Abba is an essential TRIM/RBCC protein for maintaining the integrity of sarcomeric cytoarchitecture

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**SUMMARY**

Organized sarcomeric striations represent an evolutionarily conserved hallmark of functional skeletal muscles. Here, we demonstrate that the *Drosophila* Abba protein, a member of the TRIM/RBCC superfamily, has a pivotal regulatory role in maintaining proper sarcomeric cytoarchitecture during development and muscle usage. *abba* mutant embryos initially form muscles, but F-actin and Myosin striations become progressively disrupted when the muscles undergo growth and endure increased contractile forces during larval development. Abnormal Myosin aggregates and myofiber atrophy are also notable in the *abba* mutants. The larval defects result in compromised muscle function, and hence important morphogenetic events do not occur properly during pupation, leading to lethality. Abba is localized at larval Z-discs, and genetic evidence indicates that *abba* interacts with α-actinin, kettin/D-titin, and mlp84B, genes encoding important Z-disc proteins, to provide stable myofibrillar organization and optimal muscle function. RNAi experiments and ultrastructural analysis reveal that Abba has an additional crucial role in sarcomere maintenance in adult muscles. Abba is required for ensuring the integrity and function of Z-discs and M-lines. Rescue experiments further show that Abba function is dependent upon its B-box/coiled-coil domain, NHL repeats, and RING finger domain. The importance of these presumed protein-protein interaction and ubiquitin ligase-associated domains supports our hypothesis that Abba is needed for specific protein complex formation and stabilization at Z-discs and M-lines.
INTRODUCTION

In striated skeletal muscle, the fundamental contractile units are the sarcomeres, which are composed of the thick and thin filament systems, and their associated proteins (reviewed in Clark et al., 2002). Extensive studies have established the biochemical and biophysical properties of many of the sarcomeric proteins. The analysis of genetic mutations affecting the various contractile proteins and associated proteins in model systems, such as *Drosophila* and *C. elegans*, has contributed significantly to our understanding of the function of these proteins (reviewed in Vigoreaux, 2005; Moerman and Williams, 2006). Currently, less is known about the crucial genes for muscle maturation and maintenance. One important gene is the *Drosophila* mib2, which is required for maintaining the stability of the mature musculature (Nguyen et al., 2007; Carracso-Rando et al., 2008). In particular, a recent RNAi screen in *Drosophila* primary cells has confirmed the function of known genes and identified additional new genes for diverse aspects of muscle assembly and maintenance (Bai et al., 2008). This validated cell-based approach was also used to assess the *Drosophila* orthologs of human genes that are linked to various muscle diseases. An interesting finding from these analyses is that a number of the genes that manifest a muscle phenotype encode proteins that are associated with the ubiquitin/proteosome systems, metabolic pathways, as well as basal transcriptional and translational machineries. More recently, a genome-wide screen with transgenic RNAi libraries has also uncovered a large number of new genes that are involved in determining proper morphology and sarcomeric organization in larval and muscles (Schnorrer et al., 2010).

New insights into the regulatory mechanisms that control the formation of functional sarcomeres have also been gained. Notably, the findings from a recent multi-approach study...
have indicated that the assembly of sarcomeric components into highly ordered structures, including the stereotypic striations, does not occur in a sequential manner, but rather the process is dependent upon the assembly of multiple protein complexes that are pre-formed (Rui et al., 2010). Knowledge about the mechanisms, which are responsible for maintaining the sarcomeric structures under normal mechanical stress, is however limited.

In the present study, we show that the *Drosophila* Abba (Another B-box Affiliate) protein, a new member of the TRIM/RBCC protein family, plays a key role in maintaining sarcomeric integrity under mechanical stress that is encountered during growth and usage. In the absence of *abba*, dramatic disruption of F-actin and Myosin striations occurs during larval muscle growth, leading to myofiber damage and atrophy, compromised muscle function, and pupal lethality. *abba* function is also essential for maintaining the structural integrity and function of Z-discs and M-lines in adult muscles. Lastly, the *abba* mutant phenotype shares similarities with the phenotype manifested by TRIM32-deficient mice and limb-girdle muscular dystrophy 2H (LGMD2H) patients with mutations in the TRIM32 gene.
RESULTS

Identification of abba mutant alleles.

We initially obtained Abba in a yeast two-hybrid screen with Mib2 as bait (see Materials and Methods). Another B-box Affiliate (Abba), belongs to the TRIM/RBCC protein family, whose members are involved in a variety of cellular processes and exhibit characteristic features, which include a RING finger domain, one or two zinc-binding B-boxes, and a coiled-coil domain (Meroni and Diez-Roux, 2005; O’Farrell et al., 2007). In some members, NHL repeats are found in the C-terminal region (Slack and Ruvkun, 1998). There exist four members in Drosophila: Brat and Mei-P2 function as tumor suppressors in the brain and ovary, Wech is involved in integrin adhesion, and Abba has an unknown function (Page et al., 2000; Bowman et al., 2008; Löer et al., 2008). abba mRNA is first detectable in somatic muscles in late stage embryos (data not shown). For initial analysis, we examined the deficiency Df(2R)Exel6068 that removes abba and 17 additional predicted genes. Late stage 17 embryos homozygous for Df(2R)Exel6068 show a normal muscle pattern, but the muscles in mutant larvae exhibit an aberrant striation pattern (Fig. S1). The F-actin striations are dramatically disrupted when compared to the periodic pattern in the control larvae. In order to ascribe this mutant phenotype to the loss of abba, we performed an excision screen with Mi{ET1}abba^{MB03490} to obtain abba-specific mutations (Fig. 1A). Molecular characterization, including sequencing, identified 14 excision abba alleles that differ in the size of the deleted region (see Materials and Methods for further details). The abba^{MJO-348} allele harbors a large 7.5Kb deletion that removes the translation initiation site, and hence it is likely to be an amorphic allele. Indeed, there is an absence of abba RNA transcripts and Abba protein in homozygous abba^{MJO-348} mutant embryos and larvae (data not shown).
**abba mutant larvae exhibit severe striation defects and abnormal Myosin aggregates.**

The phenotype of the \( abba^{MJO-348} \) allele was characterized, as done with the \( Df(2R)Exel6068 \). As shown in Figure 1B, late stage embryos homozygous for \( abba^{MJO-348} \) or trans-heterozygous with \( Df(2R)Exel6068 \) do not show any muscle defects and hatch normally. However, abnormalities, such as body length reduction and reduced contraction are detected during the second larval instar and become more pronounced thereafter (Fig. S2C). By late third instar, the mutant larvae are significantly slimmer and shorter by ~10% than their control counterparts (Fig. 1C and Fig. S2C). The mutant larvae are also flaccid and do not contract efficiently, thus compromising subsequent critical morphogenetic processes during pupation. As a consequence of the defects, \( abba \) mutant pupae are longer and slimmer, exhibit an abnormal “air pocket” in the posterior end, which normally is translocated anteriorly, and incomplete head eversion (Fig. 1D). Lethality occurs at early pupal stage. Taken altogether, the morphological defects show that \( abba \) function is not needed during embryogenesis for the initial formation of the larval musculature, but it is required for proper muscle growth and function during subsequent larval and pupal stages. Additionally, the comparable larval phenotype of all three mutant genotypes reinforces that \( abba^{MJO-348} \) is a recessive and functional null allele.

The apparent muscle weakness in the \( abba \) mutants suggests the occurrence of muscle detachment or destabilization of improperly formed sarcomeres. To distinguish between these two possibilities, fillets from late third instar (L3) larvae were stained for the expression of \( \alpha \text{PS2} \) and \( \beta \text{PS Integrin} \) subunits that are essential for muscle attachment sites (Brown, 1994). For both subunits, the expression pattern is normal in homozygous \( abba^{MJO-348} \) larvae, as well as in larvae trans-heterozygous for \( abba^{MJO-348} \) and the deficiency, indicating that loss of \( abba \) does not compromise muscle attachment (data not shown). However, the mutant
myofibers show signs of atrophy, being shorter and thinner, suggesting some underlying sarcomeric defects.

To help assess sarcomere formation in first instar larvae, a Kettin protein trap enhancer line was crossed into the *abba*<sup>MO-348</sup> allele. As compared to the controls, the mutant larvae exhibit relatively good sarcomeres, based upon the Kettin:GFP signals that mark Z-discs, although disruptions can be detected in a limited number of muscle fibers (Fig. S2A). Second instar larvae, which were more amenable to fillet preparation, were stained with phalloidin to visualize F-actin striations. At this stage, an increased number of muscles show disruptions in the mutant larvae (Fig. S2B). The mutant muscle fibers are also reproducibly thinner and shorter. This apparent myofiber destabilization and atrophy is consistent with the observation that L2 is the stage at which growth retardation is first detectable (Fig. S2C). To further assess the status of the sarcomeres, fillets from third instar instar larvae were stained for both Myosin and F-actin expression. The periodic F-actin striations are severely disrupted in the mutant larvae when compared to the control larvae (Fig. 2A). Upon examination of larval fillets that were double-stained with Kettin and phalloidin, we observed that F-actin is still localized at Z-discs in the mutant muscles (data not shown). The normal periodic pattern of Myosin striations is also not evident in the mutant muscles, and there is an abnormal accumulation of Myosin aggregates along the sarcolemmal membrane (Fig. 2B). Interestingly, an assessment through the z-axis of the muscles revealed that, within a muscle, more severe striation defects are associated with the internal myofibrils than with the external ones (data not shown). Of note, the documented myofibrillar defects are consistently present in all muscle groups (Fig. S3), and the positions of the nuclei are also aberrant, but this could be a secondary effect. Furthermore, it is noteworthy that the morphological and sarcomeric defects of *abba*<sup>MO-348</sup> mutants are similar to those exhibited by the *l(2)thin* mutation, which also affects *abba* gene function (Ball et al., 1985; LaBeau-DiMenna et al., 2012).
For further analysis, we also assessed the expression of Kettin/D-Titin and two other Z disc-associated proteins, α-Actinin and Mlp84B, all of which have important roles in regulating sarcomeric organization and muscle function (Fyrberg, et al., 1990; Zhang et al., 2000; Clark et al., 2007). The periodic pattern of these proteins is not dramatically affected by the loss of abba (data not shown). In the aggregate, the data indicate that abba function is critical for maintaining structural integrity of the myofibers during larval muscle growth.

**abba interacts genetically with kettin/D-titin, mlp84B, and α-actinin.**

To gain further insights into abba function, an antibody against Abba was generated and used to determine its subcellular localization. In larval muscles, Abba is expressed in periodic striations that appear to be non-overlapping with the Myosin striations (Fig. 3). Notably, Abba localization at Z-discs overlaps with Kettin/D-Titin and Mlp84B, although the Abba domain is broader and extends partially into the F-actin domain. Consistent with the aforementioned observation that loss of abba function does not abolish the localization of the αPS2 and βPS integrin subunits at attachment sites, Abba is not present at these sites. In a recent study, it was also reported that Thin/Abba is localized at Z-discs in larval muscles (LaBeau-DiMenna et al., 2012).

Although we observed that Abba does not alter the periodic pattern of Kettin, Mlp84B, and α-Actinin, the localization of Abba at Z-discs suggested that it might have functional interactions with these proteins. Kettin is encoded by the sls gene locus and plays an important role in sarcomeric assembly (Zhang et al., 2000; Kolmerer et al., 2000; Burkart et al., 2007). Previous reports documented that strong loss-of-function sallimus (sls) alleles are embryonic lethal, while specific combinations of weaker alleles die during the pupal stage. These sls mutant pupae are long and slim, resembling abba mutant pupae (Clark et al., 2007).

We assessed the functional consequences of reducing one copy of sls in the abba mutant
background. Pupae heterozygous for \textit{abba}$^{\text{MJO-348}}$ and \textit{sls}$^{\text{JID7}}$, a strong \textit{sls} allele, undergo proper pupal morphogenesis and eclosion (Fig. 4A). Notably, mutant pupae homozygous for \textit{abba} and heterozygous for \textit{sls} are slim and show a statistically significant increase in length when compared to homozygous \textit{abba} pupae (Fig. 4A-B). This result indicates an enhancement of the muscle contraction and morphogenetic defects that are associated with the \textit{abba} mutant phenotype.

It has been noted that \textit{mlp84B} mutant pupae also exhibit defects in pupal morphogenesis, resulting in more elongated pupae (Clark et al., 2007). This resemblance to \textit{abba} mutants, and their co-localization at Z-discs prompted us to test for possible genetic interactions. Since \textit{abba} mutants manifest severe defects at an earlier stage than \textit{mlp84B} mutants, we reduced one copy of \textit{abba} in the \textit{mlp84B} mutant background. F-actin striations in \textit{mlp84B}$^{\text{PA8-95}}$ mutant larvae resemble those in control larvae. By contrast, third instar larvae homozygous for \textit{mlp84B}$^{\text{PA8-95}}$ and heterozygous for \textit{abba}$^{\text{MJO-348}}$ exhibit disrupted striations and myofiber tearing, although the defects are not as severe as in homozygous \textit{abba} mutants (Fig. 4C). We also obtained fewer adult “escapers” that are homozygous for \textit{mlp84B}$^{\text{PA8-95}}$ with reduced \textit{abba} function than “escapers” homozygous for \textit{mlp84B}$^{\text{PA8-95}}$ alone (data not shown). Together, the data suggest that there is also a genetic interaction between \textit{abba} and \textit{mlp84B}.

Strong loss-of-function \textit{α-actinin} alleles are known to exhibit striation disruptions and die during early larval development (Fyrberg et al., 1990). Since the defects in \textit{abba} mutants occur at a later stage, we reduced one copy of \textit{α-actinin} in the \textit{abba} mutant background. Additionally, early third instar larvae were examined because the filament disruptions in \textit{abba} mutants are less severe at this stage, thereby allowing better detection of any enhanced effects. Larvae heterozygous for the null allele \textit{actn}$^{\text{14}}$ and \textit{abba}$^{\text{MJO-348}}$ have normal F-actin striations (Fig. 4D). By comparison, \textit{abba} mutants with reduced \textit{α-actinin} function display more extensive disorganization, indicating an enhancement of the \textit{abba} mutant phenotype.
We had observed that Mib2 is also localized at Z-discs in larval muscles (data not shown). To test for potential genetic interactions, we reduced one copy of mib2 in the abba mutant background because mib2<sup>1</sup> mutants die at an earlier stage than abba<sup>MJO-348</sup> mutants (Nguyen et al., 2007; Carrasco-Rando et al., 2008). abba<sup>MJO-348</sup> mutant larvae with reduced mib2 function do not exhibit enhanced defects when compared to abba<sup>MJO-348</sup> mutants (data not shown). Further analysis, which involved reducing both mib2 and abba function in adult muscles by using RNAi technology, does not suggest any genetic cooperation (data not shown). The exact functional relationship between abba and mib2 remains to be clarified.

Based upon the combined genetic data, we conclude that abba acts in concert with kettin, mlp84B, and α-actinin to maintain the integrity and stability of Z-discs, thereby ensuring myofibril stability and optimal muscle function.

**Abba functions to maintain the integrity of Z-discs and M-lines in thoracic indirect flight muscles (IFMs).**

To determine whether abba function is also needed in the adult muscles, adult myofibrils derived from thoracic IFMs were first double-labelled with antibodies against Abba and markers for Z-discs and M-lines. Abba is prominently localized at Z-discs, along with Kettin, and at M-lines with Zormin (Fig. 5). Zormin, an Sls isoform, is localized strongly at Z-discs and at reduced levels at M-lines (Burkart et al., 2007).

To further explore abba function at later stages of development, we used RNA interference (RNAi) technology with the GAL80<sup>T</sup>-TARGET system of McGuire et al. (2003). Temperature sensitive GAL80, a GAL4 antagonist, is active and represses GAL4-dependent gene expression at 18°C, while it becomes inactive and allows GAL4-dependent gene activity at 28°C. Two UAS-abba RNAi lines were initially tested with the mef2-Gal4 driver; both yielded larval and pupal phenotypes that are comparable to abba<sup>MJO-348</sup> mutants.
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(data not shown). In subsequent analyses, the UAS-abba RNAi 19291 line was used for crossing with the Gal80ts; mef2-Gal4 driver line. The progeny were raised at 18°C for different time periods prior to being switched to 28°C, and then maintained at 28°C until adulthood. To assess the effects of abba RNAi induction, we examined the sarcomeric organization in the dorso-longitudinal muscles (DLMs) from adult flies at one day or 11 days post-eclosion by double-staining the muscles for F-actin (phalloidin) and Kettin expression. We also conducted flight tests. RNAi induction after seven days (corresponding to ~L2) or ten days (~L3) of development at 28°C results in thin myofibrils with nondescript sarcomeres (Fig. 6A). At the ultrastructural level, short, irregular Z-discs and occasional M-lines are detected at one day post-eclosion; these structures are less evident ten days later (Fig. 6B). Consistent with the presence of severe structural defects, >90% of the tested abba RNAi flies are flightless (Fig. 7). Interestingly, induction at day 13 (~late L3/pre-pupae) initially yields misshapen sarcomeres with longer, more prominent Z-discs however fragmentation, especially at M-lines, is frequently observed at 11 days post-eclosion. ~60-70% of these abba RNAi flies are flightless. Induction at day 16, by which time the adult musculature is normally established, results in much less dramatic effects. Relatively normal sarcomeres with better defined Z-discs and M-lines are present, although occasional, limited dissolution of the M-lines is observed at the later time point. These RNAi flies do not show significant flight deficits. Based upon the RNAi studies, we suggest that abba also has a critical role in adult muscles, and a decrease in abba function results in the disintegration of sarcomeric organization when these muscles experience growth, increased contractility, and usage. The data further indicate that abba is needed for maintaining structural integrity at both Z-discs and M-lines. It is also noteworthy that the stronger effects on M-lines, which are associated with reduction of abba function, is consistent with the finding in vertebrate skeletal muscles
that, under stress, M-lines are more susceptible to disturbances than Z-discs (Gautel, 2011).

**Abba function requires the RING finger, B-box/Coiled-coil domain, and NHL repeats.**

In order to gain some insights into the mechanisms underlying Abba function, we assessed the functional importance of the various protein domains within the Abba protein. In one approach, the phenotype of two additional \( abba \) alleles was compared to the phenotype of \( abba^{MJO-348} \). In these particular alleles, \( abba^{MJO-313} \) and \( abba^{MJO-429} \), the deletion caused a frameshift, resulting in proteins that lack the region after the coiled-coil domain or after the B-box, respectively (see Fig. 1A for more details). Both mutant proteins lack the NHL repeats. Late third instar larvae were stained with phalloidin to examine the status of the F-actin striations. In both \( abba^{MJO-429} \) and \( abba^{MJO-313} \) mutant larvae, the striations are disrupted to a similar degree as in the \( abba^{MJO-348} \) mutant larvae, strongly suggesting that the region that includes the NHL repeats is crucial for Abba function (Fig. 8A). In an alternate approach, specific UAS-based constructs, encoding the Abba protein minus a particular domain, were tested for their potential to rescue the \( abba \) mutant phenotype. As shown in Figure 8B, the full-length Abba protein provides full rescue of the mutant phenotype, including survival to adulthood. By contrast, Abba without the RING finger, B-box/Coiled-coil domain or NHL repeats is not able to rescue the striation defects or to enable survival to adulthood, thereby underscoring the importance of each of these domains for Abba function. Further testing will however be needed to establish the relative importance of the individual B-box and coiled-coil domain.
DISCUSSION

Analysis of the newly generated abba alleles shows that the loss of abba activity is associated with a progressive muscle degeneration and loss of muscle function. The underlying cause is the destabilization of myofibrils and sarcomeres, most likely in response to mechanical stress that is induced by muscle growth and contractions during development and usage. Indeed, relatively normal sarcomere formation occurs in first instar mutant larvae, but then is followed by an increasing level of myofibrillar disorganization and compromised muscle function from second larval instar onwards, as well as sarcomere degeneration during aging in the adult. The filament disruptions, as well as the malformed/disintegrated Z-discs and M-lines in abba\textsuperscript{MJO-348} mutant muscles suggest that Abba is a new essential regulator of the machinery for maintaining stable mechanical links at both Z-discs and M-lines. We propose that Abba is crucial for specific protein complex formation at Z-discs and M-lines to maintain structural integrity and function of these structures. The proposed function is supported by the findings that domains within the Abba protein, which are presumed to be involved in protein-protein interactions, are required for its function.

Our genetic data indicate that, at Z-discs, abba acts in conjunction with at least three important genes, α-actinin, kettin/D-titin, and mlp84B, to ensure myofibril stability and proper muscle function. Interestingly, it has also been documented that mlp84B interacts genetically with kettin. Similar to the situation with abba\textsuperscript{MJO-348} mutants, mlp84B mutant pupae with reduced kettin function are also more elongated than mlp84B mutant pupae (Clark et al., 2007). It is conceivable that these proteins co-exist in complexes to stabilize the Z-discs, but biochemical proof is needed. In contrast to the Z-discs, the M-lines are currently less well characterized and fewer interacting proteins are known. Therefore, increased knowledge about the importance of M-lines could potentially be gained from identifying the
proteins that interact specifically with Abba at this site. Based upon their localization at the M-lines, Zormin and Obscurin could be candidates. Recent studies have shown that reduced or loss of obscurin function leads to an absence of M-lines and occurrence of asymmetrical sarcomeres (Schnorrer et al., 2010; Katzemich et al., 2012). Altogether, our data provide support for the notion that Abba has a pivotal structural role at Z-discs and M-lines.

In a recent report, it was determined that a previously-identified muscle degenerative mutation, named l(2)thin or l(2)tn, affects abba gene function (Ball et al., 1985; LaBeau-DiMenna et al., 2012). l(2)tn mutants were originally described as being “thin” (Ball et al., 1985). Based upon the recent report of LaBeau-DiMenna and colleagues, the mutant larval phenotype of the l(2)tn/abba mutation has additional common features with the phenotype of our abba mutations, namely disruptions of the F-actin striations, abnormal Myosin accumulation along the sarcolemmal membrane, and mislocalized nuclei. Importantly, LaBeau-DiMenna and colleagues also showed that costameric proteins, such as the Integrins, Talin, Spectrin, and Vinculin, are mislocalized in the l(2)tn/abba mutant larval muscles. Similarly, βPS Integrin is also not properly localized at costameric sites in abbaMJO-348 mutant larvae, although its localization at the myotendinous junctions is not affected (data not shown). It was proposed that Thin/Abba is needed to stabilize the costameres, possibly by linking Z-disc-associated proteins with costameric proteins (LaBeau-DiMenna et al., 2012). Altogether, the data support the conclusion that Thin/Abba functions primarily as a structural protein to maintain muscle stability. Interestingly, all existing abba mutations are recessive in contrast to mutations of other muscle structural proteins that are often dominant. The difference might reflect the fact that abba is not involved in the formation of muscles, as with other structural proteins, but rather abba is required for muscle stability.

To date, TRIM32 is the presumed vertebrate ortholog of Abba/Thin, based upon sequence homology and its localization at Z-discs in skeletal muscle (Kudryashova et al., 2005). It is
therefore notable that the defects in the \textit{abba}^{MJO-348} mutants resemble those present in \textit{TRIM32}-deficient mice and LGMD2H (limb-girdle muscular dystrophy 2H) patients with mutations in \textit{TRIM32}, which include streaming Z-discs, myofiber tears, and progressive muscle weakness (Kudryashova et al., 2009; and reviewed in Shieh et al., 2011). Our analysis showed that Abba function depends upon its various protein domains. Again, there exist many parallels between Abba and TRIM32 in this aspect. The importance of the NHL repeats, which may be needed for interactions with yet-to-be defined sarcomeric components, implicates Abba as a “scaffold-type” of protein for mediating complex formation. It is therefore intriguing that many human TRIM32 mutations also map to the NHL repeats, underscoring the evolutionarily conserved importance of this domain (reviewed in Shieh et al., 2011). The B-box/coiled-coil domain is also important for Abba function, but it is not yet known whether this domain interacts directly with the filaments. Biochemical studies have suggested that the coiled-coil domain of murine TRIM32 binds to the head and neck region, and possibly part of the rod, of Myosin (Kudryashova et al., 2005). The necessity of the RING finger further suggests that Abba can also function as an ubiquitin ligase, but candidate substrates have not yet been identified. \textit{In vitro} experiments have however shown that TRIM32 can ubiquinate Actin and Dysbindin (Kudryashova et al., 2005; Locke et al., 2009).

It is noteworthy that the \textit{abba}^{MJO-348} mutant phenotype also bears some resemblance to the phenotype of MuRF1/MuRF3 double knockout mice, which includes myofiber fragmentation, subsarcolemmal accumulation of myosin, and reduced muscle function (Fielitz et al., 2007). The MuRFs are also TRIM/RBCC proteins with \textit{in vitro} documented E3 ligase activity, and MuRF1 has been shown to bind to Titin (McElhinny et al., 2002). Currently, there are no apparent \textit{Drosophila} orthologs of MuRF, but the similarities in mutant phenotype of the various muscle-specific TRIM proteins reinforce the importance of this protein family in the maintenance of muscle structure and function during evolution.
In conclusion, further genetic and biochemical analyses are needed to fully elucidate the function of Abba/Thin. It would also be important to define more precisely the role of the various Abba protein domains, and to identify *bona fide* protein interactors and substrates of Abba. Based upon existing parallels between *Drosophila* and vertebrate muscle systems, greater insights into the modes of action of Abba will contribute to a better understanding of critical processes, which could be deregulated during the pathogenesis of various muscle diseases, including limb-girdle muscular dystrophy and myofibrillar myopathies.
MATERIALS AND METHODS

Yeast two-hybrid screen.

A fragment from the mib2 EST LP14687 (obtained from Berkeley Drosophila Genome Project), coding for amino acid residues 455 to 461, was cloned in-frame to the GAL4 DNA binding domain in the pGBK7 vector (Clontech, Palo Alto, CA). For screening, a 0 to 21 hour-old Drosophila embryo Matchmaker cDNA library in the pACT2 vector was used, as recommended by the suppliers. More detailed information about the molecular screen and its findings will be provided upon request.

Drosophila stocks.

The following stocks were used: Df(2R)Exel6068, Mi{ET1}^{abba[MB03490]}, sna[Sco]/SM6a, P{hsILMiT}2.4 ET1-transposase, actn^{14}, sls^{ID7} (obtained from Bloomington Stock Center); mib2^{l} (Nguyen et al., 2007); abba^{MJO-348}, abba^{MJO-429}, and abba^{MJO-313} (all from this study); mlp84B^{P58-95} (similar to mlp84B^{P5} allele but without the original P-element; provided by K. Clark, U. of Utah); Kettin protein trap line ZCL2144 (provided by L. Cooley, Yale U.; Morin et al., 2001); UAS-abba RNAi lines (Transformant ID 19290 and 19291; Vienna Drosophila RNAi Center); mef2-Gal4 (obtained from Bloomington Stock Center; Ranganayakulu et al., 1996); P{ tubP-Gal80^{r} }9;; mef2-Gal4 (provided by G. Tanentzapf, U. of British Columbia; Perkins et al., 2010). All mutations were maintained over twi>eGFP balancers.

Generation and characterization of abba mutant alleles.

abba mutant alleles were obtained by mobilizing the Minos element in Mi{ET1}^{abba[MB03490]} with the transposase from sna[Sco]/SM6a, P{hsILMiT}2.4 ET1-
transposase, using recommended conditions (Metaxakis et al., 2005). 28 putative abba mutant alleles were identified genetically, based upon lethality when tested in trans with the deficiency Df(2R)Exel6068/CyO. PCR analysis of genomic DNA from the putative mutant alleles was done with numerous pairs of primers, spanning the abba gene locus. PCR products of altered size, as compared to controls, were obtained with DNA from 14 candidates. Sequencing of these candidates identified the exact position and length of the deletion, ranging from 0.7Kb to 7Kb, within the abba gene locus. These candidates were designated as abba alleles. The remaining fourteen were presumed to harbor large deletions, which could not be detected with the PCR primer pairs that were used. More detailed information and sequence of the primers will be provided upon request.

For larval measurements, control and mutant larvae at different developmental stages were identified by the presence or absence of the GFP marker on the balancer chromosome. First instar larvae were examined with a 1x1 mm measuring grid on an Olympus compound microscope and photographed with the QCapture program. Second and third instar larvae were assessed with a 1x1 mm measuring grid on a Leica stereomicroscope, equipped with a ProgResC14 camera system. All calculations were subjected to t-tests, using the statistical analysis software in Microsoft Excel 2011.

Genetic interactions.

For testing genetic interaction between α-actinin and abba, crosses were made between actn^{14}/FM7a, twi>eGFP; abba^{MHO-348}/CyO, twi>eGFP virgin females and abba^{MHO-348}/CyO, twi>eGFP males, and progeny (early third instar larvae) were examined. To assess the potential interaction between abba and sls, crosses were done between abba^{MHO-348}/CyO, twi>eGFP; P[lacW]sls^{107}/TM3, Sb, twi>eGFP virgin females and abba^{MHO-348}/CyO,
twi>eGFP males, and progeny (pupae) were examined. For analyzing interactions between abba and mlp84B, crosses were made between mlp84B<sup>PΔ8-95</sup>/TM3, Sb, twi>eGFP virgin females and abba<sup>MOJ-348</sup>/CyO, twi>eGFP; mlp84B<sup>PΔ8-95</sup>/TM3, Sb, twi>eGFP males and progeny (late third instar larvae or adults) were examined.

In the analysis for genetic interactions between abba and sls, ~110 pupae of each genotype of interest were also measured with a 1x1 mm measuring grid on a Leica stereomicroscope, equipped with a ProgResC14 camera system. All calculations were subjected to t-tests, using the statistical analysis software in Microsoft Excel 2011.

**Rescue experiments.**

Transgenic lines were generated, containing the UAS-abba<sup>FL</sup>, UAS-abba<sup>ΔRF</sup> (Δ454-589 nt), UAS-abba<sup>AB-Box+CC</sup> (Δ703-1216 nt) or UAS-abba<sup>ΔNHL</sup> (Δ3719-4492 nt) construct. Using the EST GH06739 (Genbank accession number AY121620) as template, the entire abba coding or defined regions of abba were amplified and cloned into the pUAST vector (Brand and Perrimon, 1993). Each construct also contained a hemagglutinin tag at the 3’ end. The following “test” stocks were generated for the rescue experiments: abba<sup>MOJ-348</sup>/CyO, twi>eGFP; mef2-Gal4 (control), abba<sup>MOJ-348</sup>/CyO, twi>eGFP; UAS-abba<sup>FL</sup> (full-length), abba<sup>MOJ-348</sup>/CyO, twi>eGFP; UAS-abba<sup>ΔRF</sup> (ΔRING finger), abba<sup>MOJ-348</sup>/CyO, twi>eGFP; UAS-abba<sup>AB-Box+CC</sup> (ΔB-box/coiled-coil domain), and abba<sup>MOJ-348</sup>/CyO, twi>eGFP; UAS-abba<sup>ΔNHL</sup> (ΔNHL repeats). For analysis, abba<sup>MOJ-348</sup>/CyO, twi>eGFP; mef2-Gal4 virgin females were crossed with males from each of the “test” transgenic stocks. The progeny were assessed for the degree of rescue that was conferred by each type of construct.
Immunocytochemistry of whole-mount embryos and larval fillets.

Immunostainings of embryos were performed, according to Nguyen and Xu (1998). Larval fillets were prepared from late second or third instar larvae, essentially as described (Schnorrer et al., 2010). Larval fillets were fixed for 5 min in 3.7% formaldehyde for all antibodies, except for the α-Actinin antibody, which required fixation for 10 min in methanol. Primary antibodies included: rat anti-Tropomyosin (MAC141; 1:200; Babraham Institute, UK), rat anti-α-Actinin (MAC 276; 1:100; Babraham Institute), rat anti-Kettin (MAC 155; 1:100; Babraham Institute), rat anti-Myosin (MAC 147; 1:200 with pre-absorption; Babraham Institute), rabbit anti-Mlp84B (1:5000; gift from K. Clark and M.C. Beckerle; U. of Utah), mouse anti-βPS (1:10; Developmental Studies Hybridoma Bank), rat anti-αPS2 (1:10; gift from N. Brown; Gurdon Institute), and guinea pig anti-Abba (1:100; this study). The Abba antibody was generated against the peptide “ARYLKNKYGNKSSKDSAG”, corresponding to amino acid residues 1118 to 1134 of CG15105-PC (Peptide Specialty Laboratories, Germany). Amplification of Abba signals was obtained with the TSA system (Perkin-Elmer). Biotinylated (1:200; Vector Labs) and fluorescent (1:200; Jackson ImmunoResearch) secondary antibodies were used. To visualize F-actin and nuclei, the fillets were fixed for 5 min in 3.7% formaldehyde and incubated with Alexa Fluor®488 phalloidin (1:500; Invitrogen) and Hoechst (10mg/ml; Sigma-Aldrich).

Images of embryo and larval stainings were acquired on a Zeiss ApoTome with 20x/0.8 Plan-Apochromat and 40x/1.3 Plan-Apochromat oil objectives. The collected images were analyzed and processed with the Axiovision 4.8 and Adobe Photoshop programs. Each image corresponds to a z-projection of a single section.
Analysis of adult thoracic muscles and myofibrils.

RNAi studies were performed with *abba* RNAi lines and the GAL80°-TARGET system (McGuire et al., 2003). *UAS-abba* RNAi virgin females were mated with *P{tubP-Gal80°}9/y;; mef2-Gal4* males, and the flies were transferred daily into new vials. The progeny were raised at 18°C for different time periods before being switched to 28°C for RNAi induction. Indirect flight muscles (IFMs) in control (*GaI80°/+;; mef2-Gal4*) and RNAi flies, from each “temperature-shift time point”, were examined at one day and 11 days after eclosion. For analysis, hemi-thoraces from control and *abba* RNAi adult progeny were prepared, as described (Schnorrer et al., 2010). They were fixed in 3.7% formaldehyde before being double-stained with Alexa Fluor®488 phalloidin (1:500; Invitrogen) and a rat anti-Kettin (MAC 155; 1:100; Babraham Institute). The stained samples were scanned on a Leica SP5 II confocal microsocpe with a 40x/1.3 PL APO oil objective. Images were collected and analyzed with the Leica LAS AF program, and processed further with Adobe Photoshop. Each image corresponds to a z-projection of a single section.

For isolating myofibrils, hemi-thoraces were first incubated in relaxing buffer (20 mM sodium phosphate buffer, pH 7.0; 5 mM MgCl₂; 5 mM ATP; 5 mM DTT; 0.5% Triton X-100; 50% glycerol; proteinase inhibitor cocktail tablets). Then, the myofibrils were dissected from the IFMs and processed, essentially as described (Burkat et al., 2007). In brief, the myofibrils were fixed in 3.7% formaldehyde, washed in PBT (phosphate-buffered saline; 0.1% Tween), and incubated in blocking buffer (5% horse serum; phosphate-buffered saline; 0.1% Tween) prior to the addition of the primary antibody or Alexa Fluor®488 phalloidin (1:50). Primary antibodies included: guinea pig anti-Abba (1:25; this study), rabbit anti-Kettin (Klg34; 1:50; gift from B. Bullard; U. of York), rabbit anti-Zormin (1:50; gift from B. Bullard). Fluorescent secondary antibodies were used at 1:50. The stained myofibrils were
scanned on a Leica SP5 II confocal microscope with a 40x/1.3 PL APO oil objective. Images were captured and analyzed with the Leica LAS AF program, and processed further with Adobe Photoshop. Each image corresponds to a z-projection of a single section.

**Electron Microscopy.**

The head, wings, and abdomen of adult flies were removed with scissors, and a large cut though the thorax was made for maximal penetration of the fixative (2.5% formaldehyde; 5% glutaraldehyde; 0.06% picric acid in 100 mM cacodylate buffer, pH 7.2). The hemi-thoraces were fixed for 3.5 hours at 4°C with rotation, washed in 100 mM cacodylate buffer (pH 7.2) for 30 min at 4°C, and post-fixed with a solution of 1% osmium tetroxide, 1.5% potassium ferrocyanide (III) for 2 hours 4°C. They were further washed in 100 mM cacodylate buffer at 4°C, dehydrated at room temperature, and infiltrated with propylene oxide before being embedded in Epon 812 (Roth GmBH). Longitudinal sections were made with a diamond knife on a Reicher/Jung Ultracut E, and contrasted with uranyl acetate and lead citrate. Images were taken on a Zeiss EM10 Transmission Electron Microscope.

**Flight tests.**

~200 each of control (Gal80ts/+; mef2-Gal4) and RNAi adult progeny, from each “temperature shift time point”, were tested for their flying capability. In batches of ten, the flies were gently pushed into a two-liter glass cylinder, and the number of “fliers” (near the top of the cylinder) versus non-fliers (at the bottom) was noted.
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FIGURE LEGENDS

Figure 1. Loss of abba function results in compromised larval muscle movement and pupal morphogenesis.

(A) Schematic diagram of abba gene organization. Protein-encoding exons are in red, untranslated regions in blue, and introns as lines. Abba protein domains (e.g. RING finger, B-Box, Coiled-Coil domain, and NHL repeats) are identified below their corresponding exons. Location of Minos element, Mi[ET1]abba[MB03490], is marked. Deleted region in abba^{MJO-348}, abba^{MJO-313}, and abba^{MJO-429} is demarcated in brackets. (B) Embryos were stained with a Tropomyosin (TM1) antibody. Homozygous abba^{MJO-348} embryos or embryos trans-heterozygous for abba^{MJO-348} and Df(2R)Exel6068 do not show muscle defects. (C) Late third instar larvae, homozygous for abba or trans-heterozygous for abba and Df(2R)Exel6068 are limp, slimmer, and shorter than heterozygous abba^{MJO-348} larvae. In this and all subsequent figures, “+” denotes the CyO, twi>eGFP balancer chromosome. (D) Mutant pupae are longer and slimmer than control pupae. Abnormal “air pocket” in the posterior end (arrow) and incomplete head eversion are also observed in mutant pupae. Scale bar = 0.5 cm.

Figure 2. abba mutant larvae exhibit disrupted striations and abnormal accumulation of Myosin.

Late third instar larval fillets were triple-labelled to visualize F-Actin (phalloidin), Myosin, and nuclei (Hoechst). In representative fillets, the focus is on muscles VL3 and VL4. (A) Stereotypic F-actin striations are observed in abba^{MJO-348}/CyO, twi>eGFP control larvae, while they are severely disrupted in abba^{MJO-348}/Df(2R)Exel6068 and abba^{MJO-348}/abba^{MJO-348} mutant larvae (arrows). (B) In mutant muscles, Myosin striations are irregular, and there are
abnormal aggregates along the sarcolemmal membrane (asterisks). Mutant muscles are shorter and thinner. The positions of the nuclei in the mutant muscles are also aberrant. Each image corresponds to a z-projection of a single section. Scale bar = 50 µm.

Figure 3. **Abba is broadly localized at Z-discs in larval muscles.** Third instar larval fillets were double-labelled with antibodies against Abba and sarcomeric proteins. There is no overlap between Abba and Myosin, but partial overlap exists between Abba and F-actin (phalloidin). At Z-discs, Abba localization is coincident with Kettin/D-Titin and Mlp84B, but the Abba domain is broader. Abba is not detectable at attachment sites where αPS2 is expressed. Each image corresponds to a z-projection of a single section. Scale bar = 20µm.

Figure 4. **abba interacts genetically with kettin/sls, mlp84B, and α-actinin.** Larvae or pupae from particular mutant combinations were assessed for defects by using visual inspection or immunocytochemistry.

(A) Normal morphogenesis occurs in pupae heterozygous for abba_MJO-348 and sls_jID7, a loss-of-function allele. Mutant pupae homozygous for abba and heterozygous for sls are more elongated and show more severe head eversion defects than homozygous abba_MJO-348 mutants. Scale bar = 0.5 cm

(B) The relative length of control pupae and pupae, corresponding to specific abba and sls mutant combinations, was determined (n= 110 for each group). Control pupae, which were heterozygous for abba_MJO-348 or heterozygous for both abba_MJO-348 and sls_jID, were comparable in length. A statistically significant increase in length was noted for pupae that were homozygous for abba and heterozygous for sls, when compared to homozygous abba pupae. Error bars, s.e.m. *p <0.0025.
(C) Fillets from late third instar larvae were examined. A normal pattern of F-actin (phalloidin) striations is observed in homozygous mlp84B<sup>∆8-95</sup> larvae, a loss-of-function allele, but mild disruptions in the striation pattern are evident in mutant larvae homozygous for mlp84B with decreased abba function. Scale bar = 50 µm.

(D) Fillets from early third instar larvae were examined. A normal pattern of F-actin striations is observed in larvae heterozygous for actn<sup>14</sup> and abba<sup>MJO-348</sup>, but striations in homozygous actn<sup>14</sup> mutants are not distinct. As compared to homozygous abba<sup>MJO-348</sup> mutants, larvae homozygous for abba<sup>MJO-348</sup> and reduced α-actinin activity exhibit more severe striation disruptions. Scale bar = 50 µm.

Figure 5. Abba is detected at Z-discs and M-lines in adult myofibrils.

Myofibrils were dissected from adult thoracic muscles and double-labelled for the localization of Abba and F-actin (phalloidin), Kettin (at Z-discs) or Zormin (at Z-discs and M-lines). Abba is prominent at both Z-discs and M-lines. Each image corresponds to a z-projection of a single section. Scale bar = 5µm.

Figure 6. Decreased abba function results in thoracic muscles with sarcomeric defects, including aberrant Z-discs and M-lines.

The Gal80<sup>ts</sup>-TARGET system was used to induce UAS-abba RNAi at different developmental stages. Immunohistochemistry, using phalloidin and an antibody against Kettin (A), and electron microscopy (B) were used to examine RNAi effects on indirect flight muscles at one day and 11 days post-eclosion.

(A) RNAi induction at day 7 (~L2) or day 10 (~L3) of development results in thin myofibrils with nondescript sarcomeres, based upon F-actin (phalloidin) and Kettin-labelled Z-discs.
Induction at day 13 (~late L3/pre-pupae) yields misshapen sarcomeres, which undergo deterioration 10 days later. Induction at day 16 does not cause any significant defects. Scale bar = 7.5 µm.

(B) Short, irregular Z-discs and M-lines are present when RNAi was induced at day 7 or 10. Better-defined Z-discs and shorter M-lines are observed with RNAi induction at day 13, but they deteriorate at 11 days. With induction at day 16, the Z-discs and M-lines are initially well defined, but partial dissolution of M-lines is detectable 10 days later. Scale bar = 2 µm.

**Figure 7. Flight tests of abba RNAi flies.**

Driver line Gal80\textsuperscript{ts}; mef2-Gal4 was used to induce UAS-abba RNAi 19291 at different developmental stages. Control and abba RNAi flies were assessed for flying deficits at one day and 11 days after eclosion. When RNAi induction occurred at day 7 (~L2) or day 10 (~L3) of development, >90% of the RNAi flies are flightless. When RNAi was induced at day 13 (~late L3/pre-pupae), ~60-70% are flightless. Induction at day 16, by which time the muscles were fully formed, does not affect flying capability.

**Figure 8. Abba function requires the NHL repeats, B-box/Coiled-coil domain and RING finger.**

(A) Third instar larval fillets from abba\textsuperscript{MJO-348}, abba\textsuperscript{MJO-313}, and abba\textsuperscript{MJO-429} were stained with phalloidin to visualize F-actin. abba\textsuperscript{MJO-348} is an amorphic allele. The mutant proteins in abba\textsuperscript{MJO-313} and abba\textsuperscript{MJO-429} lack the NHL repeats (see also Fig. 1A). All three mutant abba alleles exhibit severe striation disruptions, fiber tearing, and atrophy, indicating that the NHL repeats domain is critical for Abba function. Scale bar = 50µm.
(B) UAS-based constructs, encoding Abba protein that lacks the RING finger, B-box/Coiled-coil domain or NHL repeats were introduced into the $abba^{MJO-348}$ mutant background with the mef2-Gal4 driver. Full-length abba (UAS-abba$^{FL}$) provides full rescue, including a normal F-actin (phalloidin) striation pattern and survival until adulthood. The constructs UAS-abba$^{ΔRF}$, UAS-abba$^{AB-Box+CC}$, and UAS-abba$^{ANHL}$ cannot rescue the striation defects or lethality. Scale bar = 50µm.

Figure S1. Larval muscles in $Df(2R)Exel6068$ mutants exhibit highly disrupted striations.

(A-B) Late stage 17 embryos were labelled with an anti-Tropomyosin (Tm1) antibody to visualize the somatic musculature. Homozygous $Df(2R)Exel6068$ embryos show a normal muscle pattern, as in heterozygous $Df(2R)Exel6068$ embryos. In this and all subsequent supplementary figures, “+” denotes the CyO, twi>eGFP balancer chromosome. (C-D) Muscle fillets from late third instar larvae were double-labelled with an anti-Myosin antibody and Alexa Fluor®488 phalloidin, to visualize Myosin and F-actin striations, respectively. When compared to the controls, the Myosin and F-actin striations are highly disrupted in the mutant larval muscles, and Myosin aggregates are observed along the sarcolemmal membrane. Scale bar = 50µm.

Figure S2. Body length reduction and striation defects are detectable at second larval instar.

(A) The Kettin protein trap line, ZCL2144, was crossed into the $abba^{MJO-348}$ allele to assess sarcomere formation in first instar larvae. When compared to controls, the mutant larvae exhibit relatively good sarcomeres, although the periodic pattern of Kettin:GFP is disrupted in
in a limited number of muscles. Scale bar = 50µm. (B) Second instar larvae were labelled with phalloidin to visualize the F-actin striations. At this stage, the mutant larvae have more muscles that show disrupted striations (arrows). As compared to the controls, the mutant muscles are also thinner and shorter. Scale bar = 50µm. (C) The relative length of control and mutant larvae was determined. Control and mutant first instar larvae (L1) are comparable in length (n= 60). A statistically significant decrease in length is noted for mutant second instar larvae (L2) when compared to their control counterparts (n= 50 for each group). The decrease in length becomes more pronounced, reaching ~10%, with third instar larvae (L3; n=66 for each group). Error bars, s.e.m. *p <0.0025.

**Figure S3. Striation defects and abnormal Myosin accumulation are detectable in all muscle groups in abba mutant larvae.**

L3 larval fillets were stained for F-actin (A) or Myosin (B) expression. Hoechst staining identified the nuclei. Striation defects and abnormal accumulation of Myosin are observed in all muscle groups in abbaMJO-348/Df(2R)Exel6068 and abbaMJO-348/abbaMJO-348 mutant larvae. All mutant muscles are shorter and thinner. The positions of the nuclei are also aberrant in all mutant muscles. (DA= dorsal acute, LL= lateral longitudinal, VL= ventral longitudinal). Scale bar = 50µm.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 6
Figure 7
Figure 8