A Novel Mechanism for Activation of Myosin Regulatory Light Chain by Protein Kinase C-Delta in *Drosophila*

Pooneh Vaziri,* Danielle Ryan,* Christopher A. Johnston,† and Richard M. Cripps*,1
*Department of Biology, San Diego State University, San Diego, California 92182 and †Department of Biology, University of New Mexico, Albuquerque, New Mexico 87131
ORCID ID: 0000-0001-6565-285X (R.M.C.)

ABSTRACT Myosin is an essential motor protein, which in muscle is comprised of two molecules each of myosin heavy-chain (MHC), the essential or alkali myosin light-chain 1 (MLC1), and the regulatory myosin light-chain 2 (MLC2). It has been shown previously that MLC2 phosphorylation at two canonical serine residues is essential for proper flight muscle function in *Drosophila*; however, MLC2 is also phosphorylated at additional residues for which the mechanism and functional significance is not known. We found that a hypomorphic allele of *Pkcδ* causes a flightless phenotype; therefore, we hypothesized that PKCδ phosphorylates MLC2. We rescued flight disability by duplication of the wild-type *Pkcδ* gene. Moreover, MLC2 is hypophosphorylated in *Pkcδ* mutant flies, but it is phosphorylated in rescued animals. Myosin isolated from *Pkcδ* mutant flies shows a reduced actin-activated ATPase activity, and MLC2 in these myosin preparations can be phosphorylated directly by recombinant human PKCδ. The flightless phenotype is characterized by a shortened and disorganized sarcomere phenotype that becomes apparent following eclosion. We conclude that MLC2 is a direct target of phosphorylation by PKCδ, and that this modification is necessary for flight muscle maturation and function.

KEYWORDS Drosophila; myosin light chain; PKC delta; flight muscle; phosphorylation; muscle maturation

THE interaction between actin thin filaments and myosin protein drives muscle contraction. Myosin is a large hexameric motor protein comprised of two heavy chains (MHC) and two pairs of light chains (MLC) (Weeds and Lowey 1971). The two MLCs are important for stabilizing the α-helical neck region of MHC by binding in tandem where the myosin head attaches to the myosin tail (Trybus 1994). In vertebrates, these two proteins are known as the 21.1 kDa MYL1 [myosin light-chain 1 (MLC1), the alkali light chain, or the essential light chain, ELC] and the 18.8 kDa MYL2 [myosin light-chain 2 (MLC2), the regulatory light chain or RLC].

In addition to structural roles in stabilizing myosin, the light chains contribute to the regulation of contraction. Myosin light-chain kinase (MLCK) is a Ca^{2+}/calmodulin-dependent kinase that phosphorylates MLC2 to stimulate the interaction of myosin with actin thin filaments, leading to muscle contraction (Levine *et al.* 1991). Critical targets of MLCK are Ser14 and Ser15 located at the N-terminus of MYL2 (Cole *et al.* 1985; Ikebe and Hartshorne 1985). Phosphorylation of MLC2 at these conserved serine residues is critical for myofibril assembly and muscle development in vertebrates (Sweeney *et al.* 1993). In addition, improper phosphorylation occurs in human diseases including familial hypertrophic cardiomyopathy (FHC) (Szczesna-Cordary *et al.* 2004) and muscular dystrophy, and contributes to aging (Smith *et al.* 2010).

The homolog of human muscle MYL2 in *Drosophila* is encoded by the *Mlc2* gene. *Drosophila* MLC2 contains an amino-terminal segment of 53 residues that is not present in the mammal MLC2 (Parker *et al.* 1985). This proline-alanine rich sequence is similar to the extension of MLC1 in vertebrates (Moore *et al.* 2000). As a consequence of this, the conserved Ser14 and Ser15 residues of vertebrate muscle MLC2 correspond to Ser66 and Ser67 residues in the *Drosophila* protein.

Phosphorylation of MLC2 was first detected in rabbit skeletal muscle by observing four bands on polyacrylamide electrophoresis gels (Perrie *et al.* 1973). Tohtong and associates confirmed the role of MLC2 phosphorylation in *Drosophila* flight muscle

doi: https://doi.org/10.1534/genetics.120.303540
Manuscript received April 13, 2020; accepted for publication August 3, 2020; published Early Online August 4, 2020.
[1]Corresponding author: San Diego State University, BioScience Center, San Diego, CA 92182. E-mail: rcripps@sdsu.edu

function by substituting alanines for serines at residues 66 and 67, and creating mutant flies expressing this engineered allele (*Mlc2*$^{S66A,\ S67A}$) in an *Mlc2* null mutant background. The resulting flies were viable, but had impaired flight ability and reduced power output in the indirect flight muscles (IFMs) (Tohtong *et al.* 1995). Lack of MLC2 phosphorylation at Ser66 and Ser67 also reduced the number of attached cross-bridges, leading to decreased stretch activation and net power output of the IFMs (Dickinson *et al.* 1997). In addition, Ser66 and Ser67 phosphorylation is important for orientation of myosin heads toward the thin filaments, while the N-terminal extension facilitates the prepositioning of the heads and their attachment to actin filaments (Farman *et al.* 2009).

Phosphorylation of *Drosophila* MLC2 increases myosin ATPase activity to enhance flight ability (Takahashi *et al.* 1990); however, the number and regulation of phosphorylation events is incompletely understood. *Stretchin-Mlck* (*Strn-MLCK*) is the *Drosophila* ortholog of vertebrate MLCK, and it shows high sequence homology to the catalytic and regulatory domains of vertebrate smooth muscle and skeletal muscle MLCK. The predicted Strn-MLCK protein also contains fibronectin and immunoglobulin motifs and a calmodulin (CaM)-binding domain (Kojima *et al.* 1996; Tohtong *et al.* 1997). While it is generally accepted that Strn-MLCK phosphorylates MLC2 at S66 and S67, this has not been definitively demonstrated. Related to this, high resolution two-dimensional (2D) SDS-PAGE has verified numerous phosphorylated isoforms of MLC2, more than can be accounted for by modification simply of S66 and S67 (Dickinson *et al.* 1997).

Moore *et al.* (2000) created a truncated version of MLC2 lacking amino acids 2–46 (*Dmlc2*$^{\Delta 2\text{-}46}$), and, when expressed in adult flight muscles, this isoform showed only seven isovariants, compared to 13–16 isoelectric variants in wild-type flies. They concluded from this that several novel phosphorylation sites are localized on the N-terminus of *Drosophila* MLC2. In addition, density comparison of the respective spots on 2D gels demonstrated that MLCK regulates the phosphorylation of ~25% of the native MLC2 protein, while ~70% is activated by other kinases that have yet to be identified (Dickinson *et al.* 1997).

What kinase phosphorylates these other residues on MLC2, and what is the functional significance of this modification? In mammalian nonmuscle cells, cytoplasmic MLC2 phosphorylation can be regulated by the Ca^{2+}/phospholipid dependent kinase, protein kinase C (PKC). Notably, PKC phosphorylates Ser/Thr residues (Thr9 and Ser1 or 2) other than the two major phosphorylated sites (Tan *et al.* 1992). The PKC family is divided into three subfamilies based on their activation manner: the conventional PKCs (cPKC) including α, βI, βII, γ isoforms are regulated by diacylglycerol (DAG) and Ca^{2+}; by contrast, the novel PKCs (nPkc) composed of the δ, ε, η, θ isoforms are regulated only by DAG; and the atypical PKC (aPKC), composed by the ζ and ι/λ isoforms, which are independent of DAG and Ca^{2+}, require phorbol ester for activation (Mellor and Parker 1998). However, it is not clear if these kinases phosphorylate *Drosophila* MLC2, or contribute to muscle function.

A potential regulator of MLC2 phosphorylation apart from Strn-MLCK was identified through analysis of a small deletion within polytene region 11A6-7 of the X chromosome in *Drosophila*. Hotta and associates (Mogami and Hotta 1981) named this deletion mutant *myofibrillar defect* (*mfd*) where the homozygotes were unable to jump or fly, and these mutants also lacked extensive phosphorylation of MLC2. Subsequently, Takahashi *et al.* (1990) showed that *mfd* mutant myosin has a reduced actin-activated ATPase activity compared to normal flies. They concluded that phosphorylation of MLC2 regulates the ATPase activity and has an important role in flight muscle function. The identity of the *mfd* gene was not determined, but it must be distinct from *Strn-MLCK* since *mfd* is X-linked and *Strn-MLCK* is located on chromosome 2.

While the original *mfd* mutant stocks are no longer available, in this study we have identified the gene that most likely corresponds to *mfd* as *Pkcδ*, which is also located in the 11A region of the X chromosome. We show that MLC2 is hypophosphorylated in *Pkcδ*^{e04408} mutants, and these individuals are completely flightless. We rescued the hypophosphorylated phenotype as well as the flight defect by duplication of the wild-type *Pkcδ* gene. Mass spectrometry results also identified two phosphorylated sites in control myosin samples which were not detected in *Pkcδ*^{e04408} mutants. We have also shown that the mutation in *Pkcδ* has a significant effect on the structure of the myofibrils of the indirect flight muscles resulting in shortened sarcomeres. Finally, we show, using purified PKCδ, that it directly phosphorylates, and therefore activates, MLC2.

Materials and Methods

Drosophila stocks and crosses

All flies were raised and maintained on standard fly food (Jazz mix, Fisher) at 25°. Gal4 driver crosses were carried out at 29° for optimal expression. Control *w*^{1118} flies were a gift from Kim Finley. *w*^{1118} *PBac{RB}Pkcδ*^{e04408} (Bl# 18258), *Df(1) BSC543 w*^{1118}*/FM7h/Dp(2;Y)G*, *P{hs-hid}Y* (Bl#25071), *w* Df(1)Ten-a*, *P+PBac{XP3.WH3}Ten-a* (Bl# 41562), *w*^{1118}; *Dp(1;3)DC254*, *PBac{DC254}VK00033* (Bl# 30371), and *Strn-MLCK IR* (Bl# 3189) were from the Bloomington *Drosophila* Stock Center. *MLC2 IR* (VDRC# v104621) was obtained from the Vienna *Drosophila* Resource Center. *Act88F-Gal4*, the tissue-specific Gal4 driver, was used for flight muscle-specific inducible expression (Bryantsev *et al.* 2012). See Table 1 for a full list of *Drosophila* stocks and other reagents.

Flight testing

Between 50 and 70 flies of each genotype, aged 1–2 days old, were analyzed for flight testing. Flies were examined to ensure no wing defects were present, and testing was performed in a flight chamber as described previously (Drummond *et al.*

1991), where flies were scored based on whether they flew upwards, horizontally, downward, or were flightless.

Cryosectioning, immunofluorescence, and microscopy

The protocol described by Morriss *et al.* (2012) was followed for cryosectioning and immunofluorescent staining. Flies were oriented and embedded in Tissue-Tek medium and frozen in liquid nitrogen. An HM525 NX Thermo Scientific cryostat machine was used to generate 10 μm thick sections. Sections were transferred onto slides and fixed using 3.7% formaldehyde for 8 min. Following three washes with PBS containing 0.1% Triton-X 100 (PBTx), samples were incubated in primary antibodies diluted with 1% BSA in PBTx overnight at room temperature for immunostaining. Z-lines were revealed by mouse anti-α-actinin primary antibody (Developmental Studies Hybridoma Bank 2G3-3D7) at 1:50 concentration. Rabbit anti-MLC2 antibody (this study, 1:50) was used for immunostaining of *Drosophila* myosin regulatory light chain. The sections were washed with PBTx the following day and incubated with secondary antibodies for 2 hr at room temperature. The following secondary antibodies were used: Alexa 488-linked goat anti-mouse and Alexa 647-linked anti-rabbit both from Invitrogen (A32723, A32731), diluted 1:400. Actin filaments in muscle cells were labeled with Alexa-568 conjugated phalloidin (A12380; Invitrogen) at a dilution of 1:400 in PBTx. Confocal images were obtained using a Fluoview FV3000 Confocal Laser Scanning Microscope (Olympus).

The images were processed in Adobe Photoshop CC 2018 software and Fiji Image-J version 2.0.0-rc-69/1.52p. Sarcomere lengths were measured using Image-J (Schindelin *et al.* 2012). A straight line was drawn on a 2 μm scalebar, the length of the line (in pixels) was typed in the known distance module, and the setting was saved for all the measurements. To measure the sarcomere length, a straight line was drawn between two nearby Z-lines and pixel number was used to determine length. All values were transferred to a Microsoft excel sheet for statistical analysis.

MLC2 protein expression and generation of anti-MLC2 serum

Anti-MLC2 was generated using a construct derived from the full length cDNA LD22691 (*Drosophila* Genomics Resource Center). To generate a bacterial expression plasmid for MLC2, LD22691 was subjected to PCR using the following primers:

MLC2-pDONR221 attB Forward primer: 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGCCGATGAGAAGAAGAAGGTT-3′

MLC2-pDONR221 attB Reverse primer: 5′- GGGGACCACTTTGTACAAGAAAGCTGGGTCCTAGGCGGCCTCCTCCTCCTC-3′

Gateway cloning (ThermoFisher) was used to recombine the PCR product into pDONR221 using BP clonase; and subsequently into pEXP1 using LR clonase. A sequence-verified pEXP1/Mlc2 plasmid was then transformed into BL21(DE3) competent *Escherichia coli* and cultured under ampicillin selection (100 μg/ml). Two liters of LB were inoculated with starter culture and allowed to grow to an OD_{600} of 0.5–0.7 (∼2 hr), induced with 0.1 M IPTG, and induction was continued for 1 hr. Protein purification was carried out using agarose Ni-NTA affinity purification (Invitrogen) using Pierce Columns for gravity-flow protein purification. Proteins were eluted with 5 ml of 300 mM imidazole in denaturing buffer (50 mM sodium phosphate, 300 mM NaCl, 8 M urea, pH 7.0) and concentrated with a size-exclusion column (Amicon Ultra-15 Centrifugal Filter devices). The concentrated proteins were resolved by SDS-PAGE and stained using Coomassie blue. Since there were proteins in addition to MLC2 in this purified preparation, the appropriate band was cut from the gel and sent to Pro-Sci Inc. for generating polyclonal rabbit antiserum.

Quantitative RT- PCR

Total RNA was isolated from the indirect flight muscles of 1- to 2-day-old adult w^{1118}/Y, $Pkc\delta^{e04408}/Y$, and $Pkc\delta^{e04408}/Y$; *Dp(1;3)DC25/+* males, using RNeasy Mini Kit for RNA Isolation (Qiagen). cDNA was synthesized using the iScript Adv cDNA kit for RT-qPCR (#1725038; Bio-Rad). SsoAdvanced Universal SYBR Green Supermix (#1725271; Bio-Rad) was used to perform qPCR in a CFX96 Real-Time System (Bio-Rad). Relative quantification was standardized to *Act5C* RNA expression. Primers used were as follows:

Act5C-For: 5′-GCAAGTACTCTGTCTGGATC-3′
Act5C-Rev: 5′-CCAGCAGAATCAAGACCATCC-3′
Pkcδ-RD-For: 5′-CGATCGATTGGGGTTTGCTGG-3′
Pkcδ-RD-Rev: 5′-GTGATGTGAGGATTTGTGTAGG-3′

Preparation of protein samples and western blotting

IFMs from w^{1118} and $Pkc\delta^{e04408}$ mutant combinations were dissected from 1- to 2-day-old adult flies and chemically demembranated as described by Cripps and Sparrow (1992). Briefly, the muscles were homogenized in a Triton X-100-containing solution to remove membrane and cytosolic proteins. These are referred to as skinned myofibrils. This homogenate was centrifuged for 5 min to pellet the myofibrils. After removing the supernatant, multiple washes were applied to the pellet, followed by a wash without Triton to remove the detergent from the myofibrils. 1x Laemmli sample buffer was added to the skinned myofibril pellet, heated at 95° followed by centrifugation. The samples were run on a 4−20% SDS polyacrylamide gel (Bio-Rad) and transferred to nitrocellulose membrane. Western blotting was carried out following a standard protocol. Antibodies used were as follows: rabbit anti-MLC2 (1:1000), mouse anti-α-actinin (1:1000, DSHB), rabbit anti-Fln (1:1000, a generous gift from J. Vigoreaux), horseradish peroxidase (HRP) -linked goat anti-mouse IgG (HRP; 1:5,000; Invitrogen), and HRP-linked goat anti-rabbit IgG (1:1000; Invitrogen).

The band intensity quantification was achieved using Image-lab software from Bio-Rad. The intensity of phosphorylated bands were normalized to the intensity of α-actinin bands. We used RC DC Protein assay kit from Bio Rad (#500-0121) to quantify the concentration of total protein in skinned myofibrils. The protocol was followed exactly as mentioned in the manufacturer's instructions and absorbances were read at 750 nm.

Purification of native myosin

Myosin was isolated as described previously (Rao *et al.* 2019). 250 w^{1118} and $Pkc\delta^{e04408}$ flies were aged for 1–2 days and their IFMs were dissected in York modified glycerol (YMG) buffer [20 mM potassium phosphate, pH 7.0, 2 mM $MgCl_2$, 1 mM EGTA, 20 mM dithiothreitol (DTT), protease inhibitor mixture EDTA-free (A37989-Thermo fisher) plus glycerol (50%)]. Dissected muscles were skinned using 1 ml of YMG buffer containing 2% (v/v) Triton X-100 and incubated on ice for 30 min. The myofibril pellets were then centrifuged for 5 min, washed, and resuspended in 1 ml of YMG lacking glycerol, followed by centrifugation. The high-salt myosin extraction buffer (MEB; 1 M KCl, 50 mM potassium phosphate, pH 6.8, 5 mM $MgCl_2$, 0.5 mM EGTA, 10 mM sodium pyrophosphate, 20 mM DTT, and a protease inhibitor mixture) was used for myosin extraction; 82.5 μl of MEB was added very gently and incubated for 15 min at 4°. After centrifuging for 5 min at 4°, the supernatant was transferred to an ultracentrifuge tube containing 980 μl nH_2O plus 10 mM DTT and myosin was allowed to precipitate overnight. The following day, the sample was centrifuged at 100,000 × *g* for 20 min using a Beckman TLA 100.3 rotor, and the pellet washed with wash B buffer (2.4 M KCl, 100 mM histidine, pH 6.8, 0.5 mM EGTA, and 20 mM DTT) and dissolved by pipetting up and down to dislodge any undissolved pellet. The salt concentration was then gradually reduced by adding nH_2O containing 8 mM DTT to the pellet to precipitate actomyosin. After centrifugation, the supernatant was transferred to a new ultracentrifuge tube containing nH_2O with 10 mM DTT and myosin pellets were generated after centrifuging at 100,000 × *g* for 25 min. Lastly, the purified myosin pellet was dissolved in myosin storage buffer (0.5 M KCl, 20 mM MOPS, pH 7.0, 2 mM $MgCl_2$, and 20 mM DTT). Myosin concentration was measured using a NANODROP 2000 Spectrophotometer (ThermoScientific).

Tandem mass spectrometry

Purified myosin from w^{1118} and $Pkc\delta^{e04408}$ flies was electrophoresed on standard protein gels and stained with Coomassie blue overnight. After destaining the gel with nH_2O for 4 hr, the bands corresponding to MLC2 were excised and delivered to the Biomolecular and Proteomics Mass Spectrometry Facility at the University of California, San Diego for mass spectrometry analysis. The protein coverage and posttranslational modifications including phosphorylation were assigned using Peaks Studio software.

ATPase assays

ATPase assays were performed using the EnzCheck Phosphate Assay kit (E6646-ThermoFisher Scientific). The standard protocol was followed as described by Das *et al.* (2019). In short, 10 μg of purified myosin was used for each reaction, and the final concentration of 2 μM rabbit actin (A2522; Sigma) was added. After incubating for 10 min at room temperature, 400 μM ATP (R1441-Thermo Scientific) was added to the mixture and the absorbance was measured at 360 nm at intervals of 6 sec for a 1-hr time period. The change in the absorbance at 360 nm indicates the accumulation of inorganic phosphate produced in the reaction as a result of ATPase activity. A blank reaction was carried out without the addition of actin. A Varioskan LUX plate reader from Thermo Scientific was used for this experiment. Relative ATPase activity was calculated by SkanIt Software 6.0.1, and all the data exported to Microsoft Excel.

Kinase assays

Purified myosin (2 μg) and skinned myofibrils (1.3 μg) were used as substrates for the *in vitro* kinase assays. Human *Pkcδ* enzyme (PRKCD Invitrogen A41911) was used as an active kinase. The protocol was as described by Dewey *et al.* (2015) using a PKC lipid activator (#20-133; Millipore) with a dilution of 1:5 added to the mixture. After incubating at 30° for 30 min, samples were resolved by SDS-PAGE, and dried gels were subjected to autoradiography using Kodak BioMax MS film in a Konica SRX-101A developer.

Statistical analysis

Data were analyzed using parametric, unpaired *t*-tests, using GraphPad prism software. Error bars are presented as standard error of the mean. *P*-values ≤ 0.05 are considered significant, marked by asterisks.

Data availability

The authors affirm that all data necessary for confirming the conclusions of this article are represented fully within the article and its tables and figures.

Results

Generation and validation of a MLC2 antiserum

To test the role of MLC2 phosphorylation in indirect flight muscles in *Drosophila*, we first generated an anti-MLC2 antiserum. To verify the antibody, we performed western blots of proteins from dissected and chemically demembranated "skinned" muscles, and immunofluorescence analysis of tissue sections.

For the wild-type samples in western blots, two bands were detected by anti-MLC2 (Figure 1A, lane 1). We hypothesized that the upper band should correspond to the multiply phosphorylated versions of MLC2 protein. This is based on previous observations that phosphorylation of MLC2 at one or two sites does not significantly affect its mobility in SDS-PAGE

Table 1 Regent table

Data type	Experimental species	Symbol/name used in publication	Source – public	Source – published	Source – unpublished	Identifiers	New reagent	Comments
Gene (source not applicable), strain, strain_background	*Drosophila melanogaster*	NA	NA	NA	NA			
	w^{1118}		NA		Gift from Dr. Finley			
	w1118 PBac{RB} Pkcδe04408	*$Pkcδ^{e04408}$*	Bloomington *Drosophila* Stock Center					
	Df(1)BSC543 w1118/FM7h/ Dp(2;Y)G, P{hs-hid}Y	Df(1)BSC543	Bloomington *Drosophila* Stock Center					
	w* Df(1)Ten-a, P+PBac {XP3.WH3}Ten-a	Df(1)Ten-a	Bloomington *Drosophila* Stock Center					
	w1118; Dp(1;3) DC254, PBac {DC254} VK00033	Dp(1;3)DC254	Bloomington *Drosophila* Stock Center					
	Strn-MLCKIR	StrnIR	Bloomington *Drosophila* Stock Center	PMID: 23271733				
	MLC2IR	Mlc2IR	Vienna *Drosophila* Resource Center					
	Act88F-Gal4	Act88F	NA	PMID: 22008792				
Genetic reagent (in whole organism)								
Cell line								
Transfected construct (in cell line)								
Antibody	Anti-α-actinin	a-actinin	Developmental Studies Hybridoma Bank					
	Anti-MLC2	MLC2	This study					
	Alexa-568 conjugated phalloidin	F-actin	Invitrogen					
	Alexa 488-linked goat anti-mous		Invitrogen					
	Alexa 647-linked anti-rabbit		Invitrogen					
	Anti-Fln	Fln			Gift from Dr. Vigoreaux			
	Goat anti-mouse IgG Horseradish peroxidase		Invitrogen					
	Goat anti-rabbit IgG HRP		Invitrogen					
Recombinant DNA reagent	LD22691		*Drosophila* Genomics Resource Center					
Sequence-based reagent								
Peptide, recombinant protein	PRKCD (PKC delta) recombinant human protein	Human PKCδ	Invitrogen					
	Alkaline phosphatase	AP	Sigma					
Large-scale dataset								
Commercial assay	NA							
Chemical compound, drug	NA							
Software, algorithm	NA							
Other								

gels, but multiple phosphorylation events retard its mobility (Dickinson *et al.* 1997; Takahashi *et al.* 1990). When *Mlc2* expression was knocked down by crossing the flight muscle driver *Act88F-Gal4* to *UAS-Mlc2 RNAi*, referred to as *Mlc2 knockdown (KD)*, the intensity of both bands in the western blot was strongly diminished (Figure 1A, lane 2), and the flies were flightless (Figure 1B).

Similarly, cryosections of adult flies immunofluorescently stained with anti-MLC2 showed a strong muscle-specific signal in control animals, which was located to the A-band of the

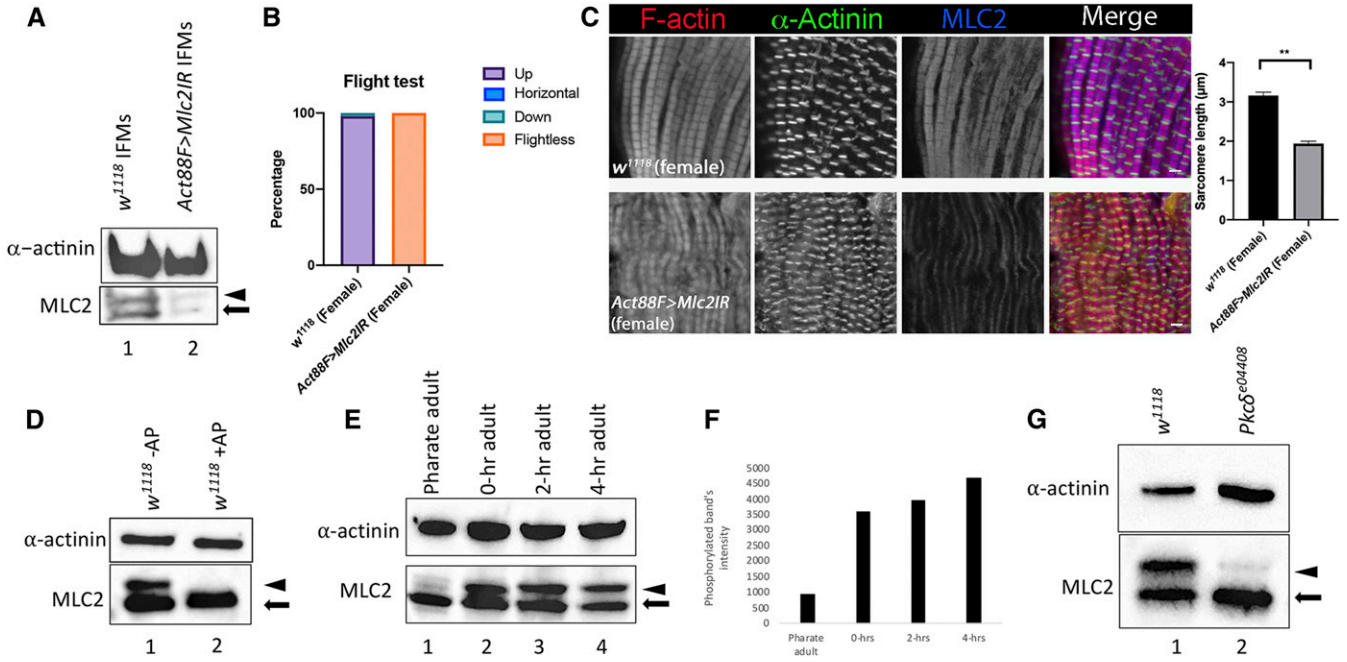

Figure 1 Generation and validation of an MLC2 antibody. (A) MLC2 antiserum detects two protein bands on western blots of wild-type *(w1118)* flight muscles (arrowhead and arrow), but the intensity of these bands is strongly reduced in *Mlc2 KD (MLC2IR)* flight muscles. (B) Percentage of flight ability in wild type and MLC2 knockdown individuals. *Mlc2 KD* adults are completely flightless. (C) Immunofluorescent labeling of control and *Mlc2 KD* adults. Expression of F-actin (Phalloidin; red), α-actinin (anti-α-actinin; green) and MLC2 (anti-MLC2; blue) in adult IFMs. Note the strong MLC2 signal in controls, which is strongly reduced by *Mlc2 KD*; also, sarcomere structure is abnormal in the knockdowns and the sarcomere length is significantly reduced (bar graph). (D) Phosphatase assay revealed that the upper (slower migrating) MLC2 band (arrowhead) disappeared following treatment with alkaline phosphatase, suggesting that the lower band (arrow) is the nonphosphorylated or hypophosphorylated isoform. (E) Western blot experiment for different stages of *Drosophila* adults indicated that MLC2 posttranslational modification occurs right after eclosion. (F) Quantitative western blot analysis of experiment in (E). (G) Western blot experiment for wild type and *Pkcδ* mutant flight muscle showed the strong reduction of phosphorylation of MLC2 in mutant flies.

myofibrils (Figure 1C). In *Mlc2 KD* samples, the intensity of MLC2 signal was once again strongly reduced. In addition, these muscles showed defects in myofibril organization: the myofibrils of knockdown adults were frayed, and the sarcomere length was significantly shorter in the knockdown animals compared to controls (Figure 1C). These studies validate the specificity of the antiserum that we have generated, and demonstrate that MLC2 is necessary for normal myofibril assembly or maintenance.

To determine if the upper band observed in the western blots corresponded to phosphorylated isoforms of MLC2, we treated purified myofibrils with alkaline phosphatase and analyzed the MLC2 pattern by western blotting. We found that the upper band disappeared after alkaline phosphatase treatment compared to control reactions in which no enzyme was added (Figure 1D), confirming that the slower-migrating band corresponded to phosphorylated MLC2, and that the antiserum recognizes both phosphorylated and nonphosphorylated MLC2 isoforms.

The next question we asked was about the developmental timing of protein modification. We carried out western blotting of control flight muscles, aged from just before eclosion to several hours after eclosion. We found that, prior to eclosion, the MLC2 was not highly phosphorylated; however, the phosphorylation was triggered immediately after eclosion, resulting in robust modification of the MLC2 with in 4 hr of emerging from the pupal case (Figure 1, E and F).

An insertion in the Pkcδ gene causes a lack phosphorylation of MLC2 protein

Hotta and associates reported that a deletion within polytene region 11A6-7 of the X chromosome leads to a flightless phenotype, and they referred to this mutation as *myofibrillar defect* (*mfd*). They also showed that myosin light chain protein lacks phosphorylation in these mutants (Mogami and Hotta 1981; Takahashi *et al.* 1990). We therefore sought to identify the gene that corresponds to *mfd*.

Examination at FlyBase.org of the annotated genes predicted to lie in the 11A region of the *Drosophila* X chromosome revealed only two genes with predicted protein kinase activity: *CG2577* and *Pkcδ*. Whereas *CG2577* is expressed almost exclusively in the adult male testes, *Pkcδ* is expressed more generally. We therefore focused on determining if PKCδ might impact MLC2 phosphorylation. PKCδ is a novel PKC isoform, which, in vertebrates, is activated by DAG (Mellor and Parker 1998). We obtained a $Pkc\delta^{e04408}$ allele with an insertion of a PBac element in an intron close to the 3′ end of the gene. We performed a western blot analysis on the skinned IFMs of control and $Pkc\delta^{e04408}$ homozygotes, and observed that the intensity of the phosphorylated band was

strongly reduced in *Pkcδ*^{e04408} mutant flies (Figure 1G). This observation prompted us to explore in more detail the relationship between *Pkcδ* and MLC2 phosphorylation.

Pkcδ mutants are flightless, and the flightless phenotype can be rescued by duplication of the Pkcδ gene

Pkcδ spans approximately 24 kb of the X chromosome, and overlaps a number of genomic deficiencies and duplications (Figure 2A). We performed flight testing and observed that *Pkcδ*^{e04408} homozygous females and hemizygous males are completely flightless (Figure 2, B and C). To confirm that the flight defect is associated with the insertion into the *Pkcδ* gene, we crossed two deficiency lines to *Pkcδ*^{e04408} flies: *Df(1)BSC543* has a deleted segment of the chromosome from X:11,921,289 to X:12,458,044, which includes the *Pkcδ* gene; and *Df(1)Ten-a* deletes from X:12,144,797 to X: 12,395,692, which does not include the *Pkcδ* gene (Figure 2A). We tested the flight ability of the offspring of those crosses and found out that *Df(1)BSC543/Pkcδ*^{e04408} females were completely flightless, while *Df(1)Ten-a/Pkcδ*^{e04408} females had normal flight ability (Figure 2B).

To rescue the flightless phenotype, we employed *Dp(1;3)DC254*, which has a translocation onto the third chromosome of a short region of the X chromosome (X:12,408,080 to X: 12,511,569) that includes a wild-type copy of *Pkcδ*. We found that *Pkcδ*^{e04408}*/Y*; *Dp(1;3)DC254/+* flies were capable of normal flight compared to *Pkcδ*^{e04408}*/Y* males (Figure 2C). These data demonstrate that the flightless phenotype of the *Pkcδ*^{e04408} strain maps to a small region of the X chromosome that contains only three protein-coding genes: *Pkcδ*, CG15927, and CG15731. *Pkcδ* is the only one of these genes that encodes a kinase.

To determine the impact of *Pkcδ*^{e04408} upon *Pkcδ* expression, we performed quantitative real time PCR experiments to measure the expression level of *Pkcδ* in wild type flies, *Pkcδ*^{e04408}*/Y* mutants, and *Pkcδ*^{e04408}*/Y*; *Dp(1;3)DC254/+* rescue animals. We found that the expression level of *Pkcδ* in *Pkcδ*^{e04408} mutants was significantly reduced compared to controls, whereas the presence of the translocated copy of *Pkcδ* in *Dp(1;3)DC254* increased *Pkcδ* expression in the mutants to greater than wild-type levels (Figure 2D). We also carried out western blotting for these genotypes and stained them with anti-MLC2 and anti-α-actinin. We found that MLC2 protein was hypophosphorylated in *Pkcδ* mutant flies, but the phosphorylation was rescued in *Pkcδ*^{e04408}*/Y*; *Dp(1;3)DC254/+* animals (Figure 2E).

The MLC2 protein of Pkcδ mutant flies is hypophosphorylated compared to wild type

Currently, the only known phosphorylation sites for MLC2 in *Drosophila* are Ser66 and Ser67 residues. Since the *Drosophila* protein has a large N-terminal extension compared to vertebrate MLC2, Ser66 and Ser67 are homologous to residues Ser14 and Ser15 in vertebrate MLC2 (Cole *et al.* 1985). Moreover, the canonical sites are thought to be phosphorylated by *Drosophila* Stretchin-MLCK (Kojima *et al.* 1996). Since numerous phosphorylated isoforms of *Drosophila* MLC2 have been documented using 2D gel electrophoresis (Dickinson *et al.* 1997), there must be additional phosphorylation sites on MLC2. To identify the other possible sites, we used tandem mass spectrometry. We purified total myosin from wild-type and *Pkcδ* mutant flight muscles, and separated the proteins by SDS-PAGE. After staining with Coomassie blue, we excised the MLC2 bands and submitted the samples for mass spectrometry analysis.

Results from the mass spectrometry of wild-type samples identified three new phosphorylated residues: Thr38, Ser55, and Ser70 (Figure 3). Whereas Ser66 phosphorylation was observed at high frequency (14 out of 27 peptides covering the region were phosphorylated), other sites were phosphorylated at lower frequencies. Lower ionization/detection efficiencies of phosphopeptides in the mass spectrometry system can be one possible reason for the low identification of phosphorylated residues. In addition, peptides corresponding to some regions of the protein were not detected (red boxes in Figure 3). Nevertheless, these studies identify new phosphorylated residues for MLC2 that might be targets of PKCδ phosphorylation.

When we analyzed MLC2 from *Pkcδ*^{e04408} mutants, we still observed phosphorylation of Ser66, Ser67, and Ser70 to the same degree as in control samples, but there was no phosphorylation of the other two sites. While on the one hand this result confirms the hypophosphorylation of MLC2 in *Pkcδ*^{e04408} mutants, we note that, since the frequency of phosphorylation for specific residues in the wild-type sample was low, it is still possible that the newly identified residues are modified to some degree in the mutants, but below the levels of detection for our experiment. Nevertheless, we can say that we have identified additional phosphorylation sites for MLC2 in wild-type muscles, that at least one of the canonical sites is still robustly phosphorylated in *Pkcδ*^{e04408} mutants, and that other sites may be hypophosphorylated in the mutants.

Pkcδ mutants show sarcomere defects after eclosion

Next, we characterized the differences at the structural level of myofibrils between wild type and *Pkcδ* pharate adults and 1- to 2-day-old adult flies. We obtained cryosections from control and mutant flies, and immunofluorescently stained them with phalloidin and anti α-actinin to visualize the sarcomeres (Figure 4A). We quantified sarcomere length using Image-J software, and, after applying a statistical *t*-test, we determined that the pharate adult sarcomere length of ~2.4 μm was not significantly different between wild type and *Pkcδ* mutants (Figure 4B). However, when we looked at eclosed adults, the myofibril structure in *Pkcδ* mutants was abnormal (Figure 4C), and sarcomere lengths were significantly shorter in mutants (~2.0 μm) compared to controls (3.5 μm) (Figure 4D). We also examined the sarcomere lengths for *Pkcδ*^{e04408}/Y *Dp(1;3)DC254/+* rescued males, where we observed that the myofibril and sarcomere length defects in adults were rescued (Figure 4, C and D).

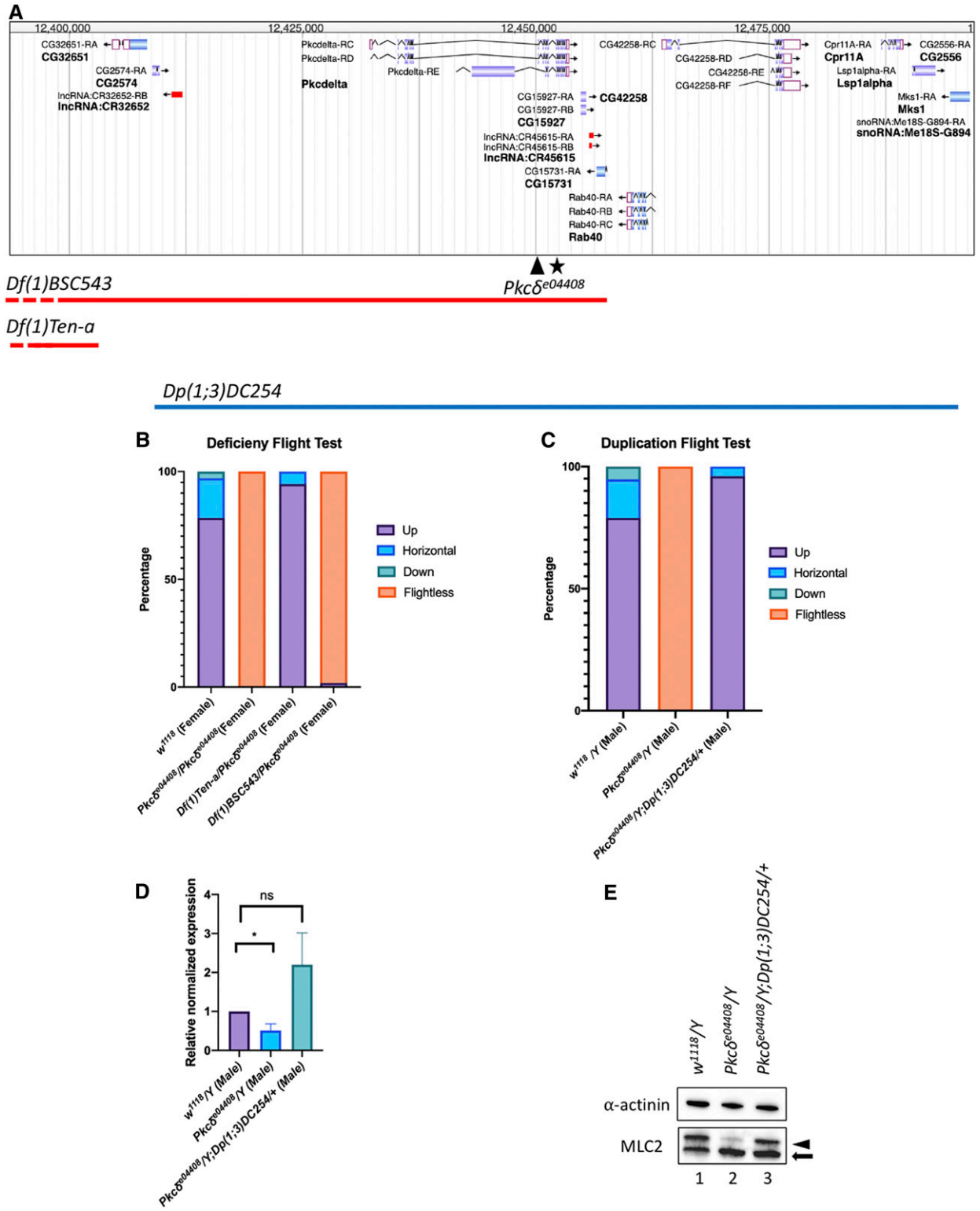

Figure 2 A *Pkcδ* mutant is flightless, which can be rescued by duplication of the *Pkcδ* gene. (A) *Drosophila melanogaster* genome map of *Pkcδ* and surrounding sequences. Red lines indicate regions eliminated by deficiencies and the blue line represents the duplication region in duplication lines. A black triangle indicates the location of the $Pkc\delta^{e04408}$ transgene insertion used in this study, and an asterisk indicates the approximate location of primers used in quantitative RT-PCR. (B) Flight test of deficiency mapping experiment, showing that female $Pkc\delta^{e04408}$ homozygotes are flightless. *Df(1)Tena*/$Pkc\delta^{e04408}$ females fly normally; however, *Df(1)BSC543*/$Pkc\delta^{e04408}$ females are flightless. (C) Duplication of the wild-type *Pkcδ* gene can rescue the flightless defect of $Pkc\delta^{e04408}$/*Y* males. (D) qPCR data using RNA from dissected flight muscles reveals that the expression level of *Pkcδ* is strongly reduced in mutant males compared to controls, and expression is rescued in $Pkc\delta^{e04408}$/*Y*; *Dp(1;3)DC254*/+ IFMs. (E) Western blot analysis of adult flight muscles shows that hypophosphorylation of MLC2 in mutant males is once again phosphorylated in the rescue experiment (arrowhead).

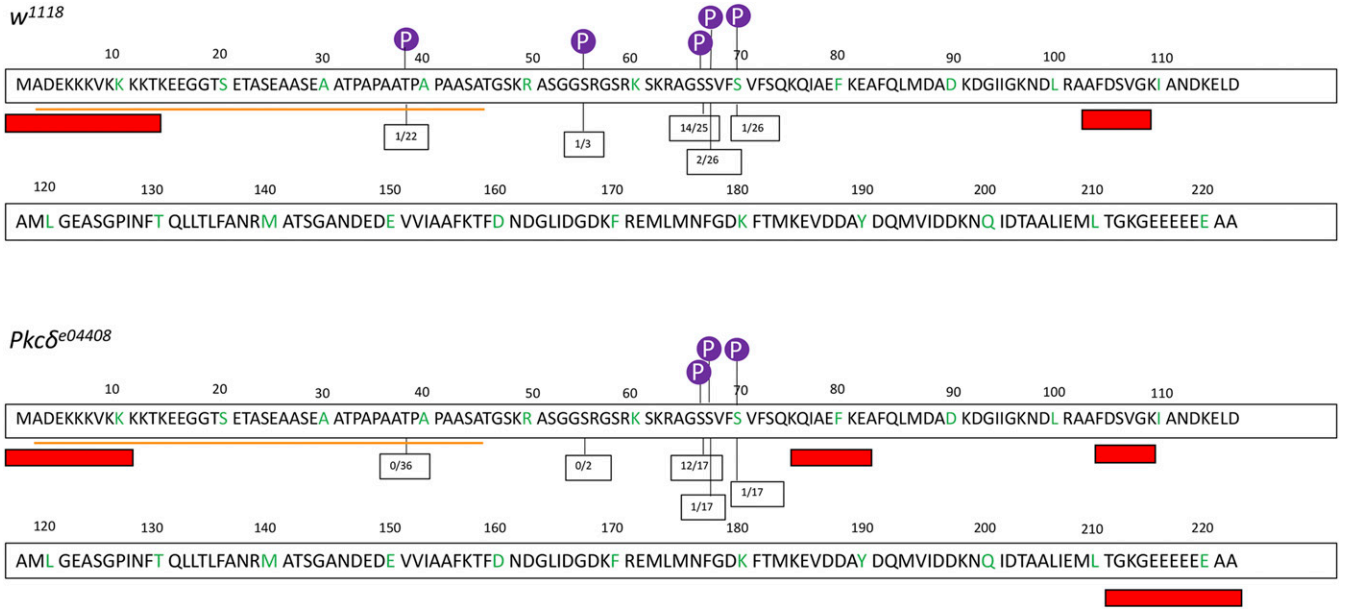

Figure 3 Identification of MLC2 phosphorylated residues in control and mutant samples. Summary of mass spectrometry results for the myosin preparation of control w^{1118} (top) and *Pkcδ* mutants (bottom). In controls, five phosphorylated residues were identified, whereas only three were observed in *Pkcδ* mutants. Red boxes indicate peptide regions that were not recovered. Orange line represents N-terminal extension relative to mammalian MLC2.

Myosin isolated from Pkcδ mutants exhibits decreased actin-activated ATPase activity

We next tested the ATPase activity of $Pkc\delta^{e04408}$ mutant myosin compared to wild-type myosin. Total myosin was extracted and purified from the IFMs of ~150 flies of control and $Pkc\delta^{e04408}$ mutants (Figure 5A). In two replicate experiments, using independently purified myosin, we found that, compared to wild-type myosin, $Pkc\delta^{e04408}$ mutant myosin had ATPase activity reduced by ~40% (Figure 5B). These results mirror the findings of Takahashi *et al.* (1990) who showed that *mfd* mutants also had reduced actin-activated ATPase activity.

Pkcδ directly phosphorylates MLC2

Since *Pkcδ* expression is necessary for MLC2 phosphorylation, we performed kinase assays to reveal if PKCδ accomplishes the phosphorylation directly. We obtained human PKCδ enzyme and PKC lipid activator mix commercially, and combined them with purified myosin from $Pkc\delta^{e04408}$ mutants or skinned IFMs from mutants as substrates in the presence or absence of [γ-^{32}P] ATP.

We tested the kinase assay under different conditions by eliminating substrate for the first reaction (Figure 5C, lane 1). In this reaction we observed a single labeled band at approximately 100 kDa. This band likely corresponds to autophosphorylation of the PKCδ, since the human PKCδ isoform used in our studies has a predicted molecular mass of 112 kDa and the labeled band was not observed in the second reaction that included myosin substrate but excluded enzyme (Figure 5C, lane 2). Next, we combined both the enzyme and purified myosin substrate (Figure 5C, lane 3), and observed a labeled band at ~30 kDa that corresponds to MLC2. The existence of this labeled product was therefore dependent upon PKCδ activity. When we carried out a similar experiment using purified myofibrils isolated from *Pkcδ* mutants, we also observed a faint but reproducible labeled band corresponding to MLC2, whose presence was dependent upon the inclusion in the reaction of purified PKCδ (Figure 5C, lanes 4–5). Overall, these data indicate that PKCδ directly phosphorylates MLC2.

Flightin is phosphorylated in Pkcδ mutant flies and Strn-MLCK is a Fln kinase

To verify if PKCδ is an MLC2-specific kinase or if it can modify other myofibrillar proteins, we analyzed the phosphorylation pattern of another highly expressed muscle protein, Flightin (Fln), in $Pkc\delta^{e04408}$ mutant flies. Fln is a flight muscle-specific thick filament protein required for thick filament assembly, which, like MLC2, is extensively phosphorylated. Interestingly, phosphorylation of Fln increases its mobility in SDS-PAGE gels (Barton *et al.* 2007) compared to MLC2, where phosphorylation reduced its mobility. We therefore tested the hypothesis that Fln may be also phosphorylated by PKCδ. We performed phosphatase assays on skinned flight muscle myofibrils isolated from w^{1118} and compared them to untreated myofibrils from $Pkc\delta^{e04408}$ mutants. The effects of these treatments were analyzed for both Fln and MLC2 by western blotting (Figure 6A). We found that, in controls, the Fln western blot showed a lower (faster migrating) band that was lost upon phosphatase treatment (Figure 6A, lanes 1 and 2, bottom panel). This was consistent with prior studies demonstrating that phosphorylated Fln migrates more rapidly than

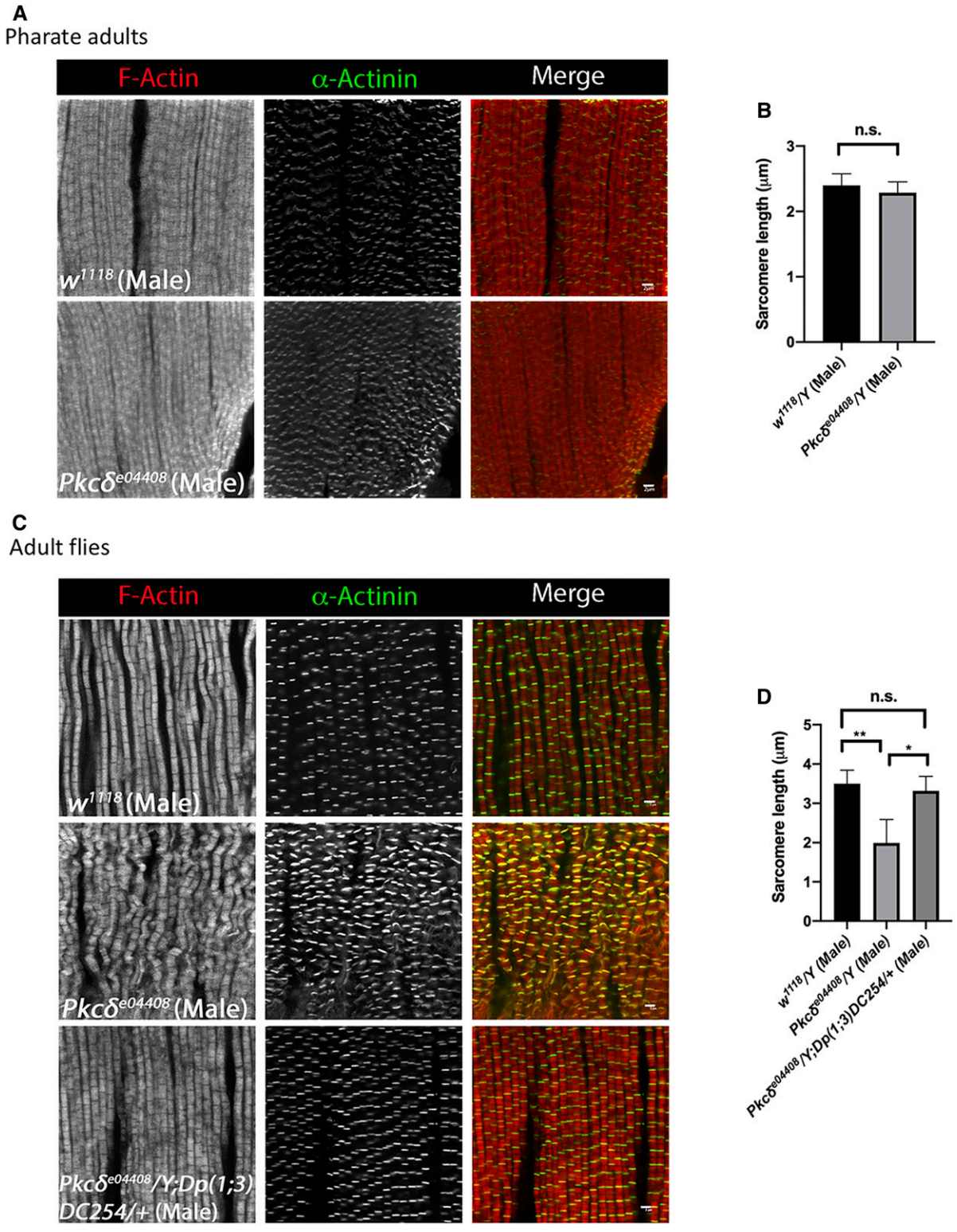

Figure 4 Myofibril defects in *Pkcδ* mutants become apparent after eclosion. (A and B) Confocal microscopy images show that there is no detectable difference at the structural level of myofibrils of indirect flight muscles (IFMs) during pharate adult stage, between w^{1118} and $Pkc\delta^{e04408}$ flies. (C and D) 1- to 2-day-old $Pkc\delta^{e04408}$ flies have shorter sarcomeres (bar graph) and wavy myofibrils. Representative IFMs are labeled by immunofluorescence for α-actinin marking the Z-disc (green), and phalloidin detecting the thin filaments (red). The myofibril and sarcomere defect is rescued in $Pkc\delta^{e04408}/Y$; *Dp(1;3)DC254*/+ males.

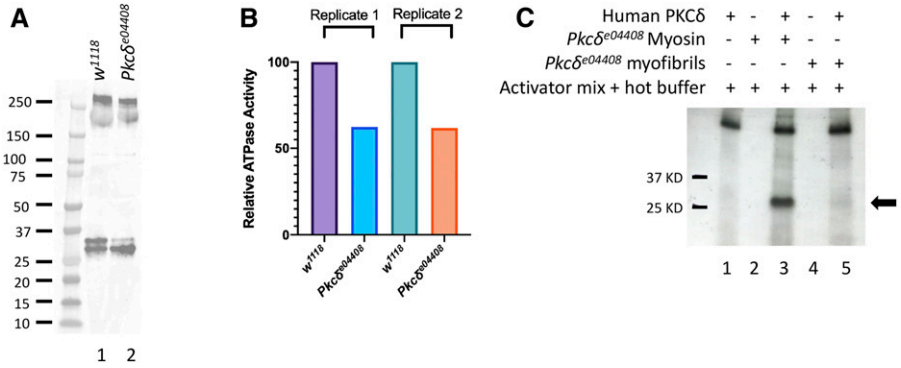

Figure 5 *Pkcδ* mutant myosin exhibits decreased ATPase activity. (A) Coomassie-stained gel of purified myosin isolated from w^{1118} and $Pkc\delta^{e04408}$ mutant flies. The myosin heavy-chain is detected at 250 kDa molecular weight. Sizes of molecular weight standards in kDa are indicated. (B) Two independent replications for ATPase activity assay in w^{1118} and *Pkcδ* mutants are depicted. ATPase activity is reduced in myosin isolated from $Pkc\delta^{e04408}$ flies. The graphs are normalized to the reaction mix without myosin. (C) Recombinant human PKCδ phosphorylates MLC2 directly (arrow). A robust band of the expected size is detected for $Pkc\delta^{e04408}$ purified myosin with addition of human PKCδ activated kinase. A fainter band of the same size was seen for the same experiment in $Pkc\delta^{e04408}$ adult myofibrils.

the nonphosphorylated isoform (Barton *et al.* 2007). We also detected the phosphorylated band in $Pkc\delta^{e04408}$ samples that were not treated with alkaline phosphatase (Figure 6A, lane 3, bottom panel), indicating that PKCδ does not play a major role in Fln phosphorylation. The same samples were also probed for MLC2 accumulation to confirm that the phosphatase enzyme was active (Figure 6, middle panel), and for accumulation of α-actinin as a loading control (Figure 6A, top panel).

Since Fln is phosphorylated in $Pkc\delta^{e04408}$ mutants, we addressed the question as to whether Strn-MLCK is the Fln kinase. We knocked down *Strn-MLCK* expression specifically in the flight muscles using an RNAi line that we had previously validated (Chechenova *et al.* 2013), and performed western blots of control (w^{1118}) and *Strn-MLCK IR* flight muscles. After staining with anti-Fln antibody, we found that the phosphorylated band observed in control samples (Figure 6B, lane 1, bottom panel) disappeared in the *Strn-MLCK* knockdown samples (Figure 6B, lane 2, bottom panel). Interestingly, MLC2 still showed significant levels of phosphorylation in knockdown animals (Figure 6B, lane 2, middle panel), indicating that phosphorylation of MLC2 by Strn-MLCK is not necessary for its phosphorylation by PKCδ.

Discussion

In this paper, we describe the identification of a novel mechanism for activating the regulatory MLC2 in *Drosophila* flight muscles, via the conserved regulatory protein PKCδ. *Pkcδ* is located on the X chromosome, encodes a serine/threonine kinase, and, based on our data, likely corresponds to *mfd* identified by Hotta and associates (Mogami and Hotta 1981). We identify phosphorylated residues in MLC2, and demonstrate that, in addition to the canonical conserved sites Ser66 and Ser67, other regions of MLC2 are phosphorylated. Moreover, these additional MLC2 phosphorylations are critical for normal sarcomere structure and for maximal myosin ATPase activity. Finally, we demonstrate that Pkcδ phosphorylates MLC2 directly and not via activation of other proteins.

Identification of new phosphorylation sites on MLC2

Human muscle myosin light chain (MYL2) has three known phosphorylation sites: Ser15, Ser16, and Thr 52, which are modified by Ca^{2+}/calmodulin-dependent MLCK. Likewise, in *Drosophila*, the two residues orthologous to Ser15 and Ser16, Ser66 and Ser67, are thought to be phosphorylated by Strn-MLCK, and are known to be phosphorylated *in vivo* (Tohtong *et al.*, 1995) (this study). However, we also identify additional modification sites, the regulation mechanism of which was not known until this study. The phosphorylation of additional MLC2 residues is not without precedent: in vertebrate cytoplasmic MLC2, Ser19 is the conserved phosphorylation site for MLCK, while *in vitro* studies have also shown that Rho kinase can phosphorylate Ser19 on MLC2 (Amano *et al.* 1996). In addition, PKC phosphorylates Ser1/2 and Thr9 of the nonmuscle MLC2 (Bresnick 1999). Whether these kinases also modify muscle MLC2 in vertebrates is not known; however, our studies here suggest that muscle MLC2 may be subject to additional regulation by kinases other than MLCK.

Is Pkcδ a direct activator of MLC2?

Our *in vitro* studies using purified human PKCδ and purified *Drosophila* myosin demonstrate that PKCδ directly regulates MLC2, and this would seem to be the most likely mechanism that occurs *in vivo*. We acknowledge the possible existence of a phosphorylation cascade, whereby PKCδ activates another kinase, which, in turn, modifies MLC2. However, this appears unlikely given our *in vitro* kinase data, unless PKCδ modifies a component that copurifies as part of the myosin preparation. Importantly, no additional phosphorylated substrates, other than MLC2 and Pkcδ itself, were observed in our kinase assays; therefore, the action of Pkcδ upon MLC2 must be direct.

These observations also raise the question as to how PKCδ activity itself is regulated. Extensive studies have been carried out investigating the regulation of mammalian PKCδ, which is activated by both small molecules such as Ca^{2+} and DAG, and by other kinases including autoactivation (Stempka *et al.* 1999; Kikkawa *et al.* 2002). We investigated whether any of these upstream regulators might control

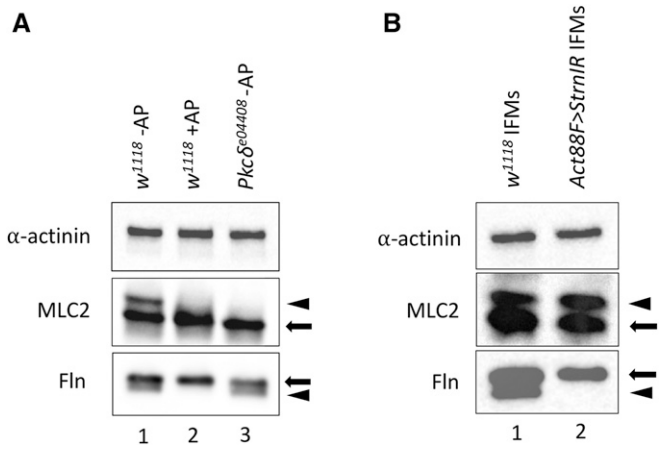

Figure 6 Fln is phosphorylated by Strn-MLCK and not PKCδ. (A) Western blot showing the effects of alkaline phosphatase treatment or *Pkcδ* mutants upon MLC2 and Fln modification. For Fln (bottom panel), there were multiple Fln isoforms in control samples (lane 1, arrow and arrowhead); however, the lower band was lost following treatment with alkaline phosphatase (lane 2, arrowhead), indicating that phosphorylation of Fln increases its migration rate. Fln phosphorylation appeared unchanged in $Pkc\delta^{e04408}$ mutants (lane 3). For MLC2 (middle panel), the more slowly migrating band (arrowhead) represents the phosphorylated isoforms, which were lost after alkaline phosphatase treatment and in the $Pkc\delta^{e04408}$ mutant background. α-actinin is included as a loading control (top panel). (B) Western blot showing the requirement of Strn-MLCK for Fln and MLC2 phosphorylation. For Fln (bottom panel), two Fln bands were detected in wild-type flight muscles (lane 1, arrowhead and arrow), but the lower phosphorylated band was lost in *Strn-MLCK KD* flight muscle (lane 2, arrowhead). The phosphorylation of MLC2 (middle panel) was largely unchanged in the knockdown compared to controls, presumably due to phosphorylation by PKCδ occurring independently of Strn-MLCK activity. α-actinin is included as a loading control (top panel).

PKCδ activity, using flight ability and MLC2 phosphorylation as a readout. However, when we used the flight muscle driver *Act88F-Gal4* to reduce DAG levels by knocking down expression of *Lipin* and *Plc21C*, we did not observe a loss of PCKδ activity. In addition, $Act88F^{KM88}$ null mutants, which show no flight muscle contraction, had normal MLC2 phosphorylation, indicating that activation of PKCδ is not dependent upon muscle use. Finally, we determined if activity of juvenile hormone (JH) at the time of eclosion might activate PKCδ. However, mutant combinations of *Met* and *ap* alleles, which reduce JH production, did not affect MLC2 phosphorylation. It is possible that some of these knockdowns or mutants did not sufficiently impact target gene expression, thereby preventing us from identifying an activator of PKCδ (data not shown). Alternatively a novel mechanism regulates PKCδ activity in the flight muscles.

Pkcδ regulates sarcomere length

During *Drosophila* flight muscle myofibril assembly, sarcomerogenesis starts ~30 hr after puparium formation, where sarcomeres with a length of ~1.7 μm are initially generated (Weitkunat *et al.* 2014). During the last 3 days of pupal development, myofibrillogenesis proceeds and developed muscles occupy much of the thorax. By a few hours following eclosion, sarcomere length of adult flies reaches ~3.5 μm, having required the incorporation of thick and thin filament proteins to extend filament lengths (Reedy and Beall 1993; Mardahl-Dumesnil and Fowler 2001; Spletter *et al.* 2018). We found that *Pkcδ* mutant adults had shorter sarcomeres than controls. One possible explanation is that *Pkcδ* is required for sarcomere growth. Indeed, some studies have reported that MLC kinases have a key role in sarcomere assembly and organization. For instance, MLCK and Rho kinase have roles in regulating the organization of sarcomeric structure in cardiac myocytes (Hoshijima *et al.* 1998; Aoki *et al.* 2000). Moreover, our data from knocking down MLC2 expression (Figure 1) demonstrate that MLC2 is required for normal sarcomere length. On the other hand, mutant sarcomeres may grow normally but the mutant myosin could be dysregulated, resulting in hypercontraction and disassembly of the myofibrils. This scenario seems less likely given that the ATPase activity of mutant myosin is reduced. We also note that PKCδ may phosphorylate other substrates that contribute to myofibril growth or stability.

In our kinase assays, we found that PKCδ could effectively phosphorylate MLC2 as a component of purified myosin; however, phosphorylation of MLC2 in intact myofibrils was not as robust. We estimated the relative amount of MLC2 in each kinase reaction (purified myosin *vs.* intact myofibrils), and found it to be 160 ng in purified myosin as opposed to 15 ng in skinned myofibrils. While the difference in labeling intensity may be due to different contaminants in the myosin *vs.* the myofibril preparations, an interesting alternative explanation is that MLC2 is more accessible to phosphorylation by PKCδ in solution rather than in the myofibril. This raises the possibility that MLC2 molecules present in the cytoplasm around the time of eclosion may be modified prior to incorporation into the myofibril. If this were the case, MLC2 that has been phosphorylated by PKCδ may be incorporated at locations of thick filament growth.

Another myofibrillar component that is extensively phosphorylated is the novel flight muscle protein Flightin (Vigoreaux *et al.* 1993). Multiple phosphorylated Fln isoforms are detected in adult flight muscles, and these isoforms sequentially appear during muscle maturation after eclosion (Vigoreaux and Perry 1994). While the observations on Fln phosphorylation are reminiscent of the modification of MLC2, there are notable differences. First, the time of phosphorylation is different between the two proteins, where Fln phosphorylation follows a much slower time-course. Second, our data demonstrate that Fln is predominantly modified by MLCK (this study), whereas MLC2 is phosphorylated by both MLCK (Kojima *et al.* 1996) and PKCδ (this study).

We have not directly tested for coordination of phosphorylation between Strn-MLCK and PKCδ upon MLC2. One possibility is that phosphorylation by one kinase is necessary for phosphorylation by the other. However, phosphorylation by PKCδ is not likely to be dependent upon Strn-MLCK phosphorylation, because the PKCδ phosphorylation occurs earlier, and phosphorylation of MLC2 is still observed in *Strn-MLKC*

knockdown muscles. Alternatively, phosphorylation of Ser66 and Ser67 by Strn-MLCK may depend on prior modification of MLC2 by PKCδ; however, in the *Pkcδ* hypomorphic mutants, there is no striking change in the levels of phosphorylation of Ser66 and Ser67 of MLC2 based upon our mass spectrometry analysis. Therefore it is more likely that these kinases function independently of each other.

Overall, our findings uncover a new level of regulation in maturing muscles, where distinct kinases function at slightly different times of development to target different proteins and regulate independent processes. How these overlapping functions are regulated to impact muscle maturation and function is not currently known.

Acknowledgments

We thank Jim Vigoreaux for anti-Flightin, Sanford Bernstein for critical reading of the manuscript, and Jennifer Suggs in the Bernstein laboratory for assistance with purification of myosin. We acknowledge technical support from the Molecular Biology Facility at the Department of Biology, University of New Mexico, supported by National Institutes of Health (NIH) grant P20 GM103452 from the Institute Development Award (IDeA) Program of National Institute of General Medical Sciences (NIGMS). This work was supported by R01 GM061738 from the NIH/NIGMS awarded to R.M.C., and R01 GM108756, from the NIH/NIGMS awarded to C.A.J.

Literature Cited

Amano, M., M. Ito, K. Kimura, Y. Fukata, K. Chihara *et al.*, 1996 Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). J. Biol. Chem. 271: 20246–20249. https://doi.org/10.1074/jbc.271.34.20246

Aoki, H., J. Sadoshima, and S. Izumo, 2000 Myosin light chain kinase mediates sarcomere organization during cardiac hypertrophy in vitro. Nat. Med. 6: 183–188. https://doi.org/10.1038/72287

Barton, B., G. Ayer, D. W. Maughan, and J. O. Vigoreaux, 2007 Site directed mutagenesis of Drosophila flightin disrupts phosphorylation and impairs flight muscle structure and mechanics. J. Muscle Res. Cell Motil. 28: 219–230. https://doi.org/10.1007/s10974-007-9120-y

Bresnick, A. R., 1999 Molecular mechanisms of nonmuscle myosin-II regulation. Curr. Opin. Cell Biol. 11: 26–33. https://doi.org/10.1016/S0955-0674(99)80004-0

Bryantsev, A. L., P. W. Baker, T. L. Lovato, M. S. Jaramillo, and R. M. Cripps, 2012 Differential requirements for myocyte enhancer factor-2 during adult myogenesis in Drosophila. Dev. Biol. 361: 191–207. https://doi.org/10.1016/j.ydbio.2011.09.031

Chechenova, M. B., A. L. Bryantsev, and R. M. Cripps, 2013 The Drosophila Z-disc protein Z(210) is an adult muscle isoform of Zasp52, which is required for normal myofibril organization in indirect flight muscles. J. Biol. Chem. 288: 3718–3726. https://doi.org/10.1074/jbc.M112.401794

Cole, H. A., H. S. Griffiths, V. B. Patchell, and S. V. Perry, 1985 Two-site phosphorylation of the phosphorylatable light chain (20-kDa light chain) of chicken gizzard myosin. FEBS Lett. 180: 165–169. https://doi.org/10.1016/0014-5793(85)81064-4

Cripps, R. M., and J. C. Sparrow, 1992 Polymorphism in a Drosophila indirect flight muscle-specific tropomyosin isozyme does not affect flight ability. Biochem. Genet. 30: 159–168. https://doi.org/10.1007/BF02399706

Das, S., P. Kumar, A. Verma, T. K. Maiti, and S. J. Mathew, 2019 Myosin heavy chain mutations that cause Freeman-Sheldon syndrome lead to muscle structural and functional defects in *Drosophila*. Dev. Biol. 449: 90–98.

Dewey, E. B., D. Sanchez, and C. A. Johnston, 2015 Warts phosphorylates mud to promote pins-mediated mitotic spindle orientation in Drosophila, independent of Yorkie. Curr. Biol. 25: 2751–2762. https://doi.org/10.1016/j.cub.2015.09.025

Dickinson, M. H., C. J. Hyatt, F. O. Lehmann, J. R. Moore, M. C. Reedy *et al.*, 1997 Phosphorylation-dependent power output of transgenic flies: an integrated study. Biophys. J. 73: 3122–3134. https://doi.org/10.1016/S0006-3495(97)78338-3

Drummond, D. R., E. S. Hennessey, and J. C. Sparrow, 1991 Characterisation of missense mutations in the Act88F gene of Drosophila melanogaster. Mol. Gen. Genet. 226: 70–80. https://doi.org/10.1007/BF00273589

Farman, G. P., M. S. Miller, M. C. Reedy, F. N. Soto-Adames, J. O. Vigoreaux *et al.*, 2009 Phosphorylation and the N-terminal extension of the regulatory light chain help orient and align the myosin heads in Drosophila flight muscle. J. Struct. Biol. 168: 240–249. https://doi.org/10.1016/j.jsb.2009.07.020

Hoshijima, M., V. P. Sah, Y. Wang, K. R. Chien, and J. H. Brown, 1998 The low molecular weight GTPase Rho regulates myofibril formation and organization in neonatal rat ventricular myocytes. Involvement of Rho kinase. J. Biol. Chem. 273: 7725–7730. https://doi.org/10.1074/jbc.273.13.7725

Ikebe, M., and D. J. Hartshorne, 1985 Phosphorylation of smooth muscle myosin at two distinct sites by myosin light chain kinase. J. Biol. Chem. 260: 10027–10031.

Kikkawa, U., H. Matsuzaki, and T. Yamamoto, 2002 Protein kinase C delta (PKC delta): activation mechanisms and functions. J. Biochem. 132: 831–839. https://doi.org/10.1093/oxfordjournals.jbchem.a003294

Kojima, S., M. Mishima, I. Mabuchi, and Y. Hotta, 1996 A single Drosophila melanogaster myosin light chain kinase gene produces multiple isoforms whose activities are differently regulated. Genes Cells 1: 855–871. https://doi.org/10.1046/j.1365-2443.1996.720272.x

Levine, R. J., P. D. Chantler, R. W. Kensler, and J. L. Woodhead, 1991 Effects of phosphorylation by myosin light chain kinase on the structure of Limulus thick filaments. J. Cell Biol. 113: 563–572. https://doi.org/10.1083/jcb.113.3.563

Mardahl-Dumesnil, M., and V. M. Fowler, 2001 Thin filaments elongate from their pointed ends during myofibril assembly in Drosophila indirect flight muscle. J. Cell Biol. 155: 1043–1053. https://doi.org/10.1083/jcb.200108026

Mellor, H., and P. J. Parker, 1998 The extended protein kinase C superfamily. Biochem. J. 332: 281–292. https://doi.org/10.1042/bj3320281

Mogami, K., and Y. Hotta, 1981 Isolation of Drosophila flightless mutants which affect myofibrillar proteins of indirect flight muscle. Mol. Gen. Genet. 183: 409–417. https://doi.org/10.1007/BF00268758

Moore, J. R., M. H. Dickinson, J. O. Vigoreaux, and D. W. Maughan, 2000 The effect of removing the N-terminal extension of the Drosophila myosin regulatory light chain upon flight ability and the contractile dynamics of indirect flight muscle. Biophys. J. 78: 1431–1440. https://doi.org/10.1016/S0006-3495(00)76696-3

Morriss, G. R., A. L. Bryantsev, M. Chechenova, E. M. LaBeau, T. L. Lovato *et al.*, 2012 Analysis of skeletal muscle development in Drosophila. Methods Mol. Biol. 798: 127–152. https://doi.org/10.1007/978-1-61779-343-1_8

Parker, V. P., S. Falkenthal, and N. Davidson, 1985 Characterization of the myosin light-chain-2 gene of Drosophila melanogaster. Mol. Cell. Biol. 5: 3058–3068. https://doi.org/10.1128/MCB.5.11.3058

Perrie, W. T., L. B. Smillie, and S. B. Perry, 1973 A phosphorylated light-chain component of myosin from skeletal muscle. Biochem. J. 135: 151–164. https://doi.org/10.1042/bj1350151

Rao, D. S., W. A. Kronert, Y. Guo, K. H. Hsu, F. Sarsoza *et al.*, 2019 Reductions in ATPase activity, actin sliding velocity, and myofibril stability yield muscle dysfunction in Drosophila models of myosin-based Freeman-Sheldon syndrome. Mol. Biol. Cell 30: 30–41. https://doi.org/10.1091/mbc.E18-08-0526

Reedy, M. C., and C. Beall, 1993 Ultrastructure of developing flight muscle in Drosophila. I. Assembly of myofibrils. Dev. Biol. 160: 443–465. https://doi.org/10.1006/dbio.1993.1320

Schindelin, J., I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair *et al.*, 2012 Fiji: an open-source platform for biological-image analysis. Nat. Methods 9: 676–682. https://doi.org/10.1038/nmeth.2019

Smith, I. C., J. Huang, J. Quadrilatero, A. R. Tupling, and R. Vandenboom, 2010 Posttetanic potentiation in mdx muscle. J. Muscle Res. Cell Motil. 31: 267–277. https://doi.org/10.1007/s10974-010-9229-2

Spletter, M. L., C. Barz, A. Yeroslaviz, X. Zhang, S. B. Lemke *et al.*, 2018 A transcriptomics resource reveals a transcriptional transition during ordered sarcomere morphogenesis in flight muscle. eLife 7: e34058. https://doi.org/10.7554/eLife.34058

Stempka, L., M. Schnölzer, S. Radke, G. Rincke, F. Marks *et al.*, 1999 Requirements of protein kinase cdelta for catalytic function. Role of glutamic acid 500 and autophosphorylation on serine 643. J. Biol. Chem. 274: 8886–8892. https://doi.org/10.1074/jbc.274.13.8886

Sweeney, H. L., B. F. Bowman, and J. T. Stull, 1993 Myosin light chain phosphorylation in vertebrate striated muscle: regulation and function. Am. J. Physiol. 264: C1085–C1095. https://doi.org/10.1152/ajpcell.1993.264.5.C1085

Szczesna-Cordary, D., G. Guzman, S. S. Ng, and J. Zhao, 2004 Familial hypertrophic cardiomyopathy-linked alterations in Ca2+ binding of human cardiac myosin regulatory light chain affect cardiac muscle contraction. J. Biol. Chem. 279: 3535–3542. https://doi.org/10.1074/jbc.M307092200

Takahashi, S., H. Takano-Ohmuro, K. Maruyama, and Y. Hotta, 1990 Regulation of Drosophila myosin ATPase activity by phosphorylation of myosin light chains–II. Flightless mfd- fly. Comp. Biochem. Physiol. B 95: 183–185. https://doi.org/10.1016/0305-0491(90)90268-X

Tan, J. L., S. Ravid, and J. A. Spudich, 1992 Control of nonmuscle myosins by phosphorylation. Annu. Rev. Biochem. 61: 721–759. https://doi.org/10.1146/annurev.bi.61.070192.003445

Tohtong, R., H. Yamashita, M. Graham, J. Haeberle, A. Simcox *et al.*, 1995 Impairment of muscle function caused by mutations of phosphorylation sites in myosin regulatory light chain. Nature 374: 650–653. https://doi.org/10.1038/374650a0

Tohtong, R., D. Rodriguez, D. Maughan, and A. Simcox, 1997 Analysis of cDNAs encoding Drosophila melanogaster myosin light chain kinase. J. Muscle Res. Cell Motil. 18: 43–56. https://doi.org/10.1023/A:1018676832164

Trybus, K. M., 1994 Role of myosin light chains. J. Muscle Res. Cell Motil. 15: 587–594. https://doi.org/10.1007/BF00121066

Vigoreaux, J. O., and L. M. Perry, 1994 Multiple isoelectric variants of flightin in Drosophila stretch-activated muscles are generated by temporally regulated phosphorylations. J. Muscle Res. Cell Motil. 15: 607–616. https://doi.org/10.1007/BF00121068

Vigoreaux, J. O., J. D. Saide, K. Valgeirsdottir, and M. L. Pardue, 1993 Flightin, a novel myofibrillar protein of Drosophila stretch-activated muscles. J. Cell Biol. 121: 587–598. https://doi.org/10.1083/jcb.121.3.587

Weeds, A. G., and S. Lowey, 1971 Substructure of the myosin molecule. II. The light chains of myosin. J. Mol. Biol. 61: 701–725. https://doi.org/10.1016/0022-2836(71)90074-X

Weitkunat, M., A. Kaya-Çopur, S. W. Grill, and F. Schnorrer, 2014 Tension and force-resistant attachment are essential for myofibrillogenesis in Drosophila flight muscle. Curr. Biol. 24: 705–716. https://doi.org/10.1016/j.cub.2014.02.032

Communicating editor: D. Andrew