitation-contraction coupling machinery of muscles, but not for synaptic vesicle endocytosis in Drosophila. Genes Dev 15: 2967–2979.

29. Wilcox M, DaMato S, Leptin M (1989) The function of PS integrins in Drosophila wing morphogenesis. Development 107: 891–897.

30. Walsh EP, Brown NH (1998) A screen to identify Drosophila genes required for integrin-mediated adhesion. Genetics 150: 791–805.

31. ervasti JM (2003) Costameres: the Achilles’ heel of Herculean muscle. J Biol Chem 278: 13591–13594.

32. Sparrow JC, Schock F (2009) The initial steps of myofibril assembly: integrins pave the way. Nat Rev Mol Cell Biol 10: 293–298.

33. Perkins AD, Ellis SJ, Ashgari P, Shamsian A, Moore ED, et al. (2010) Integrin-mediated adhesion maintains sarcomeric integrity. Dev Biol 339: 15–27.

34. MacDougall LK, Gagou ME, Leevers SJ, Hafen E, Waterfield MD (2004) Compartmental dependence of the TGF-beta homolog Dpp. Cell 103: 981–991.

35. Entchev EV, Schwabedissen A, Gonzalez-Gaitan M (2000) Gradient formation and vesicle dynamics with receptor signaling pathways. Mol Cell Biol 24: 796–808.

36. Domin J, Harper L, Aubyn D, Wheeler M, Florey O, et al. (2005) The class II phosphoinositide 3-kinase in Drosophila melanogaster reveals lipid kinase-dependent effects on patterning and interactions with receptor signaling pathways. Mol Cell Biol 25: 2096–2107.

37. Fili N, Calleja V, Woscholski R, Parker PJ, Larijani B (2006) Compartmental signal modulation: Endosomal phosphatidylinositol 3-phosphate controls endosome morphology and selective cargo sorting. Proc Natl Acad Sci U S A 103: 15473–15478.

38. Pellinen T, Arjonen A, Vuoriluoto K, Kallio K, Fransen JA, et al. (2006) Small GTPase Rab21 regulates cell adhesion and controls endosomal traffic of beta1-integrins. J Cell Biol 173: 767–780.

39. MacDougall LK, Gagou ME, Leevers SJ, Hafen E, Waterfield MD (2004) Disruption of the TGF-beta homolog Dpp. Cell 103: 981–991.

40. Powellka AM, Sun J, Li J, Gao M, Shaw LM, et al. (2004) Stimulation-dependent recruitment of integrin beta1 regulated by ARF6 and Rab11. Traffic 5: 20–36.

41. Pulipparacharuvil S, Akbar MA, Ray S, Sevrioukov EA, Haberman AS, et al. (2005) Drosophila Vps116A is required for trafficking to lysosomes and biogenesis of pigment granules. J Cell Sci 118: 3663–3673.

42. Timmers H, de Vries N, van der Sluijs M, Gonzalez-Gaitan M (2000) Gradient formation of the TGF-beta homolog Dpp. Cell 103: 981–991.

43. Bolano A, Bolis A, Prevaldi SG, Dina G, Bassini S, et al. (2004) Disruption of MtmR2 produces CMT1B-like neuropathy with myelin outfolding and impaired spermatogenesis. J Cell Biol 167: 711–721.

44. Bolano A, Bolis A, Prevaldi SG, Dina G, Bassini S, et al. (2004) Disruption of MtmR2 produces CMT1B-like neuropathy with myelin outfolding and impaired spermatogenesis. J Cell Biol 167: 711–721.

45. Lee HW, Kim Y, Han K, Kim H, Kim E (2010) The phosphoinositide 3-phosphate MTMR2 interacts with PSD-95 and maintains excitatory synapses by modulating endosomal traffic. J Neurosci 30: 5508–5518.

46. Hayashi YK, Chou FL, Engvall E, Ogawa M, Matsuura C, et al. (1998) Mutations in integrin alpha7 gene cause congenital myopathy. Nat Genet 19: 94–97.

47. Barros CS, Nguyen T, Spencer RS, Nishiyama A, Colognato H, et al. (2009) Beta1 integrins are required for normal CNS myelination and promote AKT-dependent myelin outgrowth. Development 136: 2717–2724.

48. Julhas G, Hill BH, Yaz Y, Sass M, Baerentece EH, et al. (2008) The class III PI(3)K Vps34 promotes autophagy and endocytosis but not TOR signaling in Drosophila. J Cell Biol 181: 655–666.

49. Quinones-Coello AT, Petrella LN, Ayers K, Melillo A, Mazzafero F, et al. (2007) Exploring strategies for protein trapping in Drosophila. Genetics 175: 1399–1414.

50. Wucherpfennig T, Wilch-Brauninger M, Gonzalez-Gaitan M (2003) Role of Drosophila Rab5 during endosomal trafficking at the synapse and evoked neurotransmitter release. J Cell Biol 161: 609–624.

51. Entchev EV, Schwabedissen A, Gonzalez-Gaitan M (2000) Gradient formation of the TGF-beta homolog Dpp. Cell 103: 981–991.

52. Scott RC, Shaldtiner O, Neufeld TP (2004) Role and regulation of starvation-induced autophagy in the Drosophila fat body. Dev Cell 7: 167–178.

53. Rabenosyn and Vps45. Mol Biol Cell 19: 4167–4176.

54. Bolano A, Bolis A, Prevaldi SG, Dina G, Bassini S, et al. (2004) Disruption of MtmR2 produces CMT1B-like neuropathy with myelin outfolding and impaired spermatogenesis. J Cell Biol 167: 711–721.