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Flightless-I and *Drosophila* dLRRFIP2 work together to regulate radial growth of the sarcomeres

PhD Thesis

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PUBLICATIONS

Publications related to the thesis

Péter Görög, Tibor Novák, Tamás F. Polgár, Péter Bíró, Adél Gutheil, Csaba Kozma, Tamás Gajdos, Krisztina Tóth, Alexandra Tóth, Miklós Erdélyi, József Mihály, Szilárd Szikora. Developmental remodelling of *Drosophila* flight muscle sarcomeres: a scaled myofilament lattice model based on multiscale morphometrics. OPEN BIOLOGY 15 : 250182 (2025)

IF: 3.6 (Q1)

Szikora Szilárd; **Görög Péter**; Mihály József. The mechanisms of thin filament assembly and length regulation in muscles. INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES 23 : 10 Paper: 5306 , 25 p. (2022)

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Szikora Szilárd; **Görög Péter**; Kozma Csaba; Mihály József. *Drosophila* models rediscovered with super-resolution microscopy. CELLS 10 : 8 Paper: 1924 , 22 p. (2021)

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2. Publications not directly related to the thesis

István Földi*, Krisztina Tóth*, Rita Gombos, Péter Gaszler, **Péter Görög**, Ioannis Zygouras, Beáta Bugyi, József Mihály. Molecular Dissection of DAAM Function during Axon Growth in *Drosophila* Embryonic Neurons. CELLS 11 : 9 Paper: 1487 , 20 p. (2022)

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Introduction

Muscle contraction is one of the most fundamental biological process in animals, providing the mechanical force required for movement, circulation, and a wide variety of essential physiological functions. At the heart of this process lies the sarcomere, the smallest structural and functional unit of striated muscle. The sarcomere represents a highly ordered molecular machine, the structure and dynamic regulation of which fascinated researchers for decades. Early physiological studies revealed that force production arises from sliding of the thin and thick filaments past one another. Since then, structural, biochemical and genetic approaches have expanded our knowledge of sarcomere organization and function. Despite this progress, many aspects of sarcomere development and disease remain largely unknown.

General organization of the sarcomere

Sarcomeres are defined as repeating units within myofibrils, bordered at each end by electron-dense Z-discs. Within these boundaries, three filament systems interact to generate contractile force: actin-based thin filaments, myosin-based thick filaments and the elastic filaments. The structural polarity of the thin filaments and the bipolar organization of the thick filaments enable cyclic cross-bridge interactions that underlie muscle contraction.

The thick filaments are primarily composed of type II myosin molecules. Each myosin consists of a pair of heavy chains and two pairs of light chains. The heavy chains form a long C-terminal coiled-coil, which contributes to filament assembly, and the N-terminal globular heads bind both actin and ATP. The ATPase activity of the myosin heads drives conformational changes that result in the “power stroke,” the mechanical basis of contraction. The essential and regulatory light chains, associated with the head–tail junction, further modulate myosin activity. The thin filaments consist of filamentous actin (F-actin),

formed from globular actin (G-actin) subunits, oriented head-to-tail to create structural polarity. The (+) ends of the filaments are anchored in the Z-disc, with their (–) ends extending toward the sarcomere centre. Beyond actin, thin filaments incorporate regulatory proteins, most notably tropomyosin and the troponin complex, which modulate myosin–actin interactions in response to calcium signals.

Beyond the Z-discs, sarcomeres contain other well-defined regions that reflect the specific arrangement of the filament systems. The Z-discs are flanked by the myosin-free I-bands that contain only thin filaments, whereas the A-band corresponds to the myosin-containing region, including the zones of actin–myosin overlap. Within the A-band, the H-zone represents the central region devoid of thin filaments, which contains the myosin tail domains of the bipolar thick filaments. The middle of the H-zone is defined as the M-line, where thick filaments are crosslinked by myosin associated proteins. Elastic filaments, such as titin in vertebrates and Kettin in insects, span from the Z-disc to the thick filaments, and act as molecular springs that resist overstretch and facilitate passive relaxation. Together, these structural elements establish a highly organized architecture that integrates force generation with elasticity and stability.

Models of sarcomere assembly

Although the mature sarcomere is structurally well characterized, the mechanisms by which this structure arises during development are still mostly unknown. Several models have been proposed to explain myofibrillogenesis, however, the premyofibril model is currently the most widely accepted.

This model envisions sarcomere formation as a progressive maturation process. Initially, premyofibrils arise, characterized by nascent Z-discs enriched in α-actinin, short actin filaments and non-muscle myosin II. These early structures

are dynamic and somewhat unstable, but they provide a scaffold for subsequent protein incorporation. The exchange of non-muscle myosin II for muscle-specific myosin II marks the transition toward proto-sarcomeres. As development proceeds, proto-sarcomeres undergo elongation and radial expansion, incorporating additional actin and myosin filaments as well as regulatory components.

Numerous proteins involved in this process have been identified, yet the molecular mechanisms guiding filament elongation and lateral integration remain largely unknown. Understanding these growth processes is critically important, as defects in sarcomere assembly can underlie developmental myopathies and cardiomyopathies.

Drosophila as a model system

The *Drosophila melanogaster* has emerged as a powerful system for investigating sarcomere development. Despite the evolutionary distance between insects and vertebrates, the molecular architecture of the sarcomere is highly conserved.

Several features make the indirect flight muscles (IFMs) of *Drosophila* particularly valuable for developmental studies. First, their developmental timing is well defined: immature myofibrils emerge around 36 hours after puparium formation (APF) and continue to grow until approximately 24 hours after eclosion (AE). Second, *Drosophila* genetics provides unmatched tools for manipulating gene function with temporal and spatial precision. Collectively, these attributes make the fly an excellent platform for identifying conserved molecular pathways governing sarcomere development, with direct relevance to human muscle biology.

Flightless-I: an actin-binding protein in sarcomere development

Thin filaments form the structural backbone for actin–myosin interactions. *In vitro*, actin polymerization proceeds through nucleation, elongation and steady-state phases, with distinct kinetics at the (+) and (-) ends. *In vivo*, however, regulation is far more complex, as the pool of actin monomers is tightly controlled by sequestering and exchange factors such as thymosin-β4 and profilin.

The actin-binding protein Flightless-I (FliI) has emerged as an important regulator of sarcomeric thin filaments. FliI is a highly conserved member of the gelsolin superfamily, composed of two major structural modules: an N-terminal leucine-rich repeat (LRR) region and a C-terminal series of six gelsolin-like domains. Unlike canonical gelsolin, FliI lacks calcium-binding sites in its gelsolin homology domains, indicating distinct mechanisms of actin regulation. Loss of FliI leads to lethality in mice, zebrafish and *Drosophila*. In humans, mutations in FliI are associated with cardiomyopathies, while reduced FliI activity in zebrafish and fruit flies results in disorganized muscle fibers and impaired muscle performance. At the molecular level, the precise function of FliI remains ambiguous. Some studies have suggested that FliI possesses an actin filament severing activity, however, *in vitro* assays demonstrated that both mouse and *Drosophila* FliI bind actin without severing and instead might function as (+) end regulators.

AIMS

Sarcomere development is a highly complex process, starting with formation of the pre-myofibrils, consisting of proto-sarcomeres, followed by their maturation and growth. Despite ongoing research in this area, our understanding of this process, particularly from the perspective of actin filament dynamics and regulation, remains incomplete. The lack of this knowledge hinders our ability to fully comprehend muscle development disorders and develop effective therapies for muscle diseases. To study this process, we chose the *Drosophila* IFM as our primary model system. The IFM, with its highly ordered sarcomere structure and genetic tractability, provides an excellent platform for investigating the molecular mechanisms of sarcomere development. Since there was no comprehensive research as to the developmental dynamics of myofibril growth, first, we wanted to provide a multi-scale characterization of the structural changes during development in a wild-type background to serve as a starting point for our future studies.

In addition, because the human *fliI* gene has recently been linked to childhood-onset cardiomyopathy, we wanted to revisit the function of this protein in IFM development, with the hope that a better characterization of its molecular mechanisms is helpful to understand human disease as well.

RESULTS

Development of an automated method for sarcomere measurements

Quantitative analysis of sarcomere morphology during development requires reliable and accurate tools. Traditional approaches have relied heavily on manual measuring, which is not only time consuming but also subject to user variability and error. To overcome these limitations, we developed an automated and user-friendly software tool, designated as Individual Myofibril Analyser (IMA), that detects sarcomere boundaries from Z-disc labelling and quantifies both sarcomere length and myofibril diameter across large datasets.

When tested on simulated sarcomeres of defined dimensions, the tool demonstrated high accuracy, consistently providing measurements that closely matched the true sarcomere size. In contrast, manual annotation by multiple independent users tended to underestimate myofibril diameter and showed considerable variability between annotators. The implementation of this tool also improved evaluation speed by a factor of 10 to 20. This improvement enabled us to obtain large-scale, statistically robust datasets that captured developmental trends with greater precision.

The two-phase development of sarcomeres

Using the automated analysis approach, we examined sarcomere development during defined time points of IFM formation. At 36 hours APF, proto-myofibrils contained about 100 sarcomeres per fiber. These early sarcomeres were short and narrow, they contained relatively few thick and thin filaments, and lacked a regular lattice arrangement. The Z-discs were already visible but appeared much thicker than in mature muscles. Between 36 and 48 hours APF, the number of sarcomeres per myofibril increased to 230, while individual sarcomere dimensions remained largely unchanged. During this period, filament packing

improved, and the characteristic hexagonal lattice of mature muscles became evident. From 48 to 72 hours APF, sarcomeres elongated steadily through incorporation of actin monomers at the (-) ends of the thin filaments, while peripheral filament addition occurred in parallel to the elongation. New filaments were assembled at the periphery of the existing myofibrils, coinciding with increases in myofibril diameter and Z-disc expansion. By 24 hours AE, sarcomeres had reached their mature dimensions. Together, these findings revealed a two-phase developmental program, with an initial 'Organization' phase in which sarcomeres are assembled and organized without major changes in their size, which is followed by a 'Steady Growth' phase characterized by coordinated elongation and radial expansion.

Flightless-I is required for sarcomere growth

While previous studies have identified key proteins involved in thin filament elongation, much less is known about the mechanisms and main players driving peripheral growth of the sarcomeres. FliI has emerged as a promising candidate, as it was shown to play a role in actin filament dynamics and sarcomere organization. Our analyses showed that muscle-specific knockdown of FliI, as well as the hypomorphic *fliI* alleles, exhibit severe defects in IFM organization and result in a complete loss of flight ability. Detailed examination of these muscle fibers revealed prominent myofibrillar aggregates on the surface of the fibers, along with severe impairments in myofibril formation and overall organization. High-resolution imaging demonstrated that myofibrils in FliI-deficient muscles are either severely disrupted or exhibit reduced thickness and shorter sarcomeres compared to controls. Quantitative measurements of isolated individual myofibrils confirmed these observations.

To further investigate the structural defects, we analysed the ultrastructure of the IFMs using transmission electron microscopy. Longitudinal and cross-sectional images revealed shorter and thinner sarcomeres in FliI-deficient muscles. In addition, Z-disc morphology was also altered, and the Z-discs often failed to span the entire myfibril width (remaining confined to the sarcomere core), resulting in the presence of loosely connected or completely detached peripheral myofilaments. Despite these peripheral defects, the central regions of the myofibrils maintained a normal ratio of thin to thick filaments, and the spacing within the hexagonal filament lattice was largely unaffected.

Developmental analysis of isolated myofibrils at different stages of myofibrillogenesis revealed that FliI is not required during early stages. At 36 hours APF, loss of FliI did not produce detectable defects, and sarcomere ultrastructure appeared normal. By 48 hours APF, at the end of the organization phase, a proper thin-to-thick filament ratio and hexagonal lattice were evident. Structural defects became only evident by 72 hours APF, with uneven sarcomere length and myofibril width. By 96 hours, the mutant phenotype was fully established, including reduced myofibril diameter and a significant presence of unincorporated peripheral filaments.

Taken together, these findings indicate that FliI is specifically required during the later phases of myofibrillogenesis. The formation of myofilaments occurs largely normally, but their proper incorporation into the sarcomeric lattice is impaired in the absence of FliI. This suggests that FliI plays a critical role in anchoring and organizing peripheral myofilaments at the Z-disc, ensuring proper radial growth of sarcomeres. Overall, these results highlight the importance of FliI in regulating both sarcomere size and myofibril organization during muscle development.

Localization of Flightless-I during sarcomere development

To investigate how FliI regulates Z-disc formation and myofibril thickening, we examined its localization in the IFM. In the absence of specific antibodies, we expressed a FLAG-tagged full-length FliI in a FliI-null mutant background. These tagged proteins fully rescued lethality, flight ability and normal sarcomere organization, confirming that the FLAG tags do not impair FliI function and this rescue setting is suitable for our localization studies.

Analysis of myofibers and isolated myofibrils revealed that FliI is exclusively localized to the Z-discs in mature sarcomeres. High-resolution imaging using dSTORM nanoscopy demonstrated that FliI forms a distinctive double-line pattern along the Z-disc. The measured spacing between these peaks closely corresponds to the edges of the Z-disc, where the (+) ends of the thin filaments are capped by the CapZ dimers. These findings are consistent with previous knowledge that gelsolin-like domains typically bind the (+) ends of actin filaments. In line with this, our former *in vitro* studies demonstrated that FliI behaves as a (+) end capping protein, supporting a role for FliI at the Z-disc in anchoring and organizing thin filaments during sarcomere assembly and growth.

Functional interaction of FliI with dLRRFIP2

FliI plays a key role in sarcomere development, but its molecular function in flight muscle was unclear. To gain further insights into its potential mechanisms, we performed affinity purification coupled with mass spectrometry of FLAG-tagged FliI in a protein-null background, and identified a single strong interacting partner, known as CG8578 in Drosophila. Sequence and structural analyses indicate that CG8578, hereafter referred to as dLRRFIP2, is the

Drosophila ortholog of vertebrate LRRFIP2, sharing a conserved FliI-binding region and a similar three-dimensional fold. Co-immunoprecipitation confirmed that the LRR domain of FliI directly interacts with dLRRFIP2, whereas gelsolin homology domains do not. Functionally, muscle-specific knockdown of dLRRFIP2 mirrors the lack of FliI, leading to impaired flight ability, reduced sarcomere length and diameter, disorganized myofibrils, and unincorporated peripheral filaments. Early sarcomere formation remains normal, but defects emerge during the steady growth phase, highlighting a role in radial filament incorporation. Epistasis experiments confirmed that function of the FliI protein depends on the presence of dLRRFIP2.

Molecular modelling with Alphafold predicted two distinct binding interfaces on the LRR domain that interact with the conserved FliI-binding region of dLRRFIP2. Our GST-pulldown assay revealed that dLRRFIP2 is able to form dimers through its conserved interaction domain, allowing a simultaneous occupation of both interfaces. Structural modelling further suggested the possibility of a tetrameric interaction involving the dLRRFIP2 dimer and two FliI-LRR regions. This conformation could play an important role in actin filament crosslinking or coordination during myofibrillogenesis, providing a potential mechanistic explanation for how these proteins contribute to peripheral filament addition. Together, these findings reveal that FliI and dLRRFIP2 form a conserved protein complex, that is critical for peripheral filament anchoring and radial myofibril growth.

DISCUSSION

Precise control of sarcomere size is critical for muscle function, and disruptions in thin filament organization are a defining feature of many skeletal and cardiac myopathies. While sarcomere growth appears deceptively regular, the underlying mechanisms, especially those controlling radial expansion and (-) end elongation of the thin filaments, remain poorly understood. Using the *Drosophila melanogaster* indirect flight muscle, we investigated the developmental dynamics of sarcomere size regulation with high spatial and temporal resolution.

We developed a custom Python-based analysis pipeline enabling rapid quantification of sarcomere length and width from fluorescence microscopy images. By applying this tool to wild-type IFM samples spanning 12 time points, we defined two distinct growth phases. The initial 'Organization phase' (36–48 h APF) is characterized by refinement of the sarcomere pattern without a notable increase in size, which is followed by a prolonged 'Steady growth phase' marked by coordinated elongation and lateral expansion that continues until the sarcomeres attain their fully matured size.

To understand the molecular mechanism of sarcomere growth, we focused on a conserved actin regulator protein called FliI. The *fliI* mutants are completely impaired in their ability to fly, and they display an irregular myofibrillar organization with sarcomeres that are significantly shorter and thinner than their wild type counterparts. Transmission electron microscopy revealed specific defects at the sarcomere periphery and the Z-disc, where thin filaments failed to properly integrate with the normal-looking myofibril core. These findings suggest that FliI is required specifically for lateral filament addition during radial growth. Consistent with this, nanoscopic analysis revealed that the FliI protein localizes at the edge of the Z-disc. Besides these studies, we identified

dLRRFIP2 as a prominent interaction partner of FliI. In line with a common function, the muscle-specific knockdown of dLRRFIP2 phenocopied all the key aspects of the lack of FliI. Co-immunoprecipitation and structural modelling revealed a direct interaction between the LRR domain of FliI and a dimeric form of dLRRFIP2. We propose that this interaction might facilitate the formation of a tetrameric complex that acts as an actin-crosslinking unit at the sarcomere periphery.

Altogether, our results identified FliI and dLRRFIP2 as two novel key components of peripheral sarcomere growth. We propose a model in which they stabilize newly formed actin filaments at the Z-disc, ensuring correct integration into the growing myofibril. Given the emerging links between human FliI variants and cardiomyopathy, this mechanism may represent an evolutionarily conserved mode of action, with a clinical relevance in the better understanding of muscle pathologies.

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