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RESEARCH ARTICLE

Binding partners of the kinase domains in *Drosophila* obscurin and their effect on the structure of the flight muscle

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ABSTRACT

Drosophila obscurin (Unc-89) is a titin-like protein in the M-line of the muscle sarcomere. Obscurin has two kinase domains near the C-terminus, both of which are predicted to be inactive. We have identified proteins binding to the kinase domains. Kinase domain 1 bound Bällchen (Ball, an active kinase), and both kinase domains 1 and 2 bound MASK (a 400-kDa protein with ankyrin repeats). Ball was present in the Z-disc and M-line of the indirect flight muscle (IFM) and was diffusely distributed in the sarcomere. MASK was present in both the M-line and the Z-disc. Reducing expression of Ball or MASK by siRNA resulted in abnormalities in the IFM, including missing M-lines and multiple Z-discs. Obscurin was still present, suggesting that the kinase domains act as a scaffold binding Ball and MASK. Unlike obscurin in vertebrate skeletal muscle, *Drosophila* obscurin is necessary for the correct assembly of the IFM sarcomere. We show that Ball and MASK act downstream of obscurin, and both are needed for development of a well defined M-line and Z-disc. The proteins have not previously been identified in *Drosophila* muscle.

KEY WORDS: *Drosophila*, Obscurin, Unc-89, M-line, Ball, MASK

INTRODUCTION

A stable lattice of thick and thin filaments in striated muscle is needed to maintain the optimum register of the filaments as the fibres contract. Thin filaments from neighbouring sarcomeres are anchored in the Z-disc by α-actinin and other cross-linking proteins, and thick filaments are held in position by cross-links at the M-line in the middle of the sarcomere (Agarkova and Perriard, 2005; Luther, 2009). The register of thick filaments is also maintained by elastic links between the ends of the filaments and the Z-disc (Horowits and Podolsky, 1987; Tskhovrebova and Trinick, 2003). Large modular proteins of the titin family, associated with thick filaments, contribute to both the stability and the stiffness of the sarcomere. These proteins are made up of tandem immunoglobulin (Ig) and fibronectin-like (Fn3) domains and can have one, or sometimes two, kinase domains near the C-terminus, and there can also be signalling domains (Bullard et al., 2005; Labeit and Kolmerer, 1995; Linke, 2008; Russell et al., 2002; Small et al., 2004; Young et al., 2001).

The M-line protein, obscurin, has a similar modular structure in invertebrates and vertebrates, although the number of modules in different isoforms and the position of the signalling domains vary. Both Unc-89 (the obscurin homologue in *Caenorhabditis elegans*; note that this protein is also known as Unc-89 in *Drosophila*) and obscurin in *Drosophila* have SH3 and Rho-GEF signalling domains near the N-terminus and two kinase domains near the C-terminus (Benian et al., 1996; Katzemich et al., 2012; Small et al., 2004). In vertebrate obscurin, the signalling domains are near the C-terminus; the isoform obscurin A has an ankyrin-binding domain instead of the two C-terminal kinase domains in obscurin B. Both these isoforms are at the periphery of myofibrils in the M-line region of mature skeletal fibres (Fukuzawa et al., 2008; Russell et al., 2002; Young et al., 2001). Binding of obscurin A to ankyrins creates a link between the sarcoplasmic reticulum (SR) and the myofibril (Bagnato et al., 2003; Kontrogianni-Konstantopoulos et al., 2003; Lange et al., 2009). By contrast, *Drosophila* obscurin is found throughout the M-line and there is no ankyrin-binding domain, so direct binding to the SR is unlikely (Katzemich et al., 2012). However, in the nematode, loss-of-function mutations in *unc89* result in displaced ryanodine receptor and SERCA, as well as abnormal Ca^{2+} signalling (Spooner et al., 2012). This suggests that there is a function for Unc-89 in Ca^{2+} regulation involving the SR. So far, five large isoforms of obscurin have been identified in *Drosophila* muscles: one expressed in the larva, and four expressed in the pupa and adult. All these isoforms have Ig domains in the tandem Ig region, and at least the first of the kinase domains (denoted Kin1). The indirect flight muscle (IFM) has two isoforms: a major isoform of 475 kDa and a minor isoform that is somewhat smaller (Katzemich et al., 2012). The two remaining isoforms are in other thoracic muscles. *Drosophila* obscurin is essential for the formation of an M-line, and for the correct assembly of thick and thin filaments in the sarcomere: lack of obscurin in the IFM results in asymmetrical thick filaments and thin filaments of abnormal length and polarity. Paradoxically, vertebrate obscurin is not necessary for normal sarcomere structure, given that obscurin knockout in the mouse had no serious effect on sarcomere assembly or maintenance (Lange et al., 2009).

The kinase domains of titin-like proteins often function as scaffolds binding other proteins, and might or might not be active kinases (Endicott et al., 2012; Gautel, 2011a; Mayans et al., 2013). In *C. elegans*, the Unc-89 kinase 1 domain (PK1) is predicted to be inactive because the ATP-binding site lacks essential residues. The Unc-89 kinase 2 domain (PK2) might be active, although a motif contributing to ATP-binding is atypical (Small et al., 2004). Both Unc-89 kinase domains interact with the protein small, C-terminal domain, phosphatase-like 1 (SCPL-1), which is thought to be involved in muscle-specific signalling (Qadota et al., 2008). Unc-89 PK1 also interacts with the LIM-domain protein, LIM-9; the complex of PK1, SCPL-1 and LIM-9 links Unc-89 to integrin adhesion sites at the cell surface (Lin et al., 2003; Warner et al., 2013; Xiong et al., 2009). In *Drosophila*, both obscurin the kinase domains (denoted Kin1 and Kin2) are predicted to be inactive

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because the catalytic site lacks the catalytic aspartate and other crucial residues (Endicott et al., 2012; Mayans et al., 2013). Both kinase domains in vertebrate obscurin B are predicted to be catalytically active, and can apparently be auto-phosphorylated (Endicott et al., 2012; Hu and Kontrogianni-Konstantopoulos, 2013; Mayans et al., 2013). The kinase domains are reported to interact with membrane associated proteins: kinase domain 1 (SK1) with the extracellular domain of a Na^+/K^+-ATPase at adherens junctions, which is not a substrate, and kinase domain 2 (SK2) with the cell-adhesion molecule, N-cadherin, which is an *in vitro* substrate (Hu and Kontrogianni-Konstantopoulos, 2013).

The kinase domains in titin-like proteins have sequences at the C-terminus that sterically block the active site (the C-terminal regulatory domain). This sequence can inhibit an active kinase, or regulate ligand binding; it can also be part of the structure of the kinase domain, and necessary to maintain the stability of the domain (Gautel, 2011a; Mayans et al., 2013; von Castelmur et al., 2012). Titin-like kinases are linked to stretch-activated signalling pathways in muscle. Mechano-sensing by the kinase can result in changes in the C-terminal regulatory domain and transient binding of ligands to the kinase scaffold. The precise mechanism of regulation varies in different species (Lange et al., 2005; Mayans et al., 2013; Puchner et al., 2008; von Castelmur et al., 2012).

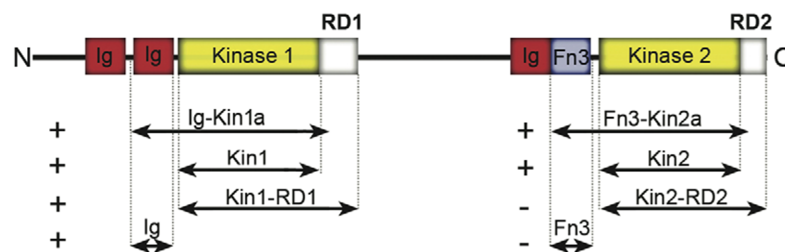
The aim of this study was to identify proteins binding to the two kinase domains in *Drosophila* obscurin, and to determine the effect of the proteins on the assembly of an ordered sarcomere in IFM. We show that Ball (a protein kinase) binds to Kin1, and MASK (an ankyrin repeat protein) binds to both Kin1 and Kin2. The kinase ligands are essential for the formation of an intact M-line and Z-disc in the IFM sarcomere.

RESULTS

Kinase domains of *Drosophila* obscurin

The arrangement of kinase domains near the C-terminus of *Drosophila* obscurin is similar to that of the kinase domains in the vertebrate and nematode proteins. Kin1 is preceded by two IgG domains, and Kin2 by an IgG and an Fn3 domain (Fig. 1A). Both kinase domains lack residues in the motifs that are present in an active kinase (Endicott et al., 2012; Hanks and Hunter, 1995; Mayans et al., 2013). Supplementary material Fig. S1 shows an alignment of *Drosophila* obscurin kinase domains with the *C. elegans* twitchin kinase, which is known to be active (Lei et al., 1994) (supplementary material Fig. S1A). Kin1 has motifs involved in binding ATP and Mg^{2+}: the glycine-rich loop, an invariant lysine in the ATP-binding motif, and the DFG motif. Kin2 has the invariant lysine and the DFG motif, but the glycine-rich loop has a single glycine. The catalytic motif in both kinases lacks the catalytic aspartate residue. However, in Kin2, the aspartate is replaced with an asparagine residue, which can result in catalytic activity through compensation by other parts of the kinase (Mayans et al., 2013). Active kinases typically have a lysine and an asparagine residue, which chelates Mg^{2+}, downstream of the catalytic aspartate residue; these are also missing in Kin1, although Kin2 does have the asparagine residue. Kin1 is predicted to be an inactive pseudokinase, whereas it is possible that Kin2 might have some activity (Mayans et al., 2013). Sequences C-terminal to Kin1 and Kin2 have little similarity with the regulatory domain of titin kinase (data not shown). The sequence following Kin1 is unusually long (~209 residues), compared with that following Kin2 (~66 residues); the function of the extra sequence is not known.

A Obscurin C-terminal domains



B Ball

C MASK

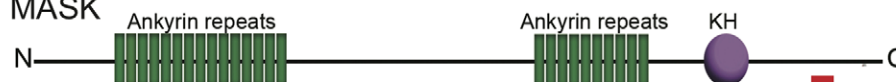


Fig. 1. Domains in the C-terminus of obscurin and their interaction with Ball and MASK. (A) There are two putative kinase domains (yellow), both of which have a C-terminal sequence that might be regulatory (RD, white). Kin1 is preceded by two Ig domains (red) and Kin2 by an Ig domain and an Fn3 domain (blue). In an initial yeast two-hybrid screen, Ig-Kin1a (with the preceding Ig domain and part of RD1) interacted with Ball. Interactions of other Kin1 constructs with Ball are shown: all interacted with Ball. In an initial Kin2 screen, Fn3-Kin2a interacted with MASK. Interactions of other Kin2 constructs with MASK are shown: Fn3 and Kin2-RD2 did not interact with MASK. (B) Ball is a kinase (yellow) of ~66 kDa, with a C-terminal non-modular tail. Kin1 bound to a region in the C-terminus. (C) MASK is a protein of ~400 kDa with multiple ankyrin repeats (green) and a C-terminal KH domain (magenta). Kin2 interacted with sequence in the C-terminus of MASK. The red bars below the Ball and MASK diagrams correspond to the sequence of prey clones identified in the yeast two-hybrid assay. Residue numbers are: Ball 448–599 (GenBank accession number AE014297); MASK 3516–3555 (GenBank accession number AF425651).

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Identification of binding partners of Kin1

In order to find ligands binding to the two kinase domains, we screened an adult *Drosophila* cDNA library with constructs from Kin1 or Kin2, using the yeast two-hybrid method. For Kin1, the bait construct included the Ig domain preceding the kinase sequence and part of a possible regulatory sequence (in the case of titin kinase, part of the regulatory domain is needed for correct folding and stability of the kinase; Lange et al., 2005). Residues included in this construct, Ig-Kin1a, are given in supplementary material Table S1. In the yeast two-hybrid screen, there were 75 positive clones. Seventeen of the clones were strongly positive in a β-galactosidase assay; plasmids from these were isolated and the inserts sequenced. Table 1 shows a selection of the positive clones that might be relevant to the development or function of muscle.

Two clones that grew in this screen contained prey plasmids with independent inserts of *bällchen* (*ball*). These clones were followed up because of their repeated identification and their apparently strong interaction with Kin1. *pACT2[ball1]* contained 570 base pairs in the 3′ end of the coding region of the gene, and *pACT2[ball2]* contained 429 base pairs that fully overlapped with the sequence of *pACT2[ball1]*. The *ball* gene encodes a 66-kDa protein kinase. C-terminal to the kinase domain, there is a tail of 271 amino acids of unknown structure (Fig. 1B). The sequence of *ball1* corresponds to a peptide of 190 amino acids and that of *ball2* to a peptide of 143 amino acids; both peptides are in the C-terminal tail (Fig. 1B and supplementary material Table S1). Unlike Kin1 and Kin2, Ball is predicted to be an active kinase homologous to the vaccinia-related kinases (VRKs), which are found in all invertebrates and vertebrates (Aihara et al., 2004; Herzig et al., 2014; Lancaster et al., 2007) (supplementary material Fig. S1B). The sequence has the glycine-rich loop, and DFG motifs needed for binding ATP and Mg^{2+}; in VRK, the phenylalanine residue in the DFG motif is replaced by a tyrosine residue. The lysine in the ATP-binding motif that is involved in correctly orienting phosphate groups of ATP is present in both Ball and VRK. The catalytic motif in both proteins has the conserved aspartate residue, as well as the downstream lysine and asparagine residues.

Table 1. Proteins interacting with obscurin kinases

Gene name	FlyBase number	Function
Kin1		
bällchen (*ball*)	CG6386	Serine/threonine kinase. Genetically linked to signalling pathway in myogenesis.
pendulin (*pen*)	CG4799	Cytoplasmic transport. Mutant phenotypes in embryonic and larval muscles.
triose phosphate isomerase (*tpi*)	CG2171	Found in IFM Z-disc and M-line and in adult heart muscle. Reacts to mechanical stimuli.
Evi5	CG11727	Rab-GTPase activator.
broad (*br*)	CG11491	DNA-binding protein. Mutant has IFM phenotype.
Kin2		
MASK	CG33106	Has ankyrin repeats and KH domain. In cytoskeleton.
Ubiquitin-specific protease 14	CG5384	Ubiquitin thioesterase. Associated with microtubules.
Not named	CG9769	Ubiquitin protease.
Not named	CG6770	Predicted to bind DNA.

Encoded proteins binding to kinase constructs were identified in yeast two-hybrid assays; the function is taken from FlyBase. Genes listed for Kin1 are relevant to muscle development or function.

In order to find out which domains of Ig-Kin1a interacted with Ball, several shorter bait constructs were co-transformed with the construct containing the longer *ball1* sequence. All the constructs tested interacted with Ball, including the Ig domain preceding the kinase domain, and Kin1-RD1, which had sequence C-terminal to the kinase (Fig. 1A). Although these C-terminal sequences in titin-like kinases can inhibit enzymatic activity or ligand binding (Mayans et al., 2013), in the case of Kin1, the sequence following the kinase is unusually long and a longer stretch might be needed to inhibit the interaction with Ball; alternatively, association of Ball with Kin1 might not be regulated in this way. In order to test for possible non-specific interaction between Ball and the Kin1 constructs, we co-transformed *ball1* with Fn3, Kin2a and Kin2-RD2 and found that Ball does not interact with any of these. This result confirms that the interaction observed between *ball1* and the Kin1 constructs, including the Ig domain, is specific.

Ball in flight muscle and the effect of reduced expression

Ball was detected in the IFM by labelling with a specific antibody (Herzig et al., 2014). The myofibril in Fig. 2A shows that Ball was present in the Z-disc and was distributed diffusely across the sarcomere of the wild-type IFM. In another sample of myofibrils, Ball was detected in the M-line as well as the Z-disc (see Fig. 5A). The Z-disc labelling was unexpected because Ball binds to obscurin, which is in the M-line. To investigate the function of Ball in the development and structure of the IFM, we looked for Ball mutants. Unfortunately, many Ball alleles are lethal in embryonic or larval stages. We therefore used small interfering RNA (siRNA) targeting Ball to reduce expression. The *GAL4-UAS* system with the driver *Mef2-GAL4,* promotes expression in all muscles. The driver line was crossed with two different *UAS* lines: *UAS-ball1(ex2)-IR*, which targets exon 2 towards the 3′ end (line 108630), and *UAS-ball2(ex1-2)-IR*, which targets exon 1 and exon 2 close to the 5′ end (line 48980). The targets do not overlap. Ball is encoded by only two exons. *UAS-ball1(ex2)-IR* flies driven with *Mef2-GAL4* at 29°C eclosed but were flightless. *UAS-ball2(ex1-2)-IR* flies with the same driver did not eclose at 29°C (91% died as early pupae); at 25°C, 12% of the pupated progeny eclosed and were flightless (82% died as early pupae). Therefore, the *UAS-ball2(ex1-2)-IR* line with the *Mef2-GAL4* driver might be more efficient in knocking down Ball expression. The lack of flight ability in the two *UAS-ball-IR* lines crossed with the *Mef2-GAL4* driver, compared with the *UAS-ball-IR* and *Mef2-GAL4* lines alone is shown in Fig. 2C. In this study, we mainly used *Mef2-GAL4;UAS-ball1(ex2)-IR* flies, in which flight was affected but not viability.

Structure of myofibrils in Ball knockdown flies

Ball was undetectable in myofibrils from the IFM of *Mef2-GAL4; UAS-ball1(ex2)-IR* flies (Fig. 2A). Myofibrils were often narrower than in the wild-type and appeared wavy. In less-affected myofibrils, the M-line and Z-disc were regularly spaced and the sarcomere length was somewhat shorter than in the wild-type (supplementary material Fig. S2). Obscurin was still present in the M-line and Kettin in the Z-disc of IFM lacking Ball (Fig. 2B). Z-discs stained with anti-Kettin antibody were frayed and diffuse at the edges, compared to the wild-type. In these myofibrils, lack of Ball had a greater effect on the Z-disc than on the M-line. The effect of Ball knockdown on the fine structure of the IFM sarcomere was seen in electron micrographs of the *Mef2-GAL4;UAS-ball1(ex2)-IR* line (Fig. 2D). In the more severe cases, rudimentary Z-discs were

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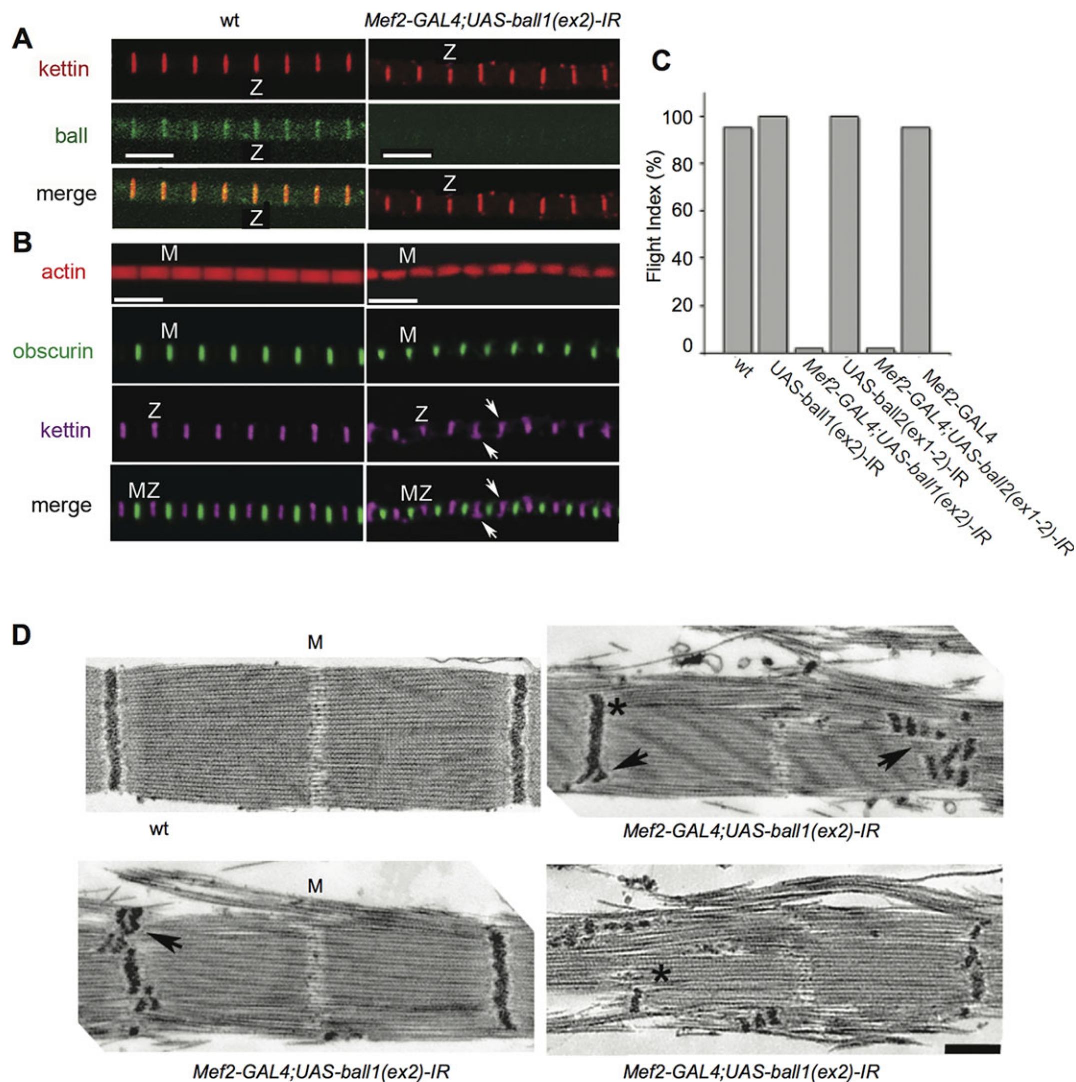

Fig. 2. The position of Ball in the IFM and the effect of reduced expression. (A) Ball in the IFM. Myofibrils were labelled with anti-Ball antibody (green) and with anti-Kettin antibody as a Z-disc (Z) marker (red). In wild-type (wt) myofibrils, Ball is labelled at the Z-disc and, more weakly, across the sarcomere. No Ball labelling is detected in myofibrils of Ball-knockdown flies. (B) Abnormal sarcomeres in IFM of Ball-knockdown flies compared with sarcomeres in wild-type IFM. Myofibrils were labelled for actin with phalloidin (red), at the M-line (M) with anti-obscurin antibody (green) and at the Z-disc with anti-Kettin antibody (magenta). In Ball-knockdown flies, labelling of actin, the M-line and the Z-disc is less regular than in the wild-type. Obscurin is present in the M-line, but labelling is narrower than in the wild-type; Z-discs in this example appear fuzzy at the periphery (arrows). Scale bars: 5 µm. (C) Flight tests of wild-type and Ball-knockdown flies. The percentage of flies able to fly is shown, n=20 for each genotype. Flies were flightless in *Mef2-GAL4*;*UAS-ball1(ex2)-IR* and *Mef2-GAL4;UAS-ball2(ex1-2)-IR* RNAi lines. Control flies with *UAS-ball-IR* or *Mef2-GAL4* alone flew normally. Crosses with *UAS-ball1(ex2)-IR* were performed at 29°C and *UAS-ball2(ex1-2)-IR* at 25°C. (D) Electron microscopy images of wild-type and Ball-knockdown flies. Z-discs of IFM in Ball-knockdown flies are often fragmented and shifted towards the core of the myofibril (arrows), leading to fraying of thick and thin filaments, and M-line disruption at the periphery. In several cases, Z-discs are broken and do not span the entire myofibril, with thick and thin filaments bypassing the Z-disc (asterisks). Scale bar: 500 nm.

shifted into the region of thick and thin filament overlap and by-passed by abnormally long filaments. The H-zone was in the middle of the sarcomere, except where Z-discs were displaced. Thus, lack of Ball has an effect on the Z-disc, as well as on the position of the M-line and H-zone. Ball knockdown in the *Mef2-GAL4;UAS-ball2(ex1-2)-IR* line resulted in fragmented Z-discs and M-lines and ragged filaments (supplementary material Fig. S3).

Identification of a binding partner of Kin2

As for Kin1, the *Drosophila* cDNA library was screened with a construct from the Kin2 region of obscurin as bait. The construct, Fn3-Kin2a, included the Fn3 domain preceding the kinase sequence and part of a potential regulatory sequence (supplementary material Table S1). There were 16 positive clones in the screen, all of which were positive in a β-galactosidase assay. Five of the clones had sequence of mitochondrial DNA and seven had sequence of the empty vector. Other clones are listed in Table 1. One clone contained 117 base pairs in the 3′end of the coding region of an 18 kb gene, *mask* (CG33106). The gene encodes the 400-kDa protein Multiple Ankyrin repeats Single KH domain (MASK), which has ankyrin repeats and a K-homology domain (KH domain, involved in RNA recognition) (Fig. 1C). The *mask* clone was followed up because of the potential importance of ankyrin repeats in ligand binding. The clone corresponds to a peptide of 39 amino acids near the non-modular C-terminus of the protein (Fig. 1C; supplementary material Table S1).

In order to find out which domains of Fn3-Kin2a interact with MASK, bait constructs were co-transformed with the *mask* prey

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plasmid. Kin2, without sequence C-terminal to the kinase domain, interacted with the MASK peptide. However, Kin2-RD2, which had sequence C-terminal to the kinase, and the Fn3 domain alone, did not interact with the MASK peptide (Fig. 1A). Thus, interaction with MASK requires the kinase domain. Although the C-terminal sequence appears to inhibit MASK binding to Kin2 in the yeast two-hybrid assay, the sequence cannot be considered regulatory without testing peptide binding *in vitro*.

MASK in flight muscle and the effect of reduced expression

MASK was detected by labelling the IFM with antibody to MASK (Smith et al., 2002). MASK was present in both the M-line and the Z-disc of wild-type IFM myofibrils, although labelling was more intense on the Z-disc than on the M-line (Fig. 3A). Existing mutant alleles of *mask* are lethal at the larval stage (Smith et al., 2002). Therefore, in order to investigate the function of MASK in IFM, we used siRNA to reduce MASK expression. The *Mef2-GAL4* driver line was crossed with two different *UAS* lines: *UAS-mask(ex1)-IR* targeting exon 1, which encodes the N-terminal region of the gene, and *UAS-mask(ex6)-IR* targeting exon 6, which encodes six of the N-terminal ankyrin repeats. The flightless phenotype of the *UAS-mask(ex1)-IR* line was previously identified in a screen for flightlessness in siRNA mutants (Schnorrer et al., 2010). Most of the *UAS-mask(ex1)-IR* flies driven by *Mef2-GAL4* at 29°C did not eclose (88% died as late pupae and 12% eclosed but were flightless). All *UAS-mask(ex6)-IR* flies driven by *Mef2-GAL4* at 29°C died as late pupae; whereas at 25°C, all pupated progeny eclosed but were flightless (Fig. 3C). We used both *UAS* lines in this study. The late-lethal phenotype of the *mask* siRNA flies is consistent with a function for MASK in the muscles of the fly that are needed for eclosure from the pupal case.

Structure of myofibrils in MASK knockdown flies

MASK was barely detectable in IFM myofibrils of *Mef2-GAL4; UAS-mask(ex1)-IR* flies (Fig. 3A). The few remaining fluorescent blobs suggest the knockdown of MASK might not generate a completely null fly. Mutant myofibrils were narrower than in wild-type and sarcomeres were shorter (supplementary material Fig. S2). Z-discs and M-lines were regularly spaced, but although Z-discs appeared normal, M-lines were crooked (Fig. 3B). The extent to which myofibrils were affected in mutant flies was variable; myofibrils with relatively normal sarcomere structure were selected for immunofluorescence because the more disrupted myofibrils did not give clear images. The sarcomere length of myofibrils from the *Mef2-GAL4;UAS-MASK(ex6)-IR* line were closer to that of the wild type (supplementary material Fig. S2).

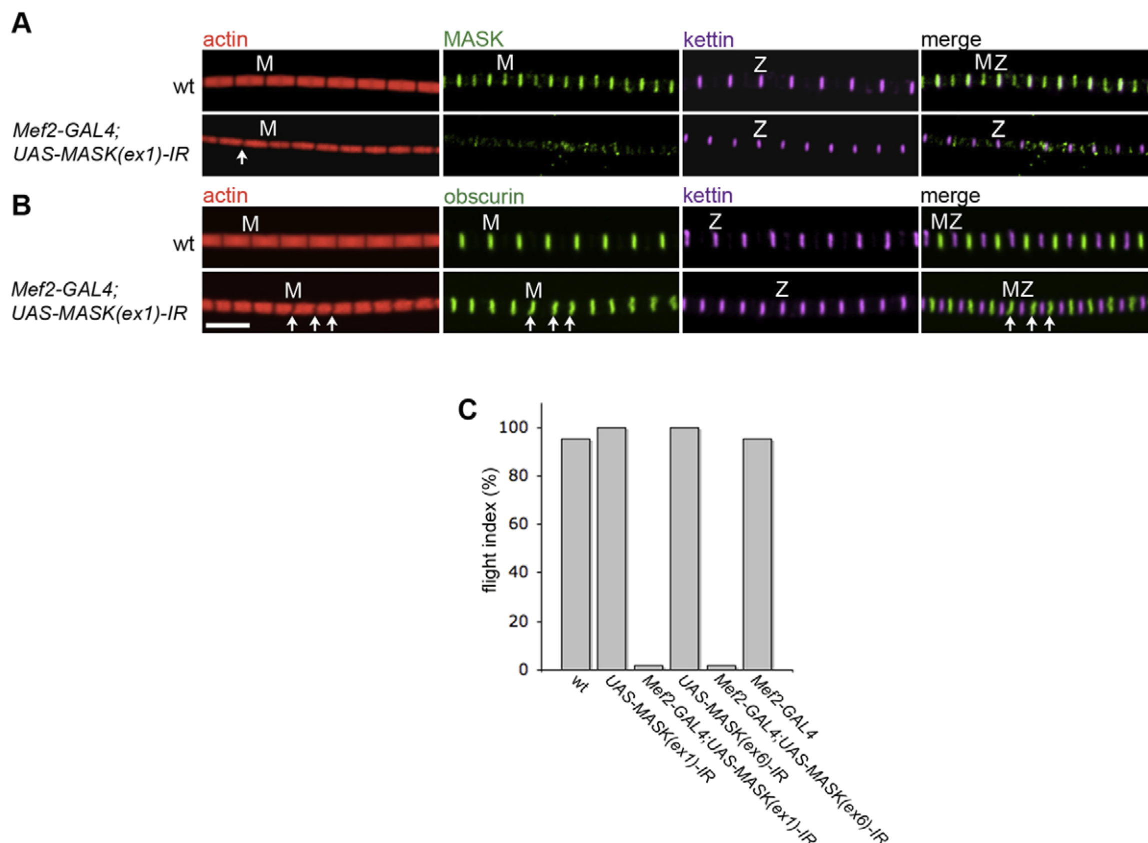

Fig. 3. The position of MASK in the IFM and the effect of reduced expression. (A) MASK in the IFM. Myofibrils were labelled with phalloidin (red), and anti-MASK (green) and anti-Kettin (magenta) antibodies. In wild-type (wt) myofibrils, MASK is in the M-line (M) and Z-disc (Z), with the Z-disc labelled more strongly by the antibody than the M-line. MASK is greatly reduced in MASK-knockdown flies. The brightness of the *Mef2-GAL4;UAS-MASK(ex1)-IR* Kettin labelling has been slightly increased in order to see Kettin clearly in the merged image. (B) Abnormal sarcomeres in IFM of MASK-knockdown flies compared with wild-type IFM. Myofibrils were labelled with phalloidin, and anti-Kettin antibodies as in A and with anti-obscurin antibody (green). In MASK-knockdown flies, M-lines and Z-discs are evenly spaced, but sarcomeres are shorter. M-lines and corresponding H-zones are often bent (arrows). MASK-knockdown flies were eclosed 'escapers'. Scale bar: 5 µm. (C) Flight tests of wild-type and MASK-knockdown flies. The percentage of flies able to fly is shown, n=20 for each genotype. Flies were flightless in *Mef2-GAL4;UAS-MASK(ex1)-IR* and *Mef2-GAL4,UAS-MASK(ex6)-IR* RNAi lines. Control flies with *UAS-MASK-IR* or *Mef2-GAL4* alone flew normally. Crosses with *UAS-mask(ex1)-IR* flies were at 29°C, and *UAS-mask(ex6)-IR* at 25°C.

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The range of abnormalities in sarcomere structure was seen in electron micrographs of mutant IFMs (Fig. 4). In less-affected myofibrils, Z-discs at the periphery of the myofibril were abnormal and the H-zone was wavy (Fig. 4B). A similar effect on the H-zone is seen in obscurin-knockdown myofibrils, where it is due to asymmetric thick filaments associated with correspondingly short or long thin filaments (Katzemich et al., 2012). In severely affected myofibrils, Z-disc structures were stacked parallel to each other in aggregates and all sarcomere structure was lost (Fig. 4C,D). Often relatively normal myofibrils were present in the same fibre as myofibrils with disrupted sarcomeres (Fig. 4D). The effect on the structure of the sarcomere in the *UAS-mask(ex6)-IR* siRNA line was similar to that of *UAS-mask(ex1)-IR* (supplementary material Fig. S4A,B). Although stacks of abnormal Z-discs were seen in the IFM of both Ball- and MASK-knockdown flies, lack of MASK had a greater effect than lack of Ball. The IFMs of pupae in MASK knockdown flies that were unable to eclose at ∼96 h after puparium formation (APF) had sarcomeres of varying length with split Z-discs and abnormal M-lines, a phenotype similar to that of IFMs after eclosion (supplementary material Fig. S4C–E). This shows that the abnormalities in the IFM of knockdown flies are due to developmental defects, rather than use of the muscle after eclosion.

The effect of lack of obscurin on the position of Ball and MASK in IFM

Given that Ball and MASK bind to obscurin, which is in the M-line, we investigated the effect of lack of obscurin on the assembly of these ligands. In the wild-type myofibrils used in this experiment,

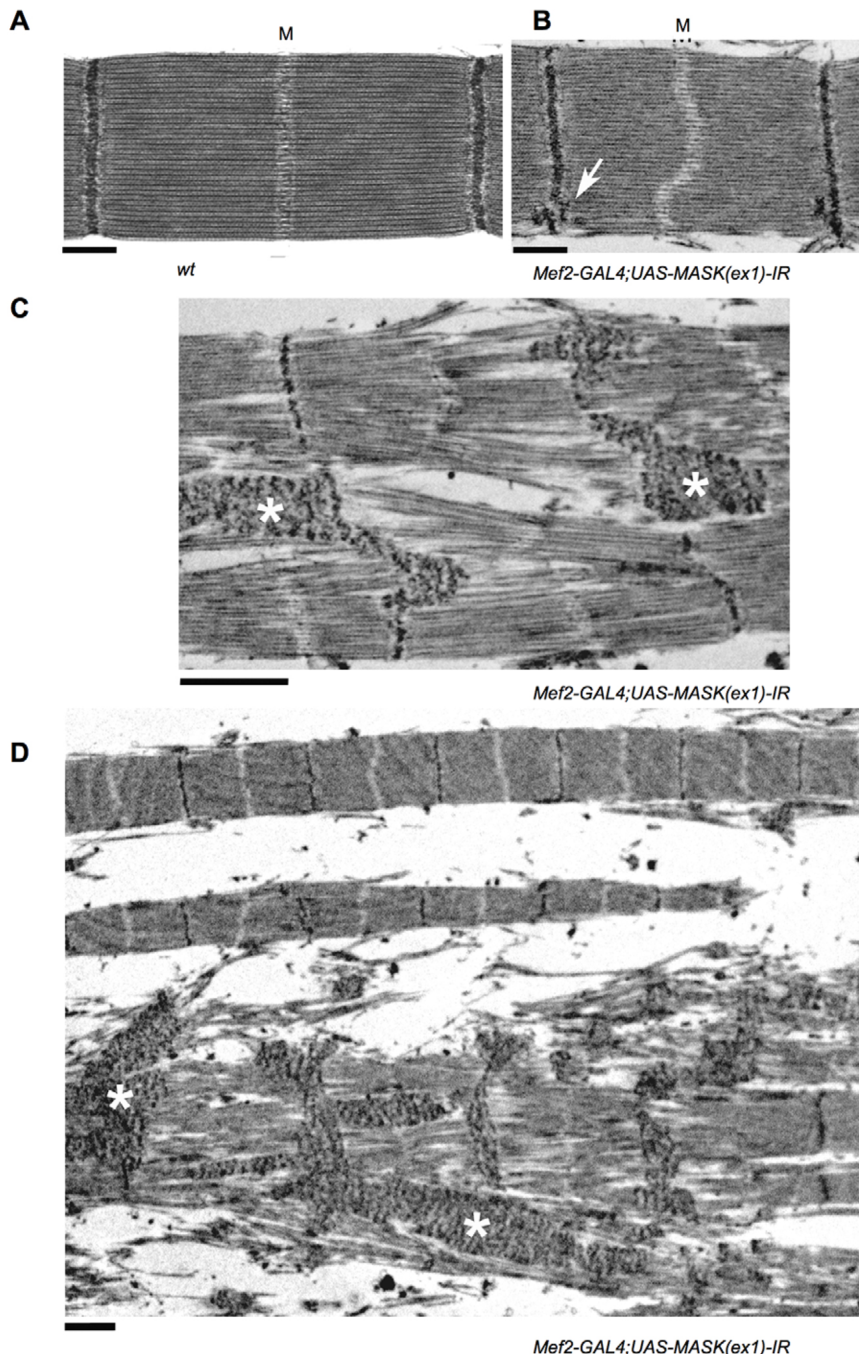

Fig. 4. Ultrastructure of IFM with reduced MASK expression. Electron microscopy images of (A) wild-type (wt) and (B–D) MASK-knockdown flies. In less affected myofibrils (B), Z-discs are often split (arrow) and H-zones are shifted, with asymmetric thick and thin filaments. In more severe cases (C,D), Z-discs form parallel aggregates (asterisks). Relatively normal and more abnormal myofibrils (D) are often seen in the same fibre. Scale bars: 500 nm (A,B); 1 µm (C,D). M, M-line.

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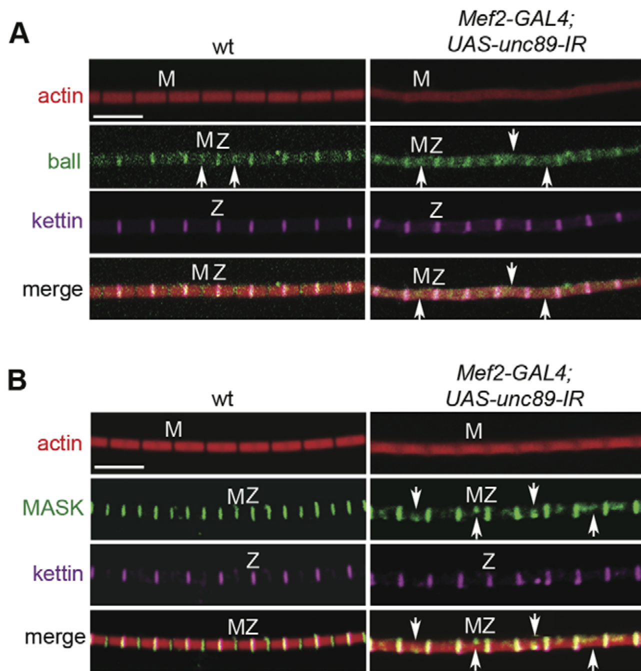

Fig. 5. The effect of reduced obscurin expression on Ball and MASK. (A) IFM myofibrils were labelled with phalloidin (red), anti-Ball (green) and anti-Kettin (magenta) antibodies. Wild-type (wt) myofibrils show clear labelling at the Z-disc (Z); Ball was also detected at the M-line (M), in addition to some labelling across the sarcomere. In obscurin-knockdown flies (*Mef2-GAL4; UAS*-unc89-*IR*), Ball is in the Z-disc, but labelling is more spread out than in the wild-type. Ball is diffusely distributed across the sarcomere and there is no clear M-line labelling. Arrows mark the M-line. (B) IFM myofibrils were labelled with phalloidin and anti-Kettin as for A, and anti-MASK (green). In obscurin-knockdown flies, MASK is present in the Z-discs, but absent or spotty in the M-lines (arrows). The brightness of the *Mef2-GAL4;UAS-unc89-IR* MASK labelling has been slightly increased in order to see remnants of MASK in the M-line of the merged image. Scale bars: 5 µm.

antibody to Ball labelled the Z-disc and diffusely labelled the rest of the sarcomere; Ball was also detected in the M-line (Fig. 5A). The M-line labelling was not seen in the myofibrils shown in Fig. 2A, suggesting that Ball can be mobile within the sarcomere, and can bind to obscurin under some conditions. In IFM myofibrils from the obscurin-knockdown fly line *Mef2-GAL4;UAS-unc89-IR* (Katzemich et al., 2012), Ball was still present in the Z-disc but misplaced, although the Z-disc itself was relatively normal, as shown by regular labelling with antibody to Kettin (Fig. 5A; Katzemich et al., 2012). Diffuse labelling across the sarcomere was more noticeable than in the wild-type and any M-line labelling was indistinct. The position of MASK in the obscurin-knockdown fly line was better defined. MASK was present in the Z-disc and spotty or absent in the M-line (Fig. 5B). Therefore, the proper assembly of Ball and MASK in the sarcomere requires the presence of obscurin, although the assembly of obscurin requires neither (Fig. 2B; Fig. 3B).

Identification of ligands of Kin1 and Kin2 by tandem affinity purification

Ligands binding to Kin1 and Kin2 in the IFM were identified by the tandem affinity purification (TAP) method. Tagged kinases were expressed in live flies and the kinases, with associated proteins, were isolated from extracts of the thorax in a two-step affinity purification. Peptides in tryptic digests of the kinases, plus any bound proteins, were analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS) and compared with digests of proteins purified from flies expressing the tags alone. The analysis of peptides in the three samples that are unique to particular proteins is shown in Table 2. The association of Ball with Kin1, found in the yeast two-hybrid assay, was confirmed in the TAP result: there are significantly more unique peptides from Ball in the Kin1 sample compared to the Kin2 and control samples. Tropomyosin-1 was associated with Kin2 but not Kin1. MASK, which was identified as a Kin2 ligand by the yeast two-hybrid assay, was not isolated with Kin2 in the TAP assay. MASK might be associated with a membrane fraction through the ankyrin repeats, and might not be soluble under the conditions used to extract proteins in the thorax.

Pulldown assay with Kin2 and MASK

A C-terminal sequence of MASK was found to bind to Kin2 in the yeast two-hybrid assay. The interaction was confirmed in a pull-down assay with extracts of thoraces from flies expressing NTAP–Kin1, NTAP–Kin2 or NTAP. NTAP–Kin1 and NTAP–KIn2 constructs have the TAP tags N-terminal to the kinase domain; NTAP has the tags without the kinase domain. Extracts were incubated with a recombinant peptide (MASK1) that included the sequence already identified as binding to Kin2; this was flanked upstream by two domains predicted to be α-helical, and downstream by a polyQ and an α-helical domain (supplementary material Table S1). Unexpectedly, MASK1 bound to both Kin1 and Kin2, although binding to Kin1 was not detected in the yeast two-hybrid assay (Fig. 6).

DISCUSSION

The kinase domains of *Drosophila* obscurin differ from those of the *C. elegans* homologue, Unc89, and the vertebrate protein. Both domains are predicted to be inactive as kinases. However, some pseudokinases can become catalytically active by replacing missing residues with residues from neighbouring domains, or from associated ligands (Endicott et al., 2012). Pseudokinases commonly act as scaffolds for binding proteins involved in signal transduction, and are often tethered to other domains, including Ig and Fn3 domains, which contribute to the binding site (Anamika et al., 2009; Boudeau et al., 2006; Scheeff et al., 2009). Titin kinase in the M-line region of vertebrate skeletal muscle forms part of a binding site for the autophagy and kinase scaffold proteins, Nbr1 and SQSTM1 (Lange et al., 2005), and the ubiquitin ligase, MuRF1 (also known as TRIM63). In the case of MuRF1, the site includes the preceding Ig and Fn3 domains, which will also bind MuRF1 without the kinase domain (Bogomolovas et al., 2014; Centner et al., 2001; Mrosek et al., 2007).

We have found that Kin1 in *Drosophila* obscurin binds Ball, which has the hallmarks of an active serine-threonine kinase. Ball differs from other kinase molecules in having a long extension C-terminal to the kinase sequence. This extension binds to Kin1 of obscurin, with or without the flanking Ig and regulatory domains. Given that Ball also binds to the Ig domain alone, it is likely the molecule spans a region of obscurin that includes the Ig domain as well as the kinase. Binding to Kin1 with the regulatory domain was unexpected. However, it is not clear how much of the sequence downstream of the kinase is included in a possible regulatory domain. In pseudokinases that are part of a larger molecule, the association of the regulatory domain with a ligand-binding site can be altered by force applied to the whole molecule (Gräter et al., 2005; Puchner et al., 2008; Mayans et al., 2013). The regulatory domain of Kin1, taken out of its usual context, might not associate with the active site in the same way as it would *in vivo*. Alternatively, the regulatory domain might be required to stabilise the kinase

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Table 2. Proteins interacting with Kin1 and Kin2 in the TAP assay

Protein	FlyBase number	Mol. Wt. (kDa)	Unique peptides		
			Control	Kin1	Kin2
Obscurin (Unc-89)	CG33519	478	7	42	47
Ball	CG6386	66	2 (2)	7 (19)	2 (2)
Tropomyosin-1	CG4898	33	0	0	5 (6)

Peptides associated with Kin1 and Kin2 and a control sample were analysed by LC-MS/MS. The number of unique peptides (as filtered by Scaffold) is shown. The total number of compounds derived from Ball or Tropomyosin-1 is given in brackets. Proteins identified with two or more unique peptides and a probability of correct identification of >95% (false discovery rate<0.5%) are included.

structure when the muscle is stretched, as suggested for twitchin kinase (von Castelmur et al., 2012).

Ball is found in the Z-disc of IFM, as well as being diffusely distributed in the sarcomere and in some samples, Ball is also detected at the M-line. Ball might migrate to bind to Kin1 in the M-line when the kinase activity of Ball is needed. There are other examples of protein migration in the muscle sarcomere. A transient translocation from the M-line to the Z-disc and cytoplasm has been observed for titin kinase ligands in cardiac muscle (Lange et al., 2005). In zebrafish, the myosin chaperone, Unc-45, is associated with myosin during myofibrillogenesis; in the adult, Unc-45 is in the Z-disc in normal fibres and it migrates to the A-band under conditions of stress, where it transiently associates with myosin again (Etard et al., 2008). Similarly, Ball might migrate from the Z-disc to the M-line under some conditions. Although we have shown that Ball is capable of binding to Kin1 *in vivo*, the conditions necessary for the association are not yet known. Ball is still present in the IFM of obscurin-knockdown flies, though with a less-ordered distribution in the sarcomere, which suggests there are likely to be other binding partners for Ball.

Kin2 binds MASK, which has two regions with ankyrin repeats, and a relatively long sequence C-terminal to a KH domain. A peptide near the end of the molecule binds to Kin2 with or without the Fn3 domain preceding the kinase. MASK does not bind to the Fn3 domain alone, nor does it bind to Kin2 with sequence C-terminal to the kinase. It is not clear at present whether the C-terminal sequence acts as a regulatory domain.

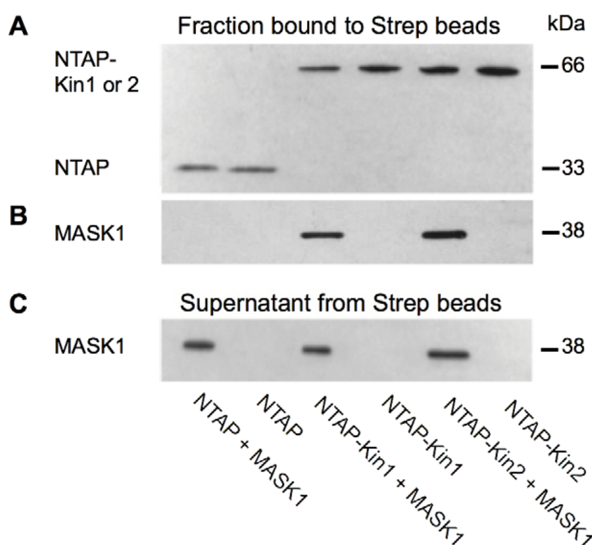

Fig. 6. Binding of MASK to Kin1 and Kin2. Extracts of thoraces containing NTAP, NTAP–Kin1 or NTAP–Kin2 were mixed with a MASK fragment (MASK1) and incubated with streptavidin beads. (A,B) Immunoblots of the fraction bound to streptavidin beads. (C) Supernatent from streptavidin beads. The blot shown in A was incubated with mouse IgG, followed by anti-mouse-IgG conjugated to HRP, to label NTAP samples; the blots shown in B and C were incubated with HRP-conjugated anti-His antibody to label MASK1. MASK1 binds to both Kin1 and Kin2, but not to the control.

The dual position of MASK in both the Z-disc and the M-line of IFM is unlikely to be due to migration of a protein of 400 kDa. The RNA coding for ankyrin-repeat proteins undergoes extensive alternative splicing, which can alter the binding sites of the different ankyrin isoforms (Cunha and Mohler, 2009). The independence of the binding sites for MASK in the M-line and Z-disc is confirmed by the finding that in obscurin-knockdown flies, MASK is almost eliminated from the M-line but still present in the Z-disc. Thus, obscurin is needed for MASK to bind in the M-line, but not to the Z-disc. The presence of obscurin in the M-line of MASK-knockdown flies is consistent with an obscurin scaffold that binds MASK.

In addition to MASK, Tropomyosin-1 was identified as a ligand associated with Kin2 expressed *in vivo*. As tropomyosin is a thin filament protein, the significance of an association with obscurin, which is at the midline of the thick filament, is not clear. Smaller isoforms of obscurin have been detected in IFM (A.K., unpublished work), and it is possible that Tropomyosin-1 could bind, outside the M-line, to a small isoform containing a Kin2 domain.

The effect of downregulating Ball or MASK on the structure of the M-line and Z-disc in IFM shows the importance of these proteins in the development of a regular filament lattice. In the IFM of Ball-knockdown flies, the shifted position of the H-zone and M-line is associated with fragmented Z-discs; where the Z-disc is normal within a sarcomere, the H-zone and M-line are at the midline. The aggregates of multiple Z-discs in the IFM of MASK-knockdown flies dominate the sarcomere and there is no regular H-zone. This phenotype differs from the effect of reducing the expression of obscurin in IFM, where the H-zone and M-line are often shifted from the midline, without a corresponding anomaly in the Z-disc. The difference might be due to the presence of Ball and MASK in the Z-disc, whereas obscurin is solely in the M-line. The high mortality rate at the larval and pupal stages of flies when either Ball or MASK is reduced differs from the survival of flies with reduced obscurin, which is unaffected in RNAi lines. Evidently, the crucial function of Ball and MASK is in binding to the Z-disc. The association of the proteins with obscurin in the M-line is not necessary for the performance of most muscles, although it is essential for the development of the precise filament lattice that is needed for the performance of the IFM. The function of obscurin in the assembly of the IFM sarcomere is not seen in vertebrates. Knockouts of obscurin in the mouse have no effect on the assembly of myosin filaments, Z-disc or M-line, but they do impair the assembly of the SR (Lange et al., 2009). Whether or not invertebrate and vertebrate obscurin-like proteins are true homologues (with the same functions, rather than just having a similar patterns of domains) is at present uncertain.

Ball and MASK are reported to be involved in cell proliferation, growth and differentiation in *Drosophila*. Ball regulates the

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proliferation and differentiation of germline stem cells and neuroblasts (Herzig et al., 2014; Yakulov et al., 2014). However, it is not clear whether this has any relevance to the function of Ball in *Drosophila* muscle. The VRK family of kinases (of which Ball is a member) phosphorylate the barrier-to-autointegration factor (BAF), which is necessary for the correct assembly of chromatin (Gorjánácz et al., 2007; Lancaster et al., 2007; Margalit et al., 2007; Nichols et al., 2006). Comparison of domains in the sequences of *Drosophila* Ball (also called NHK-1) and VRK in human, mouse, *Xenopus* and *C. elegans*, shows that *C. elegans* and *Drosophila* are the only homologues with a long C-terminal sequence extension; the other species have a relatively short stretch of sequence following the kinase domain (Aihara et al., 2004). This suggests that Ball has a different function in the invertebrates; in *Drosophila*, the C-terminal sequence binds to Kin1.

MASK was first identified in the developing eye of *Drosophila*, where it is required for proliferation and differentiation of photoreceptors (Smith et al., 2002). MASK genetically interacts with Corkscrew (CSW), a protein phosphatase that acts downstream of the epidermal growth factor receptor (EGFR) in a signalling pathway involved in myogenesis in *Drosophila* (Johnson Hamlet and Perkins, 2001; Smith et al., 2002). Through these interactions, obscurin can potentially be linked to a receptor tyrosine kinase (RTK) pathway involved in myogenesis. Obscurin and Kettin are present at an early stage in the development of pupal IFM (Katzemich et al., 2012; Weitkunat et al., 2014). Both are large titin-like proteins with tandem Ig domains, which have a dual function in myogenesis and in the mature muscle. Obscurin kinase domains appear to be scaffolds for binding MASK. Ankyrin repeats act as adaptor modules, binding cytoskeletal proteins and signalling molecules. The repeats stabilise protein networks, often together with large structural proteins (Cunha and Mohler, 2009). Therefore, an interaction between obscurin kinases and MASK could provide a platform for the assembly of signalling proteins, and this could be affected by force on the obscurin molecule. Ankyrin-repeat proteins in vertebrate skeletal muscle (MARPs) interact with the N2A elastic region of titin; in the case of Ankrd2 (also known as Arpp), expression is induced by stretch, and MARPs are thought to be involved in stretch-induced signalling pathways (Kemp et al., 2000; Miller et al., 2003). There are two smaller homologues of MASK in human cells: MASK1 (Ankhd1) and MASK2 (Ankrd17), which have the same domain structure as *Drosophila* MASK. The *Drosophila* protein (∼400 kDa) is larger than the human proteins (∼280 kDa), mainly due to a longer stretch of sequence between the ankyrin repeat domains (Sansores-Garcia et al., 2013; Sidor et al., 2013). MASK is a cofactor of *Drosophila* Yorkie (Yki) and mammalian Yes-associated protein (YAP) in the Hippo signalling pathway, which controls tissue growth. The signalling function in this pathway is similar for *Drosophila* and human MASK (Sansores-Garcia et al., 2013; Sidor et al., 2013); however, MASK isoforms have not been found it human muscle. There is a sequence in the *C. elegans* genome that codes for a protein with homology to *Drosophila*, mouse and human MASK (Smith et al., 2002). The protein, ankyrin repeat and KH domain-containing protein (listed in UniProt as R11A8 under Q21920) is predicted to be 287 kDa, so similar in size to the human protein. The function is unknown.

The presence of Ball and MASK in mature IFM suggests the proteins have a signalling function in the adult fly. There is turnover of contractile proteins in *Drosophila* muscles, including the IFM, throughout the life of a fly (Perkins and Tanentzapf, 2014). The function of obscurin kinase domains as scaffolds for the assembly of signalling proteins is likely to be important in the continual remodelling of the muscle. During contraction, the M-line experiences shearing stress, due to unbalanced forces in the two half sarcomeres, and the M-line is thought to act as a strain sensor (Agarkova and Perriard, 2005; Gautel, 2011b; Shabarchin and Tsaturyan, 2010). Ball and MASK might be recruited to the M-line in response to mechanical stress sensed by obscurin. Importantly, mutations in human titin kinase lead to a phenotype (Z-disc streaming) similar to that of Ball and MASK knockdowns, possibly by disrupting protein turnover (Chauveau et al., 2014), which supports the finding that Z-disc abnormalities can be an indirect consequence of mutations in proteins associated with the M-line.

In summary, we have identified two proteins, Ball and MASK, that are essential for the assembly of an ordered IFM. The pseudokinase domains of obscurin act as scaffolds binding the proteins. This raises the possibility of investigating the regulation of signalling pathways involved in assembly and maintenance of IFM through interaction with obscurin.

MATERIALS AND METHODS

Genetic crosses and flight tests

Wild-type flies were Oregon-R. For RNAi experiments, *UAS* responder lines directed against MASK (stock numbers 33396 and 103411) and Ball (stock numbers 10863 and 48980) were obtained from the Vienna *Drosophila* RNAi Center (Dietzl et al., 2007) (stockcenter.vdrc.at). Males of the responder lines were crossed with virgin females of the general muscle driver line *Mef2-GAL4*, at 25°C or 29°C. Adult flies were selected at 2 to 3 days after eclosion, when IFMs are fully formed. Three-day-old flies were flight-tested (Cripps et al., 1994; Katzemich et al., 2012).

Coding sequences of obscurin Kin1 and Kin2, were cloned into the *pUAST-NTAP(GS)* vector (Kyriakakis et al., 2008) and transgenic flies generated by standard microinjection procedures. Constructs and primers are listed in supplementary material Table S1. Three transgenic fly lines were established for the Kin1 and six for the Kin2 constructs, and one for the control empty vector. Adult male flies were crossed at 25°C with virgin females of the *Mef2-GAL4* line. Expression of the kinase constructs was confirmed by western blots of thoraces.

Yeast two-hybrid assay

Coding sequences of obscurin Kin1 and Kin2, with preceding Ig or Fn3 domains, and part of the regulatory sequence, were cloned into the *pGBKT7-Gal4* DNA-binding domain vector (Clontech). Constructs and primers are listed in supplementary material Table S1. Inserts were amplified using *Drosophila* cDNA as template. The constructs were transformed into the *Saccharomyces cerevisiae AH109* reporter strain, using lithium acetate (Vojtek et al., 1993). The strains containing the bait constructs were subsequently co-transformed with a *Drosophila* whole-adult fly cDNA library cloned in the *pACT2-Gal4* activation domain vector (Clontech). Selection for reporter gene activation *HIS3* was on agar plates lacking leucine, tryptophan and histidine (SD−LWH). In the case of the Kin1 screen, 2.5 mM 3-amino-1,2,4-triazole (3-AT) was added, in order to suppress background growth. Colonies that grew after 4–5 days at 30°C were tested for reporter gene *ADE2* activation on plates lacking leucine, tryptophan, histidine and adenine (SD−LWHA). Colonies were further tested for activity in a β-galactosidase filter assay. Plasmids from positive clones were confirmed in bait-dependency tests and isolated following the Matchmaker protocol (Clontech). Inserts were sequenced and subjected to BLAST analysis against the *Drosophila* genome. For confirmation and mapping of interactions, constructs of the potential interacting partners were cloned into *pGBKT7* and *pACT2* vectors, and co-transformed into yeast strain *AH109*. Growth on SD-LWHA plates was assayed.

Tandem affinity purification

The method was modified from previous work (Bailey et al., 2012; Kyriakakis et al., 2008; Veraksa et al., 2005). Thoraces were dissected from 200 flies of each of the *pUAST-NTAP(GS)* stocks expressed with the *Mef2-*

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GAL4 driver: NTAP-Kin1, NTAP-Kin2 and NTAP with the empty vector. Thoraces were stored at −20°C in 0.15 M NaCl, 50 mM Tris-HCl pH 7.0, 1 mM EDTA, 0.5% Triton X-100 and 50% glycerol with protease inhibitors. Pelleted thoraces were homogenised and extracted with 0.7 ml TAP buffer (0.15 M NaCl, 50 mM Tris-HCl pH 8.0, 1 mM EDTA and 0.1% Triton X-100) with protease inhibitors for 30 min on ice. Samples were centrifuged at 16,000 ***g*** for 30 min and the supernatants centrifuged again to clarify. Extracts were added to 50 µl rabbit-IgG agarose beads (Sigma) equilibrated with TAP buffer and rotated for 2–3 h at 4°C. Beads with protein bound were centrifuged at 1200 ***g***, washed with TAP buffer, and equilibrated with TEV buffer (0.15 M NaCl, 10 mM Tris-HCl pH 8.0, 0.5 mM EDTA 1 mM DTT, 0.1% Triton X-100). Proteins were released from the beads by cleaving the N-terminal Protein G tag with 12 µg/ml TEV protease in 0.4 ml TEV buffer, and rotating overnight at 16°C. Beads were removed by centrifugation and re-extracted with 100 µl TEV buffer. The supernatants, containing TAP proteins tagged with streptavidin-binding protein, were added to 30 µl Ultralink streptavidin agarose beads (Thermo Fisher) in TEV buffer and rotated overnight at 4°C. Beads were washed in TEV buffer and proteins eluted in 20 µl of SDS-PAGE sample buffer. The samples were run on a 4% acrylamide SDS-gel at 120 V for 7 min. The Coomassie-stained bands at the top of the gel were cut out, in-gel reduced, alkylated and digested with trypsin. Peptides were analysed by LC-MS/MS on an Orbitrap Velos Pro instrument (Thermo Fisher) and identified by Mascot (Matrix science) with a Uniprot species-specific *Drosophila* database. Scaffold (Proteome Software, Portland, OR) was used to filter common contaminants, and to give only those proteins identified with a minimum of two unique peptides per protein, with a confidence in the identification of 95% (a peptide false discovery rate of 0.5%).

Pulldown assay for MASK binding to obscurin kinases

A C-terminal sequence of MASK (MASK1) was cloned into the *pETM11* vector with an N-terminal His-tag. Primers for insert amplification from *Drosophila* cDNA are listed in supplementary material Table S1. Expression of His-tagged MASK1 was induced in Rosetta (DE3) pLysS cells for 24 h at 21°C. MASK1 was purified on a Ni-NTA agarose column (GE Healthcare) in binding buffer (20 mM Tris-HCl pH 8.0, 200 mM NaCl, 10 mM imidazole, 2 mM β-mercaptoethanol and 0.1 mM PMSF) with protease inhibitors, and eluted in buffer with 200 mM imidazole. MASK1 (250 µg) was mixed with TAP extracts from 100 thoraces (prepared as above, total protein ∼50 µg) in binding buffer (50 mM NaCl, 20 mM Tris-HCl pH 7.0, 0.1% Triton X-100, 2 mM β-mercaptoethanol) and left on ice for 2 h. Streptavidin beads (30 µl) in the same buffer were added to give a reaction volume of 400 µl. Samples were rotated for 2 h at 4°C. Beads were washed with binding buffer and bound proteins were eluted with SDS-PAGE sample buffer and identified by western blotting.

Confocal microscopy

IFMs from wild-type flies and RNAi lines were dissected from glycerinated half thoraces and washed in relaxing solution (0.1 M NaCl, 20 mM Na-phosphate pH 7.2, 5 mM $MgCl_2$, 5 mM ATP, 5 mM EGTA) with protease inhibitors (Burkart et al., 2007; Katzemich et al., 2012). Myofibrils in relaxing solution were labelled with primary antibody for 1 h, washed and labelled with secondary antibody and Rhodamine–phalloidin. Primary antibodies were: rabbit anti-obscurin (Burkart et al., 2007) and rat anti-Kettin monoclonal antibody MAC 155 (Lakey et al., 1993, 1990), diluted 1:100; rabbit anti-MASK (Smith et al., 2002), diluted 1:1; and anti-Ball (Herzig et al., 2014), diluted 1:300. Secondary antibodies were FITC-conjugated anti-rabbit-IgG and Cy3-conjugated anti-rat-IgG antibodies (Jackson ImmunoResearch). Myofibrils mounted in Prolong-Gold antifade (Invitrogen) were examined with a Zeiss LSM510 confocal microscope and images were processed with LSM software.

Electron microscopy

Half thoraces were processed as described previously (Farman et al., 2009; Reedy and Beall, 1993; Reedy et al., 1989). Thoraces were incubated in relaxing solution (0.150 mM KCl, 5 M MOPS pH 6.8, 5 mM MgCl2, 5 mM EGTA, 5 mM ATP, 5 mM NaN_3) with 1% Triton X-100 and then with 50% glycerol instead of Triton X-100. After washing in rigor solution (40 mM KCl, 5 mM MOPS, pH 6.8, 5 mM EGTA, 5 mM $MgCl_2$, 5 mM NaN_3), thoraces were fixed in 3% glutaraldehyde, 0.2% tannic acid in 20 mM MOPS pH 6.8, 5 mM EGTA, 5 mM $MgCl_2$, 5 mM NaN_3 for 2 h at 25°C. Samples were washed in 100 mM Na-phosphate pH 6.0, 10 mM $MgCl_2$. Secondary fixation, dehydration and embedding and sectioning were as described previously (Farman et al., 2009; Reedy and Beall, 1993). Sections were examined in a FEI Technai BioTWIN electron microscope at 120 kV.

SDS-PAGE and immunoblotting

Samples were analysed by 10% SDS-PAGE and proteins were transferred onto nitrocellulose for 1 h at 70 V. NTAP–Kin1, NTAP–Kin2 and NTAP control were detected by incubating blots with mouse IgG (80 µg/ml, Sigma), followed by anti-mouse-IgG conjugated to horseradish peroxidase (HRP) (diluted 1:10,000, GE Healthcare). MASK1 was detected with anti-His-HRP (diluted 1:10,000, Sigma). Immunoblots were developed with chemiluminescent substrate (Millipore).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

A.K. performed the majority of the experiments. A.K. and J.S. performed fly crosses and dissections; R.J.H.W. and S.T.S. created transgenic flies; A.K. and B.B. performed binding assays. A.F. and M.G. took part in initial yeast two-hybrid experiments. A.K. and B.B. wrote the manuscript, with contributions from other authors.

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Supplementary material

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.170639/-/DC1

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