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# Molecular analysis of the interaction between palladin and α-actinin

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**Abstract Palladin is a novel component of stress fiber dense regions. Antisense and transient overexpression studies have indicated an important role for palladin in the regulation of actin cytoskeleton. Palladin colocalizes and coimmunoprecipitates with α-actinin, a dense region component, but the molecular details and functional significance of the interaction have not been studied. We show here a direct association between the two proteins and have mapped the binding site within a short sequence of palladin and in the carboxy-terminal calmodulin domain of α-actinin. Using transfection-based targeting assays, we show that palladin is involved in targeting of α-actinin to specific subcellular foci indicating a functional interplay between the two actin-associated proteins.**


*Keywords:* Actin cytoskeleton; Dense region; EF-hand; Actin-binding protein

## 1. Introduction

Actin cytoskeleton is an essential structural and functional element that controls cell shape and movement. In order to perform its functions, the cytoskeleton must form highly organized dynamic structures, such as stress fibers and lamellipodia. When cellular needs are changed, these structures are disassembled and remodeled to meet the new requirements. The process is swift and tightly regulated by different temporal and spatial cues. The assembly, maintenance and disassembly of actin cytoskeleton is mediated by a variety of actin-associated proteins with divergent molecular interactions and functional properties.

One of the ubiquitously expressed actin-organizing molecules is α-actinin, a filament cross-linking protein. It consists of an amino-terminal actin-binding region, a rod domain with four spectrin repeats and a calmodulin EF-hand-like domain at the carboxy-terminus [1–3]. In skeletal muscle sarcomere, α-actinin is a key structural component of the Z-disc, a complex protein assembly, in which actin filaments are anchored and aligned. α-Actinin interacts with several Z-disc components, including titin, myotilin and myopalladin, all of which contain C2-type Ig-like domains [4–6]. In non-muscle cells, α-actinin is characteristically localized to focal adhesions, membrane protrusions and dense regions along actin stress fibers. Although the significance of dense regions is not well understood, they are considered to be the non-muscle equivalent of Z-discs [7]. Besides α-actinin, dense regions contain vasodilator-stimulated phosphoprotein (VASP), palladin and sometimes zyxin [8–11]. α-Actinin can also bind several transmembrane proteins such as integrin β-subunits, ICAM-1 and -2 and L-selectin [12–15]. Thus, both in muscle and non-muscle cells, α-actinin is an important cytoskeleton-organizing protein, which acts as an adaptor in multi-protein complexes associated with actin filaments [1]. The molecular interactions involved in targeting of α-actinin, e.g., to dense regions are not known.

Palladin, together with myotilin and myopalladin, forms a novel subfamily of cytoskeletal Ig-domain-containing proteins [5,6,10,11]. Myotilin contains two carboxy-terminal C2-type Ig-domains, while myopalladin has two amino-terminal and three carboxy-terminal Ig-domains and the published human and mouse palladin sequence contains three carboxy-terminal Ig-domains [5,6,10,11]. The amino-terminus of palladin contains regions with similarity to myotilin, but also unique sequence including two poly-proline stretches with putative interaction sites for other actin-organizing proteins. Myotilin and myopalladin are mainly expressed in striated muscle, whereas palladin is more widely expressed both in epithelial and mesenchymal cells. Both myotilin and myopalladin play important roles in maintaining the structural integrity of the sarcomere, and myotilin mutations lead to limb-girdle muscular dystrophy (LGMD1A), characterized by marked Z-disc alterations [16]. Myotilin and myopalladin directly interact with α-actinin, although apparently via different regions [5,6]. In addition, myotilin binds actin and bundles actin in coordination with α-actinin [17]. Based on coimmunoprecipitation and colocalization studies, palladin also is in complex with α-actinin [10].

Due to its high sequence homology to myotilin and myopalladin, palladin is presumed to play a role in maintenance and modulation of actin cytoskeleton in non-muscle tissues. In line with this idea, palladin is localized to focal adhesions and actin stress fiber dense regions, where it colocalizes with α-actinin [10,11]. Down-regulation of palladin expression leads to loss of stress fibers [18] and increased palladin expression is associated with cytoskeletal reorganization in dendritic cells, trophoblastic cells and astrocytes [10,11,19]. In order to better

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*Abbreviations:* VASP, vasodilator-stimulated phosphoprotein; EST, expressed sequence tag; GFP, green fluorescent protein

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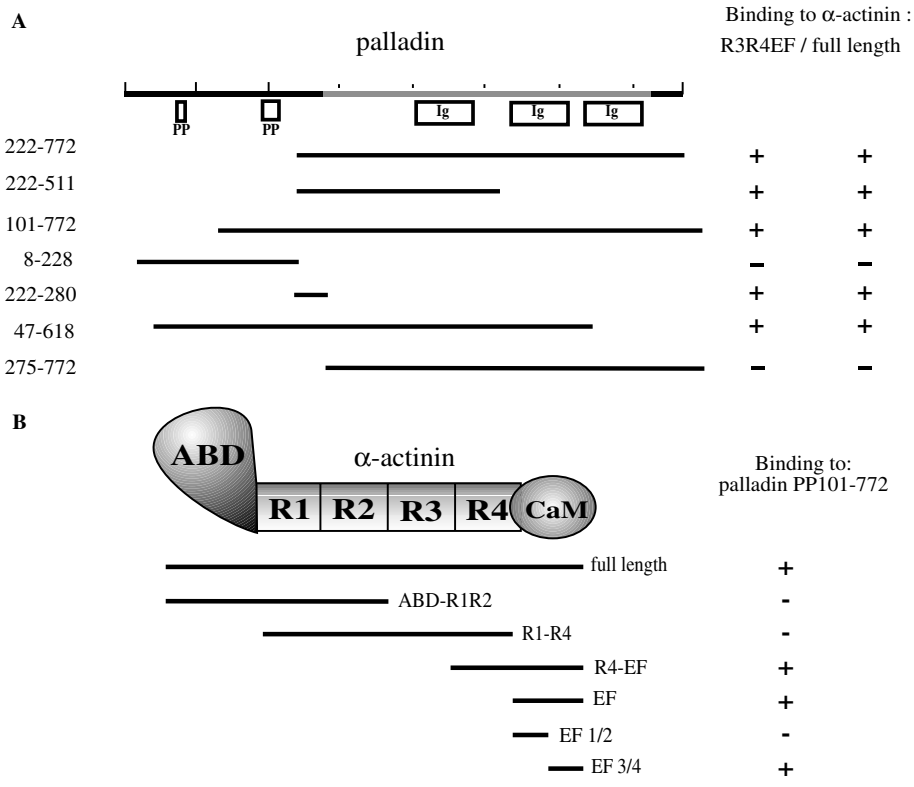


Fig. 1. Yeast two hybrid of analysis of palladin–α-actinin interaction. (A) Mapping of the α-actinin interaction site in palladin. Yeast cells were cotransformed with various palladin fragments in bait vector and α-actinin R3R4-EF or full length α-actinin in prey vector. Interactions between constructs were monitored by scoring growth on plates lacking histidine and by a filter based β-galactosidase assay. Amino acids 222–280 are necessary and sufficient for the interaction with α-actinin. The carboxy-terminal sequence (275–772) does not bind α-actinin. (B) Mapping of the palladin interaction site in α-actinin. Overlapping α-actinin constructs were tested for interaction with palladin. Results for palladin 101–772 are shown but identical results were obtained with other constructs shown to bind α-actinin in (A). The interaction is mediated by the second EF-hand (EF3/4) in the carboxy-terminal part of α-actinin.

understand palladin's biological functions, we have analyzed the molecular details and functional consequences of the interaction between palladin and α-actinin.

## 2. Materials and methods

### 2.1. Palladin constructs

Palladin constructs were PCR amplified using expressed sequence tags, EST Ab023209 and EST93367 (corresponds to NM_016081) cDNA as templates, and subcloned into pLexA vector for yeast two hybrid experiments and into pAHP or EGFP vectors for mammalian expression. For mitochondrial outer membrane (MOM) targeting, sequences encoding for palladin residues 8–387 and 222–280 were subcloned into pCDNA3-MOM-BiPro vector provided by B. Jockush [20]. For in vitro translation, palladin constructs 8–772 pAHP, 101–280 pAHP and 389–772 pAMC were used. The identity of all derived constructs was verified by DNA sequencing. Human muscle α-actinin 2A constructs (provided by P. Young and M. Gautel) for yeast two hybrid analysis were cloned into pGAD10 prey vector. A human α-actinin 1 construct, in which the rod and EF-hand-domains (R1–R4EF) are joined to green fluorescent protein (GFP), has been described [21].

### 2.2. Yeast two-hybrid analysis

Bait and prey constructs were cotransformed into *Saccharomyces cerevisiae* L40 reporter strain using a modified lithium acetate protocol. Transformants were plated onto synthetic medium lacking leucine and tryptophan. Activation of the His 3 reporter gene was assayed by restreaking yeast on plates lacking histidine, leucine, tryptophan, uracil and lysine and incubating them for 3 days at 30 °C. His+ colonies were grown on synthetic medium lacking leucine and tryptophan for 3 days at 30 °C and assayed for β-galactosidase activity by a filter assay.

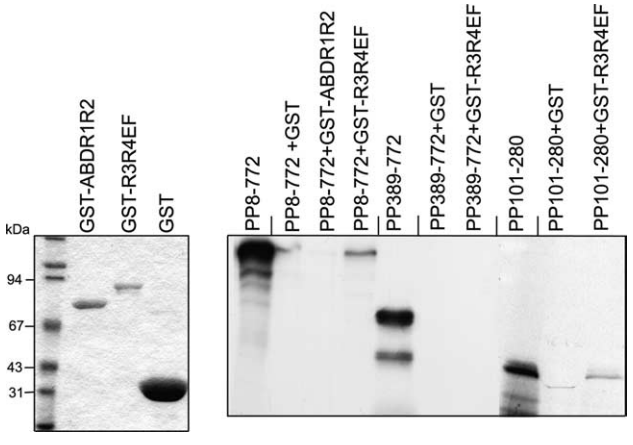


Fig. 2. Affinity precipitation analysis of palladin–α-actinin interaction. GST–α-actinin constructs coupled to glutathione–Sepharose beads were reacted with in vitro translated $^{35}$S-labeled palladin fragments. Left panel is a Coomassie blue staining of the α-actinin constructs. Right panel is an autoradiograph showing the palladin probes and material eluted from beads. Palladin 8–772 and 101–280 constructs bind to carboxy-terminal GST–α-actinin R3R4EF fusion protein, whereas PP389–772 does not bind.

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Myotilin      80 ------NPGQRVTTTYNQSPASFLSSILPSQP---DYNSSKIPSAMDSN-YQQSSA---- 125
Palladin PP 222 QASHCSSPATRFG--HGQTPAAFLSALLPSQPPPAAVNALGLPKGVTPAGFPKKASRTAR 280
                      *  *    * ***************     **  **  *    * * **
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Fig. 3. Sequence alignment of the palladin and myotilin α-actinin interacting regions. Residues 80–125 of myotilin and 222–280 of palladin were aligned using the ClustalW program. Double stars depict identical residues, stars depict conservative changes. A highly homologous 17 amino-acid segment (amino acids 89–105 of myotilin and 235–251 of palladin) can be identified and is boxed.

*2.3. In vitro translation and affinity precipitation assay*

HA- (8–772, 101–280) or Myc-tagged (389–772) palladin constructs were in vitro translated using a rabbit reticulocyte T7 kit (Promega). GST-coupled chicken gizzard α-actinin 1 constructs, ABD-R1R2 and R3R4-EF [22], were produced in *Escherichia coli* strain BL21 and purified using glutathione beads (Pharmacia). For affinity precipitation, 5 μl of $^{35}$S-Met labeled in vitro translated palladin constructs mixed with equal amount of GST–α-actinin or GST beads in binding buffer (10 mM Tris–HCl, pH7.4, 150 mM NaCl, 1 mM $MgCl_2$, and 1% Triton X-100). After 2 h incubation at +4 °C, the beads were washed three times and harvested in Laemmli buffer. Bound proteins were resolved in 10% SDS–PAGE gels and detected by autoradiography.

*2.4. Cell cultures, transfections and immunofluorescence microscopy*

U251, COS-7 and CHO cells were transfected using FuGENE 6 (Roche Diagnostics). After 24–48 h, cells grown on glass coverslips were fixed in 3.5% PFA, permeabilized with 0.1% Triton X-100 and stained. For detection of the mitochondrially targeted constructs, the Bi-Pro tag mAb 4A6 [20] was used, followed by detection with Alexa 568 anti-mouse Ig (Molecular Probes). F-actin was detected with TRITC-labeled phalloidin (Molecular Probes). Endogenous α-actinin was stained with monoclonal BM75.2 IgM antibody (Sigma) and rhodamine-conjugated anti-mouse IgM (Jackson Immunoresearch) was used for detection. Specimens were analyzed in Zeiss Axiophot fluorescence microscope equipped with AxioCam cooled CCD-camera.

## 3. Results and discussion

*3.1. Identification of the interaction sites between palladin and α-actinin*

A detailed characterization of the interaction between palladin and α-actinin was performed using different methods. Demonstration of a direct interaction and mapping of the interaction sites was initially done with the yeast two-hydrid system. Several palladin inserts were cloned in bait vector and cotransformed with α-actinin prey constructs. In these experiments a short segment, amino acids 222–280, mediated an interaction with α-actinin (Fig. 1A). None of the constructs lacking this sequence bound to α-actinin. The palladin binding site in α-actinin was located in the carboxy-terminal domain, within EF-hand repeats 3–4 (Fig. 1B).

To confirm the results with another method, we performed affinity precipitation assays with in vitro translated palladin and GST-α-actinin constructs. Palladin polypeptides 8–772, 101–280 and 389–772 were tested with several α-actinin constructs. 101–280 segment was chosen, since according to the yeast two-hybrid results the binding site is between amino acids 222–280; 389–772 was tested, because the homologous region in myopalladin has been suggested to mediate binding to α-actinin [6]. In line with yeast two-hybrid results 8–772 and 101–280 bound α-actinin, whereas the carboxy-terminal palladin construct did not show any binding (Fig. 2).

The experiments thus identified a sequence within residues 222–280 of palladin that was sufficient and necessary for binding of the carboxy-terminal EF-hands 3–4 of α-actinin. A homologous region in myotilin is also responsible for α-actinin binding (Taivainen et al., manuscript in preparation). Highest homology between the proteins is seen within a short 17 residue segment (64% identity and 94% similarity) (Fig. 3). It will be interesting to test whether this novel α-actinin binding motif interacts with EF-hand domains in other proteins. This sequence does not have features of any known structural domain, neither do homology searches identify related sequence in myopalladin or any other known protein. Interestingly, the region indicated in α-actinin binding in myopalladin (three carboxy-terminal Ig-domains) [6] was not mediating a similar interaction in palladin, although the sequences are 63% identical. The binding region in the EF-hand repeats 3–4 of α-actinin's carboxy-terminal domain is common to myopalladin, palladin and myotilin and also titin has been shown to bind the same region of α-actinin [23]. The α-actinin binding site in cardiac titin resides within Z-repeat 7, which shows no homology to the binding site in palladin and myotilin. As all these molecules are expressed in striated muscle, it will be of interest to further compare the molecular details such as binding kinetics and functional consequences of each interaction.

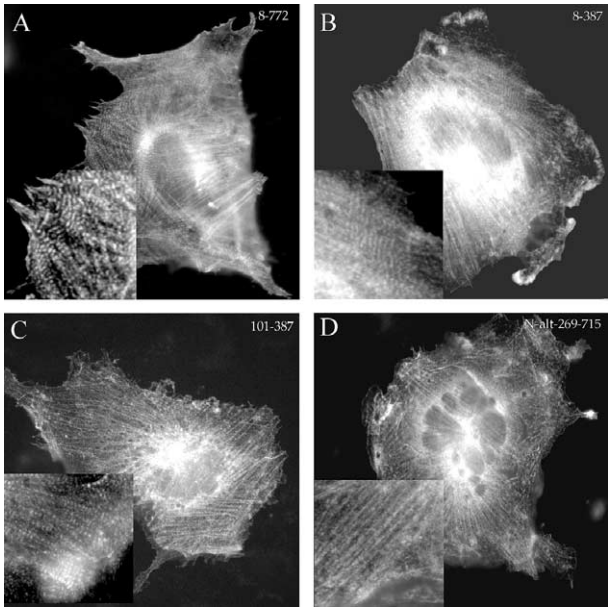


Fig. 4. Localization of GFP–palladin constructs in U251 cells. GFP-8–772 (A), GFP-8–387 (B), GFP-101–387 (C) and GFP-N-alt-269–715 (D) constructs were expressed in U251 cells. All constructs containing the α-actinin binding sequence localize to dense regions in a periodic pattern (see insets). The constructs with alternative amino-terminus lacking the α-actinin binding sequence also demonstrate stress fiber localization but do not show the periodic dense region pattern. Bar = 5 μm.

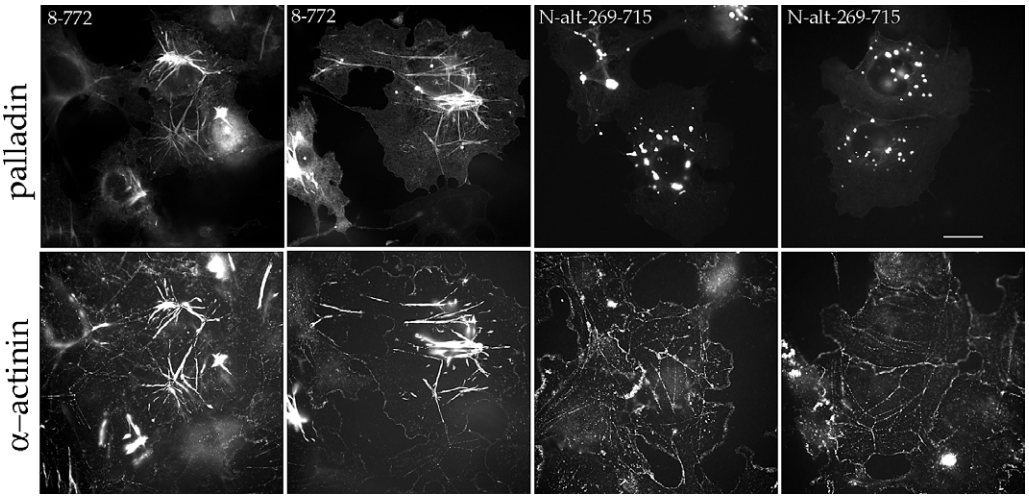


Fig. 5. Palladin 8–772 targets α-actinin to actin filaments. COS-7 cells were transfected with palladin constructs GFP-8–772 palladin or GFP-N-alt-269–715. Cells were stained for endogenous α-actinin. Two examples of palladin 8–772 expressing cells with thick irregularly shaped and oriented actin bundles are shown. Both palladin (top) and α-actinin (bottom) are recruited into the filaments. Palladin N-alt-269–715 (two right panels) induces actin-containing aggregates but does not redistribute α-actinin into these structures. Bar = 20 μm.

### 3.2. Role of interaction sites in targeting of α-actinin and palladin

To study the interaction in vivo, we used transfection-based targeting assays. First, we analyzed how different palladin constructs are targeted in cells. Three of the constructs (8–772, 8–387 and 101–387) interacted in biochemical assays with α-actinin (Fig. 4). The fourth one is a cDNA variant (N-alt-269–715), in which a unique amino-terminal sequence is fused to the published sequence at residue 269. The variant amino-terminal sequence did not bind α-actinin in yeast two-hybrid analysis (not shown). All four constructs, when expressed as GFP fusion proteins, were associated with actin filaments. However, only those containing the identified α-actinin binding site localized in dense regions suggesting that the interaction with α-actinin plays a role in targeting of palladin. In reciprocal experiments, the ability of palladin to redistribute endogenous or transfected α-actinin was tested. In one set of experiments, we used COS cells that have a poorly arranged cytoskeleton without stress fibers and which can be easily modulated with actin-organizing proteins [17]. Transfection of palladin GFP-8–772 resulted in the formation of robust actin bundles, a phenomenon seen also in astrocytes [18]. The majority of palladin 8–772 was included in these bundles (Fig. 5). Further analysis indicated that also α-actinin was targeted to these bundles and the staining patterns were highly overlapping. In COS-7 cells palladin N-alt-269–715 formed aggregates, which also contained F-actin (not shown). However, α-actinin was not targeted to these structures (Fig. 5). Since the ability of palladin to target α-actinin might be indirect and related to its actin bundling ability, we further analyzed the targeting mechanism by using a MOM-targeting vector with a birch profilin tag. In this assay, palladin constructs were co-transfected with GFP–α-actinin lacking the actin-binding domain (R1–R4EF). This α-actinin construct has been shown to retain in the cytoplasm and lead to disruption of the actin cytoskeleton [21]. The diffuse staining pattern was confirmed also in CHO cells transfected with R1–R4EF alone (Fig. 6). Short palladin inserts (8–387, 222–280) containing the identified α-actinin binding site were cloned into MOM-vector and transfected with GFP–α-actinin R1–R4EF construct into CHO cells. The proper mitochondrial targeting was verified with anti-BiPro mAb. Cotransfection of R1–R4EF together with MOM-8–387 or MOM-222–280 resulted in recruitment of the α-actinin R1–R4EF from diffuse cytoplasmic localization to the

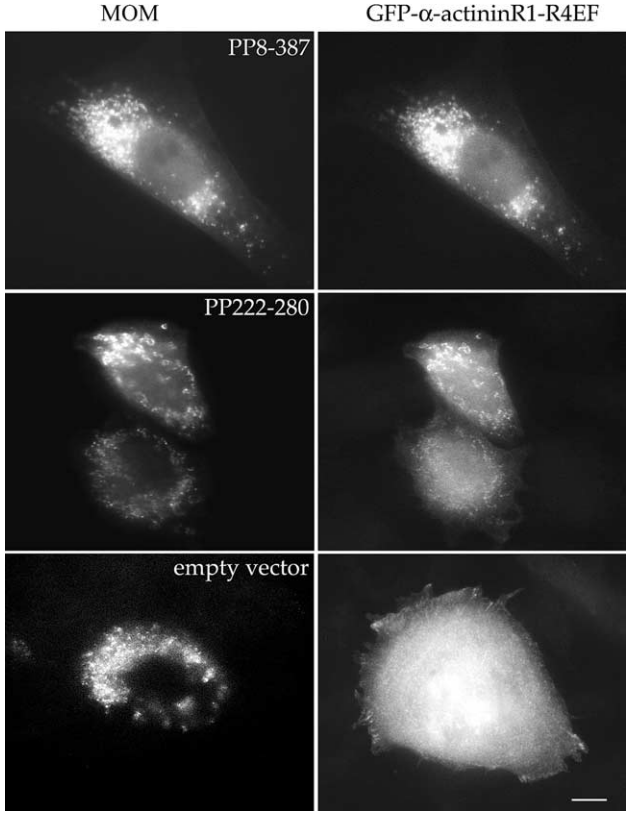


Fig. 6. Localization of α-actinin R1–R4EF in cells expressing mitochondrially targeted palladin constructs. CHO cells were cotransfected with GFP–α-actinin R1–R4EF and with palladin 8-387-MOM-BiPro, 222–280-MOM-BiPro or MOM-BiPro vector containing an outer mitochondrial membrane targeting sequence. MOM constructs were detected with BiPro tag mAb. GFP–α-actinin R1–R4EF construct transfected with the MOM-BiPro vector is distributed diffusely in the cytoplasm. When cotransfected with palladin MOM constructs, α-actinin is recruited to the mitochondrial membrane. Bar = 5 μm.

mitochondria (Fig. 6), whereas MOM vector alone did not induce any changes in the distribution of R1–R4EF.

The affinity of the interaction between myotilin and α-actinin is exceptionally high for cytoskeletal proteins ($K_D$ 6 nM) (Taivainen et al., manuscript in preparation). The binding kinetics was not measured here, but the similarity of the α-actinin binding sequence in myotilin and palladin together with the remarkable ability of palladin to target α-actinin suggests a stable and high affinity interaction. This conclusion is further supported by high degree of colocalization between palladin and α-actinin in various subcellular structures (Gorshkov et al., unpublished). In conclusion, the results indicate that the interaction between palladin and α-actinin is important for bidirectional targeting of these proteins. In the future, targeted down-regulation of palladin should clarify the functional importance of the interaction in localization and function of these proteins. The studies will also provide further information on the formation and maintenance of dense regions and other structures containing palladin and α-actinin.

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## References

[1] Otey, C.A. and Carpén, O. (2004) Cell Motil. Cytoskeleton 58, 104–111.
[2] Flood, G., Kahana, E., Gilmore, A.P., Rowe, A.J., Gratzer, W.B. and Critchley, D.R. (1995) J. Mol. Biol. 252, 227–234.
[3] Djinovic-Carugo, K., Gautel, M., Ylanne, J. and Young, P. (2002) FEBS Lett. 513, 119–123.
[4] Ohtsuka, H., Yajima, H., Maruyama, K. and Kimura, S. (1997) Biochem. Biophys. Res. Commun. 235, 1–3.
[5] Salmikangas, P., Mykkänen, O.-M., Grönholm, M., Heiska, L., Kere, J. and Carpén, O. (1999) Hum. Mol. Gen. 8, 1329–1336.
[6] Bang, M.-L., Mudry, R.E., McElhinny, A.S., Trombitas, K., Geach, A.J., Yamasaki, R., Sorimachi, H., Granzier, H., Gregorio, C.C. and Labeit, S. (2001) J. Cell Biol. 153, 413–428.
[7] Small, J.V. and Gimona, M. (1998) Acta Physiol. Scand. 164, 341–348.
[8] Crawford, A.W., Michelsen, J.W. and Beckerle, M.C. (1992) J. Cell Biol. 116, 1381–1393.
[9] Reinhard, M., Halbrugge, M., Scheer, U., Wiegand, C., Jockusch, B.M. and Walter, U. (1992) EMBO J. 11, 2063–2070.
[10] Parast, M.M. and Otey, C.A. (2000) J. Cell Biol. 150, 643–656.
[11] Mykkänen, O.M., Grönholm, M., Röonty, M., Lalowski, M., Salmikangas, P., Suila, H. and Carpén, O. (2001) Mol. Biol. Cell 12, 3060–3073.
[12] Otey, C.A., Pavalko, F.M. and Burridge, K. (1990) J. Cell Biol. 111, 721–729.
[13] Carpen, O., Pallai, P., Staunton, D.E. and Springer, T.A. (1992) J. Cell Biol. 118, 1223–1234.
[14] Pavalko, F., Walker, D.M., Graham, L., Goheen, M., Doerschuk, C.M. and Kansas, G.S. (1995) J. Cell Biol. 129, 1155–1164.
[15] Heiska, L., Kantor, C., Parr, T., Critchley, D.R., Vilja, P., Gahmberg, C.G. and Carpen, O. (1996) J. Biol. Chem. 271, 26214–26219.
[16] Hauser, M.A., Horrigan, S.K., Salmikangas, P., Torian, U.M., Viles, K.D., Dancel, R., Tim, R.W., Taivainen, A., Bartoloni, L., Gilchrist, J.M., Stajich, J.M., Gaskell, P.C., Gilbert, J.R., Vance, J.M., Pericak-Vance, M.A., Carpén, O., Westbrook, C.A. and Speer, M.C. (2000) Hum. Mol. Gen. 9, 2141–2147.
[17] Salmikangas, P., vander Ven, P.F., Lalowski, M., Taivainen, A., Zhao, F., Suila, H., Schroder, R., Lappalainen, P., Furst, D.O. and Carpen, O. (2003) Hum. Mol. Gen. 12, 189–203.
[18] Boukhelifa, M., Hwang, S.J., Valtschanoff, J.G., Meeker, R.B., Rustioni, A. and Otey, C.A. (2003) Mol. Cell. Neurosci. 23, 661–668.
[19] Boukhelifa, M., Parast, M.M., Valtschanoff, J.G., LaMantia, A.S., Meeker, R.B. and Otey, C.A. (2001) Mol. Biol. Cell 12, 2721–2729.
[20] Kaufmann, U., Zuppinger, C., Waibler, Z., Rudiger, M., Urbich, C., Martin, B., Jockusch, B.M., Eppenberger, H. and Starzinski-Powitz, A. (2000) J. Cell Sci. 113, 4121–4135.
[21] Edlund, M., Lotano, M.A. and Otey, C.A. (2001) Cell Motil. Cytoskel. 48, 190–200.
[22] Gilmore, A.P., Parr, T., Patel, B., Gratzer, W.B. and Critchley, D.R. (1994) Eur. J. Biochem. 225, 235–242.
[23] Joseph, C., Stier, G., O'Brien, R., Politou, A.S., Atkinson, R.A., Bianco, A., Ladbury, J.E., Martin, S.R. and Pastore, A. (2001) Biochemistry 40, 4957–4965.