Binding of ADAM12, a Marker of Skeletal Muscle Regeneration, to the Muscle-specific Actin-binding Protein, \( \alpha \)-Actinin-2, Is Required for Myoblast Fusion*

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ADAM12 belongs to the transmembrane metalloprotease family. ADAM12 has been implicated in muscle cell differentiation and fusion, but its precise function remains unknown. Here, we show that ADAM12 is dramatically up-regulated in regenerated, newly formed fibers in vivo. In C2C12 cells, ADAM12 is expressed at low levels in undifferentiated myoblasts and is transiently up-regulated at the onset of differentiation when myoblasts fuse into multinucleated myotubes, whereas other ADAMs, such as ADAMs 9, 10, 15, 17, and 19, are expressed at all stages of differentiation. Using the yeast two-hybrid screen, we found that the muscle-specific \( \alpha \)-actinin-2 strongly binds to the cytoplasmic tail of ADAM12. In vitro binding assays with GST fusion proteins confirmed the specific interaction. The major binding site for \( \alpha \)-actinin-2 was mapped to a short sequence in the membrane-proximal region of ADAM12 cytoplasmic tail; a second binding site was identified in the membrane-distal region. Co-immunoprecipitation experiments confirm the in vivo association of ADAM12 cytoplasmic domain with \( \alpha \)-actinin-2. Overexpression of the entire cytosolic ADAM12 tail acted in a dominant negative fashion by inhibiting fusion of C2C12 cells, whereas expression of a cytosolic ADAM12 lacking the major \( \alpha \)-actinin-2 binding site had no effect on cell fusion. Our results suggest that interaction of ADAM12 with \( \alpha \)-actinin-2 is important for ADAM12 function.

ADAMs (for “a disintegrin and metalloprotease”)³ are a family of transmembrane glycoproteins encoded by at least 30 genes identified in Caenorhabditis elegans, Drosophila, Xenopus, and various mammalian species. The extracellular portion of ADAMs share a high sequence homology and domain organization with the class III snake venom metalloprotease-disintegrins. Both ADAMs and snake venom metalloprotease-disintegrins contain a metalloprotease-like domain with an associated regulatory prodomain, a disintegrin-like domain, a cysteine-rich domain, and an epidermal growth factor-like domain. In addition, ADAMs contain a transmembrane domain and a cytoplasmic tail (1–4). ADAMS with active metalloprotease are involved in diverse and important cellular processes (5–7). ADAM17 (tumor necrosis factor converting enzyme; TACE), ADAM10 (Kuzbanian), and ADAM9 function in the shedding of the ectodomain of membrane-anchored proteins, such as the cytokine tumor necrosis factor-\( \alpha \) (8, 9), the tumor necrosis factor receptor, transforming growth factor-\( \alpha \) (10, 11), the heparin-binding epidermal growth factor-like growth factor (12), and the cleavage of the amyloid precursor protein (13–15). ADAM12 is also an active metalloprotease, which binds to \( \alpha2 \)-macroglobulin in vitro (16), but its physiological substrates have not been identified.

The other extracellular domains of ADAMs, the disintegrin and the cysteine-rich domains, also play important roles in cell-cell and cell-matrix adhesion, cell differentiation, and fusion. The disintegrin-like domains of ADAMs are the most highly conserved. They seem to have retained the ability to bind to integrins and play a role in cell adhesion processes such as fertilization, as suggested by the interaction of sperm ADAM 2 (fertilin \( \beta \)) with integrin \( \alpha6\beta1 \) on the egg (17). Two recent reports show that the disintegrin-like domain and the cysteine-rich domain of ADAM12 mediates adhesion of C2C12 cells and tumor cell lines (18, 19). ADAMs 1, 3, 12, and 14 possess in their cysteine-rich domain a motif similar to viral fusion peptides (1), and these ADAMs are expressed in cells that participate in cell fusion events, such as fertilization, myogenesis, and osteogenesis. However, the direct involvement of such a motif in any cell fusion process has not been demonstrated yet.

In contrast to the well conserved organization of the extra-cellular regions of ADAMs, their cytoplasmic tails are much less conserved. Approximately half of the known ADAM proteins have cytoplasmic tails of up to 200 amino acids containing consensus SH3-binding motifs, whereas others have shorter cytoplasmic tails that lack any identifiable motif. ADAM9 contains two SH3-binding motifs and binds to Src SH3 domains, but not to Abl SH3 domains in vitro (20). A recent report from Howard et al. (21) identified two SH3-domain-containing proteins that bind to ADAM9 and ADAM15. ADAM9 also binds to protein kinase C through its cytoplasmic tail, and this interaction regulates the metalloprotease activity of ADAM 9 toward its substrate HB-epidermal growth factor (12). Thus, these data suggest that the cytoplasmic tails of ADAMs play a role in signal transduction to regulate the activity of the extracellular

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The abbreviations used are: ADAM, a disintegrin and metalloprotease; GST, glutathione S-transferase; RT, reverse transcription; PCR, polymerase chain reaction; CHO, Chinese hamster ovary; SH3, Src homology 3; SH2, Src homology 2; gal, galactosidase.

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region. The cytoplasmic tail of ADAM12 is among the longest of all ADAM proteins and contains potential SH3-binding motifs, suggesting of interactions with cytoplasmic proteins.

Skeletal muscle differentiation and regeneration require several events, including activation and inhibition of growth factors, matrix remodeling, cell-cell interaction, and cell fusion. Some of these processes could be controlled by ADAMs. Several ADAMs are expressed in skeletal muscle. Among them, ADAM12 may play a role in muscle differentiation, as it is required for fusion of C2C12 mouse myoblasts into multinucleated myotubes (22). Moreover, a short form of a soluble splice variant of ADAM12, which is expressed in human placenta, has myogenic activity in vivo (23).

To better understand the role of ADAMs in skeletal muscle development and regeneration, we used the C2C12 myoblast cell line, derived from mouse satellite cells, as a model. We analyzed the expression pattern during C2C12 differentiation of six known ADAMs predicted to be active metalloproteases, ADAMs 9, 10, 12, 15, 17, and 19. We show that ADAM12 expression is induced at the onset of fusion, whereas the other ADAMs are constitutively expressed. We also show that ADAM12 is not expressed in normal adult muscle but is reexpressed in regenerating muscle.

We also used the yeast two-hybrid system to identify cytoplasmic proteins interacting with the cytoplasmic tail of ADAM12. We show that ADAM12 interacts with the muscle-specific α-actinin-2 in vitro and in vivo. The major site of interaction is located in the membrane-proximal region of ADAM12 tail. Disruption of interaction between ADAM12 and α-actinin-2 in C2C12 cells by overexpression of an ADAM12 cytoplasmic tail prevents cell fusion. Our results identify for the first time a potential linkage between an ADAM and the cytoskeleton mediated by an actin-binding protein and suggest that skeletal muscle regeneration requires this interaction.

### EXPERIMENTAL PROCEDURES

#### Culture of C2C12 Cells—C2C12 cells (ATCC) were grown in supplemented Dulbecco’s modified Eagle’s medium (Dulbecco’s modified Eagle’s medium containing 2% horse serum. When cells reached confluence, differentiation was induced by the addition of 5 mM EDTA and pelleted by centrifugation. Undifferentiated and differentiating C2C12 cells were detached from culture dishes with 5 mM EDTA and pelleted by centrifugation.

#### Preparation of GST Fusion Proteins—A plasmid for GST fusion proteins was sub-cloned into pGEX-5 (25). Proteins were produced in E. coli strain BL21 (DE3) containing pGEX4T-1 (Pharmacia). The entire cytoplasmic tail of muscle ADAM12 was amplified using PCR from a corresponding full-length ADAM12 cDNA library, constructed in plasmid pGAD10 containing sequences encoding the GAL4 activation domain, was obtained from CLONTECH. One and a half million transformants of the yeast strain L40 were screened according to the protocol described by Hinnen et al. (26).

#### Yeast Two-hybrid Screening—A human skeletal muscle cDNA library, constructed in plasmid pGAD10 containing sequences encoding the GAL4 activation domain, was obtained from CLONTECH. One and a half million transformants of the yeast strain L40 were screened according to the protocol described by Hinnen et al. (26).

#### In Vitro Transcription and Translation—A plasmid for in vitro transcription of the full-length α-actinin-2 was kindly provided by Dr. A. Beggs. In vitro transcription and translation was performed with the TNT kit from Promega according to the manufacturer’s instructions.

#### Transfection of C2C12 Cells with ADAM12 Cytoplasmic Tail Constructs—The entire ADAM12 cytoplasmic tail (ADAM12ct) was expressed in pCDNA3.1 vector (Invitrogen). For the constructs myr-ADAM12ct and myr-Δ45ADAM12ct, a myristoylation motif coding sequence (GGGAGTAGCAAGAGCAAG) was inserted in-frame into the 5′ end of ADAM12 cDNA or Δ45ADAM12ct, ADAM12ct cDNA
encoding a truncated ADAM12ct lacking the N-terminal 45 amino acids. C2C12 cells were plated in 12-well dishes and grown to 70% confluence. Two μg of vector containing ADAM12ct, myr-ADAM12ct, myr-Δ45ADAM12ct, or vector without insert and 0.2 μg of CMV-β-gal vector were cotransfected into cells using the Fugene 6 reagent (Roche Molecular Biochemicals) in Dulbecco's modified Eagle's medium with 10% fetal calf serum. The next day, cells were switched to differentiation medium. Staining for β-gal was performed on the cells after 4 days of differentiation. Each transfection was done in duplicate, and the number of blue myotubes was determined in 8 fields for each duplicate. The transfection efficiency was similar for each vector construct, as determined by the number of β-galactosidase-positive cells. In addition, analysis of the efficiency of expression of each construct transfected was performed by immunoblotting of cell lysates.

In Vivo Binding of ADAM12 Cytoplasmic Tail with α-Actinin-2—ADAM12ct, myr-ADAM12ct, and myr-Δ45ADAM12ct constructs were cotransfected with α-actinin-2 cDNA-plasmid in CHO cells. Cells were lysed in lysis buffer in 50 mm Tris-Cl, pH 7.5, 150 mm NaCl, 1% Triton X-100, 0.5% deoxycholate, and 10 μg/ml each of pepstatin A, leupeptin, and aprotinin. After incubation on ice for 15 min, the lysate was centrifuged at 10,000 rpm for 20 min, and the supernatant was recovered. Immunoprecipitation was performed by incubating the supernatant with 4 μl of the polyclonal mouse ADAM12ct antibody or 4 μl of the corresponding preimmune serum for 4 h at 4 °C under gentle agitation. Thirty μl of protein A-Sepharose was then added for an additional 1 h of incubation. After three washes in the lysis buffer, 25 μl of sample buffer was added to the pelleted beads, and the immunoprecipitated proteins were resolved by SDS-polyacrylamide gel electrophoresis. Immunoblotting was then performed as described above with the monoclonal sarcomeric α-actinin antibody (clone EA-53, Sigma) at dilution of 1:3000 and with the polyclonal mouse ADAM12ct antibody at dilution of 1:1000.

RESULTS
Expression of ADAMs in C2C12 Cells—C2C12 cells have been extensively used as an in vitro model of skeletal muscle differentiation. As these cells are satellite cells derived from an adult mouse, they can also be considered as a model for skeletal muscle regeneration (27). Therefore to define the roles of ADAMs in muscle regeneration, we analyzed by RT-PCR the expression of six ADAM proteins, all of which are potentially active metalloproteases. As shown in Fig. 1a, undifferentiated C2C12 myoblasts express mRNAs for all ADAMs tested, namely, ADAMs 9, 10, 12, 15, 17, and 19. During differentiation of myoblasts to myotubes, monitored here by an increase in myogenin mRNA expression, ADAM12 expression increased in the early stages and then decreased between day 2 and 3, whereas the expression of the other ADAMs tested remained constant throughout differentiation.

The changes in ADAM12 expression during differentiation of C2C12 cells were confirmed at the protein level by immunoblotting of extracts of a membrane-enriched fraction from C2C12 cells. An antibody directed against the C-terminal end of ADAM12 (Fig. 1b) detected a 100-kDa protein, presumably corresponding to the full-length ADAM12, and a 54-kDa protein, likely representing a processed form of ADAM12 lacking the metalloprotease domain, as described previously (22). During C2C12 differentiation, here monitored by the increase in β1 integrin, a marker of differentiation, was induced after 2 days in differentiation. Arrowheads indicate the two major processed forms of ADAM12: the form that lacks the prodomain, with a molecular mass of approximately 97 kDa (open arrowhead), and the fully processed form, which lacks the prodomain, with a molecular mass of approximately 54 kDa (filled arrowhead). The muscle-specific β1D integrin, a marker of differentiation, was induced after 2 days in differentiation medium. The arrow indicates the doublet forms of β1D integrin (140 and 130 kDa).

Expression of ADAM12 in Fetal, Adult, and Regenerating Skeletal Muscle—Immunolocalization of ADAM12 in adult skeletal muscle revealed no staining. In contrast, in mdx muscle, which undergoes spontaneous regeneration (28), the small, newly formed muscle fibers were strongly positive for ADAM12 (Fig. 2). These results suggest that ADAM12 expression in skeletal muscle is restricted to developing myofibers during embryonic development, as described previously (29), and during regeneration in the adult, as shown here.

Interaction of ADAM12 with the Cytoskeleton—We screened a human skeletal muscle cDNA library in a yeast two-hybrid system using the ADAM12 cytoplasmic tail as a bait (ADAM12ct). Out of 1.5 × 10^6 transformants, we isolated...
showed that proteins bound to the beads and autoradiography of the gel indicated that GST-ADAM12cyt and control fusion proteins co-translated in the presence of [35S]methionine was in vitro.

EF-hand-like repeats (Fig. 3).

ADAM12cyt with and 28 amino acids, respectively, interacted as strongly as did ADAM12cyt with two calcium-binding domain (EF-hands). Mapping of the EF-hands rod-like domain with four spectrin-like repeats, and a C-terminal domain with two calcium-binding domain (EF-hands). Mapping of the ADAM12 between the C-terminal half of repeat 3 and the C terminus.

several clones interacting with ADAM12cyt but not with an irrelevant bait, lamin C. Among them, eight distinct clones encoding the muscle-specific isoform of α-actinin, α-actinin-2, were isolated. As linkage of ADAM12 to the cytoskeleton may be important for its function, we further characterized the α-actinin binding. Based on the overlaps between the α-actinin-2 clones, it is likely that the binding site for ADAM12 is contained within the COOH-terminal half of α-actinin, between half of the spectrin-type repeat 3 and the domain with EF-hand-like repeats (Fig. 3).

Interaction of ADAM12 with α-Actinin-2 in Vitro—To confirm the interaction of ADAM12cyt with α-actinin-2, we performed in vitro binding assays. α-Actinin-2 synthesized by in vitro translation in the presence of [35S]methionine was incubated with GST-ADAM12cyt and control fusion proteins coupled to glutathione-Sepharose beads. Gel electrophoresis of proteins bound to the beads and autoradiography of the gel showed that α-actinin-2 bound to GST-ADAM12cyt but not to GST, GST-SH2, or the glutathione beads alone (Fig. 4a). The quantities of GST proteins in the assay were estimated on a separate gel (Fig. 4b).

Identification of the α-Actinin-2 Binding Sites in ADAM12—To further characterize the interaction of ADAM12cyt with α-actinin-2, we generated deletion constructs of ADAM12cyt and tested them for binding to α-actinin-2 in yeast. As shown in Fig. 5, M-C and M-G, composed of the membrane-proximal 44 and 28 amino acids, respectively, interacted as strongly as did ADAM12cyt with α-actinin-2, whereas other constructs, M-D, -E, -F, and -H, did not bind at all. The C-terminal truncated Δ2, Δ3, and Δ4, which contain all the proximal part of ADAM12cyt, bound to α-actinin-2 as expected. Surprisingly, constructs Δ5’A and Δ5’B, which lack the proximal 45 and 32 amino acids, respectively, but retain the rest of the cytoplasmic tail, bound to α-actinin-2, although less strongly (Fig. 5a). The binding affinity was estimated from β-galactosidase activity: 2.5 h of color development was required to obtain the same intensity as that obtained within 20 min with the M-C construct. This suggests that the first 30 amino acids of ADAM12cyt contain a major α-actinin-2 binding site, whereas another region, which is probably conformational, as it can not be mapped by using different deletions of ADAM12cyt, contains a minor site of interaction.

The sequence of the membrane-proximal region of ADAM12cyt is unique among the cytoplasmic domains of ADAMs and might therefore confer α-actinin-2 binding to ADAM12 only among the proteins of the ADAMs family. Two arginine residues, at positions 21 and 26 from the membrane-proximal portion, are conserved between mouse and human ADAM12. To test whether these positively charged residues could be involved in the interaction with α-actinin-2, we mutated either residue arginine 21 or arginine 26 to leucines in the M-G deletion construct and tested binding to α-actinin-2. Neither of these mutations affected the interaction of M-G with α-actinin-2 (Fig. 5b), suggesting that the binding site for α-actinin-2 is probably contained within the first residues of the membrane-proximal portion of ADAM12cyt.

The predicted structure of the 30 amino acids of the membrane-proximal region of ADAM12cyt is a helix, which is also a feature of some other longer cytoplasmic domains of ADAMs, such as ADAM15. To test the hypothesis that conformation of the membrane-proximal helix accounts for binding to α-actinin-2, we analyzed binding of the mouse ADAM15 (metargidin) cytoplasmic tail with α-actinin-2. We found that the ADAM15 cytoplasmic tail bound α-actinin-2, although less strongly than did ADAM12cyt (Fig. 5b). This result indicates that other ADAM cytoplasmic domains that possess structural homologies with ADAM12cyt can bind α-actinin.

Overexpression of ADAM12 Cytoplasmic Tail Has a Dominant-negative Effect on Fusion of C2C12 Cells—To investigate the physiological significance of ADAM12cyt binding to α-actinin-2, we disrupted this interaction by overexpressing a full-length cytoplasmic tail of ADAM12 in C2C12 cells and analyzed the effects of such an overexpression on C2C12 cell fusion. To optimize the potential competition between soluble ADAM12cyt and the endogenous ADAM12 cytoplasmic tail, we

![Schematic representation of α-actinin-2 and the α-actinin-2 clones binding to ADAM12 in the yeast two-hybrid screen.](image)

![Interaction of α-actinin-2 and ADAM12 in vitro](image)
targeted ADAM12ct to the plasma membrane by adding a myristoylation site to the N terminus of the peptide. We co-transfected a β-gal reporter plasmid (ratio, 10:1) to identify transfected cells and to score, after 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) staining, the number of myotubes resulting from fusion between ADAM12ct-transfected myoblasts. Overexpression of myristoylated ADAM12-ct reduced fusion by 70%, overexpression of ADAM12cyt without myristoylation reduced fusion by 30%, and a truncated, myristoylated ADAM12cyt, missing the proximal region containing the major binding site for α-actinin-2, had no effect on fusion (Fig. 6a). This experiment was repeated at least three times.
Immunoblotting with an antibody specific for the C-terminal of the ADAM12 cytoplasmic tail showed that all ADAM12 constructs were equally expressed in transiently transfected CHO cells (Fig. 6b). These results suggest that a membrane-targeted ADAM12cyt competes with wild type ADAM12 for binding to α-actinin-2 and that the ADAM12-α-actinin interaction is essential in cell fusion.

In Vivo Binding of ADAM12 Cytoplasmic Tail with α-Actinin-2—To confirm that the soluble ADAM12 cytoplasmic tails bind to α-actinin-2, we did immunoprecipitation experiments by cotransfecting ADAM12 cytoplasmic tail constructs and α-actinin-2-expressing plasmid in CHO cells. Immunoprecipitations were performed with polyclonal ADAM12 antibody or preimmune serum, and a monoclonal α-actinin antibody was used for immunoblotting. α-Actinin-2 was specifically coimmunoprecipitated with myristoylated ADAM12cyt (Fig. 7a). Fig. 7b shows that α-actinin-2 was better coimmunoprecipitated with the myristoylated ADAM12cyt than with the ADAM12cyt without myristoylation, suggesting that membrane-targeted ADAM12 cytoplasmic tail binds α-actinin-2 more efficiently. Moreover, much less α-actinin-2 was coimmunoprecipitated with the truncated, myristoylated ADAM12cyt, which is consistent with the fact that the truncated ADAM12cyt lacks the major binding site for α-actinin-2. These results demonstrate that ADAM12 cytoplasmic tail binds α-actinin-2 in vivo and that capacity of inhibition of fusion by the ADAM12 cytoplasmic tails is correlated with their capacity to bind α-actinin-2.

DISCUSSION

The present study shows that ADAM12 is a marker of skeletal muscle regeneration and is specifically up-regulated at the onset of myoblast fusion. We show that the cytoplasmic domain of ADAM12 interacts with the muscle-specific α-actinin-2, and we provide evidence that this interaction is necessary for ADAM12 to promote muscle cell fusion. α-Actinin is an actin binding and actin cross-linking protein that has a crucial role in linking the plasma membrane to the cytoskeleton. α-Actinin interacts with the proteins vinculin and zyxin in focal plaques (30–32) and with the members of the cysteine-rich protein family (33). α-Actinin constitutes an direct link between the actin cytoskeleton and the cytoplasmic tail of several cell surface receptors, such as β1, β2, and β3 integrins (34, 35); ζ-selectin (36); N-methyl-D-aspartate receptor (37), intercellular adhesion molecule 1 (ICAM-1) (38); and ICAM-2 (39).

The major α-actinin-2 binding site in the ADAM12 cytoplasmic domain was mapped to the first membrane-proximal 30 amino acids. By comparing all known bindings of transmembrane proteins to α-actinin, Heiska et al. (39) noted that positively charged and hydrophobic amino acids are the only common elements in the α-actinin binding site of these proteins. The sequence KRRKLMRLLFTTHKK in the membrane-proximal region of mouse ADAM12 fits this concept. A second and minor binding site for α-actinin-2 was found in the distal region of the ADAM12 cytoplasmic domain. As we could not identify a precise binding site in this region, it is possible that the site requires a particular conformational structure in this area of the ADAM12 cytoplasmic tail that is not maintained in the fragments of the tail. This second binding site could reinforce or stabilize the interaction of α-actinin-2 with the major binding site.

The amino acid sequence of the major binding site for α-actinin-2 is unique to ADAM12 in the superfamily of ADAM proteins, suggesting that the property of binding α-actinin-2 could be specific to ADAM12. However, the predicted structure of this domain is primarily helical and is, in this respect, similar to the corresponding domain of several other ADAMs with long cytoplasmic domains.2 It is therefore possible that other ADAMs might bind α-actinin. Indeed, we observed binding between the ADAM15 (metargin) cytoplasmic domain and α-actinin-2 in yeast. Although the ADAM15-α-actinin interaction was not examined in detail, this observation suggests that other ADAMs may bind α-actinin and therefore be linked to the cytoskeleton. It further suggests that the binding of ADAM12 to α-actinin is not specific to α-actinin-2, which is muscle-specific; ADAM12 may also bind to other α-actinins.

2 M.-F. Galliano and E. Engvall, unpublished observations.
Early evidence for a role for ADAM12 in muscle differentiation was presented by Yagami-Hiromasa et al. (22), when they showed that transfection of C2C12 cells with antisense mRNA encoding ADAM12 inhibited cell fusion. The same study showed that overexpression of full-length ADAM12 also inhibited myoblast fusion. In contrast, overexpression of a truncated soluble ADAM12 lacking the metalloprotease domain enhanced fusion, suggesting that the metalloprotease domain negatively regulates cell fusion (22). In addition, a truncated and soluble ADAM12 lacking the metalloprotease domain promotes ectopic muscle formation in tumor cells grown in nude mice (23). The mechanism by which ADAM12 controls myoblast fusion is unknown.

Our study shows for the first time that an ADAM is linked to the cytoskeleton and provides evidence that this linkage might be necessary for ADAM12 function during myoblast fusion. This study brings new insight to how ADAM12 functions in skeletal muscle cells and suggests that the cytoskeleton may be necessary for ADAM12 function during myoblast fusion. We thank Drs. H. Lecalvez and J. Smith for the mouse ADAM12 plasmid, Dr. A. Beggs for the generous gift of the α-actinin-2 plasmid, and Dr. N. Assa-Munt for help with structure prediction of ADAMs.

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Binding of ADAM12, a Marker of Skeletal Muscle Regeneration, to the Muscle-specific Actin-binding Protein, α-Actinin-2, Is Required for Myoblast Fusion

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