Drak is a potential mechanotransduction partner of Filamin

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Running title: Drak interaction with Filamin

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ABSTRACT

Mechanosensing involves proteins detecting mechanical changes in the cytoskeleton or at cell adhesion sites. These interactions initiate signaling cascades that produce biochemical effects such as post-translational modifications or cytoskeletal rearrangements. Filamin is a ubiquitous mechanosensing protein that binds actin filaments and senses pulling forces within the cytoskeleton. *Drosophila* Filamin (Cheerio) is structurally similar to mammalian Filamin, with roles in egg chamber development, embryo cellularization, and integrity of muscle attachment sites and Z discs in *Drosophila* indirect flight muscles (IFMs). Here we report a potential novel mechanotransduction pathway involving Filamin and Drak, a death-associated protein kinase that functions as a myosin light chain kinase. We found that Drak biochemically binds mechanically activated Filamin. During embryo cellularization, Drak-GFP localization followed that of Filamin and myosin at the initiation of the cellularization furrow. During indirect flight muscle development at the pupal stage, Drak was expressed in the tendon cells and in the developing myotubes. Drak co-localized with Filamin in the tendon cells at the time of myotube attachment site maturation and Drak expression peaked in the myotubes at the compaction stage. Drak null mutant and a Filamin mutant with reduced Drak binding caused an epistatic effect on the adult muscle attachment site morphology. These results suggest that Filamin may have a role in the regulation of Drak function in response to mechanical forces.

Key words: mechanosensing, mechanotransduction, cytoskeleton, actin, Filamin, cellularization, muscle development, *Drosophila melanogaster*

Summary statement: We discover that actin crosslinker Filamin may convert mechanical forces to biochemical signals by interacting with a serine/threonine kinase Drak.

INTRODUCTION

Mechanosensing involves proteins that change shape when force is applied (Sala et al., 2024). Mechanical force can alter protein function or expose hidden binding sites, allowing other proteins to interact and initiate a signaling cascade known as mechanotransduction. This

process can produce biochemical signals, including post-translational modifications (Sala et al., 2024). There are numerous molecules with mechanosensitive properties. There are at least four families of mechanically regulated ion channels that sense membrane pressure changes in response to forces in the cell membrane (Árnadóttir and Chalfie, 2010; Douguet and Honoré, 2019). However, many mechanosensors initiate mechanotransduction processes at cell-cell or cell-extracellular matrix attachment sites or along cytoskeletal protein filaments that are linked to these sites (Vogel and Sheetz, 2006). The cytoskeleton also provides a direct mechanical link between various parts of the cell, for instance between the cell surface and the nucleus (Fedorchak et al., 2014).

Mechanotransduction plays an important role in a wide variety of developmental processes ranging from one-cell embryo development (Duch et al., 2020), embryo compaction at 8 to 16 cell stage (Firmin et al., 2024), to control of tissue shape and size (Vignes et al., 2022). In pathological states, mechanical tissue response such as tissue stiffening and encapsulation of damaged areas are part of defence mechanisms but can also lead to extensive tissue fibrosis and functional failure (Di et al., 2023). Even though many mechanosensing and mechanotransduction pathways are known, we still do not completely understand many of the underlying molecular mechanisms. This is why we, in this paper, study the mechanotransduction partners of the actin cross-linking protein Filamin.

Filamins are dimeric, actin filament crosslinking proteins involved in mechanosensing. Three mammalian Filamins are all essential (Dalkilic et al., 2006; Feng et al., 2006; Hart, 2006; Zhou et al., 2007), and have partially distinct, partially overlapping roles in tissue development (Razinia et al., 2012; Zhou et al., 2010). Human congenital Filamin mutations cause malformations in neurons, bones, and epithelial tissues, as well as in cardiac muscle and skeletal muscle. Many of these diseases are assumed to be caused by alterations in mechanotransduction (Sutherland-Smith, 2011). Filamins are composed of an N-terminal actin binding domain and up to 24 immunoglobulin-like Filamin repeat domains (Fig. 1A). The mechanosensory region (MSR) at the C terminal part of Filamin contains two domain pairs that normally mask their protein interaction sites (Fig 1B, left image) (Heikkinen et al., 2009; Lad et al., 2007). Single molecule experiments (Rognoni et al., 2012) and live cell fluorescence-based assays (Ehrlicher et al., 2011) have demonstrated that these interaction sites can be unmasked by 3-5 pN forces or by myosin activation. When unmasked (Fig 1B, right image) , the mammalian Filamin MSR can bind cell surface adhesion receptors, such as integrins (Kiema et al., 2006) and platelet von Willebrand factor receptor (Nakamura et al., 2006), cytoskeletal regulators FilGAP (Ohta et al., 2006), migfilin (Lad et al., 2008), Fimbacin, Smoothelin (Nakamura and Wang, 2019), mRNA stabilizing proteins La related protein 4 (LARP4) (Mao and Nakamura, 2023) and Ras GTPase-activating protein-binding protein 1 (G3BP1) (Feng et al., 2023), as well as a Hippo-pathway scaffolding protein Salvador homology 1 (SAV1) (Zhang et al., 2023).

Drosophila has one Filamin gene (*cheerio, cher*) whose protein products have a similar domain structure to mammalian Filamin. The other *Drosophila* Filamin like-gene (*jitterbug, jbug*) encodes proteins that appear not to have dimerization domains similar to mammalian Filamin (Mulder et al., 2025). In this paper we call *cher* gene products *Drosophila* Filamin. The name

Cheerio comes from its function and localization in the ring-like actin structure, the ring canal, that is formed between the germ-line derived nurse cells and the oocytes during egg chamber development (Sokol and Cooley, 1999). In addition to this, Filamin is involved in other functions in *Drosophila*, including syncytial embryo cellularization (Krueger et al., 2019), epithelial cell migration (Sokol and Cooley, 2003), motor neuron axon guidance (Zheng et al., 2011), immune cell differentiation (Rus et al., 2006), as well as muscle development (González-Morales et al., 2017). For instance, during embryonic cellularization Filamin localizes to the actomyosin ring that drives invagination of the plasma membrane between the nuclei and Filamin knock down leads to cellularization failure in 80% of the embryos (Krueger et al., 2019). During *Drosophila* adult indirect flight muscle (IFM) development, Filamin knock down causes Z disc defect and flightless flies (González-Morales et al., 2017). In addition to Z disc, Filamin is also localized at the at the flight muscle attachment site and a C-terminal deletion of Filamin causes disruption of the attachment (Green et al., 2018). At least in ring canal development (Huelsmann et al., 2016) and flight muscle development (Fisher et al., 2024), mutations in the C-terminal mechanosensory region cause observable phenotypic effects.

Myosin contraction is often regulated as part of mechanotransduction pathways. Non- muscle myosin-2 is activated via phosphorylation of its regulatory light chain at residues Thr18 and Ser19 (in human and mouse) (Vicente-Manzanares et al., 2009) which correspond to residues Thr20 and Ser21 in the *Drosophila* ortholog Spaghetti squash (encoded by *sqh*). Mechanosensing at cell adhesion protein complexes activates Rho kinase (ROCK) that increases myosin regulatory light chain phosphorylation mainly via inactivation of its phosphatase or by Ca2+/Calmodulin mediated activation of Myosin light chain kinases (MLCK) (Zuidema et al., 2020). In addition to this, other serine/threonine kinases without the Ca2+/Calmodulin regulatory domains can also phosphorylate the myosin light chain. In human and mouse T-cells, Death-associated protein kinase related apoptosis-inducing protein kinase 2 (DRAK2) directly phosphorylates myosin light chain and DRAK2 deficient T-cells have reduced motility and T-cell receptor microclustering (Wilander et al., 2024).

Drosophila Drak, the single ortholog of human DRAK1 and DRAK2, has a role during *Drosophila* cellularization. Cellularization occurs during *Drosophila* early embryo development, where the embryo undergoes 14 rounds of nuclear divisions (Sokac et al., 2022). Nuclear cycles 1-7 occur in the interior of the embryo, after which the nuclei are transported to the periphery and the divisions continue. Cellularization begins at nuclear cycle 14, when the plasma membrane invaginates between each nucleus resulting in a monolayer of approximately 6000 cells (Sokac et al., 2022). Schematics of the cellularization process is shown in Fig. 1C. At the basal tip of the invaginating furrows, an actomyosin ring forms, whose contraction is tightly regulated throughout the process. Early contraction of the ring is driven by non-muscle myosin (Chougule et al., 2016; Xue and Sokac, 2016), whereas the later closure of the ring depends on actin depolymerization (Xue and Sokac, 2016). Drak null embryos show impaired ring contractility during early cellularization and reduced phosphorylation levels of non-muscle myosin (Chougule et al., 2016).

In addition to early embryos (Chougule et al., 2016) and wide expression in epithelial tissues (Neubueser et al., 2010), Drak mRNA is expressed during specific stages of *Drosophila* IFM development (Spletter et al., 2018). IFM development begins 12 hours after pupal formation (APF) with the fusion of myoblasts to form myotubes (Weitkunat and Schnorrer, 2014). The following steps include the splitting of the original IFM myotubes into two and formation of firm integrin-containing attachments to tendon cells (Fig. 1D). The attachment sites maturation between 20-24 h APF, is followed by myotube contraction which continues until 30 h APF (Weitkunat et al., 2014). This marks the transition of myotubes to immature myofibers when myofibril assembly and sarcomerogenesis is initiated (Lemke and Schnorrer, 2017; Luis and Schnorrer, 2021; Weitkunat and Schnorrer, 2014). Interestingly, the spike in Drak mRNA expression coincides with myotube compaction, occurring between 24–30 h APF (Spletter et al., 2018).

In this study we report a biochemical interaction between *Drosophila* Filamin and Drak. This interaction is facilitated with a mutant that opens Filamin's C-terminal mechanosensitive sites. We tested the hypothesis that Filamin and Drak are mechanotransduction partners during cellularization and during IFM development. We find colocalization in cellularization and in tendon cells at the IFM attachment site maturation stage. We also find an epistatic effect of *Drak* and *Filamin* mutations on adult muscle attachment sites.

METHODS

The Expression and Purification of the Recombinant Filamin and Drak Fragments

The five domain *Drosophila* Filamin protein fragments (GST-Cher15-19wt, GST-Cher15-19closedMSR, and GST-Cher15–19openMSR) (Huelsmann et al., 2016) were expressed as Glutathione S-transferase (GST) fusion proteins in *Escherichia coli* (*E. coli*) BL21 Gold cells (Agilent Technologies) at 18°C for 22 hours and purified as described earlier (Huelsmann et al., 2016). In this paper, the purified five domain *Drosophila* Filamin fragments are called Filamin-wild type, Filamin-open and Filamin-closed. C-terminal fragments of Drak (360-532, 404-532, and 435-532) cloned into pGTVL1-SCG vector were expressed at 20°C for 4 hours. The cells were harvested by centrifugation at 6,000 x g for 20 minutes at 4ºC followed by cell resuspension in PBS (1 g of cells in 10 ml) and stored in -80ºC.

Binding Assay

An aliquot (5 ml) of *E. coli* cells overexpressing Drak was thawed and supplemented with half a tablet of protease inhibitor (cOmplete ULTRA mini EDTA-free tablets, Roche Holding AG). Cell lysis was performed by sonication in three cycles, each consisting of 5 seconds on, 10 seconds off, at 10 % amplitude. The lysate was then cleared by centrifugation at 35,000 x g for 30 minutes at 4ºC and incubated with 900 µl of Protino Glutathione Agarose 4B (MachereyNagel) in PBS for 20 minutes on ice. The glutathione agarose was then washed 3 times with 5 ml of ice-cold PBS, using a centrifuge at 1000 x g for 2 minutes at 4ºC. Next, the glutathione agarose was resuspended in an equal volume of ice-cold PBS, and 100 µl was transferred into a filter column. To probe the interaction, 100 µl (0.2 -10 µM) of various Filamin fragments described above, were incubated with the GST-Drak-Glutathione agarose for 20 minutes in 20 mM Tris-HCl; pH 7.5, 100 mM NaCl and 1 mM DTT on ice. The agarose was then washed five times with 500 µl PBS, using a centrifuge at 1000 x g for 1 minute at RT. Finally, bound proteins were eluted with 50 µl of 2x SDS-electrophoresis sample buffer and separated according to molecular weight using 12% Mini-PROTEAN TGX stain-free SDS-PAGE gels (Bio-Rad Laboratories, Inc.). Triplicate experiments were performed each time for every protein concentration. Total stain-free fluorescence intensity of Drak and Cheerio bands of each sample lane were quantitated using Imagelab software (Bio-Rad Laboratories, Inc.). The relative intensity of bound Filamin fragments was obtained by comparing the ratio of the intensity of Filamin to Drak intensity for each experiment.

Yeast Two Hybrid Screen

The explore protein interacting with the open Filamin MSR, we sent a DNA fragment encoding domains 16-19 (amino acids 1518-1895 in the Genbank entry AAF55390.4) of *Drosophila* Filamin Cheerio to the Hybrigenics Services (Evry-Courcouronnes, France). This fragment contained mutations I1524E and I1710E, as the other Filamin-open constructs used in this study. The company cloned the fragment to the pB29 (N-bait-LexA-C fusion) yeast two-hybrid bait vector and conducted a yeast two-hybrid screen (Fields and Song, 1989) with *Drosophila* ovary library RP1 using 100 mM 3-aminotriazole to inhibit autoactivation of the bait. The screen resulted 249 hits that were sequenced. 11 of the hits corresponded to Drak fragments and 4 of them were identical.

Fly Strains and Upkeep

We used genomic GFP insertion of the *cher* locus, *cher*[WT GFP] (w;;P{ry[+t7.2]=neoFRT}82B GT{*cher*[GFP_mGFP6-2]}) and corresponding *cher*[closed MSR] and *cher*[open MSR] mutations, all expressing cher-GFP fusion proteins (Huelsmann et al., 2016). The *cher*[WT mCherry] was generated by integrating a *cher*-mCherry fusion construct to the same founder cher attP-insertion line ($cher^{s24}$) that was used for the generation of *cher*-GFP lines (Huelsmann et al., 2016). The *spaghetti-squash* (*sqh)* -mCherry fly stock (#99923) carrying an extra genomic copy of *sqh* linked to mCherry in the second chromosome was obtained from the Bloomington Drosophila Stock Center. $Drak^{KO}$ line lacking most of the kinase domain, was a gift from David R. Hipfner (Department of Medicine, University of Montreal, Canada) (Neubueser et al., 2010). The *Drak*-GFP allele was generated by creating a double stranded DNA break 17 nucleotides before the *Drak* STOP codon and using a homologous recombination template in the pHD-sfGFPScarlesDSred (Addgene) vector containing a red fluorescent protein marker induced in eye tissues. The template vector contained a 1 kb 5' *Drak* genomic fragment linked to GFP with a 12 amino acid linker: GGSGGSGGSGGS and a 2 kb 3' *Drak* genomic fragment. The guide RNA target PAM motif was mutated with a silent substitution in the homologous recombination template. The TH-attp40 *Drosophila* embryos carrying nanos-Cas9 in chromosome 2 were microinjected with the *Drak* guide-RNA plasmid pCFD3 (Addgene) and the homologous recombination template plasmid at the University of Cambridge, Department of Genetics Microinjection service facility. The adults surviving after microinjection were crossed with w^- flies and offspring with red fluorescent eyes were selected. A single fly line harbouring *Drak*-GFP was verified by sequencing. Flies were maintained at 22°C on Nutri-Fly Bloomington media (Genesee Scientific).

Embryo Preparation and Imaging in Cellularization

For embryo collection, flies were kept on grape agar dishes (FlyStuff Grape Agar Premix, Genesee Scientific) with a drop of yeast. Dishes were changed 2–3 times daily for 2–3 days. Embryos were collected after allowing the flies to lay eggs for 2–3 hours. Halocarbon oil 27 (Sigma-Aldrich) was added on the agar plate to better visualize the embryos. Embryos close to final nuclear divisions were picked and excess oil removed. Embryos were dechorionated using household bleach for 1.5 minutes and rinsed in three drops of water. Embryos were placed on 35 mm high glass-bottom dish (ibidi) in PBS, 0.1 mM ascorbic acid.

Imaging was carried out using Leica SPX8 Falcon confocal microscope with an HC PL APO 63×/1.20 water immersion objective. For actomyosin ring dynamics study, embryos were imaged at 3-minute intervals over a $20 \times 20 \times 40$ µm³ 3D section with a voxel size of $0.08 \times 0.08 \times 0.35$ µm³. For localization studies, the time interval was 90 seconds, with dimensions of $20.5 \times 20.5 \times 30.8$ µm³ and a voxel size of $0.16 \times 0.16 \times 0.50$ µm³. In the colocalization studies pixel size was 0.14×0.14 µm^2.

Pupal Preparation and Imaging of Developing Flight Muscles

Flies were transferred to fresh vials and kept for 6–8 days until white prepupae appeared. Prepupae were collected and aged for 20–24 hours. Pupae were secured on a double-sided tape, and the dorsal side of the pupal case was removed near the head and thorax using a fine needle. Pupae were detached from the tape using water, dried, and arranged in a row on a 35 mm glass-bottom dish, with the dorsal abdomen adhered to double-sided tape and the thorax towards the glass. The pupae were firmly secured in place by placing a tape on top of them and tightened until thoraces were pressed against the glass. For high-resolution imaging, a small drop of halocarbon oil 27 was added before mounting each pupa.

Low-resolution images were acquired using a Nikon A1R laser scanning confocal microscope with a Plan Apo 20× air objective. Imaging dimensions were 570 × 570 × 34 μm^3 with a voxel size of 0.56 × 0.56 × 2 μm^3, captured at 1-hour intervals. High-resolution images were obtained using the same microscope with a Plan Apo 60× oil objective or a Leica SPX8 Falcon confocal microscope with an HC Plan Apo 63× glycerol objective. For the Nikon, high-res imaging dimensions were 100 × 100 × 15 μm^3 with a voxel size of 0.099 × 0.099 × 3 μm^3. For the Leica, dimensions were 92 × 92 × 3 μm^3 with a voxel size of 0.075 × 0.075 × 3 μm^3. The time interval for both setups was 1 hour. The pupa IFM development movie was done with 0.099 × 0.099 μm^2 pixel size and 1 min time interval. Colocalization studies were done using Leica with pixel size of 0.14 × 0.14 μm^2.

Adult Flight Muscle Preparation and Imaging

IFMs were dissected from adult flies 1–2 days after eclosion. After removing the head and legs, the thorax was gently punctured with spring scissors. Thoraces were fixed in 4% paraformaldehyde PBS for 30 minutes at room temperature and then washed twice using PBS with 0.1 % triton X-100 for 5 minutes each. The thorax was cut in half with a longitudinal incision. The abdomen was removed, and the half thoraces were stained with 0.5 U/ml Alexa Fluor 555 phalloidin (Thermofisher) overnight at 6°C.

The half-thorax was submerged in a drop of Vectashield Vibrance (VectorLabs) mounting medium on a microscope slide, and the flight muscles were dissected using a fine tungsten needle (Fine Science Tools). A coverslip was placed on the sample and sealed with nail polish. Muscles were imaged using a Nikon A1R confocal microscope with a 60× oil objective. For each muscle sample, we captured 2–3 images covering a 50 × 50 μm^2 area of muscle attachment sites, yielding approximately 20–30 individual sites for measurement. The Filamin-GFP signal was used to confirm the presence of intact attachment sites.

Western blot

Embryos were collected approximately half an hour after final nuclear division into 1x Laemmli sample buffer containing 2-mercaptoethanol, 1 mM vanadate, 10 mM sodium fluoride, and 2 mM beta-glycerophosphate to a concentration of 2.5 embryos/µl. All embryo samples were prepared in technical duplicates. Phosphorylated Sqh was detected with 1:1000 dilution of Anti-Myosin pS19/pS20 (Rabbit) antibody (#600-401-416, Rockland). To quantify total Spaghetti Squash (Sqh), a custom-ordered Sqh antibody from Boster Biological Technology CO., Ltd. Full length Sqh (Uniprot ID: P40423) expressed in *E. coli* was used for

rabbit immunization and for affinity purification. Sqh antibody was used at a 1:3000 dilution. The secondary antibody, Anti-Rabbit HRP (#ab6721, abcam), was applied at a 1:3000 dilution for phosphorylated Sqh detection and at a 1:6000 dilution for total Sqh. Chemiluminescent detection was performed using SuperSignal West Pico PLUS substrate (Thermo). Samples were run on 12% stain-free gels (Bio-Rad), and proteins were transferred onto a 0.2 µm PVDF membrane using the Trans-Blot Turbo Transfer Pack kit (Bio-Rad). The transfer was carried out using the Trans-Blot Turbo Transfer System (Bio-Rad) for 5 minutes at 1.3 A and 25 V.

Image Analysis

Confocal images of cellularization were analysed using Fiji (Schindelin et al., 2012). XY projections of the cortical region were obtained by taking five slices starting 1 µm from the edge of the embryo. For deeper regions within the embryo, XY projections were created by summing three slices around the actomyosin ring. A Gaussian blur with sigma = 1 was applied. Minimum and maximum gray values were set consistently for each time point. XZ projections were generated by using "Reslice" function over a 7.5 µm thick area across three nuclei using 15 slices and creating a sum projection and applying Gaussian blur with sigma = 2.

In cellularization analyses, the samples were aligned temporarily by setting zero to the last time frame where the nuclei still appeared undivided at the onset of the final nuclear division. Background values for Drak-GFP, Filamin-mCherry, and Sqh-mCherry in cellularization and pupal flight muscle intensity-based analyses were obtained as follows: Single-fluorophore flies were imaged, and the intensity was measured on the un-tagged channel with identical imaging parameters as the double-fluorophore flies.

Apical intensity profile in cellularization (Fig. 3B) was calculated by measuring the mean intensities of a 1.13 µm thick section starting 0.16 µm from the embryo surface. Background values were subtracted in each respective time point. Vertical fluorescence intensity profiles (Fig. 3D, bottom panel) during cellularization were generated by extracting intensity data from each XY plane of the 3D image. Each sample was aligned by using the z-coordinate of the actomyosin front in time points that was closest to the assigned actomyosin ring ingression depths measured from the embryo surface (5.5, 8.5, 15.5, and 21 µm). The intensity profile data was normalized within each sample. Background samples were aligned the same way and the mean background was calculated for each XY plane in each ring ingression depth.

The shape of the actomyosin ring was analysed using Filamin-GFP signal at the peak position of the vertical intensity profiles. Samples that showed no contraction after passing the basal level of the nuclei were excluded. Noise was reduced using the Noise2Void plugin (Krull et al., 2019) with a model trained on ~10 projections of the ring per genotype. The slice was thresholded using the Huang method (Huang and Wang, 1995) and smoothed with the Shape Smoothing plugin (https://github.com/thorstenwagner/ij-shape-smoothing) (relative FDs = 8). Circularity was measured for each ring by selecting outlines and using Analyze → Measure.

The relationship between circularity and time in cellularization for each genotype was fitted using generalized additive mixed model using the gamm() function from the R mgcv package (https://CRAN.R-project.org/package=mgcv). Average circularity was calculated in each time point and to account for non-linear trends in circularity over time, we used a smooth term for

time, s(Time). We included a random intercept for each sample to account for baseline differences in circularity across samples. The final model had the form: gamm(Circularity ~ s(Time), random = list(Sample = ~ 1), data = genotype_data).

Low-resolution images of the pupal IFMs (Fig. 4A) were created by sum projecting four imaging planes. High-resolution images (Fig. 4B, C) were obtained from single slices, and a Gaussian blur with sigma = 1 was applied.

Changes in Drak intensity in developing IFMs were measured from a 20 µm thick section selected at the middle of the muscle. Using "Reslice" function, this section was computationally reoriented so that 10 longitudinal slices could be obtained across the myotubes and tendons. These slices were then z-projected using the "sum slices" option. The mean intensity of the Drak-GFP signal in the visible cytoplasmic area was measured. Background was subtracted at each time point, and each sample was min-max normalized. Displacement of the myotubes was measured by tracking the x-coordinate of the tendon-myotube junction and subtracting these coordinates from the first time point. Single dorsal-longitudinal IFMs from three pupae were analysed. Time points between samples were aligned by adjusting the time axis so that the minimum x-coordinates matched, and the first time point was set to 24 h APF for all samples, corresponding to the time when Drak intensity begins to increase. Normalized Drak intensity and displacement values were plotted to show the relationship between Drak intensity and myotube compaction and elongation, and both were fitted with a local regression model in R.

Muscle attachment site images for measurement were processed as follows: For the GFP channel, a Gaussian blur with sigma = 1 was applied, followed by contrast enhancement with 0.1% saturated pixels. For the phalloidin channel, a Gaussian blur with sigma = 2 was applied, and contrast was enhanced with 1% saturated pixels. To further enhance edge features, a Laplacian filter with smoothing set to 10 using the FeatureJ plugin (https://imagescience.org/meijering/software/featurej/) was applied. Attachment site length was manually measured from the inner side of the terminal Z-line to the tip of the myofiber using the line selection tool.

To compare the muscle attachment site length in different fly genotypes, we fitted a generalized linear mixed model, using function glmer() from the lme4 R package (https://CRAN.R-project.org/package=lme4). The response variable, attachment site length, was modelled with gamma distribution and a log link function to account for positively skewed distribution. Genotype was included as a fixed effect to estimate how attachment site length differed among genotypes. To account for non-independence of images from the same sample, we included a nested random intercept for image number within each sample, allowing for baseline variation among samples and their respective images. The final model was specified as: glmer(att_site_length ~ genotype + (1 | sample/image_number), data = att_site_length_df, family = Gamma(link = "log")). The wild type genotype (Filamin-GFP) was designated as the reference level so that all other genotype effects would be interpreted relative to it. To evaluate the effects of the other genotypes, custom contrasts were specified to test three comparisons: 1) wild type vs. Filamin-closed, 2) wild type vs. Drak-KO, and 3) double mutant (Drak-KO,

Filamin-closed) vs. the additive effects of the individual Filamin-closed and Drak-KO genotypes. P-values for the comparisons were derived from the model's summary output.

To quantify co-localization for Drak-GFP and Filamin-mCherry during the cellularization priming phase and tendon-myotube attachment site maturation we measured thresholded Manders' co-localization coefficients (M1 and M2) and the thresholded Pearson correlation coefficient (tPCC). Single-fluorophore flies were imaged with the same settings as double-fluorophore flies to determine the background levels of each channel. Global threshold was set to the weighted mean plus three standard deviations. M1 is the sum of above-threshold Drak-GFP pixel intensity values that overlap with above-threshold Filamin-mCherry signal, divided by the sum of above-threshold Drak-GFP pixel intensity values. M2 is the analogous value for Filamin-mCherry intensities. tPCC is the linear intensity correlation coefficient between Drak-GFP and Filamin-mCherry channels calculated from the above-threshold pixel intensity values. M1, M2, and tPCC were calculated separately for each image. Means and standard deviations were weighted by the number of informative pixels in each image: M1 by the count of Drak pixels above threshold, M2 by the count of above-threshold Filamin pixels, and tPCC by the count of pixels that exceeded the threshold in both channels.

As image areas with either no expression of the fluorophores or with high autofluorescence can skew co-localization analysis, we created an ImageJ macro to define ROI for the embryo colocalization analysis. The embryo edge areas that had high autofluorescence were manually excluded. Nuclear areas were excluded from the ROI as both fluorophores were mostly absent inside nuclei as expected. The ROI surrounding the nuclei was segmented by using Filamin-mCherry signal only as it defined the boundaries more consistently: The background value was first subtracted, then mean filter was applied with radius of 4 px, and finally using Auto Threshold with method "Otsu". Additionally, the imaging area had some high intensity autofluorescent spots in both channels that were automatically excluded: Spot masks for each channel were created by first subtracting background with rolling ball method using 50 px radius. Then mean filter with radius of 3 px was applied and the resulting image was subtracted from the background-removed image. Finally, Auto Threshold with method "MaxEntrophy" was used to segment the bright spots and the spots were dilated once with run("Dilate"). The spot mask that was excluded from the final ROI. The ROIs for tendon-myotube attachment site samples were defined manually by including the visible tendon signal and avoiding myotube tissue and highly autofluorescent areas. Representative colocalization images and ROI masks of each sample studied are given in Fig. S3 & Fig. S4.

AI tools were used to improve ImageJ and R code, and to rephrase individual sentences to increase clarity and to check for grammatical errors.

RESULTS

Drak binds to open Filamin

To identify mechanically regulated binding partners of Filamin, we ordered a yeast two hybrid screen with the *Drosophila* Filamin mechanosensor region. As bait, we used *Drosophila* filamin 16-19 domains with previously characterized open mechanosensor mutation exposing binding sites in domains 17 and 19. The screen identified 8 overlapping hits of the Death

associated protein kinase related protein Drak. All hits mapped to the COOH-terminal region of Drak and the minimal common sequence was residues 360-476 (Fig. 2A).

To biochemically verify the yeast two-hybrid interaction, we attached short GST-Drak fragments on glutathione Sepharose and used these in a pull-down system to test the interaction with Filamin. In this assay we used the previously characterized five-domain fragment of *Drosophila* Filamin (Huelsmann et al., 2016), because both the five-domain wild type fragment and the open and closed mechanosensory mutants could be readily expressed and were soluble. The file domain Filamin fragment was also readily distinguished in SDS-PAGE from the GST-Drak and its degradation products. The minimum Drak fragment that could be expressed and interacted with the Filamin-open protein contained amino acids 435-532 (Fig. 2A). At 10 µM concentration, Filamin-wild type showed weaker interaction with this Drak fragment than Filamin-open, whereas Filamin-closed did not interact (Fig. 2B). This difference could be reproduced with concentrations between 0.2 µM to 10 µM (Fig. 2C), but accurate Kd could not be determined, because of experimental variation.

Drak and Filamin in early embryo development

As Drak has a known function during cellularization, we next tested if it has similar localization with Filamin or its substrate myosin regulatory light chain, Spaghetti squash, in early *Drosophila* embryos. To do this, we genomically inserted Green fluorescent protein (GFP) in frame to the COOH-terminus of Drak and followed Drak-GFP in live time-lapse confocal imaging simultaneously either with Filamin-mCherry or Spaghetti squash-mCherry. At the onset of cellularization all three proteins were recruited to the cortical region of the embryo (Fig. 3A, XZ view). Drak recruitment followed Filamin and Spaghetti squash with a slight delay and remained high at the cortex for longer than the two others (Fig. 3B). This recruitment was linked to the last (14th) nuclear division. At the beginning of the division, Filamin and Spaghetti squash weakly marked the transient metaphase furrows at the plasma membrane between nuclei, but Drak was not detected in these structures (Fig. 3A, 0 min and 3 min). However, after the division Drak was seen in the forming cellularization furrows together with Filamin and Spaghetti squash (Fig. 3A, 6 min and 9 min, marked with white arrowhead in Drak XY view). When the furrows proceeded, Drak remained at the cortical region (Fig. 3A, black arrow on the right, XZ view, 21 min) whereas Spaghetti squash strongly localized to the basal region of the furrow (Fig. 3A, black arrowhead on the right, XZ view, 21 min) and Filamin was seen in both cortical and basal areas.

Later during cellularization, as the basal tip of the cellularization furrow containing the actomyosin ring advanced between the nuclei, Drak-GFP remained mainly diffuse in the cytoplasm. However, a small fraction of Drak-GFP was detected in the actomyosin ring together with Filamin (Fig. 3D). This partial localization of Drak was weakly seen in the confocal z-projections (Fig. 3D, middle row arrowheads on the left), and in the intensity profiles (Fig. 3D, 8,5 µm and 15,5 µm). When the actomyosin ring contracted below the nuclei, Drak fluorescence was not associated with the ring (Fig. 3D, 21 µm).

As the images from time lapse confocal recording had too low signal intensity for rigorous pixel-to-pixel colocalization analysis, we used higher laser power to take individual confocal

images of the embryos right after the 14th nuclear division, corresponding to the 6 min time point in Fig. 2A. We combined 37 images from 9 individuals for colocalization analysis and found that Drak-GFP and Filamin-mCherry showed high pixel-to -pixel overlap (Mander's coefficients M1= 0.997 ± 0.0017 (standard deviation, SD) M2=0.958 ± 0.039 (SD) and moderate intensity correlation with Pearson's correlation coefficient of 0.347 ± 0.094 (SD). Fig. 4 shows a representative image pair with the corresponding intensity dot-plot. Representative images from all samples and the control samples with individual fluorophores that were used for thresholding the intensities are shown in Supplemental Fig. S3. Correlation coefficient values from all images are given in supplemental Table SI. Colocalization analysis was validated by randomizing pixels in the Drak-GFP channel, resulting in close to zero tPCC values (supplemental Table S1).

To test if reducing Filamin-Drak interaction would cause similar changes in cellularization dynamics as Drak depletion, we compared the actomyosin ring circularity in flies expressing Filamin-closed MSR mutation to flies expressing wild type Filamin-GFP or Filamin-GFP with Drak-KO. We were able to reproduce the previously reported non-circular actin ring phenotype with Drak-KO, but Filamin-closed MSR actin rings did not differ from Filamin-wild type rings (Fig. 5A, and B). In line with this, myosin light chain phosphorylation was not reduced in Filamin-closed embryos, even though a reduction was detected in Drak-KO embryos (Fig. 5C).

In summary, we showed that Drak, Filamin and the Myosin light chain, Spaghetti squash partially overlap at the forming cellularization furrows. Later, Filamin strongly localizes to the actomyosin ring, while Drak becomes more diffuse but still slightly localizes there. Drak-KO flies showed defects in cellularization actomyosin ring circularity and the amount of phosphorylated myosin was reduced, but this was not observed with Filamin-closed MSR.

Drak and Filamin in *Drosophila* flight muscle development

We next studied the potential Filamin - Drak interaction in *Drosophila* developing muscles as Drak mRNA is detected during IFM development (Spletter et al., 2018). Living pupae were imaged by taking a confocal time series of the fly thorax. At 24-26 h APF Drak-GFP was detected in tendon cells and in the developing myotubes (Fig. 6A, B, C). At this time point, co-inciding with the time of muscle-tendon attachment site maturation, Filamin was mainly expressed in the tendon cells (Fig. 6D). Colocalization between Drak-GFP and Filamin-mCherry was detected in the tendon cells at the myotube-tendon attachment sites with structured Filamin distribution (Fig. 6E). Individual confocal slices from several samples were used for pixel-to-pixel colocalization analysis in tendon cells. The analysis showed high overlap between the fluorophores with weighted Mander's coefficients M1=1.000 ± 0.001 (SD) M2= 0.858± 0.193 (SD), and moderate intensity correlation, with weighted, thresholded Pearson's correlation coefficient of 0.444 ± 0.125 (SD) (Fig. 6F, Supplemental Fig. S4, and Supplemental Table S2).

During myotube compaction, Drak-GFP expression increased in myotubes (Fig. 7A,B,D) and suppl. Movie 1). Highest Drak-GFP intensity was observed at 35 h APF, coinciding with the maximum compaction of the developing myotube between 33-35 h APF (Fig. 7D). A weak Drak-GFP signal remained in the tendon cells through these stages (arrowhead Fig. 7B), but

Drak-GFP distribution was diffuse and no concentration to attachment sites or filamentous structures was observed either in myotubes or tendon cells. Filamin, however, was detected in the tendon cells in filamentous structures (Fig. 7C, arrowhead) and later appeared in the developing myotubes with a patterned distribution apparently coinciding with sarcomerogenesis (Fig. 7C 35 h and 37 h). Sqh-mCherry was detected in myotubes and weakly at the tendon cells (Fig. 7A).

To study if *Drak* and *Filamin* participate in the same processes during IFM development, we examined *Drak* knockout, *Filamin*-closed MSR, and a double mutant combining both mutations. We measured the length of the actin-rich muscle attachment sites in adult flies, as earlier studies have shown increased muscle attachment site length in flies with defective Filamin mechanosensory region (Green et al., 2018). Filamin-closed MSR mutant showed significantly increased attachment site length, while Drak-KO mutant had a slightly shorter length (Fig. 8A, B). Interestingly, the double mutant showed significantly longer attachment sites compared to additive effects of Drak-KO and Filamin-closed genotypes, showing an epistatic interaction.

In conclusion, Drak-GFP allowed us to follow Drak expression and localization in tendon cells and myotubes during IFM development. During most of the process Filamin is expressed in tendon cells and Drak in myotubes. However, during the myotube-tendon attachment maturation Drak and Filamin colocalize in tendon cells at the myotube attachment sites. In addition, we find that both proteins contribute to the normal morphology of mature muscle attachment site.

DISCUSSION

In this study, we report a potential mechanotransduction pathway involving Filamin and Drak. Filamins are known mechanosensors that change conformation in response to piconewton magnitude pulling force. Their mechanosensory region (MSR) is typically masked, making it challenging to identify binding partners using conventional screening methods. To overcome this, we have used Filamins with altered MSRs that expose two masked binding sites. We show that biochemically Drak preferentially bound to the open MSR of Filamin, less to the wild type Filamin MSR fragment and did not bind to closed MSR of Filamin. By inserting GFP in frame to the *Drak* locus, we demonstrate that Drak and Filamin have similar localization at the priming phase of cellularization and in tendon cells at the time of IFM attachment site maturation in pupa. We also found that Drak is expressed in the developing myotube at the time of maximal compaction. Both Drak knock-out and Filamin closed MSR alleles change the length of the actin-rich layer at the muscle attachment site. Since Drak is a known myosin light chain kinase, we suggest that opening Filamin's MSR either targets or activates Drak to phosphorylate myosin and thus forms a feed-forward loop of actomyosin contraction.

Below, we discuss the implications of the direct interaction between Drak and Filamin, and Drak-GFP localization studies in early embryo cellularization and in IFM development.

Filamin-Drak interaction

By combining yeast two hybrid and biochemical pull-down experiments, we were able to map the sequence required for Filamin binding to a 42 amino acid (aa) (435-476) stretch within the 380 aa long C-terminal, unstructured part of Drak. This was the overlap between the shortest interacting fragments in yeast two hybrid and pull-down experiments. Apparently the 42 aa is not sufficient for binding or not sufficiently stable for interaction, since the minimal interacting fragment in yeast two hybrid screen was 129 aa and in pull down experiment 97 aa. We observed that wild-type, open-MSR, and closed-MSR variants of Drosophila Filamin interact differently with C-terminal fragment of Drak.

While the wild type Filamin fragment binding was just detectable over the background at 10 μM protein concentration, the open-MSR mutant bound about 10 times more, and the closed-MSR did not bind (Fig. 1B and C). This suggests that the interaction of Drak and Filamin is specific and mechanically regulated.

Drak can be grouped to the family of death associated protein kinase (DAPK). Typically, this family proteins contain autoregulatory motifs C-terminal to the kinase domain (Temmerman et al., 2013). Among mammalian death associated protein kinases, DRAK1 and DRAK2 are the only members of the family whose C-terminal parts have no known motifs (Temmerman et al., 2013). In DRAK1 and DRAK2 the 80-90 aa long C-terminal parts are predicted to be intrinsically disordered (Uniprot Q9UEE5, human DRAK1; O94658 human DRAK2). In the genus *Drosophila* this disordered C-terminal part is even longer, 380 aa. According to AlphaFold prediction (identifier: AF-Q0KHT7-F1) and by protein disorder prediction programme such FoldIndex and IUPred this area shares features of intrinsically unfolded protein sequences, because it contains an exceptionally high proportion of polar and charged residues (Uniprot Q0KHT7, *Drosophila* Drak). Mapping the Filamin interaction to the C-terminal part of Drak raises the possibility that Filamin binding could regulate Drak kinase activity analogously to how Ca2+/Calmodulin regulates the kinase activity of DAPKs and myosin light chain kinases containing an autoregulatory domain C-terminal to the kinase domain (Temmerman et al., 2013). Alternatively, the role of Filamin-Drak interaction may be to recruit Drak to a specific subcellular location. Interestingly, Filamin and myosin are members of a large multiprotein complex, where some interactions with a transmembrane transporter are dependent on the Filamin MSR (Külshammer et al., 2022).

Drak in cellularization

Our results suggest that Drak has a role at the priming phase of cellularization. This is supported by three observations: 1) Drak-GFP was recruited to the cortical region after the last nuclear division before cellularization. 2) Drak-GFP transiently marked the developing cellularization furrows at this stage and colocalized with Filamin. 3) Drak KO allele causes a delay in the cellularization ring contraction, and the delay starts at the assembly state of the cellularization ring ((Chougule et al., 2016) and Fig. 5).

Filamin, myosin-2, and Drak are all known components of the actomyosin regulation function during cellularization and all three are translated from maternal mRNA (Sokac et al., 2022). However, they are just a part of the large protein repertoire involved in cellularization, which

requires spatial and dynamic coordination of various mechanisms such as membrane sculpting, endocytosis, vesicle transport and fusion, microtubules and actomyosin filaments (Sokac et al., 2022). Similar delay of the cellularization contraction as with the Drak mutant, has also been observed with inactive Sqh^{AA} mutation, where the phosphorylated residues are mutated to Ala (Xue and Sokac, 2016). All this is consistent with Drak being a myosin light chain kinase activating myosin contraction at the priming phase and at the early phase of cellularization (Chougule et al., 2016). Interaction of Drak with open Filamin MSR mutation and transient localization at priming phase suggest that Filamin might be involved in Drak function at the forming cellularization furrow. Unfortunately, we could not confirm the role of Filamin in Drak function by using the Filamin closed MSR mutation in vivo. We could not observe a consistent change in the actin ring dynamic in embryos with this Filamin mutation. We were able to reproduce the reduced myosin light chain phosphorylation with Drak knock out mutation, but we did not detect similar change in phosphorylation with the Filamin closed MSR mutant. It is possible that our Filamin closed MSR mutant is still partially functional in vivo. Stronger mutants, such as Filamin ΔMSR lacking six Ig-like domain at the MSR site or Filamin ΔC, lacking the entire C-terminus, could not be used for the live time-lapse recording or timed embryo collection, because they caused significant delay and irregular cellularization, even though some of the embryos eventually survive (data not shown).

Drak in Flight Muscle Development

To study the possible role of Drak and Drak-Filamin interaction in IFM development we studied Drak-GFP expression pattern during pupal developmental stages and combined Drak and Filamin mutant alleles. Drak-GFP protein expression was observed in the tendon cells at the time of tendon-myotube attachment site maturation and then peaks at developing IFM myotubes at the stage of maximal myotube compaction. The attachment site initiation includes formation of filopodial structures by the tendon cells that form integrin-containing contacts with the myotubes (Weitkunat et al., 2014). Apparently, the cells attach to each other via extracellular matrix, but the cellular cytoskeletal structures associated with integrins and attachment sites may be different in myotubes and tendon cells. It is interesting that Filamin and Drak appear to be components of the attachment site on the tendon side, but not in the myotube. Later, in mature muscle, Filamin is a component of the actin-rich layer between the integrin-associate juxta membrane complex and the first Z-line resembling structure (Green et al., 2018).

In our experiments, the maximal myotube contraction occurred between 33-35 h APF at 24°C. This corresponds to the 30 h APF point reported earlier at 27°C (Weitkunat et al., 2014). This compaction was associated with increased Drak-GFP signal. Earlier, similar increase of Drak mRNA has been observed with transcriptomics sequencing (Spletter et al., 2018). Our analysis confirms the transcriptomics results at protein level, provides better temporal resolution of protein expression changes, and shows the expression also in tendon cells. This suggests that Drak has some role in myotube and tendon cell compaction.

Earlier we reported defects in adult IFM caused by Filamin ΔC mutation and Filamin RNAi (Green et al., 2018). By extending the analysis with more samples, we now report that Filamin closed-MSR also causes a lengthening of the actin-rich layer of the IFM attachment site. Drak

KO alone caused a minor decrease in the attachment site. However, when the combined effect Filamin closed MSR and Drak KO mutation was statistically larger than the sum of the individual effects. This suggests that Drak and the Filamin mechanosensory region have a role in the same process. Co-localization of Drak and Filamin in the tendon cells at the time of myotube attachment suggests that this might be the event, when the two proteins have synergistic function, and the activation of tendon cells contraction by myosin might be partially regulated by filamin and Drak. Although Drak-KO flies had apparently normal muscle development, a defect in the muscle attachment site was seen in adult attachment sites. Combined with our colocalization data, this suggests a role for Drak in muscle-tendon attachment development which does not become apparent until the vigorous stress of adult muscle contractions is applied to the attachment sites.

In conclusion, we found direct biochemical interaction between Drak and the Filamin mechanosensory region. Mutations that open the mechanosensory sites, enhanced the interaction and mechanosensory site closing mutations diminished the interaction. Our studies with Drak-GFP shed new light to Drak expression and localization. Notably, Drak localization pattern transiently followed Filamin and myosin in the apical region of the forming cellularization furrows during the early stages of cellularization. Furthermore, Drak and filamin colocalized in tendon cells at the time of IFM myotube -tendon cell attachment. Drak binding to mechanically opened Filamin may be a novel mechanotransduction signalling pathway that fine-tunes myosin activity during tissue development.

Figures:

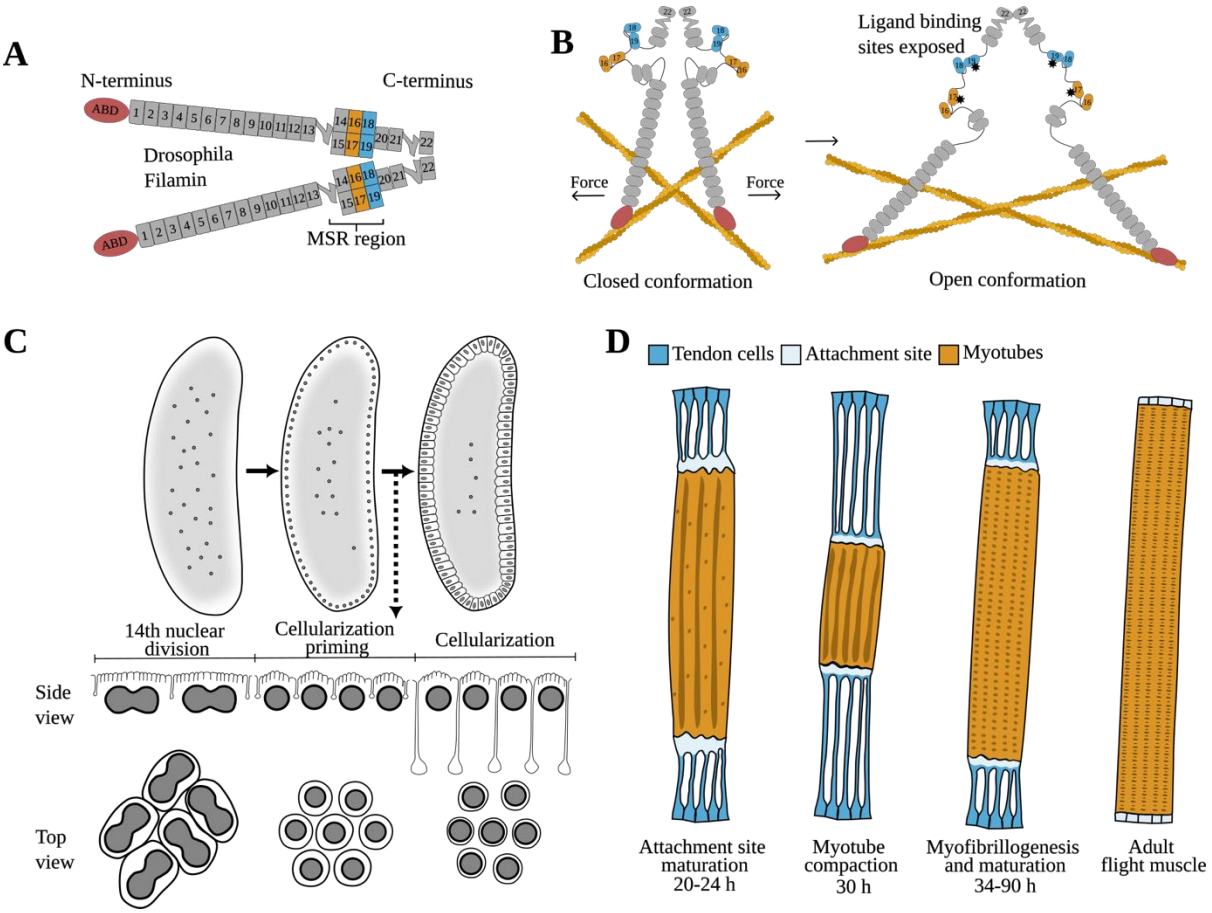

Figure 1: **Schematics of Filamin protein structure and the Drosophila tissue development events studied in this report.** A) Drosophila Filamin (Cheerio) and mammalian Filamins share dimeric structure and domain composition with the main difference that mammalian filamins have 24 filamin repeats and the Drosophila has 22. The domain numbering of Drosophila filamin is shown. Actin binding domains (ABD) are shown in red, prototypical filamin repeats in grey, and mechanically regulated domain pairs at C-terminal mechanosensory region (MSR) in orange and light blue. The two flexible hinge regions of each monomer are indicated. B) Model of the mechanically opening of the interaction sites at the mechanosensory region. In the closed conformation (left), protein binding sites are masked by protein sequences from the neighbouring domains. Actomyosin contraction favours the to open conformation (right) with exposed interaction sites (marked with asterix). C) Drosophila cellularization takes place after syncytium formation (upper left) and organization of nuclei close the to the plasma membrane (upper middle). After the 14th nuclear division of the embryo (bottom left), initial plasma membrane invaginations are formed between each nucleus, and an actomyosin ring is formed at the tip of the invaginations (bottom middle). The ring moves in between the nuclei with the membrane and eventually contracts to close the membrane under the nuclei (bottom right). Filamin is localized at the actomyosin ring shown in the top view. D) During pupal indirect flight muscle development, myoblast fuse to form myotubes that extend and attach to tendon

cells linking to the thorax epithelium. At the time of attachment site maturation (20–24 h APF), the contractile forces within the myotube-tendon cell system are low but gradually increase leading to myotube compaction (30 h APF). After the compaction (34-90 h APF), myotube sarcomere maturation and myofibrillogenesis continue until the time of adult enclosure (right image).

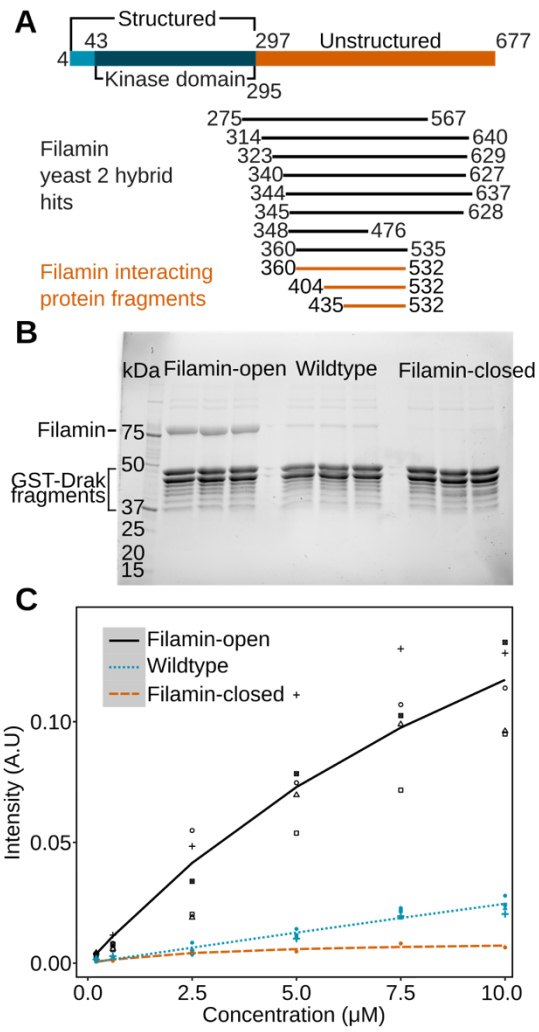

Figure 2. **Drak binds to open Filamin.** A) Schematic diagram of Drak kinase as depicted by Alphafold structure (Identifier: AF-Q0KHT7-F1), highlighting structured (blue) and unstructured (orange) regions, the kinase domain (dark blue), and their amino acid positions. Drak fragments interacting with Filamin are shown in black (yeast two-hybrid data), and orange (protein binding assay data). B) SDS-PAGE analysis of a pull-down experiment showing binding of Drak to Filamin-open (lanes 2-4), Filamin-wild type (lanes 6-8), and Filamin-closed (lanes 11-12). The positions of five domain Filamin fragments and GST-Drak fragments are shown on the left. Three technical replicates of the pull-down experiment with GST-Drak (435-532) and Filamin-open, wild type and closed are shown. C) Quantitation of Filamin binding with different concentrations. All measurement points are mean of triplicate measurements and replicate experiments, and the line shows the mean over separate experiments. All SDS-PAGE gels used for the analysis are shown in Suppl. Fig. S1.

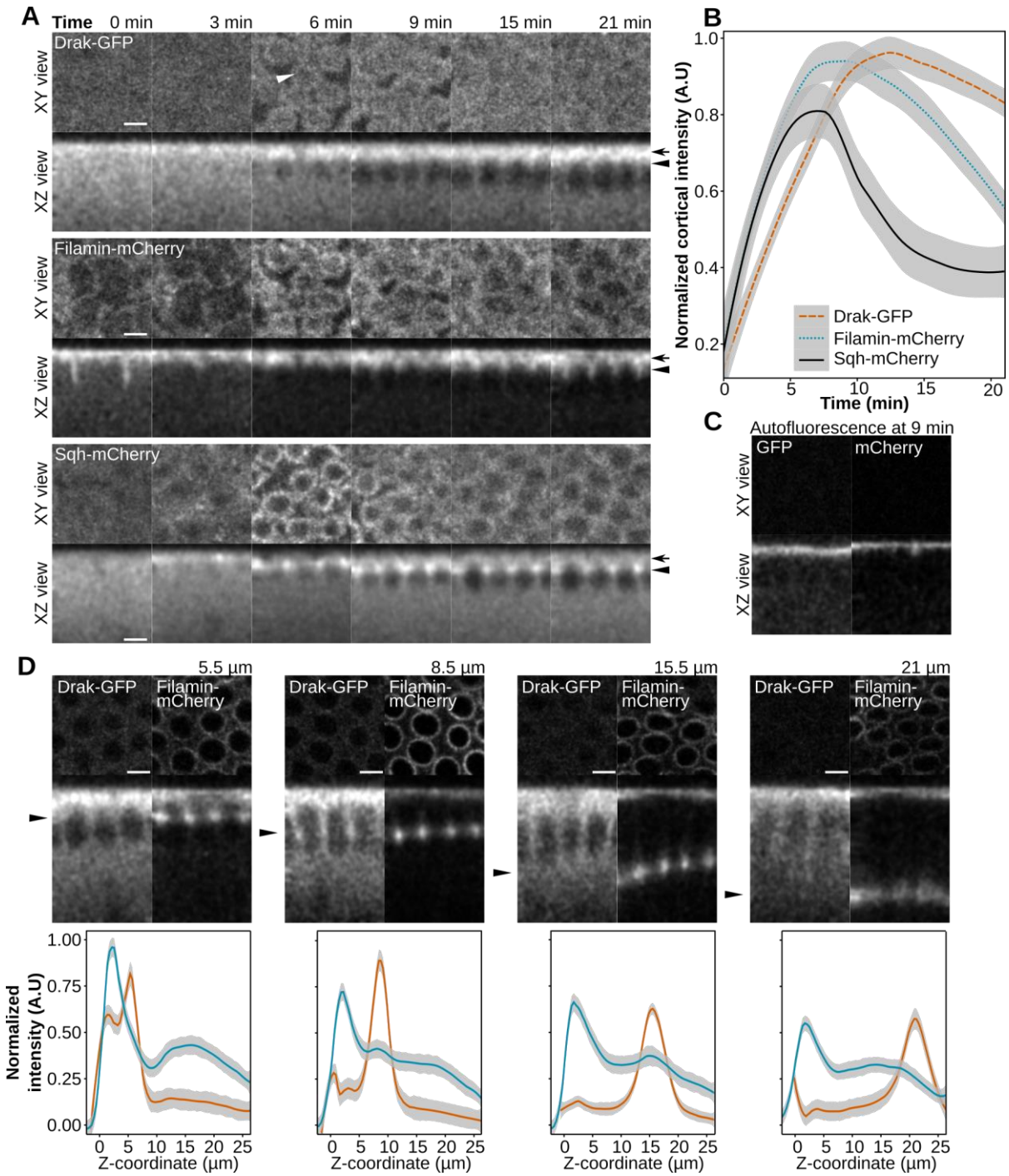

Figure 3. **Drak, Filamin and Spaghetti squash (Sqh) localization during cellularization.** A) Distribution of Drak-GFP, (top panel), Filamin -mCherry (middle panel), and Sqh-mCherry (bottom panel) during the first 0-21 minutes starting from the last nuclear division. Confocal microscope fluorescence signals are shown in top down (XY view: five confocal planes) and sagittal sections (XZ view; projection of 7.5 µm thickness). Drak-GFP and Filamin-mCherry images are from the same sample. The arrows on the right point to the cortical region and the triangles to the basal region of the cellularization furrows. B) Normalized and background corrected cortical intensity of Drak-GFP, Filamin-mCherry, and Sqh-mCherry during early stages of cellularization. C) Detected autofluorescence signal at 9 min time point with the same microscope and images setting as in GFP and mCherry channels. D) Top down and sagittal

sections of Drak-GFP and Filamin-mCherry from later stages of cellularization (top panel), during actomyosin ring ingression of 5.5 µm 8.5 µm, 15.5 µm and 21 µm from the surface of embryos. Arrows in the sagittal sections point to the location of the actomyosin ring. The bottom panel shows the normalized and background corrected intensity profiles of Filamin-mCherry and Drak-GFP from three timelapse recordings that were aligned using the Filamin-mCherry signal peak at the actomyosin ring position. All scalebar are 4 µm.

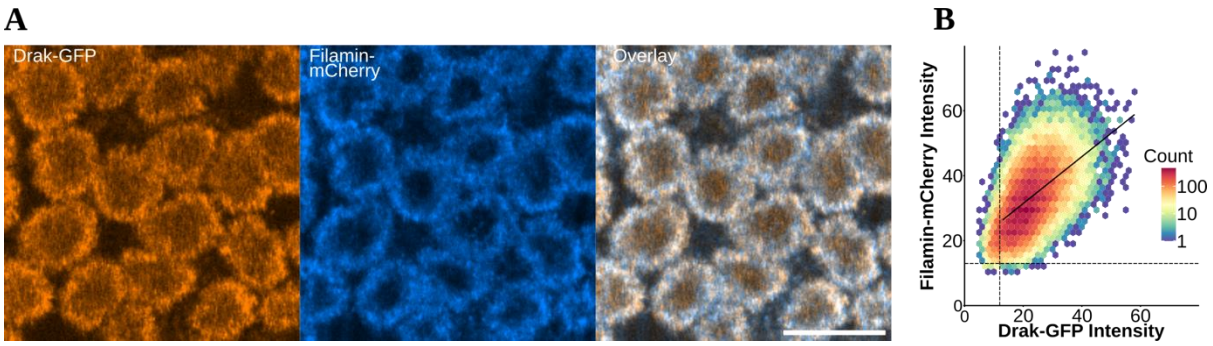

Figure 4. Co-localization analysis during the priming phase of cellularization show strong overlap between Drak and Filamin. One representative image (sample 8, image 1, see Supplementary Fig. S3) is shown. A) Drak-GFP (orange) and Filamin-mCherry (blue) show distinct distribution around the newly formed nuclei. Scale bar is 10 µm. B) Density dot plot of Drak-GFP and Filamin-mCherry pixel intensities. Dashed vertical line shows threshold for Drak-GFP channel and horizontal line for Filamin-mCherry. The linear regression line is plotted using above-threshold pixel values.

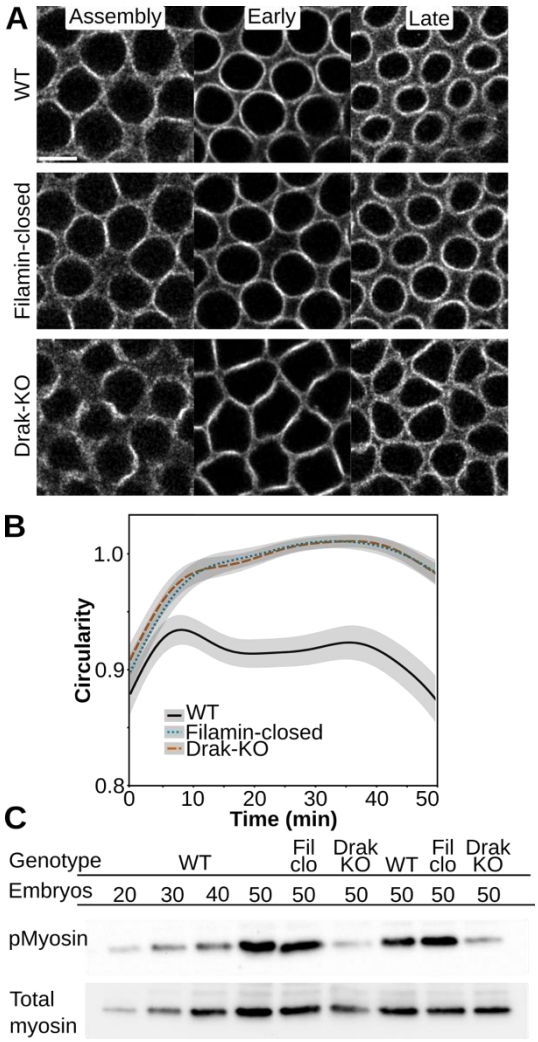

Figure 5. Drak knockout causes defected cellularization dynamics and reduced myosin phosphorylation. A) Representative confocal images of actomyosin rings during timepoints 6 min (left, assembly), 21-27 min (middle, early), and 39-45 min (right, late) in Filamin-wild type (WT), Filamin-closed, and Drak-KO flies, visualized with Filamin-GFP. Note that in Drak-KO embryos the actomyosin rings remain hexagonal, while in the other samples they became rounded already at the early cellularization. Scalebar is 5 µm. B) Circularity change of actomyosin rings over time in Filamin-wild type, Filamin -closed, and Drak knockout (Drak-KO) flies. C) Western blot of phosphorylated Sqh (pMyosin) and total myosin in genotypes Filamin-wild type, Filamin-closed, and Drak-KO. The number of embryos is shown. All gels of replicate analyses are given in Supplementary Fig. S1.

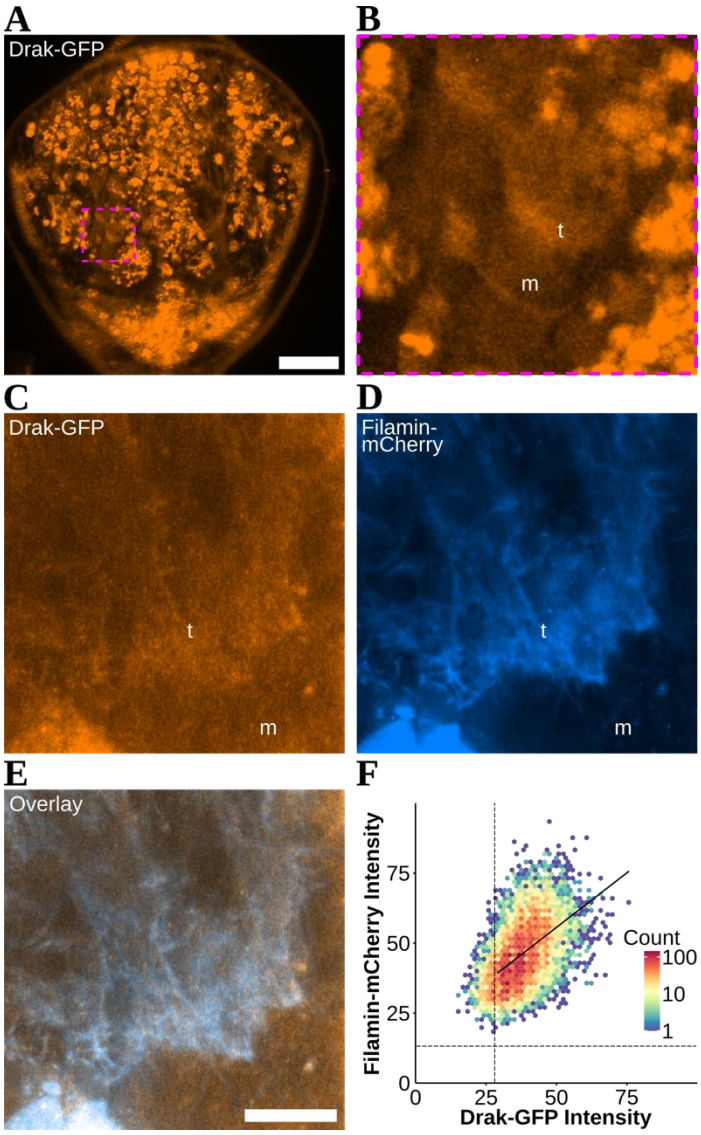

Figure 6. Drak and Filamin co-localize at tendons during tendon-myotube attachment site maturation in developing indirect flight muscles. A) Overview of Drosophila thorax during attachment site maturation 24-26h after pupal formation visualized by Drak-GFP (orange). Scalebar is 100 µm. Boxed area is enlarged in B. B) Close-up of the boxed area. t = tendon, m = myotube. C-E). Representative images used for quantitative colocalization analysis of single confocal slices corresponding to the same time point as in A and B are shown. Drak GFP-channel is shown red (C), Filamin-mCherry channel in blue (D) and the overlapping signal in white (E). Scalebar is 10 µm. F) Density dot plot of Drak-GFP and Filamin-mCherry pixel intensities. Dashed vertical line shows threshold for Drak-GFP channel and horizontal line for Filamin-mCherry. A linear regression line was plotted using only pixels that were above the threshold in both channels. Representative images of each sample are given in Supplementary Fig. S4. The images in C-F are from sample 3 image 1.

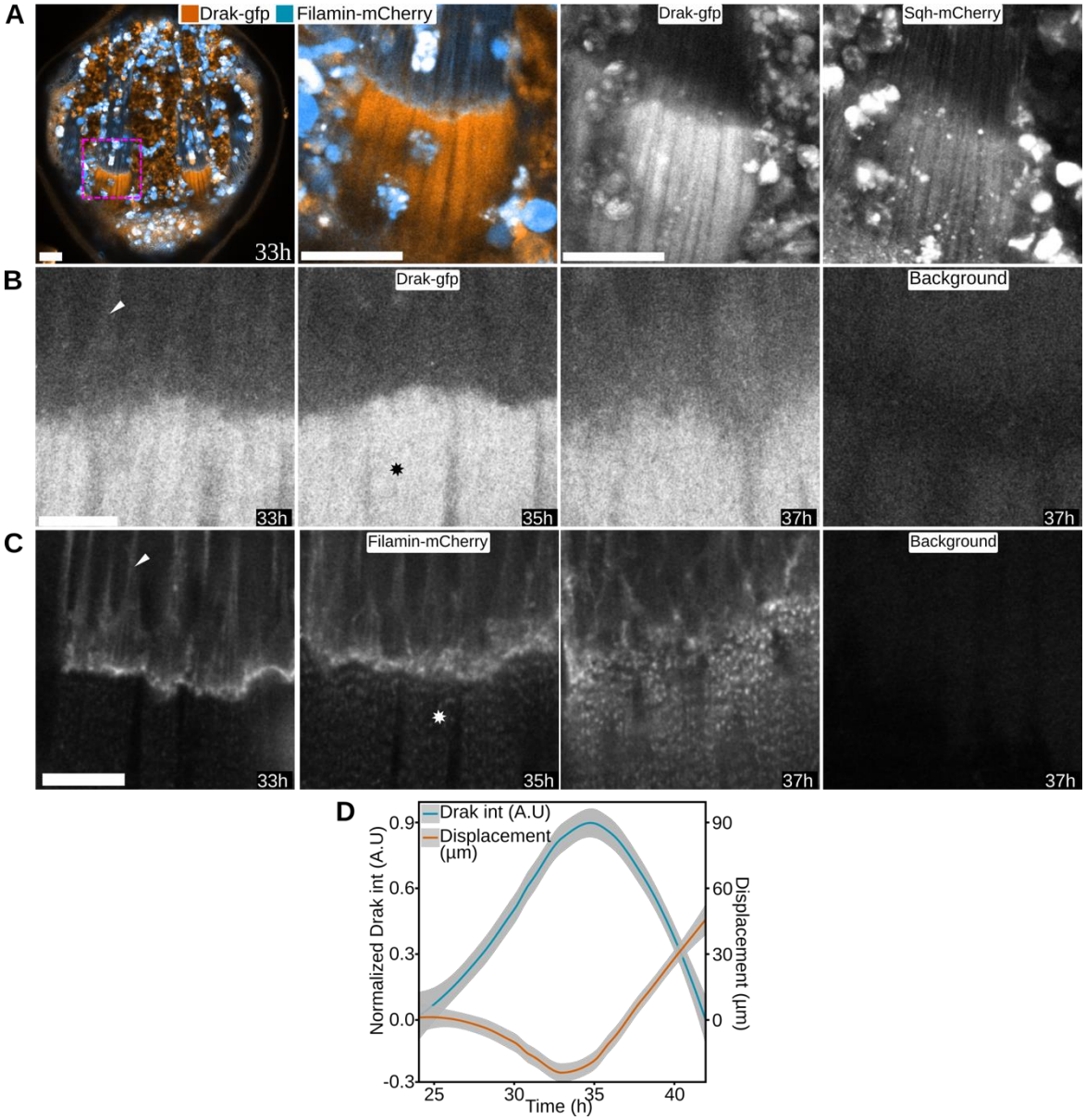

Figure 7. Drak, Filamin, and Spaghetti squash (Sqh) localize in muscle and tendon cells during indirect flight muscle (IFM) development. A) Confocal images of IFMs taken with 20x objective. Colored images on the left show Drak-GFP (orange) and Filamin-mCherry distribution (blue) in composite images at 33 h after pupal formation (APF). First image on the left shows the fly thorax, with IFMs on each side. Second image from the left shows a zoomed-in image of the compacting myotubes (zoom area highlighted in magenta). Third and fourth images show dual imaging of Drak-GFP and Sqh-mCherry from a pupa expressing both marked alleles. Scalebars are 50 µm. High-resolution single-channel images show the distribution of B) Drak-GFP, imaged with 63x objective, and C) Filamin-mCherry, imaged with 60x objective, in timepoints 33-37 h. Arrowheads indicate location of tendon cells, stars indicate myotubes. The rightmost panel image in both panels show the autofluorescence with same imaging parameters. Scalebars are 10 µm. D) Normalized Drak-GFP intensity in myotubes (blue, y-axis on the left) and displacement of the developing myotubes (orange, y-

axis on the right) plotted against average time after pupal formation (APF). The data represents mean ± standard error, n = 3.

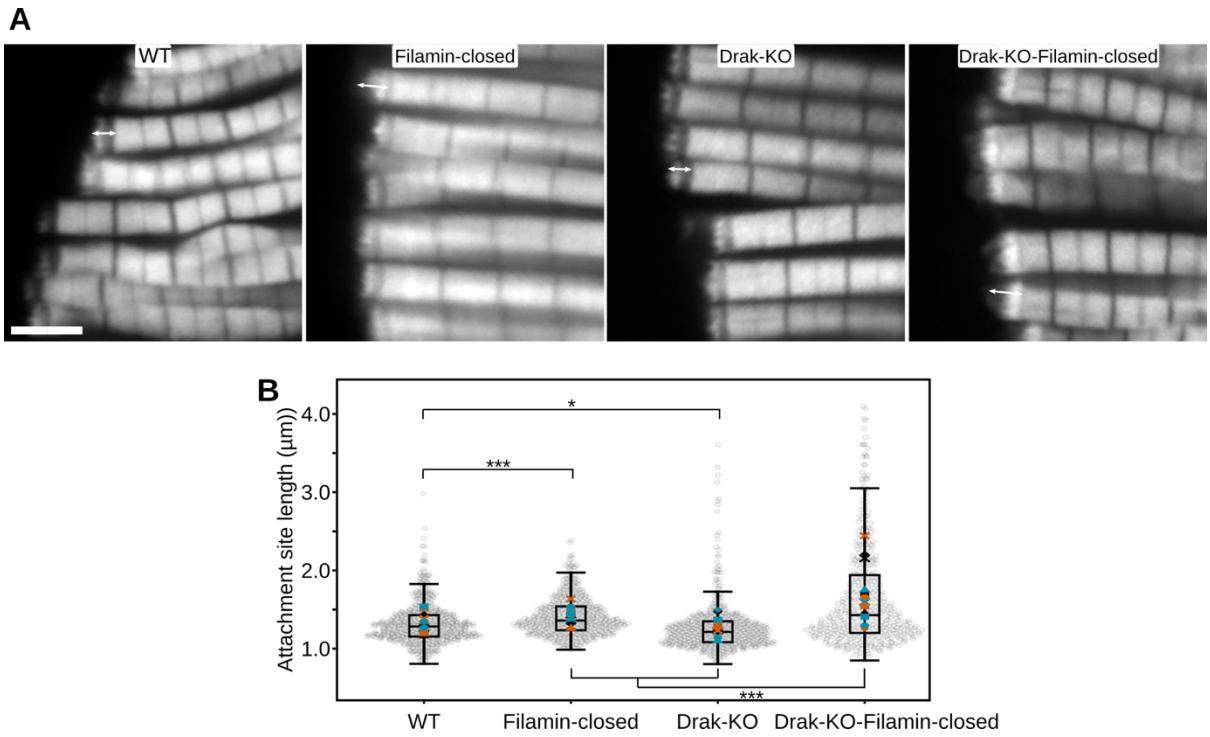

Figure 8. Attachment site length in adult flies. A) Representative confocal images of dissected adult IFMs in Filamin-wild type, Filamin-closed, Drak-KO, and Drak-KO Filamin-Closed flies. Double arrow shows the location of muscle attachment sites. Scale bar is 5 µm. B) Distribution of attachment site lengths across genotypes. Each genotype included 12-14 flies, with approximately 20 attachment sites measured per fly. Grey dots represent individual attachment site measurements, while coloured symbols indicate the mean values for each fly. The boxplot's central horizontal line represents the median for each genotype, the box edges mark the lower and upper quartiles, and the whiskers extend to the minimum and maximum data points, excluding outliers. *$P \leq 0.05$, ***$P \leq 0.001$

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Author contributions

Conceptualization: R.O.K, C.T., J.Y.; Methodology: R.O.K, C.T., H.J.G, J.Y.; Experiments and analysis: R.O.K, C.T., J.Y.; Writing - original draft: R.O.K, C.T., J.Y.; Writing review & editing: R.O.K, C.T., H.J.G, J.Y.; Funding acquisition: J.Y.

Conflict of interest statement

The authors declare no competing or financial interests.

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Data availability

All original data and software code will be made openly available in JYX (Jyväskylä Digital Repository). Editors or reviewers can ask for access from the correspondence author.

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