


Taking flight: an educational primer for use with “A novel mechanism for activation of myosin regulatory light chain by protein kinase C-delta in *Drosophila*”

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Abstract

Muscles are required for animal movement, feeding, heartbeat, and reproduction. Disruption of muscle function can lead to mobility impairments and diseases like muscular dystrophy and cardiac myopathy; therefore, research in this area has significant implications for public health. Recent work by Vaziri and colleagues has taken genetic, cell biological, and biochemical approaches to identify Protein kinase C-d ($Pkc\delta$) as a novel regulator of the essential myosin light chain 2 (MLC2) by phosphorylation. The authors determine which residues of MLC2 are modified by $Pkc\delta$ and show that phosphorylation by $Pkc\delta$ is required for proper sarcomere assembly and function. This study underscores the importance of *Drosophila melanogaster* as a model system for muscle function and highlights how protein phosphorylation is a vital part of post-translational gene regulation.

Keywords: primer article; muscle; sarcomere; myosin; *Drosophila*; MLC2; $Pkc\delta$; post-translational modification; phosphorylation

Introduction

The animal muscular system is necessary for proper heart function and locomotion, including walking, waving, lifting, smiling, and chewing. This movement is achieved by an underlying scaffold in muscle cells: the sarcomere, an evolutionarily conserved structure which both generates and withstands the force of muscle contraction. The sarcomere is made of a network of proteins including actin and myosin. Myosin is a large, multi-subunit motor protein containing two subunits of myosin heavy chain (MHC), two subunits of myosin light chain 1 (MLC1), and two subunits of myosin light chain 2 (MLC2) (Weeds and Lowey 1971). Vaziri et al. (2020) focuses on the phosphorylation of MLC2 and identifies Protein kinase C-d ($Pkc\delta$) as a key regulator of MLC2. The authors show that loss of $Pkc\delta$ leads to defects in muscle structure and function.

MLC2 and human muscle disease

MLC2 is a protein of interest because, in humans, it is encoded by a gene (*MYL2*) that has been found to be mutated in patients with familial hypertrophic cardiomyopathy (Sheikh et al. 2015; Marston 2018). Familial hypertrophic cardiomyopathy is the most common genetic heart disease in the United States, affecting approximately 1/500 people (Kimura 2008). Hypertrophic cardiac myopathy is characterized by an enlarged heart muscle and disrupted sarcomeres. It can cause heart palpitations, shortness

of breath, fainting, chest pain, and—if left untreated—death. Modification of MLC2 has also been implicated in limb girdle muscular dystrophy, a genetic disease which leads to debilitating decreases in strength and mobility (Liu et al. 2020). In a broad sense, research into the genes and proteins required for muscle function like *MYL2* provides a window to understanding muscle disease and identifying targets for therapies. Studies such as Vaziri et al. (2020) underscore the usefulness of model organisms like *Drosophila*, not just for understanding the basic biology of cells, but also for performing key mechanistic studies that can have important implications for human health.

Examining muscle in *Drosophila*

Flies make an ideal system for the study of muscle function because they share many characteristics and genes with human muscle, combined with all the advantages of a genetically tractable, rapidly reproducing model system (Hales et al. 2015). In both systems muscles are multinucleated fibers innervated by motor neurons, the architecture and proteins of the sarcomere are conserved, and muscle contraction depends upon the release of intracellular calcium (Sweeney and Hammers 2018). Flies provide an especially robust *in vivo* system for quantitatively testing muscle function at multiple developmental stages: for example, larval crawling and adult flying (Drummond et al. 1991; Brooks et al. 2016). This makes it efficient to determine the effects of specific

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genetic, regulatory, or structural changes to the sarcomere on locomotion.

Fruit flies have two sets of muscles during their lifespan: the larval muscles used for crawling, which develop during embryogenesis; and the adult muscles used for flying, walking, and jumping, which develop during pupation (Dobi et al. 2015). The indirect flight muscles (IFMs) develop during the pupal stage and are located inside the thorax. *Drosophila* flight has different mechanics compared to vertebrates like birds. Contrary to preconceived notion, flies do not “flap” their wings like birds or bats do—using their muscles to raise and lower their wings directly (Conley and Lindstedt 2002). IFMs are not attached to the wings; rather, the oscillating contractions of two sets of IFMs deform the thorax itself, leading to the displacement of the wings and the beating motion required for flight. Insect flight muscles are particularly fascinating because they have greater mechanical power needs than other types of animal movement, including bird or mammalian flight, per gram body weight (Maughan and Vigoreaux 2005). The authors of this study take advantage of the IFMs’ robust sarcomere structure which can be easily dissected for visualization with microscopy or for use in biochemical experiments.

Sarcomere structure

Disruption of sarcomere structure has long been shown to be an indicator of both underlying protein defects and poor muscle function (Marston 2018). Sarcomeres are made up of two long, fibrous protein aggregates interwoven together: the actin filaments and myosin motor proteins (reviewed in Henderson et al. 2017). Actin is a cytoskeletal protein that mediates cellular movement and shape changes in addition to muscle contraction. Actin filaments (F-actin) are made up of actin monomers (G-actin) assembled and twisted into a fiber (Figure 1A). In sarcomeres, these

actin filaments are bound by the troponin complex and tropomyosin, which regulate myosin binding to the actin filament in response to calcium. Actin, troponin complex proteins, and tropomyosin, together with additional proteins, comprise the thin filament of the sarcomere.

Myosins are multi-subunit proteins. The largest subunit, MHC, consists of a globular head domain and a helical tail domain (Figure 1B). The head domains of MHC bind in an ATP-dependent manner to actin during muscle contraction. The tail domains of two MHC molecules twist together to make a dimer. At the spot on each MHC where the head and tail domains meet, also known as the “lever arm” region, two smaller MLC proteins bind to stabilize the molecule (Squire 2019). MLC1 is also known as the essential light chain (ELC) and is encoded in vertebrates by *MYL1*. MLC2 is the regulatory light chain (RLC) and is encoded by *MYL2* in vertebrates. Many myosin molecules intertwine to form the thick filament of the sarcomere (Figure 1C). Additional proteins serve to anchor actin and myosin together and add tensile strength to the structure, including alpha-actinin, Zasp, and Titin.

The thin and thick filaments interlace within the sarcomere, their patterning creating dark (electron-dense) and lighter “bands” visible via electron microscopy. The region where thin filaments of one sarcomere are anchored to the thin filaments of the adjacent sarcomere is known as the Z-line, where alpha-actinin and Zasp are localized (see Figure 1D). The thick filament, or A-band, is bidirectional, with the heads toward the Z-line, and the tails pointed toward the center M-band. The M-band is marked by the proteins Myomesin and Obscurin. The region between the Z-line and the edge of the thick filament is the I-band; it is the region where the actin filaments do not overlap with the myosin filaments. During muscle contraction, as the thin filaments slide between the thick filaments, the length of the I-band

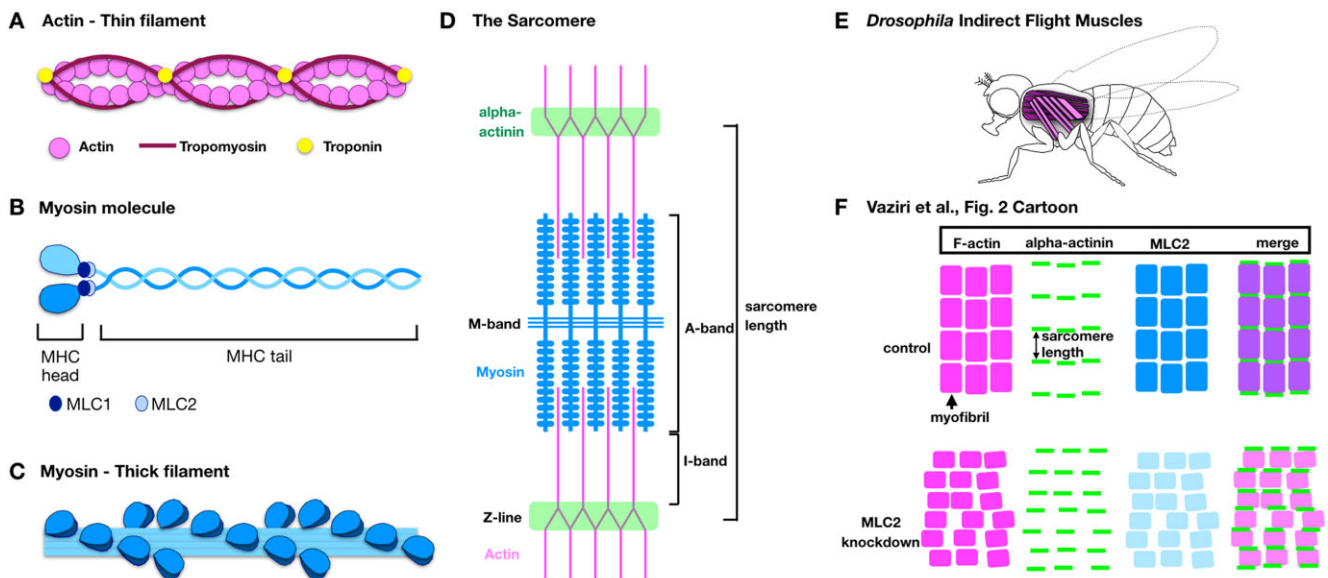


Figure 1 Actin thin filaments and myosin thick filaments make up the sarcomere. (A) Actin monomers (magenta spheres) assemble into a helical filament associated with tropomyosin (dark red) and troponin complex members (yellow). (B) The myosin monomer is a hexamer made up of a dimer of MHC (shades of blue) and two subunits each of myosin light chains 1 and 2 (MLC1 and MLC2). (C) The myosin molecules assemble into a larger filament. The MHC head domains can be seen projecting from the filament; the head domains interact with actin filaments. (D) The sarcomere is an intercalated structure of myosin thick filaments (blue) and actin thin filaments (magenta), anchored and associated with other proteins such as alpha-actinin (green). (E) The indirect flight muscles of the adult fruit fly thorax are shown in shades of magenta (Hartenstein 1993). (F) Cartoon depicting imaging analysis of Vaziri et al., Figure 1C. Myofibrils are stained for actin (magenta), alpha-actinin (green), and MLC2 (blue). In control, myofibrils actin and MLC2 overlap, and the myofibrils appear purple. MLC2-knockdown myofibrils have reduced MLC2 staining and are disorganized. The sarcomere lengths (distance between alpha-actinin bands) are also shorter in these flies.

decreases, while the A-band length remains the same. Thin and thick filaments are held together by the protein Titin. Multiple, repeated units of the sarcomere make up a myofibril, and many myofibrils bundle together to make up an individual muscle cell (sometimes known as a myofiber).

Muscle contraction

Muscle contraction is regulated by both calcium and the ATPase activity of the myosin head (Vandenboom 2016). When a muscle is at rest, tropomyosin and the troponin complex block the myosin binding sites along the actin filament. After signaling releases intracellular calcium from a specialized smooth endoplasmic reticulum, calcium ions bind to troponin. This binding leads to a conformational change exposing the myosin binding sites for actin. ADP-bound myosin heads then bind to actin along the thin filament. Release of ADP and inorganic phosphate causes the myosin heads to rotate, pulling the thin filament along in the “power stroke.” Following this movement, ATP binds to the myosin heads and they unbind from actin. The ATPase activity of myosin cleaves ATP into ADP and inorganic phosphate, readying myosin for the next round of binding and movement. Phosphorylation of MLC2 and its orthologs has been shown to be integral to myosin’s role in muscle contraction and has therefore been implicated as a potential target for disease therapeutics (Takahashi et al. 1990a,b; Takano-Ohmuro et al. 1990; Sweeney et al., 1993; Sheikh et al. 2015). In vertebrates, phosphorylation of MLC2 by myosin light chain kinase (MLCK) on Serines 14 and 15 is necessary for proper myofibril assembly and muscle function (Sweeney et al. 1993). In *Drosophila*, the MLC2 protein is longer and Ser 14 and 15 in vertebrates correspond to Ser 66 and 67 in *Drosophila*. Studies in *Drosophila* have demonstrated that the absence of MLC2 phosphorylation at Serines 66 and 67 leads to a change in the angle of the myosin head with respect to the actin filaments, a reduction of actin binding and an impairment of muscle function (Tohtong et al. 1995; Farman et al. 2009). Evidence suggests that the *Drosophila* ortholog of MLCK, Stretchin-Mlck, phosphorylates Ser 66 and 67, but these are not the only phosphorylated amino acid residues of MLC2 (Dickinson et al. 1997). In mammalian non-muscle cells, cytoplasmic (that is, non-sarcomere localized) MLC2 can be phosphorylated by the Protein Kinase C at Threonine 9 and Ser 1 or 2 (Tan et al. 1992). The authors hypothesize that Pkc δ is an additional kinase that is able to phosphorylate MLC2 in *Drosophila*.

Understanding assays and research techniques

Vaziri and colleagues took a number of experimental approaches to examine the proteins, structure and function of the sarcomeres in indirect flight muscles.

The flight assay

Drosophila provides the opportunity to directly test the effect of genetic mutations on muscle function. In this paper, the authors examined IFM function by testing the ability to fly. One type of flight assay takes advantage of the property of adult fruit flies to generally move toward a light source (also known as positive phototaxis) (Kain et al. 2012). In the flight assay, flies are released into a square, transparent box (flight chamber) from a middle height (Drummond et al. 1991). Flies with good motor ability are expected to fly above their release height, toward the light at the top of the chamber, and they are marked “up.” Flies that remain at approximately the same level as their release are marked

“horizontal,” while flies that go below are marked “down.” Flies that are completely unable to fly, and instead land in a dish at the bottom of the box, are marked “null.” To rule out the possibility that flight may be negatively affected by the age of the flies, all of the experiments are conducted on flies that are 1–2 days old. Large sample sizes (50–70 flies) are tested for each background to control for variability across individuals, and all flies are tested using the same conditions. Knockdown of factors in IFMs to assay muscle function is a useful technique for testing essential genes, since the ability to fly is not required for viability in the laboratory (Bernard et al. 2003; Schnorrer et al. 2010).

Western blotting

Western blotting is a method that allows for the detection of a protein of interest, either as part of a bulk preparation, or following purification or enrichment of a particular protein or protein complex (Jensen 2012; SENS Foundation 2020). In this article, the authors were interested in determining both the protein levels and the phosphorylation state of MLC2. Typically, a tissue or cell sample is homogenized using mechanical homogenization and then the sample is treated with a strong denaturing buffer to remove the protein’s secondary/tertiary structure. The vast majority of Western blotting is performed with denatured protein samples. In the case of Vaziri and colleagues, they dissected whole muscle tissue from adult flies and homogenized the tissue using a pestle in buffer containing strong detergent, which removes membrane and cytosolic proteins. The remaining myofibrils were pelleted in a centrifuge and washed several times to remove contaminating proteins and the strong detergent. Then the myofibrils were denatured in Laemmli sample buffer, which contains high concentrations of a reducing agent (e.g., urea) and a detergent, sodium dodecyl sulfate (SDS). The reducing agent eliminates disulfide structures in the protein, and the SDS alters the surface charge of the protein. The end result is that the proteins in the sample are reduced to a globular shape whose cross-section is directly proportional to its molecular weight. These reduced proteins are then loaded onto a polyacrylamide gel for electrophoresis (the PAGE of SDS-PAGE) and are then resolved as they migrate through the electric field according to the size of this globular shape.

Dyes like Coomassie Blue and Congo Red permit the visualization of all proteins in the gel, the same way ethidium bromide labels nucleic acids in an agarose gel. Both of these dyes allow one to generically discriminate proteins based on size only. To identify specific proteins that are present in the protein gel, however, they must first be transferred, or “blotted,” to a membrane. For the blotting, once the proteins are separated in the gel, the gel is laid on top of a nylon membrane, and a different (perpendicular) electric field is applied to the gel/membrane sandwich. This field migrates the proteins out of the gel and onto the membrane, where they are immobilized and ready for detection.

Detection of proteins in both Western analysis and immunohistochemistry takes advantage of the vertebrate immune system’s defense mechanism for recognizing foreign bodies. For research use, antibodies are raised against an antigen (protein) of interest in animals like rabbits, mice, or goats. Researchers harvest the antibodies produced by the immune cells following injection of the protein of interest. Antibodies recognize and bind to short protein sequences, also called epitopes. The antibody that binds your protein of interest is known as the primary antibody. Bound antibodies can be visualized using either fluorescent or enzymatic methods.

For Western protein detection, the membrane with its immobilized proteins is first immersed in a solution containing an inert protein (e.g., bovine serum albumin) to coat the membrane and immobilized proteins and “block” any nonspecific antibody binding to the proteins on the membrane. Following this blocking step, the membrane is incubated in a solution containing a primary antibody specific for the protein of interest (Figure 3). After a period of time, the membrane is washed to remove excess antibody, leaving behind only those antibodies bound tightly to their targets. After this washing, the membrane is then incubated in a second solution containing a secondary antibody. This secondary antibody is specific for the animal in which the primary antibody was raised. For example, if the primary antibody was raised in a rabbit (like the MLC2 antibody in the Vaziri *et al.* study), then the secondary antibody would be a generic “anti-rabbit protein” antibody.

After the incubation in the secondary antibody solution, the membrane is again washed to remove unbound secondary antibody, and the target protein/primary antibody/secondary antibody complexes are then detected (Figure 3). This method can vary depending on the particular setup in the laboratory. Typically, the secondary antibody is chemically conjugated to a detection molecule. This could be either an enzyme-like horseradish peroxidase, or a fluorescent molecule like fluorescein. For secondary antibodies containing an enzyme (e.g., horseradish peroxidase), the membrane is then incubated in a chemical substrate that is cleaved by the enzyme and the cleaved product is deposited on the membrane, allowing for visualization of the protein. If the secondary antibody uses a fluorescent molecule, then the membrane is scanned using a specialized scanner that can detect the secondary antibody on the membrane by its emission.

It is possible to quantify proteins detected in Western blotting. To enable a quantitative analysis primary antibodies that detect a reference protein—a protein whose level is not expected to change in either control or experimental samples—are included in the primary antibody step. In this case, Vaziri and colleagues used alpha-actinin as their reference protein and observed similar levels of alpha-actinin in both control and experimental samples. Correspondingly, when comparing the level of your protein of interest in either control or experimental samples, a relative difference in protein levels will be reflected in either an increase or decrease in band intensity on the Western blot across the two samples. This difference in band intensity can be quantified using analysis software to quantify relative pixel intensity in the Western blot bands.

Immunofluorescence

Immunofluorescence allows researchers to determine where and when in a tissue a particular protein is expressed. Immunofluorescence is very similar to Western blot detection, using a primary antibody to detect a protein of interest. Unlike Western blots that require grinding up tissue to make a lysate, immunofluorescence is performed on an *in vivo* tissue specimen—either a whole embryo or intact piece of tissue. The tissue is first “fixed” using heat or a chemical like formaldehyde or glutaraldehyde to link proteins to other proteins and set them in place in the specimen. The tissue is then incubated in a solution containing the primary antibody, washed like a Western blot membrane, and incubated with a solution of secondary antibodies, each linked to a fluorescent molecule. After washing off excess secondary antibody, the tissue piece is then mounted on a microscope slide and imaged using light of specific wavelengths to show only the target protein of interest as well as white light to

visualize the entire tissue. This method allows the researcher to see exactly where in the tissue or cell their protein of interest is located. In addition to immunofluorescence, cellular structures can also be visualized using fluorescently labeled dyes that bind molecules directly. In this article, Vaziri and colleagues use fluorescently-conjugated phalloidin (a mushroom toxin that binds actin filaments) to visualize the actin cytoskeleton of muscles.

Mass spectrometry

Protein modification is an important regulatory step in an array of cell processes. Proteins may be modified post-translationally via addition of small molecules (phosphate, acetyl, or methyl groups) or other short proteins (ubiquitin). The addition of phosphate groups to particular amino acid residues of a given protein can modify the activity of that protein’s properties in a reversible manner. While phosphorylation can often be detected as a change in apparent size on a Western blot, this method does not permit identification of the sites where phosphorylation has occurred. A common method to identify such sites is protein mass spectrometry (Creative Proteomics 2018). This powerful technique allows researchers to analyze and identify several protein features based on the mass properties of the protein or fragments of a protein. This technique has a wide variety of applications, ranging from determining the amino acid sequence of an unknown protein, to identifying stable post-translational modifications, such as phosphorylated amino acid residues, in a known protein. The protein of interest is first purified and then cut into a number of smaller peptide fragments. These peptides are then electrically charged, or ionized, in the gas phase by one of several methods, and these ionized peptides are then subjected to an electromagnetic field that separates them based upon mass. Each unique peptide has a distinct mass to charge (m/z) ratio and not every peptide will be observed with the same frequency. Tandem mass spectrometry, the technique used by Vaziri and colleagues, adds a second round of fragmentation, ionization, and separation steps to increase specificity of the experiment and aid in the detection of protein fragments. Protein modifications like phosphorylation alter the mass of the peptide fragment. If the sequence of the protein (MLC2 in this study) is known, then mass variants in the peptides can be detected and modifications can be assigned to particular residues.

Experimental summary

Vaziri and colleagues were interested in examining MLC2 function in indirect flight muscles. Prior work had identified at least one kinase regulating MLC2 in *Drosophila*: Stretchin-Myosin Light Chain Kinase (Strn-MLCK), which phosphorylates MLC2 at the Serine residues located at positions 66 and 67 in the amino acid sequence of the protein (Kojima *et al.* 1996; Tohtong *et al.* 1997; Dickinson *et al.* 1997). However, work from a number of laboratories suggested that there were other modified residues in the amino terminus of MLC2. The central objective of Vaziri *et al.* was to identify the kinase that phosphorylates these additional serine and threonine residues. Previous genetic work in *Drosophila* pointed the authors toward *Pkcδ*, which encodes a member of the Protein Kinase C family. This family of proteins had been shown to be required for MLC2 phosphorylation in mammalian non-muscle cells. This research paper explores the interaction between *Pkcδ* and MLC2 in indirect flight muscle cells.

The authors’ first task was to generate an antibody to detect the fruit fly version of the MLC2 protein and show that the antibody

was specific. They showed that their antibody bound two bands on a Western blot, corresponding to the size and mobility of MLC2 with and without phosphorylation. The authors next wanted to examine the localization of MLC2 using immunofluorescence and show that it is present in IFM sarcomeres. In Vaziri et al. Figure 1C, the authors dissect IFMs from adult flies and use immunofluorescence to visualize the myofibrils of control and MLC2 RNAi knockdown flies. Each IFM is made up of multiple vertically-oriented myofibrils, which are themselves composed of sarcomeres. These myofibrils have been stained with fluorescently-conjugated phalloidin to visualize the actin cytoskeleton, and an antibody that recognizes alpha-actinin to visualize the Z-bands of each sarcomere (diagrammed in Figure 1F). The authors see disruptions in myofibril structure, including an overall wavy and disorganized appearance, and reduction in sarcomere length, calculated as the distance between the short, horizontal Z bands. Taken together, these data indicate an important functional role for MLC2 in sarcomere structure.

Analysis of the prior literature and a search of FlyBase.org (Larkin et al. 2021) strongly suggested Pkc δ as a potential MLC2 kinase in fly muscle. To test this hypothesis, the authors prepared proteins from Pkc δ hypomorphic mutants and showed that MLC2 phosphorylation was significantly reduced in these flies. Moreover, the authors used flight assays to demonstrate that Pkc δ mutants lacked IFM muscle function and were flightless. They consulted a map of the Pkc δ genomic region showing the location of the Pkc δ ^{e04408} mutation, as well as surrounding protein-encoding genes (e.g., Rab40 and Cpr11A) and long noncoding RNAs (lncRNA) (Vaziri et al., Figure 2A). They identified two deletion mutations—also known as deficiencies in *Drosophila*—that remove the Pkc δ coding region and surrounding sequences and performed genetic crosses with stocks carrying these deficiencies. The authors performed a thorough genetic analysis to confirm that the phenotypes they observed were directly due to a loss of Pkc δ function.

To identify the sites of MLC2 phosphorylation in *Drosophila*, the authors purified myosin from control (*w*¹¹¹⁸) and Pkc δ ^{e04408} mutants, separated the MLC2 protein by electrophoresis and performed tandem mass spectrometry analysis on MLC2. These experiments identified peptide fragments of MLC2 where phosphorylation had occurred. The authors additionally

demonstrated that Pkc δ mutants are unable to fly, and showed that these mutants have reduced phosphorylation of MLC2 and aberrant sarcomere structures. These results confirmed that Pkc δ and MLC2 phosphorylation serve important roles in muscle structure. Further tests confirmed the enzymatic role of Pkc δ .

Protein kinases can have multiple targets, even within the same tissue. Having shown that Pkc δ is a direct regulator of MLC2 in muscle, the authors next explored whether Pkc δ modifies any other proteins. One potential target was Flightin (Fln), a muscle-specific thick filament protein that is known to be phosphorylated; however, the authors showed that the phosphorylated form of Fln was still present in Pkc δ mutants (Vigoreaux et al. 1993; Vigoreaux and Perry 1994; Barton et al. 2007). Next, the authors used RNAi-mediated knockdown of the kinase Stretchin (Strn) to show that it, instead of Pkc δ , is required for phosphorylation of Fln. Strn is the *Drosophila* homolog of human myosin light chain kinase (MLCK), and there is evidence to support its regulation of MLC2 by phosphorylation of Ser residues 66 and 67 (Kojima et al. 1996; Tohtong et al. 1997). Intriguingly, the phosphorylated form of MLC2 was still present in a Western analysis of Strn-knockdown flies. Future work will be required to show whether Strn-MLCK phosphorylates MLC2 directly, and to identify any other alternative targets for Pkc δ .

Determining whether a phosphorylation target is direct or indirect is difficult. *In vivo*, the presence of other kinases and regulatory cascade members complicates assignment of functions. Moreover, the interactions between kinases and their targets are brief, and so these interactions resist methods (such as immunoprecipitations or “pull downs”) that would reveal more stable associations, like protein complex formation. Most assays examining direct phosphorylation, then, must be done *in vitro*, controlling for the presence of only the proteins of interest. However, a significant caveat is that because a kinase can phosphorylate a target *in vitro* does not mean that it always does so *in vivo*. Kinases can have different sets of targets and different specificities depending on cell/tissue type and time during the development, and by their very nature *in vitro* assays are performed without this cellular context. Short of complicated assays to detect transient interactions directly, the best approach is to combine evidence of *in vivo* requirement with proof that the enzyme is able to add a phosphate to a particular substrate *in vitro*. Taken together, the work of Vaziri and colleagues demonstrates that Pkc δ regulates MLC2 by phosphorylation on Thr38 and Ser55, and that this regulation is critical for sarcomere assembly and muscle function. Without MLC2 phosphorylation, muscle contraction will be impaired, and flies will not be able to fly. Conservation of this role with human PKC δ positions this as an important protein for further investigation in human cardiac and somatic muscle diseases.

Suggestions for classroom use

This primer is designed to enable undergraduate students to critically read the original research of Vaziri et al. (2020); it can be supplemented by Hales et al. (2015) “Genetics on the Fly: A Primer on the *Drosophila* Model System” for courses in Molecular/Cellular Biology, Genetics, Biochemistry, Development, Physiology, or Biotechnology. It could be assigned to accompany discussions about complementation testing, cytoskeletal proteins and motors, sarcomere structure, or muscle contraction.

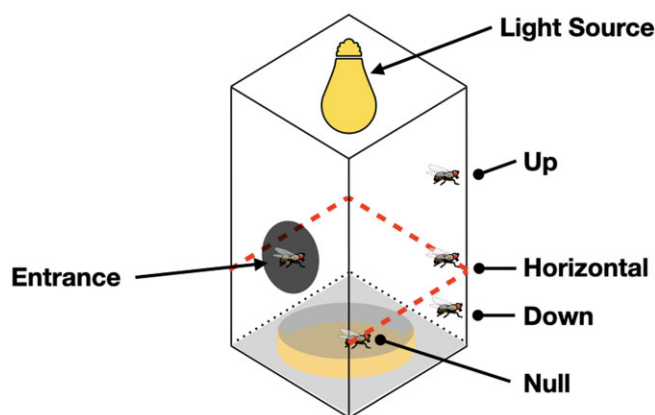


Figure 2 Diagram of a flight assay (described in Drummond et al., 1991). A transparent chamber is set up with a light at the top and a collection plate on the bottom. For each genotype, 50–70 flies, 1–2 days old, enter through a hole in the side of the box. Flies that stay at the same level as the entrance are marked “horizontal”, while flies that go above that line are marked “up” and flies that go below are marked “down.” Flies that fall to the bottom of the box are collected in the plate and marked “null” for flightless.

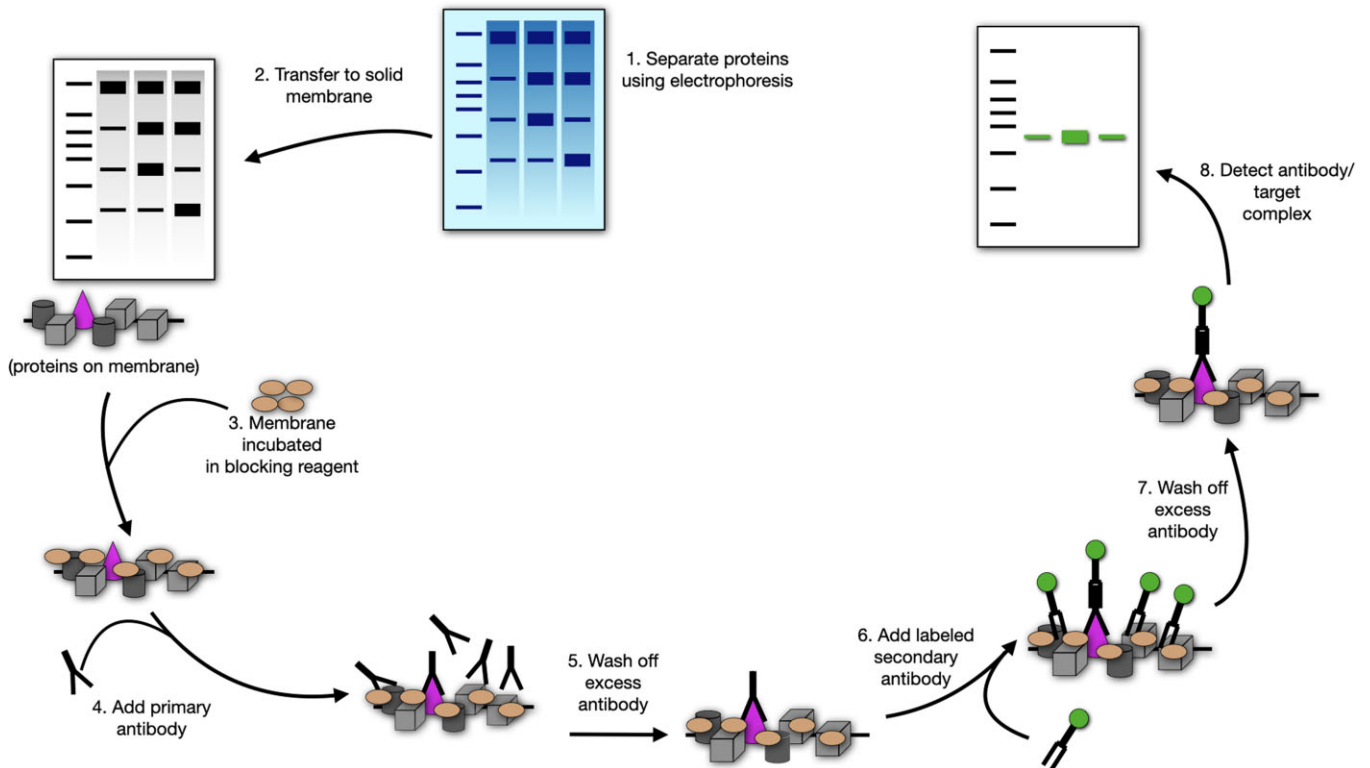


Figure 3. Outline of Western blotting and detection. (1) A protein sample is prepared and separated using SDS–polyacrylamide gel electrophoresis. (2) Proteins in the gel are transferred to a solid nylon membrane using wet electrical transfer. (3) The membrane is incubated in blocking reagent, typically an inert protein (e.g., bovine serum albumin), to reduce off-target antigen/antibody complexes for forming. (4) The membrane is incubated in a solution containing primary antibody. (5) Following incubation, excess antibody is washed off, and (6) the membrane is incubated in a solution containing a labeled secondary antibody, which detects the primary antibody. (7) The excess secondary antibody is washed off, and the target/antibody complex is detected (8) to locate the protein of interest.

Questions for discussion of the research paper

- How did the authors use previous *Drosophila* mutant and genome information to identify *Pkcδ*?
- What genetic technique do the authors use to reduce the levels of MLC2 protein in the IFMs? How do they demonstrate that this technique was successful?
- Why did the authors treat their protein extracts with alkaline phosphatase in Figure 1D? What did they conclude from this experiment?
- At what life stage is phosphorylation of MLC2 at its highest levels? Does this timing make sense based on what you have learned about MLC2 function?
- What type of mutant is *Pkcδ^{se04408}*? How did the authors obtain this mutant? How would you go about constructing such a mutant?
- How did the authors carry out their analysis of *Pkcδ* deficiency mutants? How did their results with each genotype support their phenotypic observations?
- How did the authors confirm that the flightless phenotypes they observed were specifically due to loss of *Pkcδ* function?
- Before doing their own mass spectrometry analysis, what data led the authors to conclude that there were more than two phosphorylation sites on MLC2?
- How would you expect the mass of a phosphorylated peptide fragment to change with phosphorylation?
- How do you read the map in Vaziri et al. Figure 3? What do the numbers assigned to each purple “P” mean? What differences in phosphorylation do you see between the control and mutant fly samples, and what did the authors conclude from this experiment?
- What effect does loss of *Pkcδ* have on myofibril structure?
- How do the authors genetically rescue the *Pkcδ* mutants in Figure 4?
- Describe the experiments performed in Vaziri et al. Figure 5, and what conclusions the authors drew from their results. In Figure 5C, why do the authors use myosin purified from *Pkcδ^{se04408}* mutants? Why might the authors have used recombinant human PKCδ protein in this experiment?
- Do the authors show that the only muscle function for *Pkcδ* is through the phosphorylation of MLC2? Why do you think they provided both biochemical and immunohistochemical imaging evidence?
- Strn-MLCK* modifies MLC2 on Ser66 and Ser67. Why do you think the authors not detect changes to MLC2 phosphorylation in the *Strn-MLCK* knockdown flies?
- What part of the skeletal muscle contraction cycle is likely disrupted in *Pkcδ* mutants?

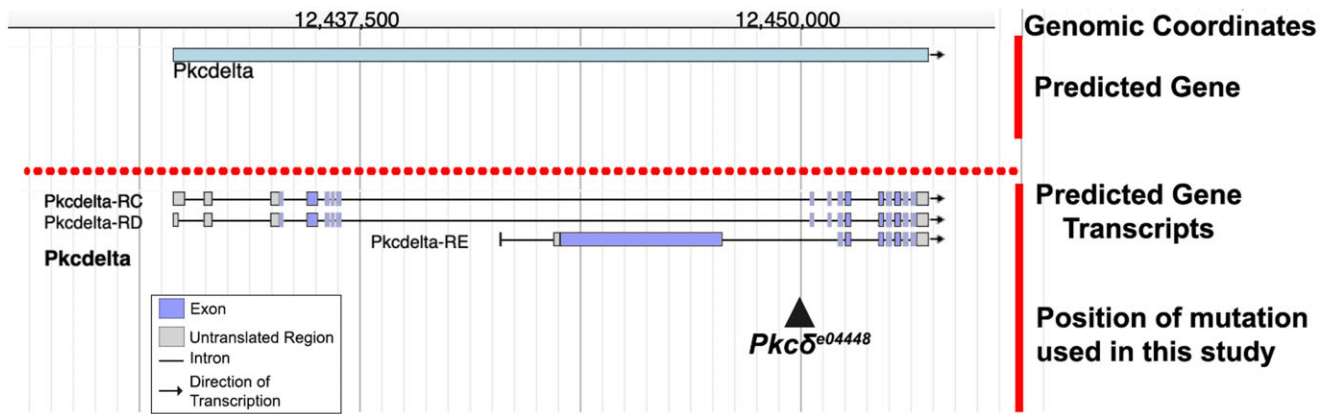


Figure 4 Simplified genomic map of *Pkcδ* locus. Shown is a modification of Figure 2A from Vaziri *et al.* In this simplified genomic map, the gene locus for *Pkcδ* is shown in pale blue. Arrowhead indicates the direction of transcription. *Pkcδ* has three alternative transcripts, indicated in lower portion of figure. For each transcript, exons are shown as boxes, translated portions of exons are shown as purple boxes, introns are represented as intervening black lines, and untranslated regions of exons are represented as gray boxes. The position of the mutation that created the *Pkcδ*^{e04448} mutant allele is indicated with a black arrowhead.

- 17) How did the authors of Vaziri *et al.* ensure reproducibility of their data?
- 18) How could the authors be certain that their quantitative measurements (for example, sarcomere lengths) were significantly different between samples/genotypes? Are there any examples where quantitative methods could be improved, or new computational approaches applied to strengthen their conclusions?

Suggestions for additional classroom assignments

The experiments described in the research article lend themselves to active learning exercises to provide practice with basic computational skills including working with a genome browser and sequence analysis. These exercises would be suitable for distance-based or hybrid learning environments.

The first exercise familiarizes students with genome browsing software. Students should look up *Pkcδ* (CG42349/FBgn0287828) on FlyBase.org (Larkin *et al.* 2021), scroll down to “Genomic Location” and “Genomic Maps” and click on “JBrowse.” Get started with understanding what’s on the screen.

- 1) What chromosome are you looking at? What are the gene’s coordinates? How did you determine this information?
- 2) What is the direction of transcription?
On the left hand side of the screen, multiple “tracks” are available for display. Click to make tracks visible or invisible.
- 3) What are the differences between the “RNA” and “CDS” tracks? What types of information are available in the other tracks?
- 4) How many transcripts are synthesized from the *Pkcδ* gene? What number of exons do each of these transcripts have?
Now scroll down the panel of available tracks to learn more about *Pkcδ*, and ask questions specific to your unit or course; these might include:
- 5) Where and when is this gene expressed?
- 6) What transcription factors bind in this region?
- 7) What types of mutations have been mapped in this gene?
- 8) What other genes are nearby?

Once familiar with this system for *Pkcδ*, students can use the genome browser to learn more about other genes of interest.

To identify specific amino acid residues and their modifications within the Mlc2 protein, download the sequence (CG2184/

FBgn0002773) from www.ensembl.org (Accessed: 2021 February 11) (http://useast.ensembl.org/Drosophila_melanogaster/Info/Index) and visualize the protein using a three-dimensional modeling program like the free, open-source program PyMol (<https://pymol.org> (Accessed: 2021 February 11)). Now locate the following amino acid residues: Thr38, Ser55, Ser66, and Ser67.

- 1) How are serine and threonine similar?
- 2) What makes them suitable for modification by a phosphate?
- 3) What will you predict will happen to the protein if you add phosphates to these amino acid residues?
Now use the “Molecular Builder” tool within PyMol to place phosphates on these residues.
- 4) What happens to the protein after you have added the phosphates?

Finally, *Drosophila* is an ideal model organism for hands-on student laboratory activities since they are easy to work with and inexpensive to rear. Flight and other mobility assays are visible, quantifiable measures of phenotype that make it easy for students to conduct a screen of mutants (Drummond *et al.* 1991; Brooks *et al.* 2016; Chechenova *et al.* 2017). These accessible laboratory activities give students the opportunity to collect, organize, analyze and present their data, as well as potentially allowing the students to identify novel genes required for muscle function.

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Conflicts of interest

The authors declare that there is no conflict of interest.

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