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Phosphoinositide Regulation of Integrin Trafficking Required for Muscle Attachment and Maintenance

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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.

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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 hypotrophic myofibers [9,10]. Disruption of related *MTMR2* leads to Charcot-Marie-Tooth neuropathy (CMT4B1, OMIM #601382) with abnormal morphology and plasma membrane outfoldings in

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. Myotubularin (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.

myelinating Schwann cells [11]. Both MTM1 and MTMR2 partially localize to endosomal compartments and are attributed with PI(3)P and PI(3,5)P₂ turnover [12–18]. However, it is not clear how disruption of MTMs and potential regulation of endosomal phosphoinositides might lead to the morphological defects found in MTM-related disease. The kinase(s) that coregulate the relevant phosphoinositide pool(s) for specific MTM functions in muscle have not been explored. Candidates include both Class II and Class III PI3-kinases (PI3KC2 and Vps34, respectively) that generate PI(3)P [19]. Vps34 has well established conserved roles at endosomal membranes. In contrast, PI3KC2 has less-understood roles mostly related with functions at the plasma membrane [18,20–23].

Here we show that *mtm* (GenBank NM_078765), encoding the sole *D. melanogaster* homolog of human MTM1/MTMR2, acts with Class II *Pi3K68D* (GenBank NM_079304) to maintain attachments upon myofiber remodeling. We found that *mtm* controls β -integrin turnover and trafficking from perinuclear compartments to maintain spatially restricted adhesions at MTJs and costameres, reflecting a broad *mtm* requirement for integrin-mediated adhesion also needed in the wing. The defects discovered in flies were substantiated by observing similar integrin mislocalization in human XLMTM myopathy, suggesting a conserved *MTM1* function in membrane trafficking and roles for integrin adhesions in maintenance of myofiber organization. Altogether, our results identify specific phosphoinositide regulation important for endocytic recycling and dynamic control of cell compartmentalization.

Results

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

Given the role for myotubularins 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 larvae, which remained mobile and exhibited normal body wall muscle formation, attachments and growth (Figure 1A and 1B–1B', 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 (DMef2-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'–1E; 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 rethickening (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*-depleted IOM cells. The peripheral

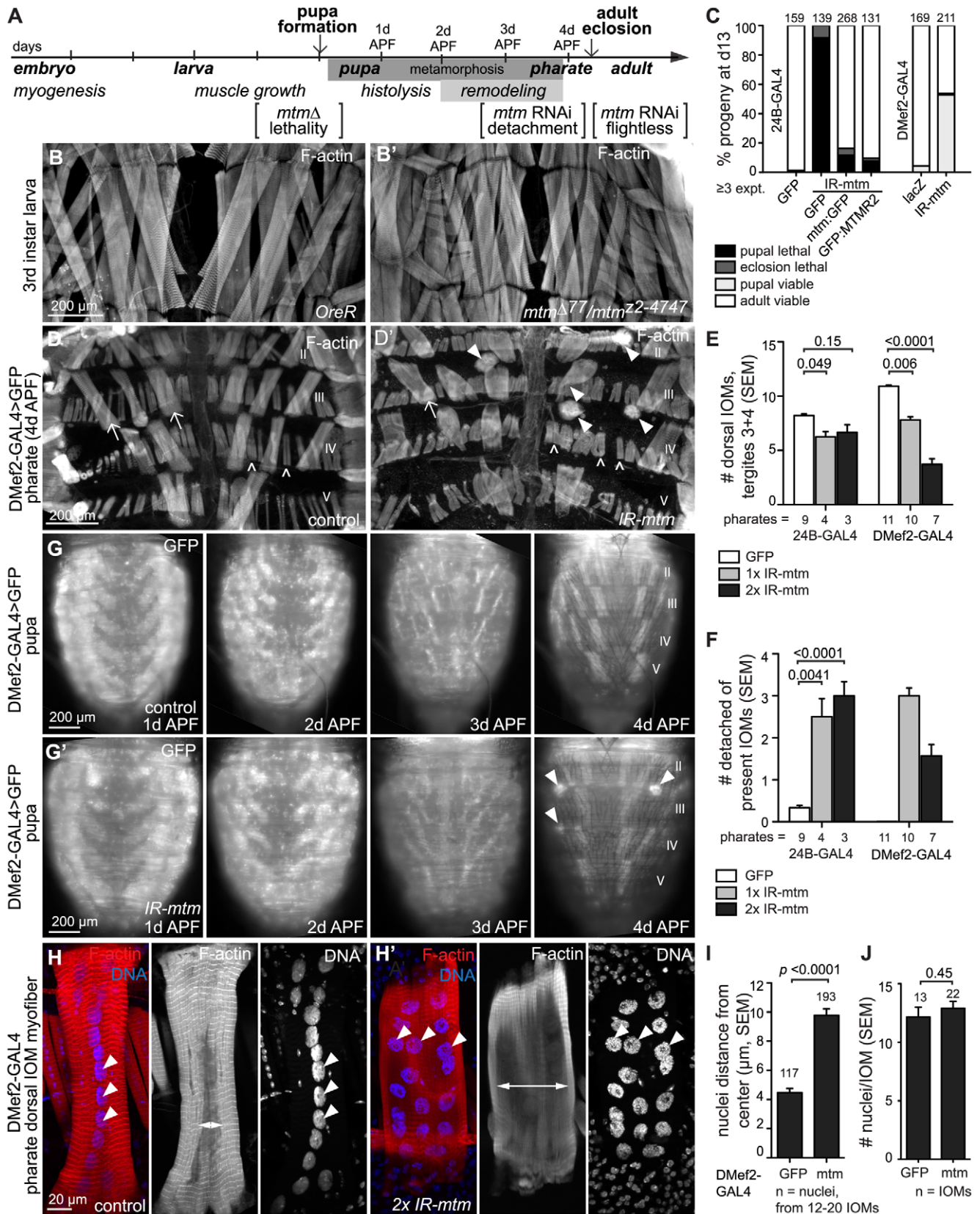


Figure 1. Mtm depletion leads to myofiber detachment and morphological defects common with human myotubular myopathy. (A) Timeline of fly and muscle development, indicating stages of *mtm* requirements; days after puparium formation (APF). (B–B') Normal body wall muscles in *OreR* and *mtm Δ ⁷⁷/mtm^{z2-4747}* third instar larvae. F-actin. (C) Percent viable and lethal progeny 13 days after egg lays with 24B-GAL4 and DMef2-GAL4 muscle-targeted *mtm* RNAi. (D–D') Pharate dorsal abdominal muscles F-actin. Large IOMs (arrows) and smaller adult muscles (open

arrowheads) span tergites (numerals). (D') Detached IOMs (arrowheads) seen with *mtm* RNAi. (E–F) IOMs in filleted abdomens with 24B-GAL4 or DMef2-GAL4 expression of 1 or 2 copies of *mtm* RNAi hairpins. (E) Number IOMs present in tergites 3 and 4, including detached but present IOMs. (F) Number of present, visibly detached IOMs. (G–G') Timelapse microscopy of GFP in IOMs imaged in same animals 1, 2, 3 and 4 days APF. (G') With *mtm* RNAi, normal IOM formation (1d APF), survival upon histolysis of non-persistent muscles (2d APF), myofiber thinning (3d APF) and rethickening (4d APF) preceded detachment (4d APF, arrowheads). (H–H') Individual IOMs. F-actin, red; DNA, blue. Projections, merged and nuclei images; central z-sections, F-actin. (H) Contractile myofibrils are normally tightly packed (double arrow) around linear aligned nuclei (arrowheads). (H') With *mtm* RNAi, intact peripheral myofibrils surround expanded central area (double arrow) with unaligned nuclei (arrowheads). (I) Distance (μm) of nuclei from IOM midline. (J) Number nuclei per IOM. Scale bar 200 μm , except H–H' 20 μm . doi:10.1371/journal.pgen.1001295.g001

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 found that both the Amphiphysin (Amph) 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 Amph and Dlg 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; $n \geq 21$). 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 β PS-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 β PS-integrin, the single *D. melanogaster* β -integrin subunit encoded by *mys* (GenBank NM_080054), was dramatically mislocalized in *mtm*-depleted muscles (Figure 2B'). In contrast to wildtype muscle, β PS-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 β PS-integrin protein was detected as small punctae within wildtype myofibers (Figure 2D), upon *mtm* knockdown, β PS-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, α PS2-integrin and Talin, were both detected at MTJs but not at the inclusions, suggesting β PS-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 β PS-integrin localization at earlier developmental stages. In *mtm* null or RNAi depleted larval muscles, normal integrin localization was detected at myofiber attachments, without any β PS-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, β PS-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 β PS-integrin:YFP along MTJs in intact larvae [6]. The mobile fraction of β PS-integrin:YFP was significantly increased in *mtm* mutant larval muscles (Figure 2F), indicating that *mtm* is required to stabilize sarcolemmal β PS-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 pupal 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 GAL80^{ts}, 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

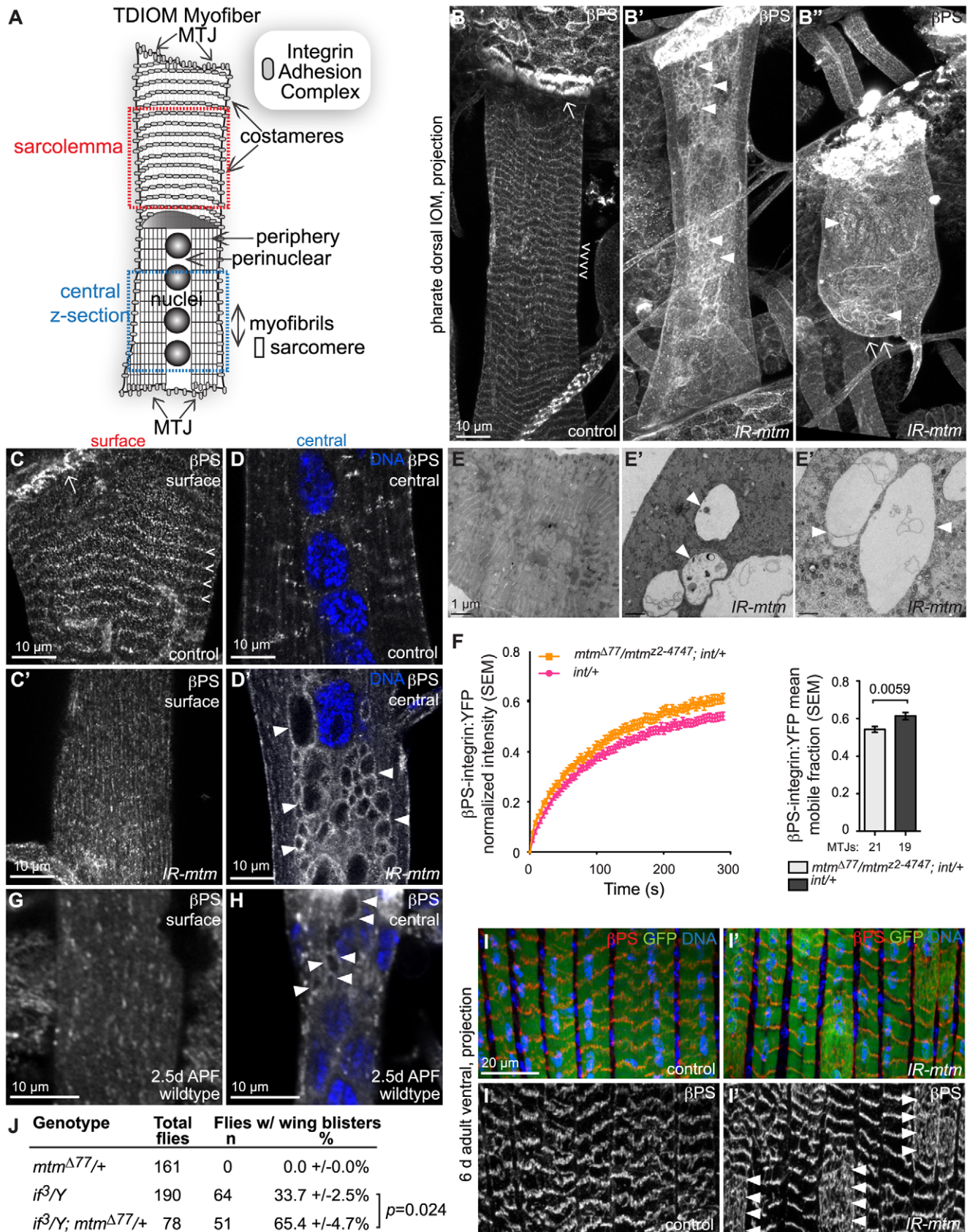


Figure 2. Mtm is required for β PS-integrin flux from intracellular compartments and localization at sarcolemmal adhesions. (A) Schematic of individual pharate IOM and regions imaged. MTJ, myotendinous junction. (B-B'') β PS-integrin in IOM z-projections. (B) β PS-integrin at MTJs (arrow) and costameres (open arrowheads) in control. (B'-B'') With *mtm* RNAi, β PS-integrin was absent from detached ends (B'', arrow) and

costameres, and detected on abnormal inclusions (arrowheads). (C–C') IOM sarcolemma highlighting β PS-integrin at costameres in control (C, open arrowheads), absent with *mtm* RNAi (C'). (D–D') IOM central z-sections revealing β PS-integrin punctae in control (D), and accumulation on abnormal inclusions with *mtm* RNAi (D', arrowheads). DNA, blue. (E–E') Transmission electron microscopy of IOM cross-sections, showing densely packed central regions in control (E) and large lucent membrane compartments with *mtm* RNAi (E', arrowheads). (F) Averaged FRAP recovery curves and mean mobile fraction for larval β PS-integrin:YFP (*int/+*) in wildtype background (pink) and trans-heterozygous null *mtm* ^{Δ 77}/*mtm*²²⁻⁴⁷⁴⁷ (orange). (G) Little to no β PS-integrin present at the sarcolemma in wildtype pupal IOM, 2.5 days APF. (H) β PS-integrin on central inclusions (arrowheads) detected in wildtype pupal IOM, 2.5 days APF. DNA, blue. (I–I') β PS-integrin (red, and single channel below) at costameres in z-projections of adult abdominal lateral transversal muscles (I), and sporadically absent from costameres and dispersed in regions of myofibers with *mtm* RNAi (I') in 6 day old adult flies. GFP, green; DNA, blue. *DMef2-GAL4*. (J) Heterozygous *mtm* ^{Δ 77}/+ enhanced frequency of adult wing blisters in hemizygous *if* ^{β} /Y flies. Scale bars 10 μ m, except E–E' 1 μ m.

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resulting from a hypomorphic allele of α PS2-integrin, *if*³ [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 β PS-integrin, appeared along abnormal central inclusions (Figure 3B, 3B'). To characterize the membrane identity of the inclusions, we first tested whether β PS-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 β PS-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, β PS-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.

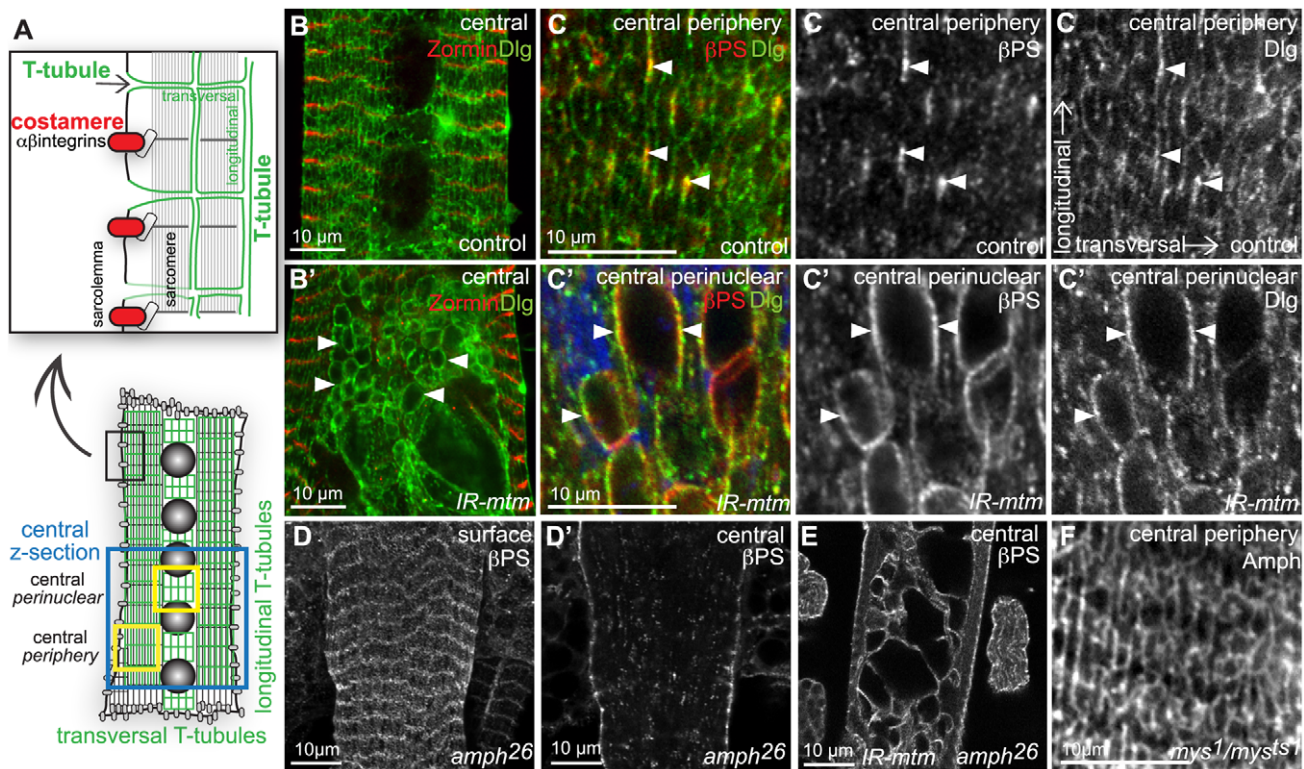


Figure 3. Integrin adhesions are independent of T-tubules, but share an *mtm* function for maintained organization. (A) IOM longitudinal section schematic of alternating sarcolemmal structures: T-tubule membranes (green) marked by Amph and Dlg; costameres (red) with IACs linked to Z-lines of peripheral 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') β PS-integrin (red) and *dlg*: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 β PS-integrin localization at costameres and (D') internal punctae in *amph*²⁶ mutants that lack T-tubules. (E) Persistent β PS-integrin-inclusions with *mtm* RNAi in *amph*²⁶ mutant. *amph*²⁶, *UAS-IR-mtm*^{3.1}/*amph*²⁶, *DMef2-GAL4*/+. (F) Normal transverse tubule formation and Amph localization in *mys*¹/*mys*⁵⁵ mutants reared at non-permissive temperature, with reduced β PS-integrin function. Scale bars 10 μ m.

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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 [28], we observed normal muscle attachments, normal β PS-integrin localization to costameres, and no β PS-integrin- or Dlg-inclusions (Figure 3D–3D'). This suggests that T-tubules are not required for β PS-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 β PS-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 β PS-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 β PS-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

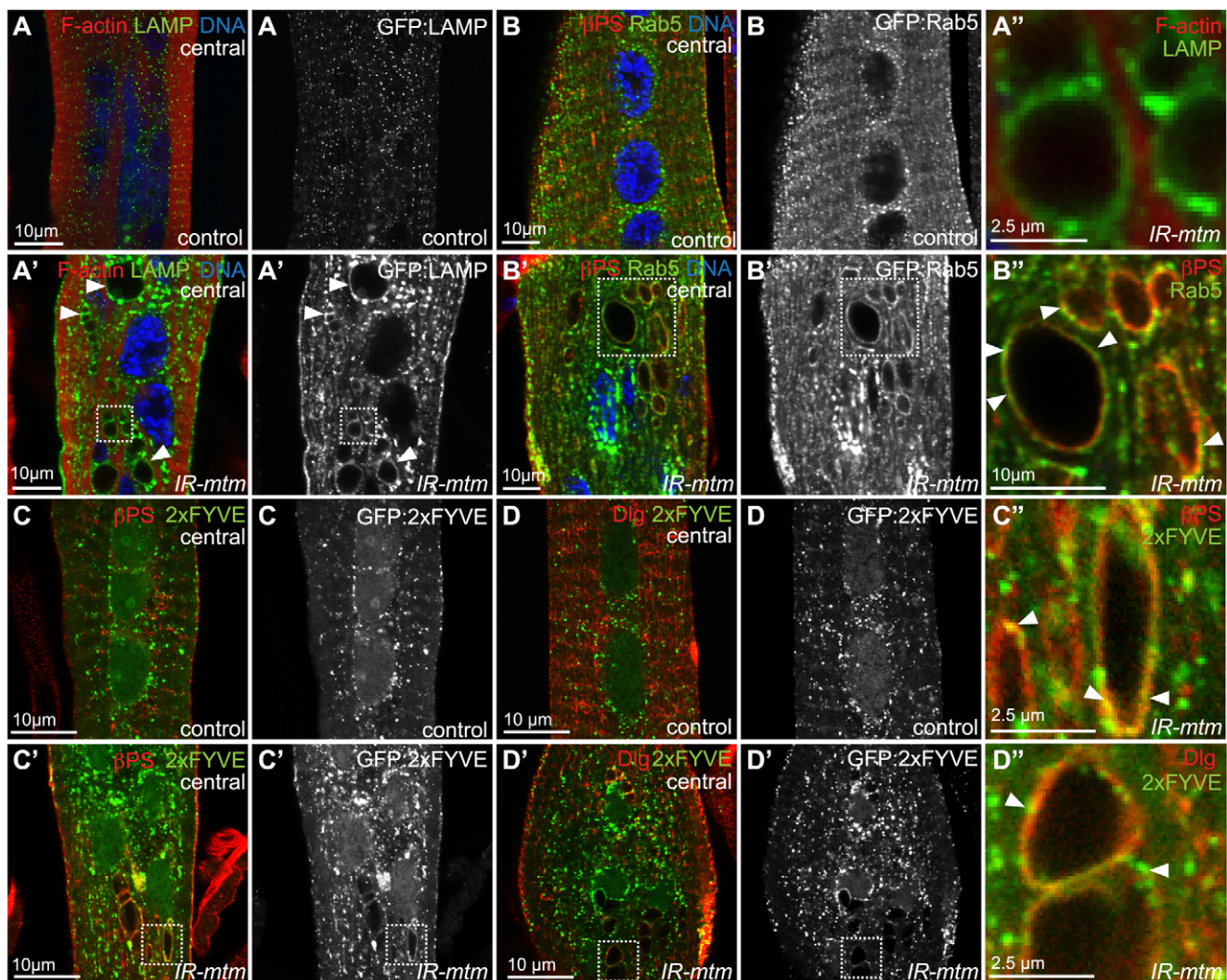


Figure 4. Mtm depletion disrupts integrin trafficking at endosomal compartments. (A–A'') GFP:LAMP (green, and single channels) found as punctae throughout IOM controls (A) and localized to inclusions with *mtm* RNAi (A', arrowheads; A''). F-actin, red; DNA, blue. (B) GFP:Rab5 (green, and single channels) found as punctae with normally little overlap with β PS-integrin (red) throughout IOM controls. (B'–B'') Rab5 partially co-localized with β PS-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 β PS-integrin (red) exhibited little overlap in control (C), but co-localized on inclusions with *mtm* RNAi (C'–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'–D'', arrowheads). Scale bars 10 μ m, except zooms A'', C'', D'' 2.5 μ m. doi:10.1371/journal.pgen.1001295.g004

endosomes, GFP:Rab5 (Figure 4B–4B''), and infrequently by the Rab5 effector, Rbsn5 (Figure S5H–S5H''), but not an indicator of late endosome identity, GFP:Rab7 (Figure S5I–S5I'). 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 homeostasis 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 β PS-integrin (Figure 4C) or Dlg (Figure 4D). Upon *mtm*-depletion, enlarged and more erratically positioned PI(3)P-containing compartments were detected (Figure 4C', 4D'). In addition, GFP:2xFYVE co-localized with β PS-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 β PS-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 (*Pi3K68D* and *Vps34*, respectively), known to synthesize PI(3)P, to abdominal muscle maintenance. Muscle-targeted knockdown of *Pi3K68D* or expression of dominant negative kinase-dead *Vps34-KD* did not individually disrupt eclosion or animal viability. However, *Pi3K68D* depletion in combination with *mtm* RNAi was able to rescue the lethality and delayed development; in contrast, *Vps34-KD* expression enhanced lethality in combination with *mtm* depletion (Figure S6A, S6B). Neither *Pi3K68D* 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 *Pi3K68D*-independent and dependent *mtm* muscle functions required for normal T-tubules and viability, respectively.

A similar functional relationship was seen between *Pi3K68D* and *mtm* for roles related to integrin adhesions, as with viability. Importantly, *Pi3K68D*, but not *Vps34*, depletion rescued muscle detachment (Figure 5A, 5B) and loss of β PS-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 *Pi3K68D*, and not *Vps34*, also eliminated the β PS-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 testis visceral muscle function was also restored to normal with *Pi3K68D* and *mtm* co-depletion, implicating turnover of integrin-mediated adhesions in the gonadal muscle. Altogether, these results signify that *Pi3K68D* function mediates *mtm* RNAi mutant defects in maintenance of IACs, and suggest that Pi3K68D 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 β PS-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 phosphoinositide-mediated steps in IAC maintenance, distinct from trafficking

points that involve antagonistic Pi3K68D 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. β 1-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, β 1-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 β 1-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 β PS-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 *Pi3K68D* and *mtm* co-depletion could revert both an imbalance in PI(3)P and defects in cortical

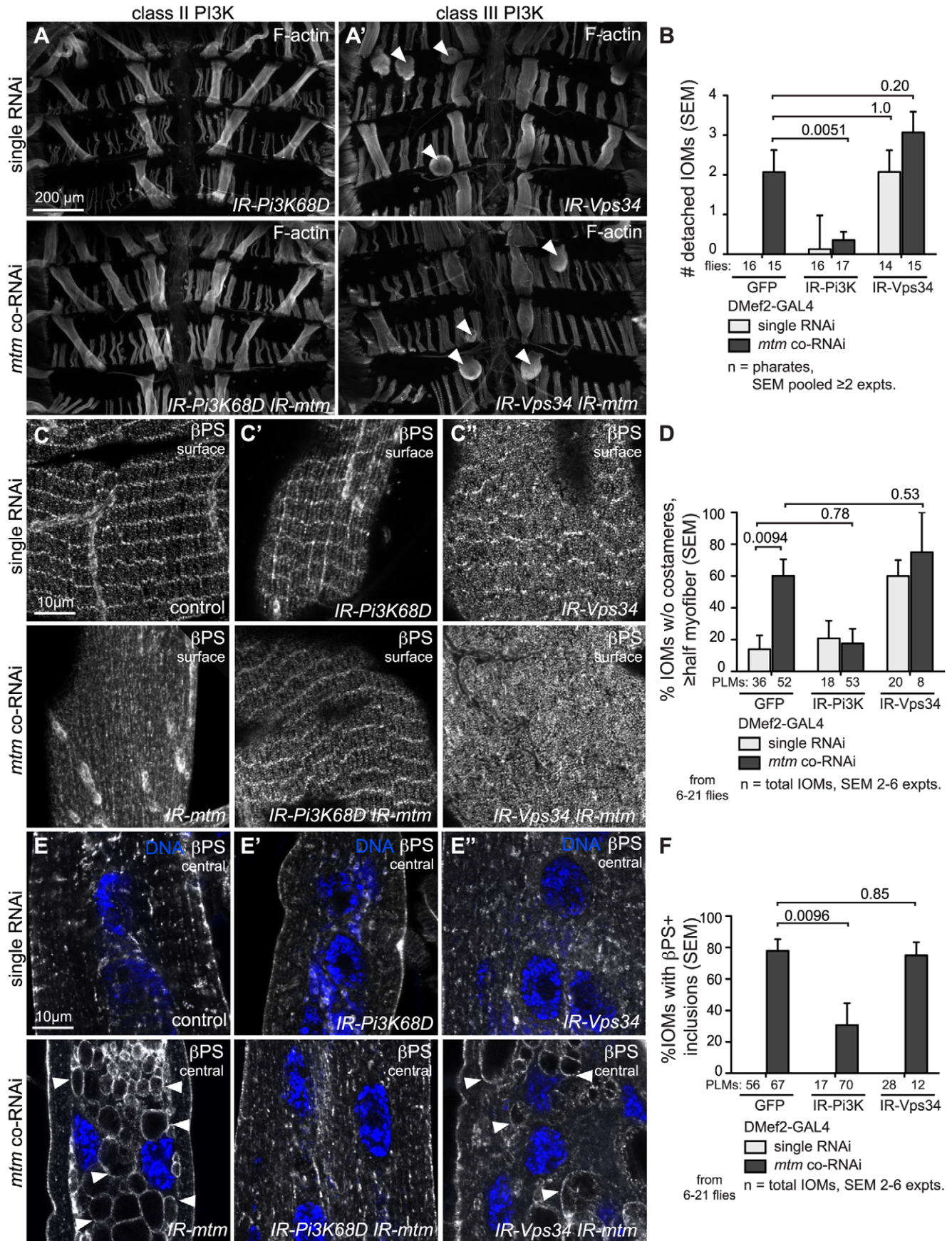


Figure 5. Class II and Class III PI3-kinases affect *mtm*-dependent integrin adhesions differently. (A–A') Pharate abdominal muscles, F-actin. (A) *IR-Pi3K68D* and (A') *IR-Vps34* single RNAi (top) and *mtm* co-RNAi (bottom). Arrowheads, detached IOMs. (B) Number of visibly detached IOMs. (C, C', C'') Sarcolemmal β PS-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 β PS-integrin at costameres. (D) Percentage IOMs that lack costameres \geq half of myofiber surface. (E, E', E'') β PS-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 β PS-integrin-inclusions. (F) Percentage IOMs with β PS-integrin on inclusions. (B,D,F) IOMs in single RNAi (light bars) and *mtm* co-RNAi (dark bars) conditions. Scale bars 10 μ m, except A–A' 200 μ m.
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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.

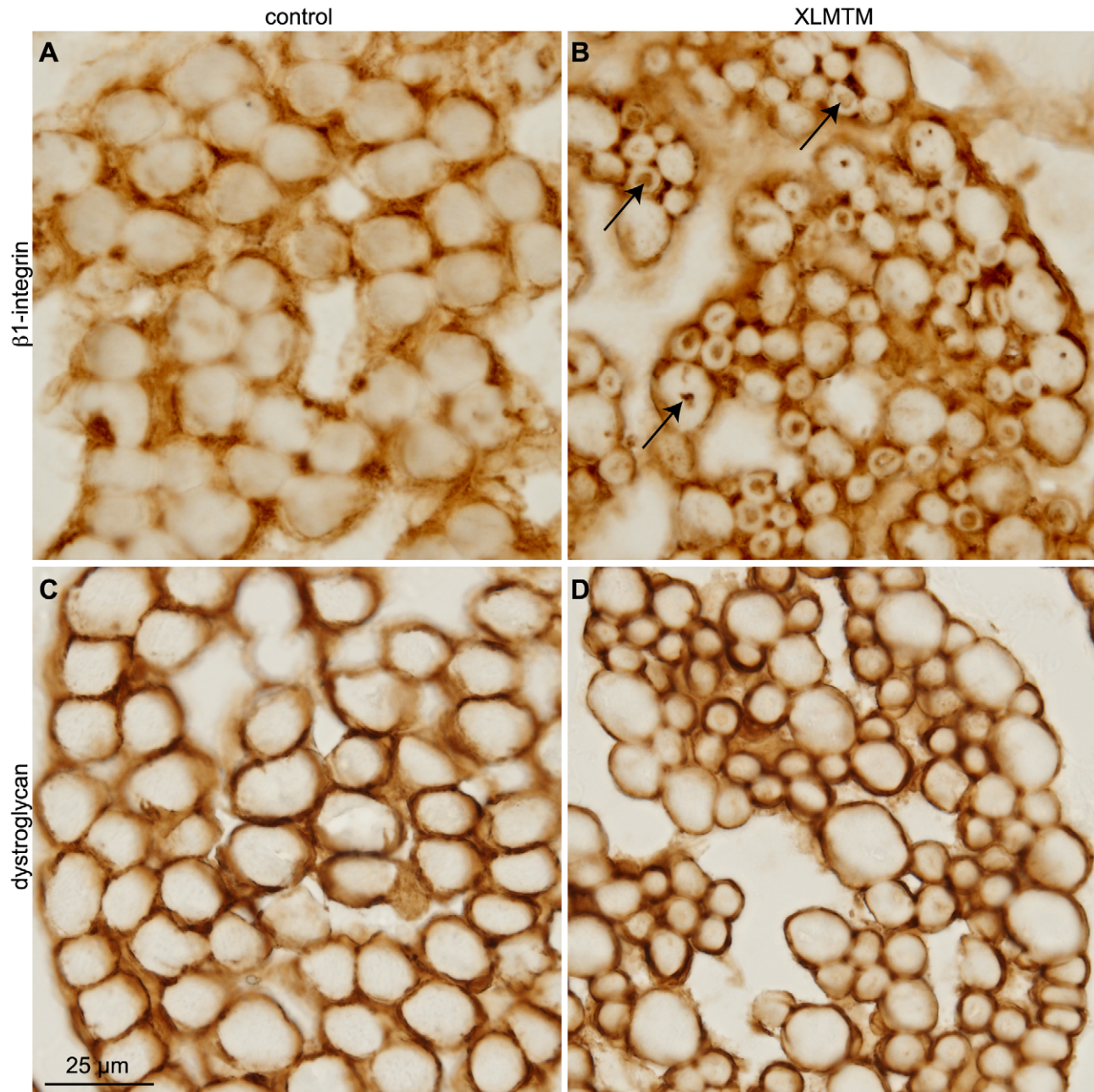


Figure 6. β 1-integrin is mislocalized to perinuclear inclusions in XLMTM myopathy. Cryosections from XLMTM (n=3) and age-matched control human muscle biopsies were immunostained either with anti- β -dystroglycan or with anti- β 1D-integrin. (A–B) β 1D-integrin was found only along the sarcolemma in 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 μ m.
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* depletion (this study) or *Pi3K68D* 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 *Pi3K68D*, in conjunction with PI3KC2 ability to create PI(3)P *in vivo* [18,20–23], supports the possibility that Pi3K68D could generate a PI(3)P substrate pool acted on by Mtm phosphatase. Alternatively, Pi3K68D could act more distantly on an interrelated phosphoinositide pool. We envision that Pi3K68D 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 β 1-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 PKC ϵ 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 that 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 β PS-integrin at T-tubules, and the dual requirements for *mtm*, we found that normal integrin adhesions and abnormal β PS-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 with Dlg1/SAP-97 and Dlg4/PSD-95 to promote postsynaptic function [45,46]. Thus, the shared accumulation of β PS-integrin, Dlg and Amph on central membrane

inclusions in *mtm*-depleted myofibers, and their elimination with *Pi3K68D* co-depletion, points to a possible role for a 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 β PS-integrin turnover through endocytic trafficking led us to uncover a previously untested defect in β 1-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 *Pi3K68D* 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 CMT4B 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: *mtm* ^{Δ 77}, *mtm*²⁻⁴⁷⁴⁷ and RNAi hairpins *w*; *UAS-IR-mtm*³⁻¹ and *w*; *UAS-IR-mtm*³⁻⁵ interchangeably or in combination when “2x IR-mtm” noted, *w*; *UAS-mtm:GFP*⁷ and *w*; *UAS-GFP:MTMR2* [18]; *UAS-IR-Pi3K68D*¹⁶²⁴⁰ and *UAS-IR-Vps34*¹⁰⁰²⁹⁶ (VDR); *UAS-Vps34-KD*^{m8} [49]; *How*^{24B}-*GALA*, *DMef2-GALA*, *UAS-Dcr2*; *DMef2-GALA*, *UAS-2xeGFP*^{1H2}, *mys*¹ and *mys*^{ts1} (Bloomington); *ij*³ (F. Schöck); *Ubi- β PS-integrin:YFP* [6]; *amph*²⁶ [28]; *w*, *dlg1:GFP*^{1C0005} (FlyTrap, [50]); *UAS-GFP:LAMP* [51]; *UAS-GFP:Rab5* and *UAS-GFP:myc:2xFYVE* [52]; *UAS-GFP:Rab7* [53]; *UAS-mRFP:PH-FAPPI* (G. Polevoy and J. Brill). *UAS-GFP:Atg8a* [54] and *Ubi-Talin:GFP* [55]. To reduce β PS-integrin function, *mys*¹/*mys*^{ts1} 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-GALA*, *tubulin-Gal80*^{ts}/*+* and *UAS-EGFP*, *UAS-IR-mtm*^{3.1}/*+*; *DMef2-GALA*, *tubulin-Gal80*^{ts}/*+* 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,

UAS-Dcr2; Mef2-GAL4/UAS-EGFP and *UAS-Dcr2; Mef2-GAL4/UAS-EGFP, UAS-IRmtm^{3.1}* flies raised at permissive 18°C were shifted to 29°C at eclosion until adults were analyzed at 6 or 10 days old.

Muscle preparations and immunofluorescence

Staged pharate adults were removed from pupal case fastened to double-sided tape and pinned on a sylgard 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 Fluorsave. Antibodies included Amph Ra29 (C. O'Kane), Dlg 4F3 and βPS-integrin CG.6G11 (Developmental Studies Hybridoma Bank), α-tubulin (Sigma-Aldrich), Zormin B1 (B. Bullard), muscle myosin (D. Kiehart), αPS2-integrin 7A10 [56], anti-Rabenosyn-5 [57] and KDEL (Babraham Institute). Propidium iodide (PI) staining was done on tissue. Pharate adult and adult abdomens were dissected in 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 throughout imaging. Images of abdomen fillets were taken with a Leica DMI 6000B inverted microscope using semi-apochromat 5× objective (N.A. 0.15), of live IOMs with a Zeiss Axiovert 200M using a LD-Plan NeoFluar 20× (N.A. 0.4) objective and of individual myofibers with FV1000 Olympus point scanning confocal using 60× Plan Apo N (N.A. 1.2) and 100× Plan Apo (N.A. 1.45) objectives. Exported TIFFs were handled by Adobe Photoshop or ImageJ software.

Timelapse microscopy

White pupae were positioned on double-sided tape on a coverslip, placed in a petri dish with water-soaked filter and incubated at 25°C. At each time point, the coverslip was flipped over for imaging on Leica DMI 6000B, as above.

FRAP

FRAP was carried out as described [6] in living 3rd instar larvae. Integrin:YFP was heterozygous (*Int/+*) in all experiments. A total of 21 and 19 individual FRAP experiments from multiple larvae were carried out for *int/+* and *mtm^{z2-4747}/mtm^{Δ77};int/+* respectively.

Electron microscopy

Pharate adults dissected and pinned flat were fixed 1 hr. RT (0.1 M sodium phosphate, 3% paraformaldehyde, 2% glutaraldehyde, 2 mM sodium EGTA, 0.1 M sucrose, pH 7.2), postfixed 1 hr. (1% osmium tetroxide in 0.1 M cacodylate buffer) and stained 1 hr. (1% uranyl acetate). Abdomen fillets were embedded in epoxy resin and 70 nm sections were collected on Formvar and carbon-coated copper grids.

Viability and flight assays

To assay viability, pre-cleared vials were counted for surviving adults, dead adults post-eclosion on food and mid-eclosion, dead pharates and dead pupae at 13 or 17 days after egg laying. Flies 1–6 days old were tested for flight at least 24 hours after CO₂ anesthesia, by releasing 12 females in a 2 L cylinder (50.8 cm

high). Flies that landed below 0.6 L (14.6 cm) were scored flightless.

Human muscle studies

8 mM cryosections were obtained from muscle biopsies from 3 genetically confirmed cases of XLMTM and age matched cases without obvious histopathology (Carsten Bonnemann), per institutional IRB accepted protocol. Sections were stained using manufacturers instructions (NovoLink kit, Novocastra) and as previously described [14] using primary antibodies to beta1D integrin (Chemicon; 1:25) and dystroglycan (Novocastra; 1:20).

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. (D,G,H,I,J) Control, *UAS-EGFP/+; DMef2-GAL4/+*. (D',G',H',I,J) RNAi: *UAS-EGFP, UAS-IR-mtm^{3.1}/+; DMef2-GAL4/+*. (C) 24B-GAL4. Control: *UAS-EGFP/24B-GAL4*. RNAi: *UAS-EGFP, UAS-IR-mtm^{3.1}/24B-GAL4*. RNAi with *mtm* cDNA: *UAS-mtm:EGFP, UAS-IR-mtm^{3.1}/24B-GAL4*. RNAi with human *MTMR2* cDNA: *UAS-EGFP:huMTMR2, UAS-IR-mtm^{3.1}/24B-GAL4*. *DMef2-GAL4*. Control: *UAS-EGFP/+; DMef2-GAL4, UAS-LacZ/+*. RNAi: *UAS-EGFP/+; DMef2-GAL4, UAS-IR-mtm^{3.5}/+*. (E, F). 24B-GAL4: Control: *UAS-EGFP/24B-GAL4*. 1x RNAi: *UAS-IR-mtm^{3.1}/24B-GAL4*. 2x RNAi: *UAS-IR-mtm^{3.1}/24B-GAL4; UAS-IR-mtm^{3.5}/+*. *DMef2-GAL4*. Control: *UAS-EGFP/+; DMef2-GAL4/+*. 1x RNAi: *UAS-IR-mtm^{3.1}/+; DMef2-GAL4/+*. 2x RNAi: *UAS-IR-mtm^{3.1}/+; DMef2-GAL4/UAS-IR-mtm^{3.5}*.

Figure 2. (B,C,D,E) Control: *UAS-EGFP/+; DMef2-GAL4/+*. (B',C',D',E') RNAi: *UAS-EGFP, UAS-IR-mtm^{3.1}/+; DMef2-GAL4/+*. (F) Control: *Ubi-βPS-integrin:YFP/+*. mutants: *mtm^{Δ77}/mtm^{z2-4747}/Ubi-βPS-integrin:YFP*. (G,H) *dlg1:GFP*. (I) Control: *UAS-Dcr2; Mef2-GAL4/UAS-EGFP*. RNAi: *UAS-Dcr2; Mef2-GAL4/UAS-EGFP, UAS-IRmtm^{3.1}*.

Figure 3. (B,C) Control: *UAS-EGFP/+; DMef2-GAL4/+*. RNAi: *UAS-EGFP, UAS-IR-mtm^{3.1}/+; DMef2-GAL4/+*. (D, D') *amph²⁶*. (E) *amph²⁶/amph²⁶, UAS-IR-mtm^{3.1}/+; DMef2-GAL4/+*. (F) *mys¹/mys^{Δ51}*.

Figure 4. (A) Control: *UAS-GFP:LAMP/+; DMef2-GAL4/+*. (A'–A") RNAi: *UAS-GFP:LAMP, UAS-IR-mtm^{3.1}/+; DMef2-GAL4/+*. (B) Control: *UAS-GFP:Rab5/+; DMef2-GAL4, UAS-lacZ/+*. (B'–B") RNAi: *UAS-GFP:Rab5/+; DMef2-GAL4, UAS-IRmtm^{3.5}/+*. (C,D) Control: *UAS-GFP:myc:2XFYVE/+; DMef2-GAL4/+*. (C',D',C'',D'") RNAi: *UAS-GFP:myc:2XFYVE, UAS-IR-mtm^{3.1}/+; DMef2-GAL4/+*.

Figure 5. (B,C,D,E,F) Control: *UAS-EGFP/+; DMef2-GAL4, UAS-lacZ/+*. RNAi: *UAS-EGFP/+; DMef2-GAL4, UAS-IR-mtm^{3.5}/+*. (A,A',B,C',C''D,E',E'',F) Single RNAi: *DMef2-GAL4, UAS-lacZ/UAS-IR-Pi3K68D* or *UAS-IR-Vps34/+; DMef2-GAL4, UAS-lacZ/+*. *mtm* co-RNAi: *DMef2-GAL4, UAS-IR-mtm^{3.5}/UAS-IR-Pi3K68D* or *UAS-IR-Vps34/+; DMef2-GAL4, UAS-IR-mtm^{3.5}/+*.

Supporting Information

Figure S1 Mtm maintains myofiber attachments and visceral muscle function. (A) RT-PCR of *mtm* RNAi depletion detected in whole adult thorax. *Rp32L* 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 hairpin. Muscle myosin. (D–D') Pharate ventral abdominal muscles, detached PLMs. F-actin. (A,B,C) Control: *UAS-EGFP/+; DMef2-GAL4/+*. (A',B,C') *mtm* RNAi: *UAS-EGFP, UAS-IR-mtm^{3.1}/+; DMef2-GAL4/+*. (E–F) Ventral PLMs quantified in filleted abdomens with 24B-GAL4 or DMef2-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 ensheathed 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-mtm^{3.1}*. Scale bar 200 μ m.

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 5–7 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–E') 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 (μ m), as measured in Zormin-stained myofibrils. IOMs, control: *UAS-EGFP/+; DMef2-GAL4/+*. RNAi: *UAS-EGFP, UAS-IR-mtm^{3.1}/+; DMef2-GAL4/+*. Scale bars 10 μ m, except D–D', G–G' 1 μ m.

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') Dlg 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/+; DMef2-GAL4/+*. RNAi: *UAS-EGFP, UAS-IR-mtm^{3.1}/+; DMef2-GAL4/+*. Schematic indicat-

ing peripheral central region of myofiber where microscopy z-sections imaged. Scale bars 10 μ m, except D–D' 1 μ m.

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Figure S4 β PS-integrin inclusions specifically accumulate due to a requirement for an *mtm* function during metamorphosis. (A–A') Normal α PS2-integrin localization at IOM MTJs (arrows; green and single channels) and undetected on Dlg-containing inclusions (red) with *mtm* RNAi. Control: *UAS-EGFP/+; DMef2-GAL4, UAS-lacZ/+*. RNAi: *UAS-EGFP/+; DMef2-GAL4, UAS-IR-mtm^{3.5}/+*. (B–B') Normal GFP:Talin localization at IOM MTJs (arrows; green and single channels) and undetected on β PS-integrin inclusions (red) with *mtm* RNAi. DNA, blue. Control: *Ubi-GFP:Talin/+; DMef2-GAL4, UAS-lacZ/+*. RNAi: *Ubi-GFP:Talin/+; DMef2-GAL4, UAS-IR-mtm^{3.5}/+*. (C–C') Normal β PS-integrin localization at larval MTJs (arrows; green and single channels), along peripheral cell surface and in small internal punctae in *OreR* and *mtm^{A77/mtm^{z-2-4747}}* third instar larvae. F-actin, red; DNA, blue. (D–D') *UAS-EGFP, UAS-IR-mtm^{3.1}/+; DMef2-GAL4/+* pharates. (D) Normal IOM attachments when flies raised continuously at 18°C, due to low levels of GAL4 activity. (D') Pharate adults show IOM detachment (arrowheads) when upshifted from 18°C to 29°C for 1–2 days, due to *mtm* RNAi during pupal stages. EGFP. (E) Percent pharate adults with one or more detached IOMS. (F–F') *UAS-EGFP, UAS-IR-mtm^{3.1}/+; DMef2-GAL4, tub>Gal80^{ts}/+* pharates. (F) Normal β PS-integrin localization (red, and single channel) in pharate adult IOM in flies raised continuously at 18°C, due to GAL80^{ts}-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 μ m, except D–D' 200 μ m.

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Figure S5 Membrane inclusions in *mtm*-depleted myofibers are an endosomal-related compartment. (A) Co-localization of β PS-integrin and Amph increased along longitudinal T-tubules in IOM central periphery regions with *mtm* knockdown, as determined by Pearson correlation. (B–B') β PS-integrin (red) and Amph (green) infrequently co-localized (arrowheads) in control IOMs (B). With *mtm* RNAi, β PS-integrin and Amph became continuously co-localized along longitudinal T-tubules in central periphery (B', β PS-integrin single channel at right) and showed punctuated co-localization along inclusions (B'', arrowheads). Control: *dlg1:GFP; DMef2-GAL4, UAS-lacZ/+*. RNAi: *dlg1:GFP; DMef2-GAL4, UAS-IR-mtm^{3.5}/+*. (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–E') 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/+; DMef2-GAL4/+*. RNAi: *UAS-EGFP, UAS-IR-mtm^{3.1}/+; DMef2-GAL4/+*. (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; DMef2-GAL4/+*. RNAi: *UAS-EGFP, UAS-IR-mtm^{3.1}/UAS-mRFP:PH-FAPP1; DMef2-GAL4/+*. (E–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/+; DMef2-GAL4, UAS-lacZ/+*. RNAi: *UAS-GFP:Atg8a/+; DMef2-GAL4, UAS-IR-mtm^{3.5}/+*. (H–H') Small punctae positive for Rbsn5 (green) throughout IOMs, with infrequent Rbsn5 co-localized with integrin (red) on inclusions with *mtm* RNAi (H'). Control: *UAS-EGFP/+; DMef2-GAL4/+*. RNAi: *UAS-EGFP, UAS-IR-mtm^{3.1}/+; DMef2-GAL4/+*. EGFP not depicted. (I) In control IOMs, GFP:Rab7 (green) detected as small particles throughout myofiber periphery and in larger clusters in perinuclear region. (I') In *mtm* RNAi IOMs, GFP:Rab7 is absent from periphery and does not colocalize on inclusions. Control: *UAS-GFP:Rab7/+; DMef2-GAL4, UAS-lacZ/+*. RNAi: *UAS-GFP:Rab7/+; DMef2-GAL4, UAS-IR-mtm^{3.5}/+*. β PS-integrin, red; DNA, blue. Scale bars 10 μ m. Found at: doi:10.1371/journal.pgen.1001295.s005 (6.43 MB TIF)

Figure S6 Lethality and Dlg inclusions with muscle-specific *mtm* RNAi are rescued by *Pi3K68D* co-depletion. (A) Percent viable and lethal staged progeny at 17 days after staged egg lays for *Dcr2; DMef2-GAL4* single and double RNAi hairpin conditions, as shown; ≥ 3 experiments. Disruption of class II or III PI3-kinases alone (*IR-Pi3K68D* hairpin and *Vps34-KD*, kinase dead) did not alter viability. Co-disruption of *Pi3K68D* or *Vps34* with *mtm* co-RNAi, respectively, restored or enhanced lethality and delayed development. (B) RT-PCR of *Pi3K68D* depletion and *GAPDH2* control detected in adult abdomens with *DMef2-GAL4* muscle-driven *Pi3K68D* 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) Percentage of flightless adults, aged 1–6

days old, from ≥ 5 experiments (semi-lethality prevented equivalent numbers from *IRmtm, Vps34-KD*). (E) Percentage 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–F') Pharate abdominal muscles, F-actin. (F) *IR-Atg1* alone and (F') with *mtm* co-RNAi. Arrowheads, detached IOMs. (G–G') Sarcolemmal β PS-integrin no longer detected at costameres; (G) *IR-Atg1* alone and (G') with *mtm* co-RNAi. (H–H') β PS-integrin central z -sections; (H) no inclusions detected with *IR-Atg1* alone and (H') inclusions present with *mtm* co-RNAi. (F–H') Control: *UAS-IR-Atg1/+; DMef2-GAL4, UAS-lacZ/+*. RNAi: *UAS-IR-Atg1/+; DMef2-GAL4, UAS-IR-mtm^{3.5}/+*. Scale bars, F–F' 200 μ m, G–H' 10 μ m.

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Author Contributions

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

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