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Integrin-mediated adhesion maintains sarcomeric integrity

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ABSTRACT

Integrin-mediated adhesion to the ECM is essential for normal development of animal tissues. During muscle development, integrins provide the structural stability required to construct such a highly tensile, force generating tissue. Mutations that disrupt integrin-mediated adhesion in skeletal muscles give rise to a myopathy in humans and mice. To determine if this is due to defects in formation or defects in maintenance of muscle tissue, we used an inducible, targeted RNAi based approach to disrupt integrin-mediated adhesion in fully formed adult fly muscles. A decrease in integrin-mediated adhesion in adult muscles led to a progressive loss of muscle function due to a failure to maintain normal sarcomeric cytoarchitecture. This defect was due to a gradual, age dependent disorganization of the sarcomeric actin, Z-line, and M-line. Electron microscopic analysis showed that reduction in integrin-mediated adhesion resulted in detachment of actin filaments from the Z-lines, separation of the Z-lines from the membrane, and eventually to disintegration of the Z-lines. Our results show that integrin-mediated adhesion is essential for maintaining sarcomeric integrity and illustrate that the seemingly stable adhesive contacts underlying sarcomeric architecture are inherently dynamic.

Introduction

In multicellular organisms, cell adhesion plays an important role in the development and morphogenesis of highly complex three-dimensional patterns and tissues (Hynes, 2002). In addition, following embryonic development, cell adhesion is essential for maintaining the integrity of differentiated tissues throughout the life of the organism. To achieve and maintain complex tissue organization, cells must form both transient and stable adhesive contacts with each other (Cell–Cell adhesion) and with their extracellular environment (cell adhesion to the extracellular matrix (ECM), or Cell–ECM adhesion). The most common family of adhesion receptors that mediate Cell–ECM adhesion in animals are the integrins (Hynes, 2002). Integrins are essential for establishing tissue integrity in animals, and depletion of integrins at early stages of animal development results in early lethality (Bokel and Brown, 2002; Brown et al., 2000; Hynes, 2002). This has meant that analyzing the roles of integrin-mediated adhesion in the long-term maintenance of tissues requiring integrins to form and differentiate has remained a challenge.

Integrin-mediated adhesion is required during muscle development and integrins are the core components of two different types of adhesive structures in muscles, myotendinous junctions (MTJs) and costameres (Mayer, 2003). MTJs are specialized sites at the surface of muscle fibers where force is transmitted between muscle and tendon. Costameres are a complex protein network that forms the physical attachment between the Z-lines of the muscle cell and its surrounding ECM (Samarel, 2005).

Depletion of integrin-mediated adhesion during muscle development results in impaired MTJs and defective adhesion between muscles and tendons (Bokel and Brown, 2002; Schwander et al., 2003). The fly equivalent of the MTJs, muscle attachment sites, also require integrin-mediated adhesion during muscle development (Brown et al., 2000). Flies that lack integrins as well as integrin-associated proteins such as talin, ILK, and PINCH, exhibit detachment of muscles from each other and their epidermal attachment sites (Bokel and Brown, 2002; Brown et al., 2000). Moreover, integrin-mediated adhesion at the costamere is implicated in the development of the sarcomeric architecture (Sparrow and Schock, 2009; Volk et al., 1990). Work in vertebrate cell culture and knockout mice showed that integrins play an important role in the formation of Z-lines (Fassler et al., 1996; Samarel, 2005; Schwander et al., 2003). Similarly, during muscle formation in fly embryos, integrins concentrate at Z-lines, and Z-line formation is defective in the absence of integrins (Sparrow and Schock, 2009; Volk et al., 1990).

The role of integrins in maintaining muscle integrity is less clear. Knockouts of talin1 (Conti et al., 2008), ILK (Gheyara et al., 2007; Wang et al., 2008), and α7 integrin (Mayer et al., 1997) in mouse muscles lead to post-embryonic myopathy due to impairment of function of the MTJs. However, in these studies the gene knockout occurs before muscle development is complete and therefore defects

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can arise during muscle formation that manifest later on. This means that it is difficult to discern roles of integrins in maintenance versus development. To address this problem and study the role of integrins in maintaining muscle integrity, we have utilized RNAi driven gene knockdown coupled with an inducible tissue specific expression system to deplete integrin-mediated function in adult flies after muscle development is complete. This approach is especially powerful since fly muscles express only a single ortholog of the α and β integrins as well as other integrin-associated genes (Brown et al., 2000).

Here we show that integrin-mediated adhesion is essential for maintaining muscle tissue integrity throughout the life of the organism. Moreover, we show that integrin and components of the integrin adhesion complex must be constantly replenished in muscle. Our main finding is that depletion of integrin-mediated adhesion leads to disintegration of the sarcomere. These results illustrate that costameres are dynamic structures that have an ongoing role in maintaining sarcomeric integrity. Our experiments allow for clear delineation of roles for integrins in tissue formation and tissue maintenance and point to potential disease mechanisms that underlie human myopathies caused by loss of integrin-mediated adhesion in muscles.

Materials and methods

Fly stocks and genetics

The RNAi lines were obtained from the VDRC (Dietzl et al., 2007). These include UAS-*rhea* RNAi (Transformant IDs 40399 and 40400, Construct ID 12050); UAS-*ilk* RNAi (Transformant ID 16062, Construct ID 6996); UAS-*mys* RNAi (Transformant ID 29612, Construct ID 15002); and UAS-*if* RNAi (Transformant ID 44885, Construct ID 1175). Some RNAi lines were obtained from the RNAi collection at the National Institute of Genetics (Kyoto, Japan) including UAS-*rhea* RNAi (stock IDs 6831R-1, 6831R-2); UAS-*mys* RNAi (stock IDs 1560R-1, 1560R-2); UAS-*if* RNAi (stock IDs 9623R-1, 9623R-2); and UAS-*Actn* RNAi (stock IDs 7762R-1, 7761R-1). To combine the RNAi lines with the ts-GAL80 construct (McGuire et al., 2003), we crossed the virgin females from the RNAi lines to males with the genotype: P{tubP-GAL80^{ts}}9/y ;; Mef2-GAL4 / Mef2-GAL4. The F1 female flies from this cross were raised at 18 °C for the duration of the their development, once the adult flies eclosed they were collected and transferred to 29 °C to induce RNAi expression. NLS-GFP flies were obtained from the Bloomington stock center (BL-1691). For rescue experiments, the lines used were UAS-*rhea* #8 and UAS-*mys* (Tanentzapf et al., 2006).

Immunohistochemistry and microscopy

Antibody staining was carried out according to standard procedures. Experimental and control fly thoraxes were dissected and IFMs exposed. Thoraxes were fixed in 6% formaldehyde in PBS, washed with PBT 4× for 15 min, blocked in 0.2% BSA in PBT, and incubated with primary antibodies O/N at 4 °C. Thoraxes were washed, blocked in BSA, and incubated with the secondary antibodies O/N. IFMs were dissected and mounted on glass slides. For phalloidin staining, thoraxes were fixed in 6% formaldehyde in PBS, washed 4× for 15 min with PBS, and incubated with phalloidin O/N at 4 °C. The primary antibodies used were anti-kettin (KIg16 rat pAb, 1:200, gift of Dr. Belinda Bullard), anti-B1 zormin (KQ07 rabbit pAb, 1:200, gift of Dr. Belinda Bullard), anti-α-actinin (MAC276 rat mAb, 1:200, gift of Dr. Belinda Bullard), and phalloidin Alexa Fluor 488 (Invitrogen). ILK was visualized using the GFP trap line ZCL3111 (Morin et al., 2001). Confocal images were obtained using an Olympus IX81/FV1000 microscope with 10×/0.40, 40×/1.30 oil, and 60X/1.33 oil lenses. Images were processed with Adobe Photoshop.

Measurement of ts-GAL80 function using NLS-GFP

Larvae with the genotype w^{-}; P{w^{+}, UAS-NLS eGFP} ^{ID2} / P{w^{+}, ubiP-GAL80^{ts}}; P{w^{+}, UAS-NLS EGFP}^{ID3} / Mef2-GAL4 were grown at both the permissive and non-permissive temperature for the TARGET system. The larva were collected and washed in PBT. As a control, we used larva of genotype w^{-}, UAS-NLS EGFP ^{ID2} / +; UAS-NLS EGFP ^{ID3} / Mef2-GAL4. Whole 3rd instar larvae were mounted and images were taken of the nuclei.

Viability, geotaxis, and flight assays

For viability assays, 20 food vials with 10–20 freshly eclosed flies were set up at 29 °C and passed daily to fresh vials, and the number of dead flies was recorded. The survival index is the ratio of flies alive at a given data point to the number of flies alive in the vial before being switched to the permissive 29 °C temperature.

Geotaxis assays were carried out as previously described (Leal and Neckameyer, 2002) with modifications. Briefly, approximately 100 flies were tested for each line. The flies were kept in vials containing 10 flies each and regularly passed to fresh vials. Flies were transferred into an empty vial and tapped lightly to knock the flies down to the bottom, and the number of flies that climbed 7 cm within 8 s was recorded. The test was repeated 4 times per vial and an average score per vial was determined. The climbing index is the ratio of a given data point to the score recorded prior to the shift to the permissive temperature of 29 °C.

Flight assays were carried out as previously described (Benzer, 1973) with slight modifications. Briefly, approximately 25 flies per line were analyzed for each data point. The flies were dropped into a 500 ml graduated cylinder marked at 1 cm intervals and coated with paraffin on the inner walls to immobilize the flies. The number of flies stuck in the paraffin at each interval was counted and an average score was calculated, reflecting the flying ability of the flies. The flight index is the ratio of the score from a given data point to the score recorded before switching the flies to the permissive temperature 29 °C.

Statistical analysis of the data was done in the same in way for all of the three assays. Using *t*-tests, or the Mann–Whitney *U*-test if the data were not from a normal distribution, each data point from the various RNAi knockdowns was compared to its appropriate counterpart in the control. The resulting *p*-value indicates how significant the difference is between the control and RNAi knockdown at that time point. All statistical analysis was carried out using the statistical analysis software Prism (GraphPad, La Jolla CA).

Quantitative western blots

Total protein extract was prepared from adult talin RNAi and Mef2-GAL4/+ flies. Blots were probed with anti-talin antibody (Mouse mAb (Brown et al., 2002), 1:500) and anti-actin antibody (Mouse mAb, Ab 8224, abCAM, Cambridge, UK, 1:2000). Quantification of western blots was carried using IR 800 conjugated anti-mouse (LI-COR). Blots probed in this fashion were analyzed using the LI-COR Odyssey linear detection system.

qPCR

Total RNA was isolated from whole flies using TRIzol (Invitrogen) and treated with RNase free DNase (Fermentas). 1000 μg of total RNA was converted to cDNA using the qScript™ cDNA Synthesis Kit (Quanta Biosciences). Subsequently, qPCR was performed using the PerfeCTa™ SYBR® Green FastMIX™ ROX kit (Quanta Biosciences) and primer pairs 5′-TGTGACCGAAAGTATCAATC-3′ and 5′-GTTCTACG-CAATCGAAGTATCC-3′ for talin; and 5′-ACAAACAACTGGGGTTATCAT-3′ and 5′-AGTAGGTTTGTCCTTGCCAATA-3′ for αPS2 integrin. Levels of talin or αPS2 integrin mRNA were normalized to actin5c mRNA using