The role of α-actinin in Z-disks assembly: a morphological point of view

V. Baldassarri,[1] S. Salucci,[1] P. Ferri,[1] E. Falcieri,[1,2] S. Burattini[1]

[1]*DiSTeVA, Università degli Studi di Urbino "Carlo Bo", Urbino*
[2]*Istituto di Genetica Molecolare, CNR, Istituti Ortopedici Rizzoli, Bologna*

Corresponding author: Dr. Valentina Baldassarri
Dipartimento di Scienza della Terra, della Vita e dell'Ambiente (DiSTeVA), Università degli Studi di Urbino "Carlo Bo"
Campus Scientifico "E. Mattei", via Ca' le Suore 2, 61029 Urbino (PU), Italy
Tel. +39.0722.304311 - Fax: +39.0722.304244
E-mail: valentina.baldassarri@uniurb.it

Summary
Sarcomeric α-actinin is a multitasking protein that provides structural integrity of the sarcomeres in skeletal muscle cells. Furthermore it can modulate receptors and channels and it serves as a scaffold for several signaling pathways. α-Actinin is crucial for connecting together actin filaments from adjacent sarcomeres, forming the Z-disk and then contributing to proper muscle physiology. The aim of this work was to clear up the role of sarcomeric α-actinin in Z-disk formation during myogenic differentiation. For this purpose, C2C12 murine skeletal muscle cells were analyzed at three time points of differentiation. Confocal laser scanner microscopy and transmission electron microscopy have been utilized for α-actinin immunolocalization.
Our results suggest that, when differentiation is induced, α-actinin links at first membrane-associated proteins, then it aligns longitudinally across the cytoplasm and finally binds actin, giving rise to the Z-disks. A failure in this multistep process can lead to several myopathies involving aberrant accumulation of myofilaments (*e.g.* nemaline rod myopathy), called Z-discopathies.
So, further study of α-actinin behavior could be a useful tool to better understand myofilament organization during myogenic differentiation and in correlated pathologies.

Key words: α-actinin, myogenic differentiation, Z-disk, immunofluorescence, immunogold.

Introduction

α-Actinin belongs to the spectrin superfamily of proteins, which includes a wide range of cytoskeletal proteins and in humans is encoded by four genes, which give rise to at least six protein isoforms. The cytoskeletal isoforms (α-actinin-1 and -4) can be observed along microfilament bundles and in adherent junctions, where they are involved in binding actin to the plasma membrane. They are ubiquitously expressed and calcium sensitive. The muscle-specific isoforms (α-actinin-2 and -3) are necessary for actin filament attachment to the Z-disks in skeletal muscle fibers and to the analogous dense bodies in smooth muscle cells. These isoforms have lost their ability to bind calcium, so their binding affinity to actin is regulated by phosphoinositides (Sjöblom *et al.*, 2008). In the Z-disks, actin filaments from adjacent sarcomeres overlap and are held together to form an highly stable structure (Figure 1), composed by a variable number of α-actinin cross-links, determining Z-disk width and depending on muscle-type. In fact, slow oxidative fibers show more links compared to fast glycolytic ones (Gautel, 2011), because thinner Z-disks produce higher sarcomere-shortening velocities.

Literature evidences that, besides actin-binding, sarcomeric α-actinins are able to bind other structural proteins, including nebulin and titin. Furthermore, they provide membrane integrity during muscle contractions through their bond to vinculin, integrins and dystrophin, they can modulate membrane receptors and channels and they interact with metabolic proteins (Lek *et al.*, 2009). Not least, they serve as a scaffold to connect the sarcomere to several signaling pathways (Otey and Carpen, 2004).

Thus, α-actinin is a multitasking protein that contributes to proper muscle physiology and it has been shown to be involved in several Z-disk diseases (called Z-discopathies), such as hypertrophic and dilated cardiomyopathy, congenital nemaline myopathy and intranuclear nemaline rod myopathy (Chiu *et al.*, 2010; Ilkovski, 2008).

In our previous studies we have already discussed the myogenic differentiation, particularly that of C2C12 cells (Burattini *et al.*, 2004); in this work we wanted to investigate the Z-disk formation, in the same cell model, which is closely related to α-actinin behavior. For this purpose, cells were analyzed at three time points of differentiation and carefully monitored by means of inverted microscopy (IM). Confocal laser scanner microscopy (CLSM) and transmission electron microscopy (TEM) were utilized to carry out the immunolocalization of the protein.

Materials and Methods

Cell culture and treatment

C2C12 adherent myoblasts were grown, in dishes containing a coverslip, in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine and 1% antibiotics, and they were maintained at 37°C in humidified air with 5% CO_2. To induce myogenic differentiation, after reaching at least 80% cell confluence, growth medium was replaced with differentiation medium supplemented with 1% FBS (Lattanzi *et al.*, 2000).

Cells were observed at undifferentiated stage (T_0), after 4 days of differentiation (T_1) and finally after 7 days of differentiation (T_2), using a Nikon Eclipse TE 2000-S IM equipped with a DN 100 Nikon digital camera system. To ensure cellular viability the trypan blue exclusion assay was carried out (Luchetti *et al.*, 2003; Salucci *et al.*, 2010).

Immunofluorescence (IF)

For CLSM analysis, IF techniques were carried out on coverslips. Samples were rinsed with 0.1 M PBS pH 7.4 and fixed *in situ* with 4% paraformaldehyde in PBS, for 30 minutes at room temperature (R.T.). After a further washing with PBS, cells were permeabilized with 0.2% Triton X-100 in PBS, for 10 minutes at R.T., and rinsed again with PBS.

For α-actinin labeling (D'Emilio *et al.*, 2010), samples were treated with 2% bovine serum albumin (BSA) in PBS and 5% normal horse serum (NHS) in the PBS-BSA mixture, for 30 minutes at R.T., and then incubated with a mouse primary antibody against sarcomeric α-actinin (Sigma; 1:500 in the PBS-BSA-NHS mixture), overnight at 4°C. The next day, samples were rinsed with PBS and incubated with a FITC-conjugated horse anti-mouse secondary antibody (Vector Laboratories; 1:50 in the PBS-BSA-NHS mixture), for 45 minutes at R.T. in the dark. Specimens were stained with 0.5 µg/mL propidium iodide (PI) in PBS, for 5 minutes at R.T., to visualize cell nuclei, and finally mounted with the Vectashield mounting media for fluorescence. IF analysis was performed, at T_0, T_1 and T_2.

Images were collected with a Leica TCS-SP5 connected to a DMI 6000 CS inverted microscope (Leica Microsystems CMS GmbH) and analyzed using the software Leica Application Suite Advanced Fluorescence (LAS AF). Samples were examined using oil immersion objective lenses (40x N.A. 1.25; 63x N.A. 1.40). Excitation was at 488 nm (FITC) and 543 nm (PI); emission signals were detected at 525 nm and 617 nm, respectively. CLSM images are presented as maximum intensity projections or single-plane images.

Immunogold

C2C12 myoblasts and myotubes, grown in dishes with coverslips, were washed and immediately fixed *in situ* with 1% glutaraldehyde in 0.1 M phosphate buffer pH 7.4, for 40 minutes. Monolayers were partially alcohol dehydrated and embedded, directly on coverslips, in London Resin White (LRW) acrylic resin (TAAB, England, UK), at 0°C (Luchetti *et al.*, 2002). Coverslips were taken off by dipping in liquid nitrogen. Thin sectioning was preceded by the analysis of toluidine blue-stained semithin sections. Thin sections were collected on 400 mesh nickel grids.

For immunogold technique (Ferri *et al.*, 2009), after distilled water washing, grids were rinsed with BSA 0.1% and Tween 0.05% in 0.05 M Tris buffered saline 1 (TBS1) and treated with normal goat serum (NGS; Sigma; 1:20 in TBS1-BSA mixture), for 15 minutes at R.T. in the dark. Subsequently, samples were incubated with a mouse primary antibody against sarcomeric α-actinin (Sigma; 1:10 in the TBS1-BSA-Tween mixture), overnight at 4°C in the dark. The next day,

samples were rinsed with the TBS1-BSA-Tween mixture, with BSA 0.1% in TBS1 and then with BSA 0.1% in 0.02 M Tris buffered saline 2 (TBS2); finally they were incubated with a 30 nm colloidal gold particle-conjugated goat anti-mouse secondary antibody (BBInternational; 1:25 in the TBS2+BSA mixture), for 1 hour at R.T.

Grids were finally stained with 3% uranyl acetate in distilled water and lead citrate, 1 minute each, and analyzed with a Philips CM10 transmission electron microscope.

Conventional transmission electron microscopy

Cells were previously fixed for 40 minutes with 2.5% glutaraldehyde in 0.1 M phosphate buffer. Monolayers were additionally fixed with 1% OsO_4, for 1 hour, then they were alcohol dehydrated and embedded, directly on coverslips, in araldite. Coverslips were taken off as described above. Thin sectioning was preceded by the analysis of toluidine blue-stained semithin sections. Thin sections were stained with uranyl acetate and lead citrate and analyzed with a Philips CM10 electron microscope (Baldassarri *et al.*, 2011).

Results

All samples showed a good cellular viability at IM, CLSM and TEM.

Undifferentiated stage (T_0)

At IM, myoblasts appear fusiform or star-shaped, tightly adherent to the substrate and with a smooth surface; they have a central nucleus with prominent nucleoli (Figure 2A).

At CLSM, most myoblasts show a fairly uniform cytoplasmic labeling pattern, with punctate α-actinin Z-bodies, apparently more concentrated in the perinuclear area (Figure 2B). Instead, a few myoblasts seem to have α-actinin organized in filaments in close relationship with the plasma membrane (Figure 2C). In both cases, Z-disks are not visible yet. Moreover, in Figure 2C which represents a single-plane image, a certain nuclear labeling can be observed.

The punctate cytoplasmic localization of α-actinin, especially around the nuclear envelope (Figure 2D) and at the focal adhesions (Figure 2E), is confirmed by TEM analysis. Even immunogold evidences a positivity inside the nucleus, which seems to be mainly located in the euchromatin domains (Figure 2F).

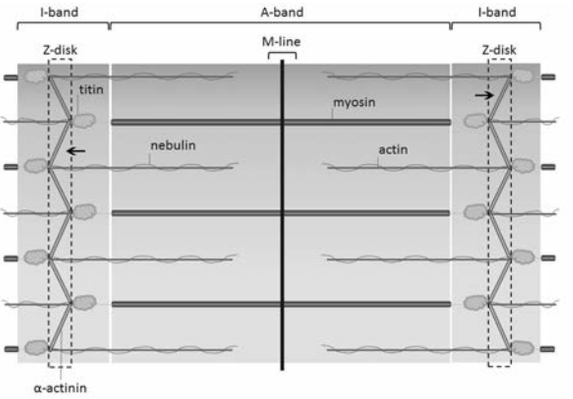

Figure 1. Schematic representation of the sarcomere structure: α-actinin is shown, by arrows, in the Z-disks.

Intermediate stage of differentiation (T_1)

After 4 days in differentiation medium, cells observed at IM appear more elongated compared to myoblasts and they begin to align themselves with each other. These early myotubes are still tightly adherent to the substrate, they show a smooth surface and a variable number of centrally placed nuclei (Figure 3A).

At CLSM, syncytia with few nuclei (2-5) reveal an intense labeling under the plasma membrane and at both ends, but filaments crossing the whole cell are rarely visible (Figure 3B). When the nuclei number increases (>5), α-actinin filaments become evident along the cytoplasm and, sometimes, nascent Z-disks in the form of cytoplasmic streaks initially assembling below the plasma membrane, can be revealed (Figure 3C).

At TEM, it is possible to observe early myotubes with few nuclei and myofilament bundles clearly visible all along the cytoplasm, in a lateral position with respect to the centrally placed nuclei (Figure 3D). High magnification images reveal α-actinin staining in these structures (Figure 3E) and just beneath the plasma membrane (Figure 3F). Some nuclear gold particles can also be observed.

Differentiated stage (T_2)

After 7 day of differentiation, myotubes cling to each other and, at IM, they appear elongated, with a regular membrane surface and numerous

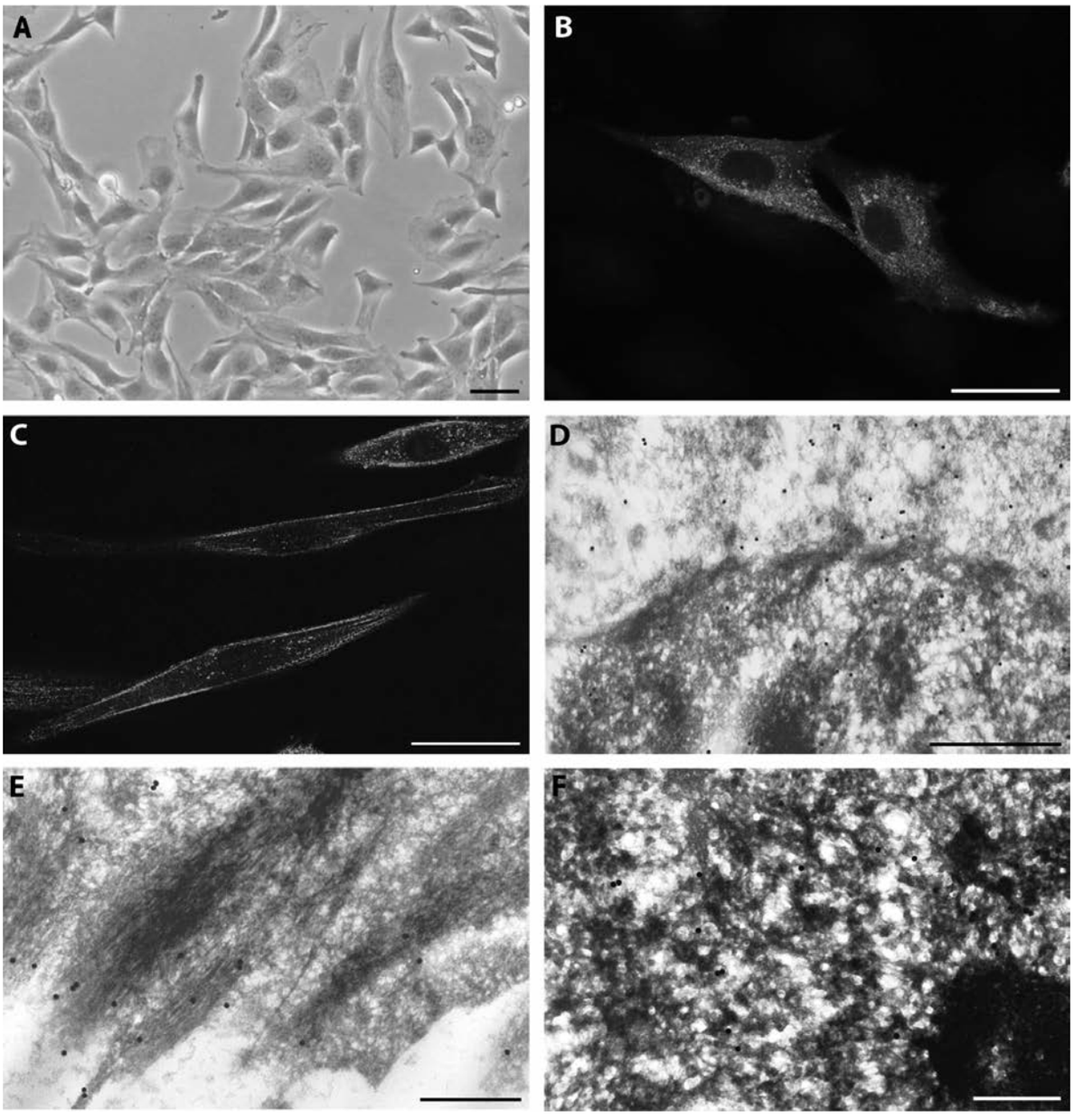

Figure 2. T_0. (A) Myoblasts observed at IM; (B) maximum intensity projection of myoblasts observed at CLSM; (C) single-plan image at CLSM; (D-F) myoblasts observed at TEM, using the immunogold technique. A, B, C bar = 25 µm; D bar = 1 µm; E, F bar = 0.5 µm.

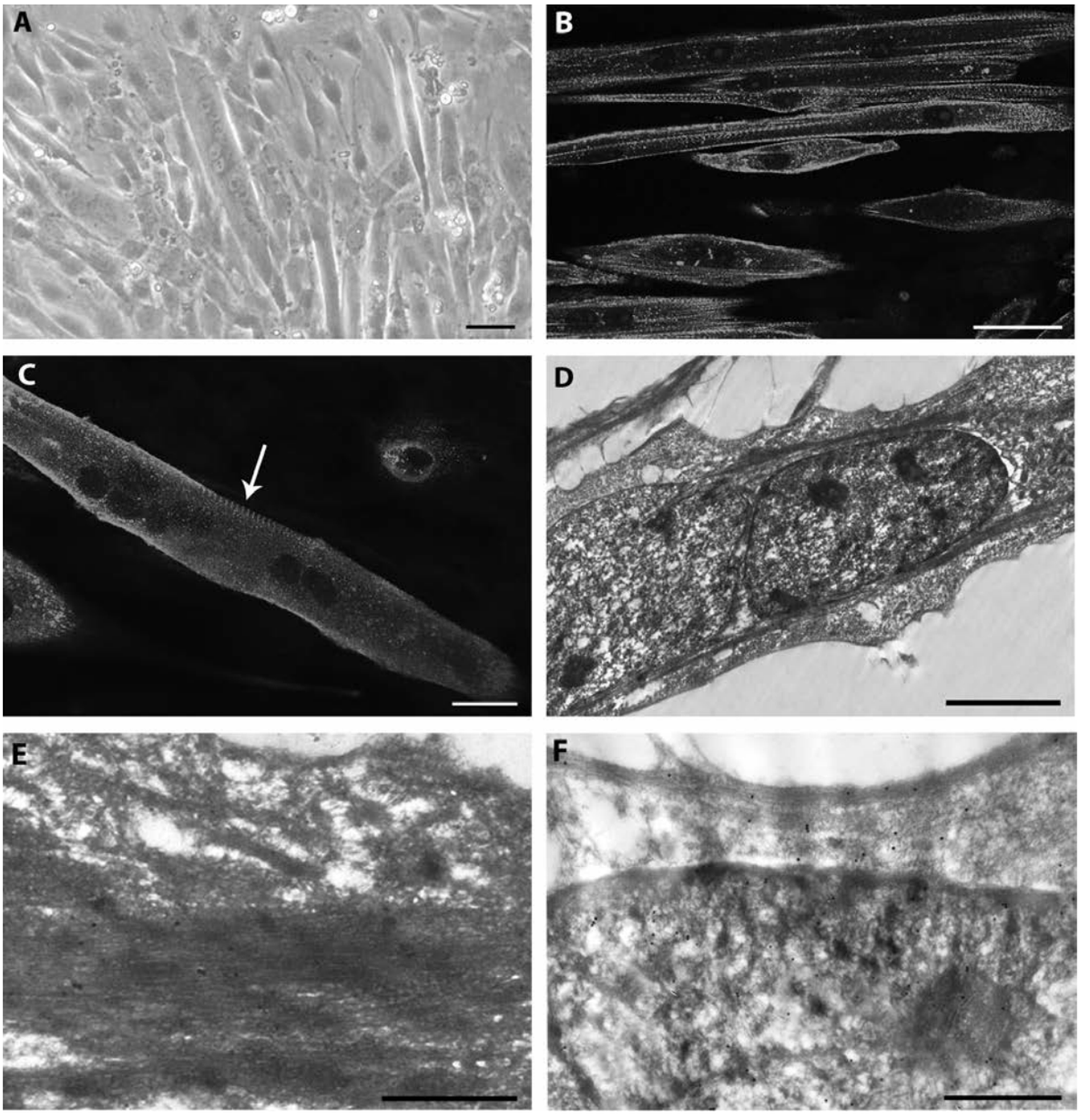

Figure 3. T_1. (A) Early myotubes, after 4 days of differentiation, observed at IM; (B) single-plan image at CLSM; (C) maximum intensity projection at CLSM; (D-F) cells at intermediate stage of differentiation observed at TEM, using the immunogold technique. (*arrow*) Nascent Z-disks. A, C bar = 25 µm; B bar = 50 µm; D bar = 5 µm; E, F bar = 1 µm.

central nuclei disposed in a single or double row (Figure 4A).

At CLSM, they appear to be characterized by numerous longitudinal α-actinin filaments crossing the cell (Figure 4B). The labeling is particularly marked just beneath the plasma membrane and at both cell ends; Z-disks are clearly visible in most myotubes (Figure 4C). It is noteworthy that in myoblasts that do not undergo differentiation and constitute the monolayer below myotubes, α-actinin labeling appear weak or even absent (Figure 4B).

Occasionally, myotubes reveal cytoplasmic areas not labeled (or weakly labeled), nor did they show PI staining. On the other hand, α-actinin seems to be located along the outlines of this areas (Figure 4D). We can hypothesize that these structures are autophagic vacuoles. In fact, as we can see at TEM (Figure 4E), they contain cellular debris and membranous material.

In general, TEM observation of myotubes confirms the labeling under the plasma membrane and in the myofilament bundles (Figure 4F), although Z-disks are not clearly visible. Even at this stage we can observe a certain nuclear labeling.

Discussion

Sarcomeric α-actinin, together with several structural and signaling proteins, is fundamental to maintain proper muscle physiology. In particular, in skeletal muscle tissue it plays a pivotal role in providing structural integrity of the sarcomeres and in anchoring the latter to the plasma membrane; it also contributes to connect many other proteins involved in stretch sensing and signaling.

So, highlighting the multistep mechanisms that result in myofibril and Z-disk assembly is crucial to understand the complex interactions between Z-disk proteins and the molecular basis of the various Z-discopathies.

Our results reveal that in myoblasts (T_0) α-actinin is uniformly distributed throughout the cytoplasm; spot-like α-actinin Z-bodies can be observed, but Z-disks are not yet visible. This is in accordance to the fact that in assembling premyofibril structures α-actinin appears in the form of closely spaced dots and it is recruited to previously polymerized actin fibrils (Crawford and Horowits, 2011).

Few days after differentiation induction (T_1), myoblasts become spindle-shaped and fuse together to form early myotubes with few nuclei. They show filamentous α-actinin molecules, probably as a result of the lateral fusion of Z-bodies. At CLSM and TEM, the labeling is evident especially beneath the plasma membrane and at the cell ends, according to the fact that *de novo* sarcomere formation or new sarcomere addition in response to stretch or growth signals begin from the cell periphery (Crawford and Horowits, 2011). With the numerical increase of nuclei, α-actinin becomes more evident and better organized and occasionally it forms nascent Z-disks.

After 7 days in differentiation medium, myotubes (T_2) appear intensely labeled; α-actinin is arranged into longitudinal arrays across the cytoplasm and almost all myotubes show evident Z-disks at CLSM.

Since α-actinin is responsible for sarcomere anchorage to the plasma membrane, probably this is why we observe an intense membrane labeling in each differentiation stage. So we can hypothesize that when differentiation is induced α-actinin, which is uniformly distributed in myoblasts, links at first membrane-associated proteins, such as vinculin and integrins, then it forms punctate Z-bodies, it aligns longitudinally across the cytoplasm and finally it links actin, nebulin and titin, thereby giving rise to the Z-disks.

α-Actinin presence inside the nucleus has been observed, in this work, at all time points, both at CLSM and TEM; probably, this is related to α-actinin involvement in several signaling pathways (Lin *et al.*, 2010) and in chromatin remodeling (Young and Kothary, 2005) and to its role in the enhancement of nuclear receptor transcriptional activation (Huang *et al.*, 2004). Furthermore, this supports the idea that α-actinin participates to signaling and transcription in each step of myogenic differentiation.

It is well known that during myogenic differentiation a portion of cells undergo spontaneous apoptosis; interestingly, myoblasts which do not undergo differentiation nor apoptosis and remain visible as a monolayer below myotubes, do not show α-actinin labeling at CLSM. A probable explanation is that in these quiescent cells α-actinin organization undergoes involution, because it is no longer required. Furthermore, it will be interesting to confirm the presence of autophagic vacuoles, observed by means of both CLSM and TEM, in conjunction with α-actinin

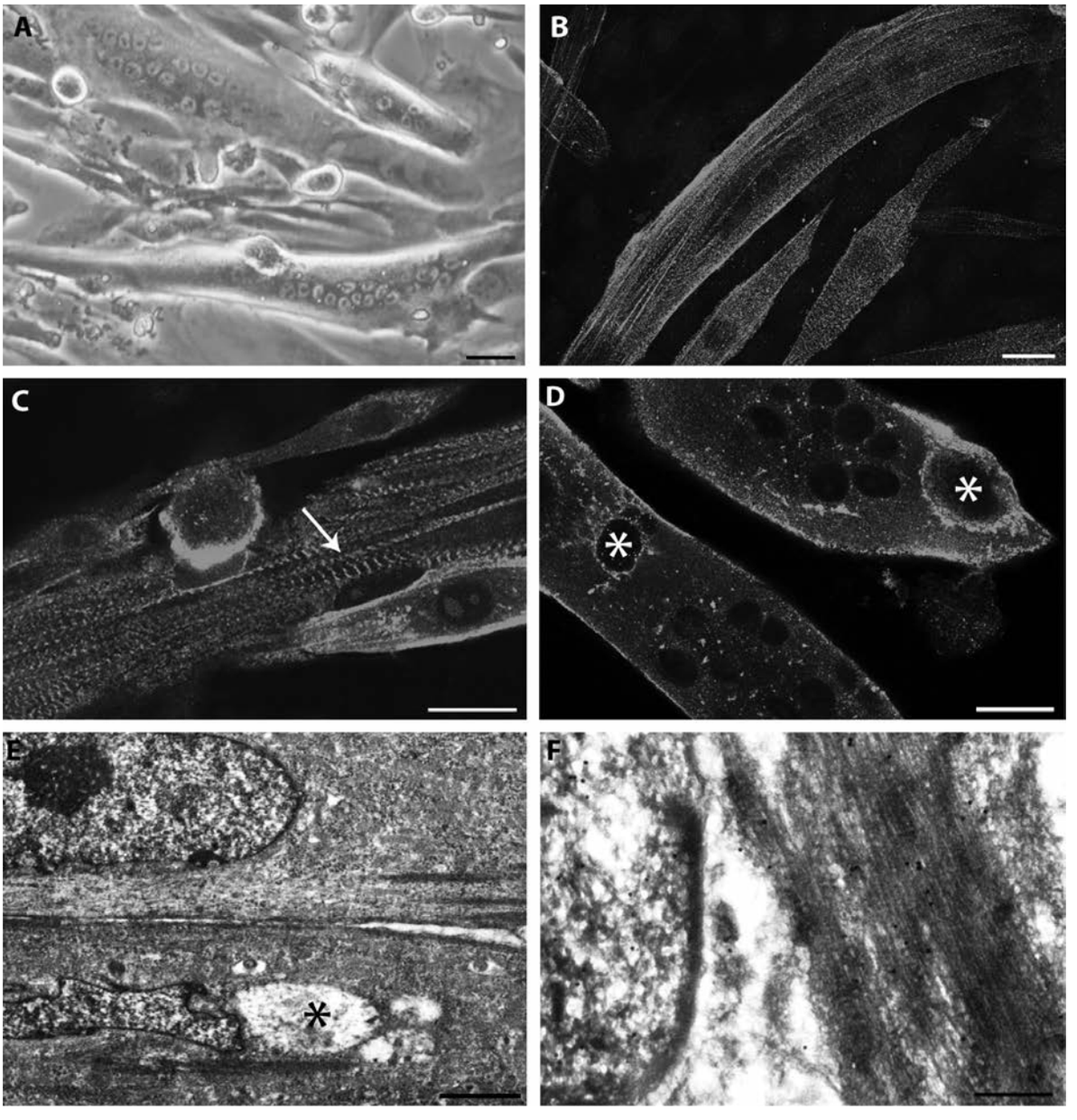

Figure 4. T_2. (A) Myotubes observed at IM; (B, D) maximum intensity projections of myotubes at CLSM; (C) single-plan image of myotubes at CLSM; (E) myotubes analyzed by means of conventional TEM; (F) myotubes observed at TEM, using the immunogold technique. (*arrow*) Z-disks; (*asterisk*) autophagic vacuoles. A, B, C, D bar = 25 µm; E bar = 2 µm; F bar = 0.5 µm.

labeling and to identify their content.

Altogether, our data outline a probable role for α-actinin during Z-disk (and sarcomere) assembly, but further experiments are needed to better characterize this process at the molecular level. Moreover, it would be useful to test this research on skeletal muscle biopsies too, because myotubes constitute a differentiated and functional model but they are not muscle fibers yet.

The characterization of α-actinin behavior during myogenic differentiation is of great importance, given also that a failure in the multistep process of Z-disks assembly could be involved in the pathogenesis of several Z-discopathies, like the nemaline rod myopathy.

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