

# Association of ARVCF with Zonula Occludens (ZO)-1 and ZO-2: Binding to PDZ-Domain Proteins and Cell-Cell Adhesion Regulate Plasma Membrane and Nuclear Localization of ARVCF

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ARVCF, an armadillo-repeat protein of the p120<sup>cas</sup> family, associates with classical cadherins and is present in adherens junctions, but its function is poorly understood. Here, we show that ARVCF interacts via a C-terminal PDZ-binding motif with zonula occludens (ZO)-1 and ZO-2. ARVCF and ZO-1 partially colocalize in the vicinity of the apical adhesion complex in polarized epithelial Madin-Darby canine kidney cells. ARVCF, ZO-1, and E-cadherin form a complex and are recruited to sites of initial cell-cell contact in sparse cell cultures. E-cadherin binding and plasma membrane localization of ARVCF require the PDZ-binding motif. Disruption of cell-cell adhesion releases ARVCF from the plasma membrane and an increased fraction of the protein localizes to the nucleus. Nuclear localization of ARVCF also requires the PDZ-binding motif and can be mediated by the PDZ domains of ZO-2. Thus, the interaction of ARVCF with distinct PDZ-domain proteins determines its subcellular localization. Interactions with ZO-1 and ZO-2, in particular, may mediate recruitment of ARVCF to the plasma membrane and the nucleus, respectively, possibly in response to cell-cell adhesion cues.

## INTRODUCTION

Tight junctions (TJs) and adherens junctions (AJs) are composed of distinct families of transmembrane adhesion proteins and associated cytosolic proteins that link the transmembrane proteins to the actin cytoskeleton. The possibility to assemble different combinations of transmembrane and cytosolic adhesion plaque proteins allows for the formation of different adhesion complexes, each presumably bringing particular structural and functional properties to sites of cell-cell contact (Borrman *et al.*, 2000). At TJs, the zonula occludens (ZO) proteins ZO-1, ZO-2, and ZO-3 bind to claudins, junction adhesion molecule, and occludin via their PDZ domains (claudins and junction adhesion molecule) or the guanylate kinase homology domain (occludin) (Stevenson and Keon, 1998; Cerejido *et al.*, 2000; Gonzalez-Mariscal *et al.*, 2000). ZO-1 can further heterodimerize with ZO-2 or ZO-3 through PDZ domain interactions (Itoh *et al.*, 1997; Ando-Akatsuka *et al.*, 1999; Wittchen *et al.*, 1999). ZO proteins bind actin filaments (Fanning *et al.*, 1998; Wittchen *et al.*, 1999; Fanning *et al.*, 2002) and several signaling molecules, suggesting that they serve as platforms for the recruitment or assembly of signaling complexes in response to cell-cell adhesion (Zahraoui *et al.*, 2000; Angst *et al.*, 2001).

In AJs, classical cadherins assemble different combinations of  $\alpha$ -,  $\beta$ -,  $\gamma$ -catenin (plakoglobin), p120<sup>cas</sup>,  $\delta$ -catenin, and Armadillo-repeat gene deleted in Velo-cardio-facial syn-

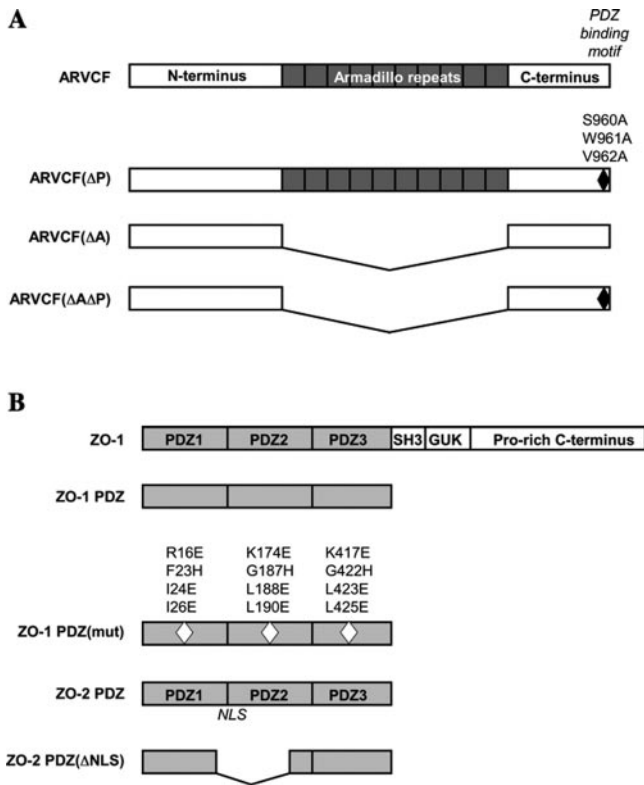
drome (ARVCF) (Marrs and Nelson, 1996; Borrman *et al.*, 2000; Angst *et al.*, 2001; Nagafuchi, 2001).  $\beta$ - and  $\gamma$ -catenin bind directly and competitively to the membrane distal region of the cytosolic domain of cadherins (Mathur *et al.*, 1994; Staddon *et al.*, 1995).  $\alpha$ -catenin associates with  $\beta$ - or  $\gamma$ -catenin to form either  $\alpha$ -/ $\gamma$ - or  $\alpha$ -/ $\beta$ -catenin complexes (Borrman *et al.*, 2000).  $\alpha$ -catenin binds F-actin directly (Rimm *et al.*, 1995) or through the interaction with the F-actin binding proteins  $\alpha$ -actinin and vinculin (Nieset *et al.*, 1997; Weiss *et al.*, 1998).

ARVCF and two other members of the p120<sup>cas</sup> family (Peifer *et al.*, 1994), p120<sup>cas</sup> itself (Reynolds *et al.*, 1992; Staddon *et al.*, 1995) and  $\delta$ -catenin/NPRAP (Paffenholz and Franke, 1997; Zhou *et al.*, 1997), associate with the membrane proximal region of the cytosolic tail of classical cadherins (Ozawa and Kemler, 1998; Yap *et al.*, 1998; Lu *et al.*, 1999; Kaufmann *et al.*, 2000). ARVCF can associate with E-, M-, and possibly N-cadherin (Kaufmann *et al.*, 2000; Waibler *et al.*, 2001). Although widely expressed (Sirotkin *et al.*, 1997), ARVCF is generally present in very low amounts compared with p120<sup>cas</sup> (Mariner *et al.*, 2000), suggesting that high levels of expression are either not required for its function or that they are temporally or spatially restricted.  $\delta$ -catenin, for example, is brain specific, expressed highest during brain development and only present in low amounts in adult neuronal tissues (Paffenholz and Franke, 1997; Zhou *et al.*, 1997).

ARVCF and p120<sup>cas</sup> bind to cadherins via their armadillo-repeat domains in a mutually exclusive manner (Mariner *et al.*, 2000), presumably competing for the same binding site (Kaufmann *et al.*, 2000). However, due to the low abundance of ARVCF compared with p120<sup>cas</sup> in most cells and tissues (Mariner *et al.*, 2000), ARVCF is unlikely to function solely as a competitive binding protein, but may rather confer distinct

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**Figure 1.** Schematic diagrams of ARVCF, ZO-1, and ZO-2. (A) Domain structure of ARVCF. The amino acid substitutions that abolish binding to PDZ-domains [ARVCF( $\Delta$ P); black diamond] and the deletion of the armadillo domains (ARVCF( $\Delta$ A)) are indicated. (B) Domain organization of ZO-1. Truncated ZO-1 and ZO-2 proteins carrying only the three PDZ domains (ZO-1 PDZ, ZO-2 PDZ) are depicted and amino acid substitutions in the PDZ domains of ZO-1 that abolish binding to C-terminal peptide [ZO-1 PDZ(mut), white diamonds] and the deletion of the nuclear localization signal in ZO-2 PDZ (ZO-2 ( $\Delta$ NLS)) are shown. Diagrams are not to scale.

properties to AJs. Consistent with the notion that the two members of the p120<sup>ctn</sup> family mediate different functions, ARVCF lacks the cellular branching activity induced by p120<sup>ctn</sup> when overexpressed in fibroblasts (Mariner *et al.*, 2000). *Xenopus laevis* ARVCF, however, mediates branching via the RhoA and Rac pathways (Fang *et al.*, 2004). Nuclear

localization of ARVCF has been reported previously (Mariner *et al.*, 2000), the functional relevance of which is unclear.

In polarized epithelial cells, ZO proteins are generally restricted to TJs and absent from AJs. However, in nonepithelial cells that lack TJs, ZO-1 predominantly localizes to AJ, where it binds to  $\alpha$ -catenin (Itoh *et al.*, 1991, 1993, 1997). In cardiomyocytes, ZO-1 is found in intercalated discs (Barker *et al.*, 2002), regions of intimate contact between adjacent myocytes that also contain N-cadherin and ARVCF (Itoh *et al.*, 1991; Toyofuku *et al.*, 1998; Kaufmann *et al.*, 2000). Even in epithelial cells, ZO proteins colocalize and associate with AJ markers under certain circumstances. For example, in the initial stages during the establishment of apico-basal polarity in Madin-Darby canine kidney (MDCK) cells, ZO-1 and E-cadherin colocalize at sites where adjacent cells establish contact with each other (Yonemura *et al.*, 1995; Rajasekaran *et al.*, 1996; Adams *et al.*, 1998; Ando-Akatsuka *et al.*, 1999; Vasioukhin *et al.*, 2000). As polarization proceeds, these contact zones mature into TJ by the gradual recruitment of occludin and the segregation of E-cadherin from ZO-1 to form AJs (Ando-Akatsuka *et al.*, 1999).

Here, we characterize novel interactions of ARVCF with ZO-1 and ZO-2. ARVCF and ZO-1 form a complex with E-cadherin and the three proteins colocalize at sites of initial cell-cell adhesion and, in polarized cells, to a region adjacent to the apical adhesion complex. Membrane recruitment as well as nuclear localization of ARVCF is regulated by cell-cell adhesion and the interaction with PDZ-domain proteins. ZO-1, in particular, may regulate plasma membrane localization of ARVCF, whereas ZO-2 may play a role in localizing ARVCF to the nucleus.

**MATERIALS AND METHODS**

**Antibodies**

The following commercial primary antibodies were used: rabbit polyclonal anti-ZO-1 and ZO-2 (Zymed Laboratories, South San Francisco, CA), rat monoclonal anti-hemagglutinin (HA) (Roche Diagnostics, Indianapolis, IN), mouse monoclonal anti-E-cadherin (BD Biosciences Transduction Laboratories, Lexington, KY), goat polyclonal anti-ARVCF (Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-Xpress (Invitrogen, Carlsbad, CA), and mouse anti-glutathione S-transferase (GST) (Santa Cruz Biotechnology). Horseradish peroxidase (HRP)-labeled secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA), Pierce Chemical (Rockford, IL), or Bio-Rad (Hercules, CA), and fluorescently labeled (Alexa 488 and 594) secondary antibodies were from Molecular Probes (Eugene, OR).

**Yeast Two-Hybrid Screen**

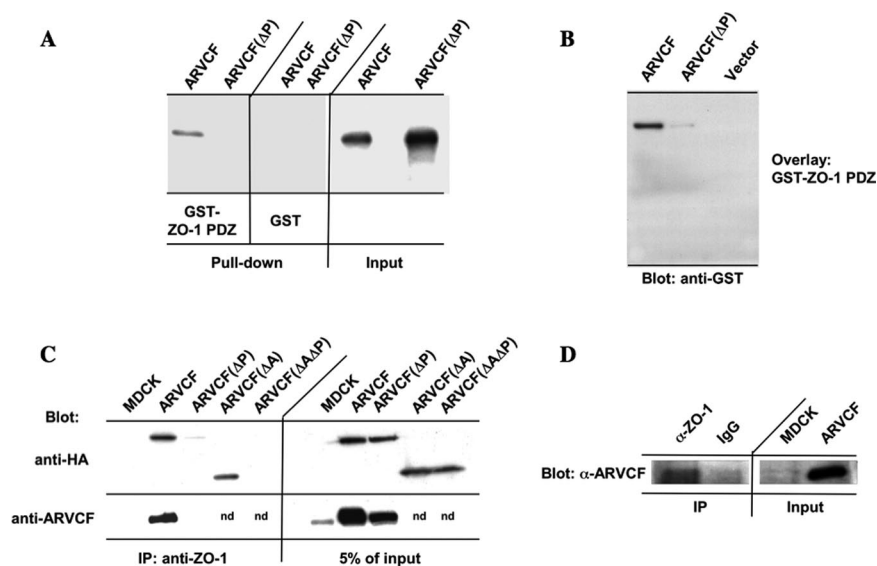
The screening of a pretransformed mouse 17-d embryo cDNA library by using the N-terminal three PDZ domains of human ZO-1 (amino acids 1–507)

**Table 1.** Interaction of the PDZ domains of ZO-1 and ZO-2 with a construct encoding the C-terminal region of ARVCF (amino acids 670–893) or a mutant thereof (ARVCF( $\Delta$ P)) in which the SWV amino acids encoding a putative PDZ-binding motif were changed to alanines

	ARVCF	ARVCF( $\Delta$ P)	Cx-45	Library vector	Laminin	T-Ag
ZO-1 PDZ	+++	–	+++	–	–	nd
ZO-2 PDZ	+++	–	–	–	–	nd
ZO-3 PDZ	–	–	+++	–	–	nd
Bait vector	–	–	–	–	–	–
p53	–	–	–	–	–	+++

Interactions were determined by monitoring growth of cotransformed yeast on selective media and  $\beta$ -galactosidase activity. Similar results were obtained under low and high stringency conditions. Empty bait (pGBKT7) and library (pACT2) plasmids and a lamin construct served as negative controls. T-antigen (T-Ag) and p53, two known interaction partners, were used as a positive control. Growth on dropout media and  $\beta$ -galactosidase activities for yeast coexpressing T-Ag and p53 (positive control) were similar for clones coexpressing the C-terminus of ARVCF and the PDZ-domains of ZO-1 and ZO-2. Since the ZO-3 bait can interact with the PDZ-motif in Cx-45, the lack of an interaction with ARVCF was not due to the experimental set up. + + +, interaction; –, no interaction; nd, not determined.

**Figure 2.** Binding of ARVCF and ZO-1. (A) In vitro-translated ARVCF binds to a GST fusion protein containing the PDZ domains of ZO-1. GST or GST-ZO-1 PDZ fusion proteins were coupled to glutathione beads and incubated with in vitro-translated, radioactively labeled ARVCF or ARVCF( $\Delta$ P). Protein bound to the beads (pull-down) was analyzed by SDS-PAGE and autoradiography. An aliquot (5%) of the in vitro translated material was directly analyzed to confirm that similar amounts of the in vitro-translated proteins were added to the binding reaction. (B) ZO-1 PDZ domains bind directly to ARVCF in blot overlays. In vitro-translated ARVCF or ARVCF( $\Delta$ P) were fractionated by SDS-PAGE, transferred to PVDF membranes, and incubated with ZO-1 PDZ domains fused to GST. Bound GST-ZO-1 PDZ was detected with a labeled anti-GST antibody. (C) ARVCF coprecipitates with ZO-1 from transfected MDCK cells. Control cells (MDCK) or cells expressing ARVCF, ARVCF( $\Delta$ P), ARVCF( $\Delta$ A), or ARVCF( $\Delta$ A $\Delta$ P) were lysed and equal amounts of total protein used to immunoprecipitate ZO-1. Precipitates were blotted to detect ARVCF (anti-HA and anti-ARVCF) that was bound to ZO-1. An aliquot of the cell lysate (5%) was directly blotted to determine the amount of wild-type and mutant ARVCF present in the lysates. Untransfected cells served as a control for the specificity of the precipitation; nd, not determined. (D) Endogenous ARVCF and ZO-1 coprecipitate from MDCK cells. Endogenous ZO-1 was immunoprecipitated from lysates of MDCK cells and precipitates were analyzed by Western blot using an anti-ARVCF antibody to detect associated ARVCF. Beads coated with an irrelevant antibody (IgG) failed to precipitate ARVCF. An aliquot of the MDCK cell lysate (5%), or for comparison cells transfected with ARVCF, was directly blotted. Approximately 20 $\times$  the amount of protein used in C was used for the coprecipitation of endogenous proteins.



and bait vectors carrying the three PDZ domains of canine ZO-2 (amino acids 1–591) and ZO-3 (amino acids 1–467) have been described previously (Kausalya *et al.*, 2001).

### Plasmid Constructs

The full-length human ARVCF cDNA was obtained by reverse transcription-polymerase chain reaction of testis cDNA library (BD Biosciences Clontech, Palo Alto, CA) by using suitable primers covering the 5' and 3' coding region of the cDNA. The primers were designed to introduce a 5' EcoRI restriction site followed by an N-terminal HA-epitope tag and a 3' XhoI restriction site. To generate a mutant ARVCF with an inactivated PDZ-binding motif [ARVCF( $\Delta$ P)], the codons for the C-terminal serine-tryptophane-valine (SWV) were changed to code for alanines. Mutants lacking the armadillo repeats (amino acids 336–782) were generated in the background of the wild-type [ARVCF( $\Delta$ A)] or PDZ-binding motif mutant [ARVCF( $\Delta$ A $\Delta$ P)] constructs. The ARVCF constructs were subcloned into the pCDNA3 vector for expression in mammalian cells and for in vitro translation. Myc-tagged ZO-1 and ZO-2 constructs carrying only the three PDZ domains (ZO-1 PDZ, ZO-2 PDZ) have been described previously (Reichert *et al.*, 2000b; Jaramillo *et al.*, 2004). To inactivate the binding capacity of the PDZ domains in the context of the ZO-1 PDZ construct [ZO-1 PDZ(mut)], suitable overlapping primer pairs were used to generate substitutions in amino acids predicted to contact C-terminal interacting peptides (Doyle *et al.*, 1996) in PDZ1 (R16E, F23H, I24E, and I26E), PDZ2 (K174E, G187H, L188E, and L190E) and PDZ3 (K417E, G422H, L423E, and L425E). Plasmids for Xpress-tagged ZO-2 PDZ domains (ZO-2 PDZ) or a ZO-2 PDZ mutant with an inactivated nuclear localization signal [ZO-2 PDZ( $\Delta$ NLS)] were obtained from Dr. Gonzalez-Mariscal (Jaramillo *et al.*, 2004). To generate GST-ZO-1 PDZ fusion protein, a cDNA encoding the N-terminal three PDZ domains of human ZO-1 (amino acids 1–507) was cloned into pGEX4T to obtain a C-terminal in frame fusion to the GST cDNA. An expression vector encoding the cytosolic tail of E-cadherin fused to GST was as described previously (Müller *et al.*, 1999). The sequences of the primers used are available upon request, a schematic representation of the proteins expressed by the different cDNA constructs is provided in Figure 1.

### Cell Culture and Transfection of MDCK Cells

MDCK strain II cells were cultured as described previously (Honing and Hunziker, 1995; Reichert *et al.*, 2000a; Mauro *et al.*, 2001). Wild-type and mutant human ARVCF cDNAs in the pcDNA3 expression vector were transfected into MDCK cells by using LipofectAMINE (Invitrogen). MDCK cell lines stably expressing ARVCF were selected in G418 and expressing clones identified by immunofluorescence or Western blot analysis. MDCK cells were grown on permeable Transwell polycarbonate filter units (Costar, Cambridge, MA) to obtain polarized cell monolayers (Reichert *et al.*, 2000a). MCF7 cells

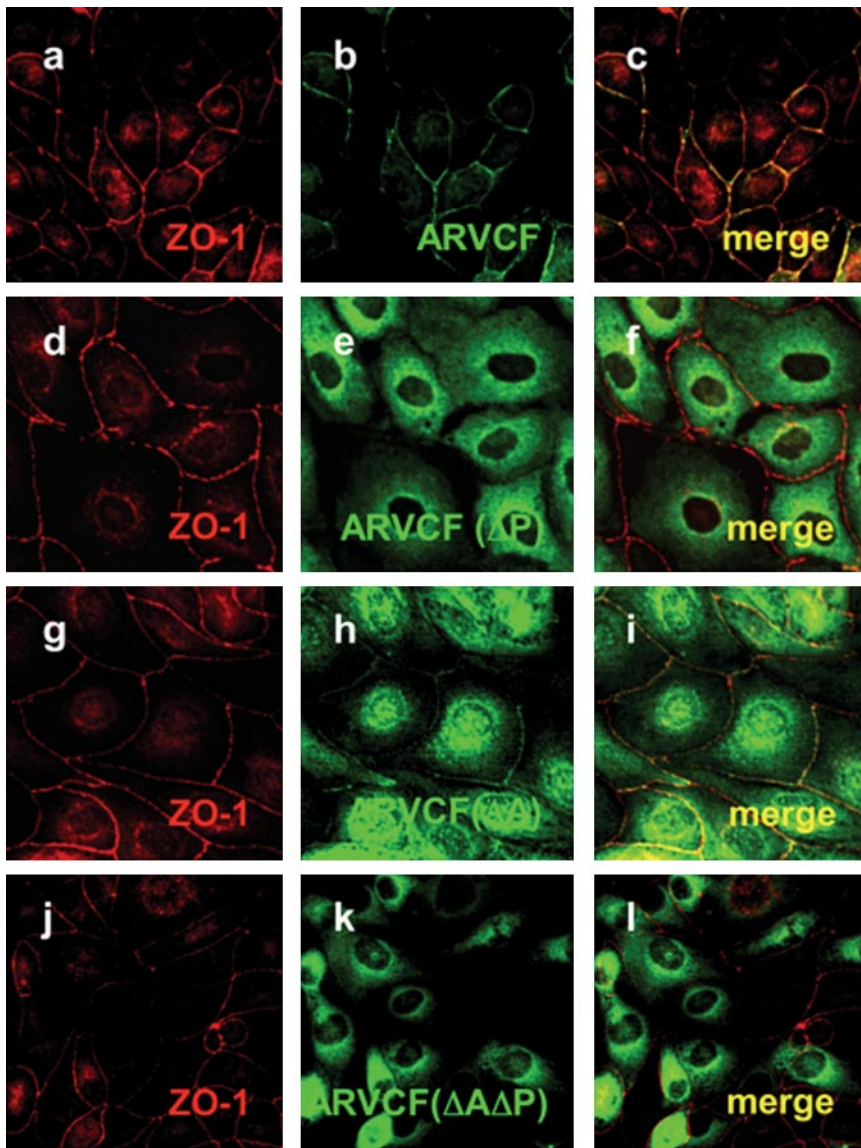
were transiently transfected with different cDNA constructs using LipofectAMINE (Invitrogen).

### GST Pull-Down

GST, GST-ZO-1 PDZ, GST-ARVCF, or GST-E-cadherin tail fusion proteins were produced, purified, and bound to glutathione Sepharose-4B (Amersham Biosciences, Piscataway, NJ) following standard protocols. Bound proteins were quantified by SDS-PAGE by using known amounts of BSA as standards. Beads carrying 2–10  $\mu$ g of GST or GST fusion proteins were incubated with 5–10  $\mu$ l of in vitro translated (Quick Coupled T7 TNT; Promega, Madison, WI) wild-type, or mutant proteins in binding buffer (25 mM Tris, pH 7.5, 50 mM NaCl, 0.1% Tween 20 or Triton X-100, 20 mM MgCl<sub>2</sub>, and 1 mM dithiothreitol [DTT] for GST-ZO-1-PDZ fusions or 20 mM HEPES, pH 7.9, 60 mM NaCl, 6 mM MgCl<sub>2</sub>, 8.2% glycerine, 0.1 mM EDTA, and 0.5% CHAPS for GST-E-cadherin tail fusion) for 2 h at 4°C. The beads were washed with washing buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20 or Triton X-100, 20 mM MgCl<sub>2</sub>, and 1 mM DTT for GST-ZO-1-PDZ fusions or 20 mM Tris-HCl, pH 8.8, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40 for GST-E-cadherin tail fusions), resuspended in SDS sample buffer, and bound proteins analyzed by SDS-PAGE, autoradiography, and densitometry.

### Coimmunoprecipitations

Control MDCK cells or transfected cells expressing either wild-type or mutant ARVCF were washed with cold phosphate-buffered saline (PBS) and lysed on ice in lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% deoxycholate, and supplemented with a protease inhibitor cocktail). The postnuclear supernatant was precleared and immunoprecipitated either with anti-ZO-1 (Zymed Laboratories), anti-HA (Roche Diagnostics), or anti-E-cadherin (BD Biosciences Transduction Laboratories) antibodies and protein G-Sepharose (Pharmacia, Uppsala, Sweden). Immunoprecipitates were washed with lysis buffer, fractionated by SDS-PAGE (10% acrylamide), blotted onto polyvinylidene difluoride (PVDF) membranes, and probed with antibodies to HA, ARVCF (Santa Cruz Biotechnology), ZO-1 (BD Biosciences Transduction Laboratories and Zymed Laboratories) followed by suitable HRP-labeled secondary antibodies (Jackson ImmunoResearch Laboratories and Pierce Chemical) and chemiluminescence (Super Signal West Pico; Pierce Chemical). Autoradiographs were analyzed by densitometry. Alternatively, different wild-type or mutant ARVCF and ZO-1 forms were cotranslated in vitro as detailed above. After immunoprecipitation of either ARVCF or ZO-1, the proteins were fractionated by SDS-PAGE, the gels were dried, and radioactively labeled proteins were visualized by autoradiography.



**Figure 3.** Colocalization of ARVCF and ZO-1 in transfected MDCK cells. MDCK cells transfected with wild-type ARVCF (a–c) or mutant ARVCF( $\Delta$ P) (d–f), ARVCF( $\Delta$ A) (g–i), or ARVCF( $\Delta$ A $\Delta$ P) (j–l) were grown on glass coverslips, fixed, permeabilized, and stained for ZO-1 (red, a, d, g, and j) and ARVCF (green, b, e, h, and k). Confocal images of the ZO-1 and ARVCF labeling were merged to show regions of colocalization (yellow, c, f, i, and l).

**Blot Overlay**

In vitro-translated ARVCF or ARVCF( $\Delta$ P) (20–50  $\mu$ l of TNT reaction) was fractionated by SDS-PAGE and transferred to PVDF membranes. Blots were incubated with a purified GST-ZO-1 PDZ domain fusion protein (75 nM), followed by a mouse anti-GST antibody and HRP-labeled goat anti-mouse antibodies, all in PBS with 0.2% Tween 20.

**Immunofluorescence Labeling**

MDCK cells grown on glass coverslips or 0.4- $\mu$ m Transwell filters (Costar) were fixed with either cold methanol (2.5 min;  $-20^{\circ}$ C) or paraformaldehyde (PFA) (3.7%; 30 min), and, in the case of PFA fixation, permeabilized with Triton X-100 (0.2% in PBS). After blocking in 10% goat serum (Invitrogen), cells were incubated with rat anti-HA, rabbit anti-ZO-1 or ZO-2, or monoclonal mouse anti-E-cadherin or anti-Xpress (Invitrogen) antibodies (5  $\mu$ g/ml) followed by suitable, fluorescently labeled secondary antibodies (1:1000; Molecular Probes). Images were acquired using a confocal laser scanning microscope (Bio-Rad) and IMARIS software (Bitplane, Zurich, Switzerland).

**Calcium-Switch and Detection of Cell-Cell Contact Protocols**

Disassembly and resealing of TJs and AJs was essentially carried out as described previously (Lawrence *et al.*, 2002). Briefly, filter grown MDCK cells were incubated for 45 min at  $37^{\circ}$ C in 2 mM EDTA in PBS to chelate calcium. Cells were then washed and either fixed or incubated for 2 h at  $37^{\circ}$ C in complete media before fixation. To analyze cell-cell adhesion in MCF7 cells, a

day after transient transfection the cells were plated on coverslips at an appropriate density and fixed after contact formation was observed.

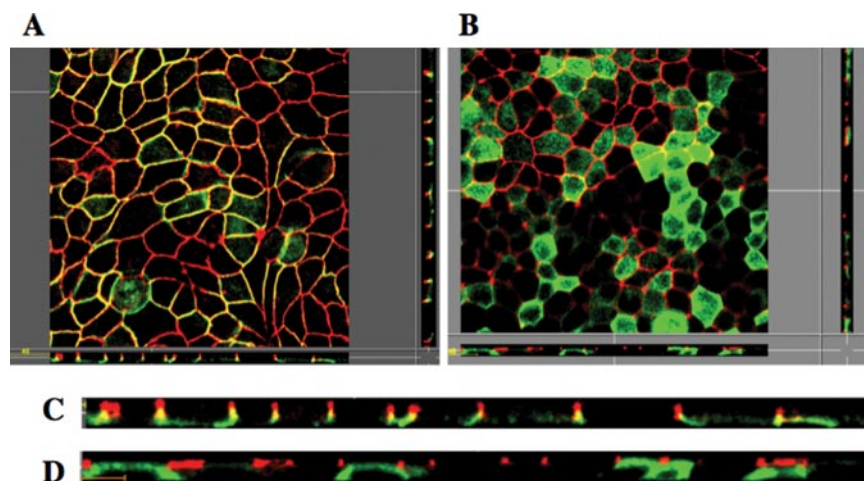
**Cytochalasin D treatment**

Cells were incubated for 1 h at  $37^{\circ}$ C with 20  $\mu$ g/ml cytochalasin D (Sigma-Aldrich, St. Louis, MO) in culture media and then washed with cold PBS and processed for immunoprecipitation or immunofluorescence labeling as described above. Labeling with rhodamine-phalloidin was used to confirm disruption of F-actin by the drug.

**RESULTS**

**ARVCF Interacts with ZO-1 and ZO-2 but Not with ZO-3**

Proteins that interact with the PDZ domains of ZO-1 were identified in a yeast two-hybrid screen of a mouse 17-d embryo library by using the three PDZ domains of human ZO-1 as a bait (Kausalya *et al.*, 2001). Sequencing of positive clones identified cDNAs encoding the C-terminal region of ARVCF. A cDNA clone encoding amino acids 670–893 of mouse ARVCF was selected for further characterization of the interaction with the PDZ-domain of different ZO proteins by using the two-hybrid assay (Table 1). In addition to



**Figure 4.** ARVCF partially colocalizes with ZO-1 to a discrete region between the lateral plasma membrane and TJ of polarized MDCK cells. MDCK cells transfected with ARVCF (a and c) or ARVCF( $\Delta$ P) (b and d) were grown on polycarbonate filters to obtain polarized cell monolayers. Cells were fixed, permeabilized and stained for ARVCF (green) and ZO-1 (red). Confocal images corresponding to horizontal sections at the height of TJ (a and b) or to vertical sections along the apico-basal axis of the monolayer (c and d) were acquired and merged. Yellow denotes regions where ARVCF and ZO-1 colocalize. Bar, 10  $\mu$ m.

the PDZ domains of ZO-1, the ARVCF construct interacted with the PDZ domains of ZO-2 but not with those of ZO-3. The interaction of the C terminus of Connexin-45 with ZO-1 and ZO-3 (Kausalya *et al.*, 2001), and p53 with T-Ag served as positive controls, whereas lamin and the empty library vector were used as negative controls, confirming the specificity of the observed associations.

PDZ domains generally interact with short C-terminal amino acid sequences and the C-terminal SWV in ARVCF conforms to a class I PDZ-binding motif (Sheng and Sala, 2001). Mutation of the S, W, and V amino acids to alanine (A) abolished the interaction with the PDZ domains of ZO-1 and ZO-2 (Table 1), showing that the SWV sequence is a functional PDZ-domain binding motif that mediates the observed interactions.

Because ARVCF was initially identified using the PDZ domains of ZO-1 as bait, subsequent experiments focused on the characterization of the binding between ARVCF and ZO-1. A GST fusion protein carrying the three PDZ domains of ZO-1 was analyzed for its ability to bind full-length in vitro-translated ARVCF or a mutant with a destroyed PDZ-binding motif [ARVCF( $\Delta$ P)] (Figure 1A). Confirming the yeast two-hybrid results, the GST-ZO-1 PDZ bound to ARVCF but not ARVCF( $\Delta$ P) (Figure 2A). To further establish that the association was direct, in vitro-translated ARVCF and ARVCF( $\Delta$ P) were fractionated by SDS-PAGE, transferred to PVDF membranes, and the blot overlaid with GST-ZO-1 PDZ. As shown in Figure 2B, GST-ZO-1 PDZ bound to ARVCF but not ARVCF( $\Delta$ P), consistent with a direct interaction.

These results thus establish a direct association between the PDZ-domains of ZO-1 and a C-terminal PDZ-binding motif in ARVCF.

#### **ARVCF and ZO-1 Interact In Vivo and Partially Colocalize in Polarized MDCK Cells**

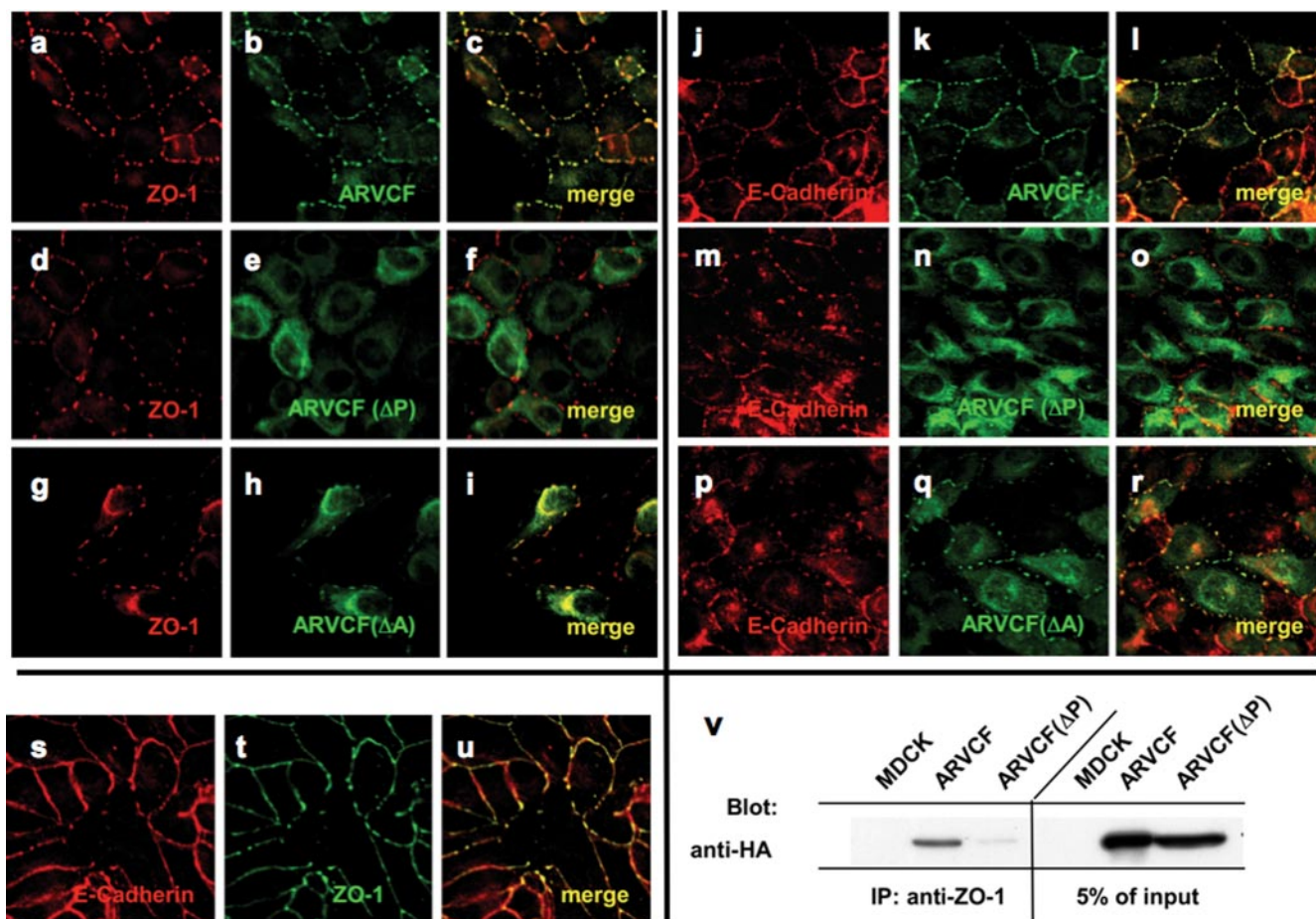
To determine whether full-length ZO-1 and ARVCF interact in vivo, epithelial MDCK cells, which endogenously express ZO-1 and low levels of ARVCF (Figure 2C), were stably transfected with cDNAs coding for wild-type or mutant human ARVCF constructs carrying an N-terminal HA-tag.

To analyze whether ZO-1 and ARVCF associate in vivo, endogenous ZO-1 was immunoprecipitated and precipitates blotted with antibodies to HA or, as a control, ARVCF. ARVCF but not ARVCF( $\Delta$ P) coprecipitated with ZO-1 (Figure 2C). Similarly, an ARVCF mutant lacking the armadillo

repeats [ARVCF( $\Delta$ A); Figure 1A] coprecipitated with ZO-1, but the association was lost if the PDZ-binding motif was mutated [ARVCF( $\Delta$ A $\Delta$ P); Figure 1A]. Furthermore, endogenous ARVCF coprecipitated with ZO-1 from MDCK cells (Figure 2D), which express low levels of ARVCF (Figure 2C; Kaufmann *et al.*, 2000), validating the coprecipitation experiments from cells overexpressing the tagged protein.

To determine whether ZO-1 and ARVCF colocalize, MDCK cells expressing wild-type or mutant ARVCF were stained with antibodies to ZO-1 and HA and analyzed by confocal immunofluorescence microscopy (Figure 3). In cells grown on coverslips, ARVCF was present at the plasma membrane at sites of cell-cell contact, where it extensively colocalized with ZO-1 (a–c). In contrast, ARVCF( $\Delta$ P) was cytoplasmic and absent from the plasma membrane and the nucleus (d–f). Compared with wild-type ARVCF, the amount of ARVCF( $\Delta$ A) at the plasma membrane was reduced but still detectable, with an increased fraction found in the cytoplasm and nucleus (g and h). The fraction of ARVCF( $\Delta$ A) present on the plasma membrane extensively colocalized with ZO-1. Similar to ARVCF( $\Delta$ P), ARVCF( $\Delta$ A $\Delta$ P) was absent from the plasma membrane and excluded from the nucleus (j–l).

The colocalization of ARVCF and ZO-1 was confirmed in polarized MDCK cell monolayers grown on permeable polycarbonate filters (Figure 4). Cells were stained for ZO-1 and ARVCF, and images of horizontal sections at the height of the TJ (Figure 4, A and B) or vertical sections along the apico-basal axis, c and d) of the cell monolayer were acquired. Although ARVCF (green, A and C) predominantly localized to the lateral plasma membrane, ARVCF( $\Delta$ P) was absent from the plasma membrane (B and C), consistent with the results for cells grown on coverslips (Figure 3). Although ARVCF was found along the length of the lateral membrane, a significant degree of colocalization with ZO-1 (red) was observed only in the apical region of the lateral membrane (yellow). In contrast, ARVCF( $\Delta$ P) did not colocalize with ZO-1. Interestingly, in contrast to the characteristic and well localized punctuate staining for ZO-1 in control cells (our unpublished data) or cells expressing ARVCF( $\Delta$ P) (D), the ZO-1 staining extended further down into the lateral membrane in cells expressing ARVCF (C). Expression levels of endogenous ARVCF in MDCK cells were too low to be detected by immunostaining (Mariner *et al.*, 1999).



**Figure 5.** Colocalization and coprecipitation of ARVCF and ZO-1 or E-cadherin in transfected MDCK cells treated with cytochalasin D. (a–u) Colocalization of ZO-1, E-cadherin, and ARVCF. MDCK cells transfected with wild-type ARVCF (a–c and j–l) or mutant ARVCF( $\Delta$ P) (d–f and m–o) or ARVCF( $\Delta$ A) (g–i or p–r) were fixed, permeabilized, and stained for ZO-1 (red, a, d, and g), E-cadherin (red, j, m, and p), and wild-type or mutant ARVCF (green, b, e, h, k, n, and q). Confocal images of the ZO-1 or E-cadherin and ARVCF labeling were merged to show regions of colocalization (yellow, c, f, i, l, o, and r). Colocalization of E-cadherin (red, s) and ZO-1 (green, t) in cytochalasin-treated cells is shown in yellow (u). v, ARVCF coprecipitates with ZO-1 from cytochalasin D-treated MDCK cells. Control cells (MDCK) or cells expressing ARVCF or ARVCF( $\Delta$ P) were lysed, and equal amounts of total protein were used to immunoprecipitate ZO-1. Precipitates were blotted to detect ARVCF that was bound to ZO-1. An aliquot of the cell lysate (5%) was directly blotted to determine the amount of wild-type and mutant ARVCF present in the lysates. Untransfected cells served as a control for the specificity of the precipitation.

In conclusion, ARVCF and ZO-1 associate and partially colocalize in vivo, with both the interaction and colocalization being dependent on the presence of an intact PDZ-binding motif in ARVCF.

**Colocalization and Association of ARVCF with ZO-1 Does Not Require an Intact Actin Cytoskeleton**

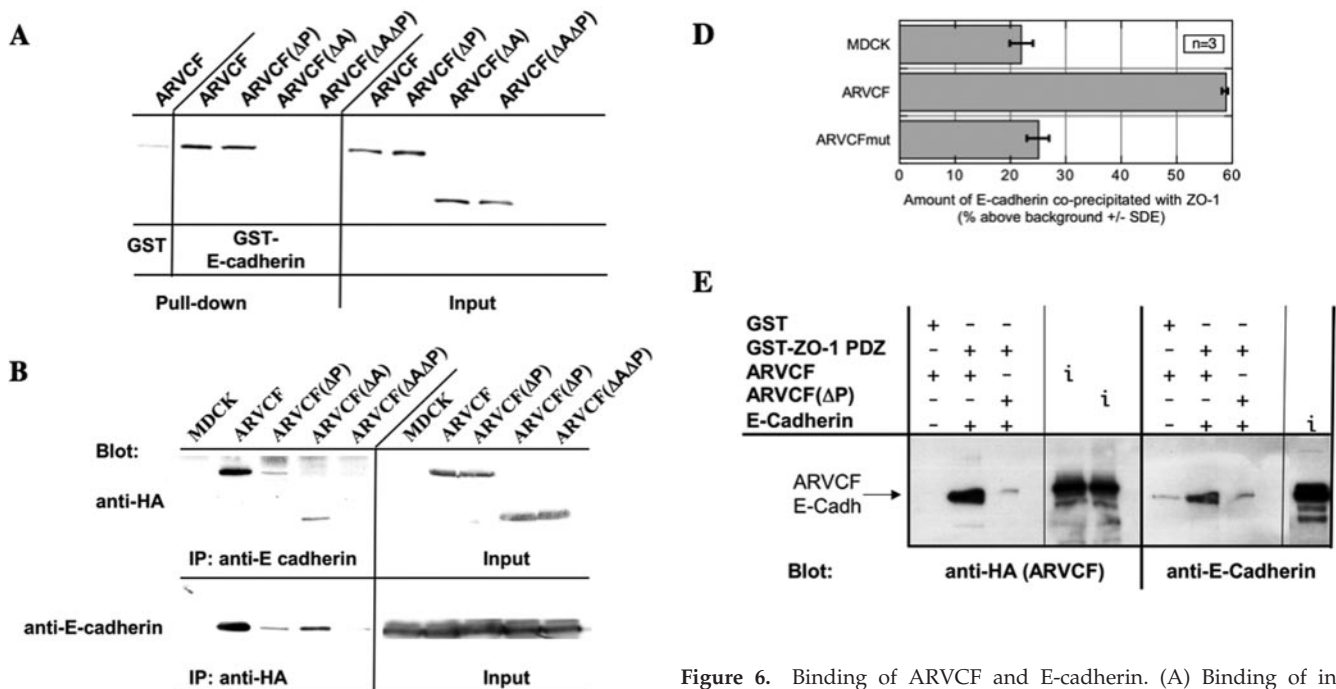
Because ZO-1 and ARVCF are found in adhesion complexes that link to F-actin, we analyzed whether compromising the integrity of the actin cytoskeleton by using cytochalasin D altered the colocalization and association of the two proteins. Cytochalasin D severely disrupted the normal organization of the actin cytoskeleton in MDCK cells (our unpublished data) and the contiguous localization of ZO-1 and ARVCF to areas of cell-cell contact (Figure 2) was broken up into strings of patches where ZO-1 and ARVCF extensively colocalized (Figure 5, a–c). The cytoplasmic localization of ARVCF( $\Delta$ P) was not altered by the drug (d–f). ARVCF( $\Delta$ A) showed a punctuate staining pattern similar to ARVCF, but the fraction present in the cytosol was increased in drug-treated cells (g–i).

As expected from their known association, also ARVCF and E-cadherin extensively colocalized in cytochalasin D-treated cells (j–l). Whereas ARVCF( $\Delta$ P) did not colocalize with E-cadherin (m–o), a detectable fraction of ARVCF( $\Delta$ A) was present in E-cadherin-containing patches (p–r). As expected from the colocalization of ARVCF with ZO-1 as well as with E-cadherin, also ZO-1 and E-cadherin colocalized in cytochalasin D-treated cells (s–u). Furthermore, ZO-1 and ARVCF but not ARVCF( $\Delta$ P) coprecipitated from cytochalasin D-treated cells (v).

Thus, the colocalization and interaction of ZO-1 with ARVCF does require an intact actin cytoskeleton.

**ARVCF Can Associate with E-Cadherin via Its Armadillo Domains or through Binding to PDZ-Domain Proteins**

Intriguingly, the PDZ binding motif seemed to be critical for plasma membrane localization of ARVCF in MDCK (Figures 2–4), 293T (our unpublished data) and MCF7 (see below) cells, whereas deletion of the armadillo repeats, which have been shown to mediate the interaction between ARVCF and M-cadherin (Kaufmann *et al.*, 2000), only partially affected



**Figure 6.** Binding of ARVCF and E-cadherin. (A) Binding of in vitro-translated wild-type and mutant ARVCF to a GST fusion protein containing the cytosolic domain of E-cadherin. GST or GST-E-cadherin tail fusion proteins were coupled to glutathione beads and incubated with in vitro translated, radioactively labeled, ARVCF, ARVCF( $\Delta$ P), ARVCF( $\Delta$ A), or ARVCF( $\Delta\Delta$ P). Protein bound to the beads (pull-down) was analyzed by SDS-PAGE and autoradiography. An aliquot (5%) of the in vitro-translated material was directly analyzed to confirm that similar amounts of the in vitro translated proteins were added to the binding reaction. (B) Coprecipitation of wild-type or mutant ARVCF with E-cadherin. Control cells (MDCK) or cells expressing ARVCF, ARVCF( $\Delta$ P), ARVCF( $\Delta$ A), or ARVCF( $\Delta\Delta$ P) were lysed, and equal amounts of total protein were used to immunoprecipitate E-cadherin. Precipitates were blotted to detect ARVCF (anti-HA) that was bound to E-cadherin. Alternatively, wild-type or mutant ARVCF was precipitated with the anti-HA antibody, and precipitates were blotted to detect bound E-cadherin. An aliquot of the cell lysate (5%) was directly blotted to determine the total amount of ARVCF or E-cadherin, respectively. (C) Coprecipitation of ZO-1 and E-cadherin. Control cells (MDCK) or cells expressing ARVCF or ARVCF( $\Delta$ P) were lysed, and equal amounts of total protein were used to immunoprecipitate ZO-1. Precipitates were then analyzed by Western blot to detect E-cadherin that was bound to ZO-1. An aliquot of the cell lysate (5%) was directly blotted to determine the amount of E-cadherin present in the lysate. (D) Quantification. The relative amounts of E-cadherin that coprecipitated with ZO-1 from control cells or those expressing wild-type or mutant ARVCF was quantified. (E) In vitro association of ZO-PDZ, ARVCF, and E-cadherin. Immobilized GST or GST-ZO-1 PDZ domain fusion proteins were incubated with in vitro translated ARVCF or ARVCF( $\Delta$ P) and E-cadherin. Proteins bound to the beads were then identified by Western blot. An aliquot of the in vitro translation reaction was directly blotted (i; input) to monitor the amount of ARVCF, ARVCF( $\Delta$ P), and E-cadherin added to the reaction.

present in the lysates. Untransfected cells served as a control for the specificity of the precipitations. (C) Coprecipitation of ZO-1 and E-cadherin. Control cells (MDCK) or cells expressing ARVCF or ARVCF( $\Delta$ P) were lysed, and equal amounts of total protein were used to immunoprecipitate ZO-1. Precipitates were then analyzed by Western blot to detect E-cadherin that was bound to ZO-1. An aliquot of the cell lysate (5%) was directly blotted to determine the amount of E-cadherin present in the lysate. (D) Quantification. The relative amounts of E-cadherin that coprecipitated with ZO-1 from control cells or those expressing wild-type or mutant ARVCF was quantified. (E) In vitro association of ZO-PDZ, ARVCF, and E-cadherin. Immobilized GST or GST-ZO-1 PDZ domain fusion proteins were incubated with in vitro translated ARVCF or ARVCF( $\Delta$ P) and E-cadherin. Proteins bound to the beads were then identified by Western blot. An aliquot of the in vitro translation reaction was directly blotted (i; input) to monitor the amount of ARVCF, ARVCF( $\Delta$ P), and E-cadherin added to the reaction.

plasma membrane localization (Figures 4 and 5). We therefore analyzed the contributions of the armadillo repeats in binding of ARVCF to E-cadherin, both in vitro and in vivo. An immobilized GST-fusion protein carrying the cytosolic domain of E-cadherin bound in vitro-translated ARVCF (Figure 6A), confirming the direct interaction of ARVCF with cadherins (Kaufmann *et al.*, 2000; Mariner *et al.*, 2000). Deletion of the armadillo domains in ARVCF abolished the interaction, similar to the results obtained for M-cadherin (Kaufmann *et al.*, 2000). The interaction of ARVCF( $\Delta$ P) with the GST-E-cadherin fusion protein was comparable with that of wild-type ARVCF, indicating that the PDZ-binding motif is dispensable for E-cadherin binding in vitro.

In vivo, however, the association of ARVCF( $\Delta$ P) with endogenous E-cadherin was severely impaired compared with wild-type ARVCF (Figure 6B). ARVCF( $\Delta$ A), on the

other hand, retained E-cadherin binding activity, which was abolished only if the PDZ-binding motif also was mutated. The ability of the different ARVCF constructs to coprecipitate with E-cadherin in vivo therefore correlated with the extent of their colocalization with E-cadherin (Figure 4) and their presence at the plasma membrane (Figures 3–5). Thus, in vivo, the armadillo domains as well as the C-terminal PDZ-binding motif contribute to the plasma membrane localization of ARVCF and its association with E-cadherin.

Given the interaction of ARVCF with cadherins and ZO-1, we next analyzed whether E-cadherin coprecipitated with ZO-1 and whether the association was affected by overexpressing ARVCF. E-cadherin was detected in ZO-1 precipitates from control cells or cells expressing ARVCF or ARVCF( $\Delta$ P) (Figure 6C). Interestingly, however, significantly more E-cadherin coprecipitated with ZO-1 from cells

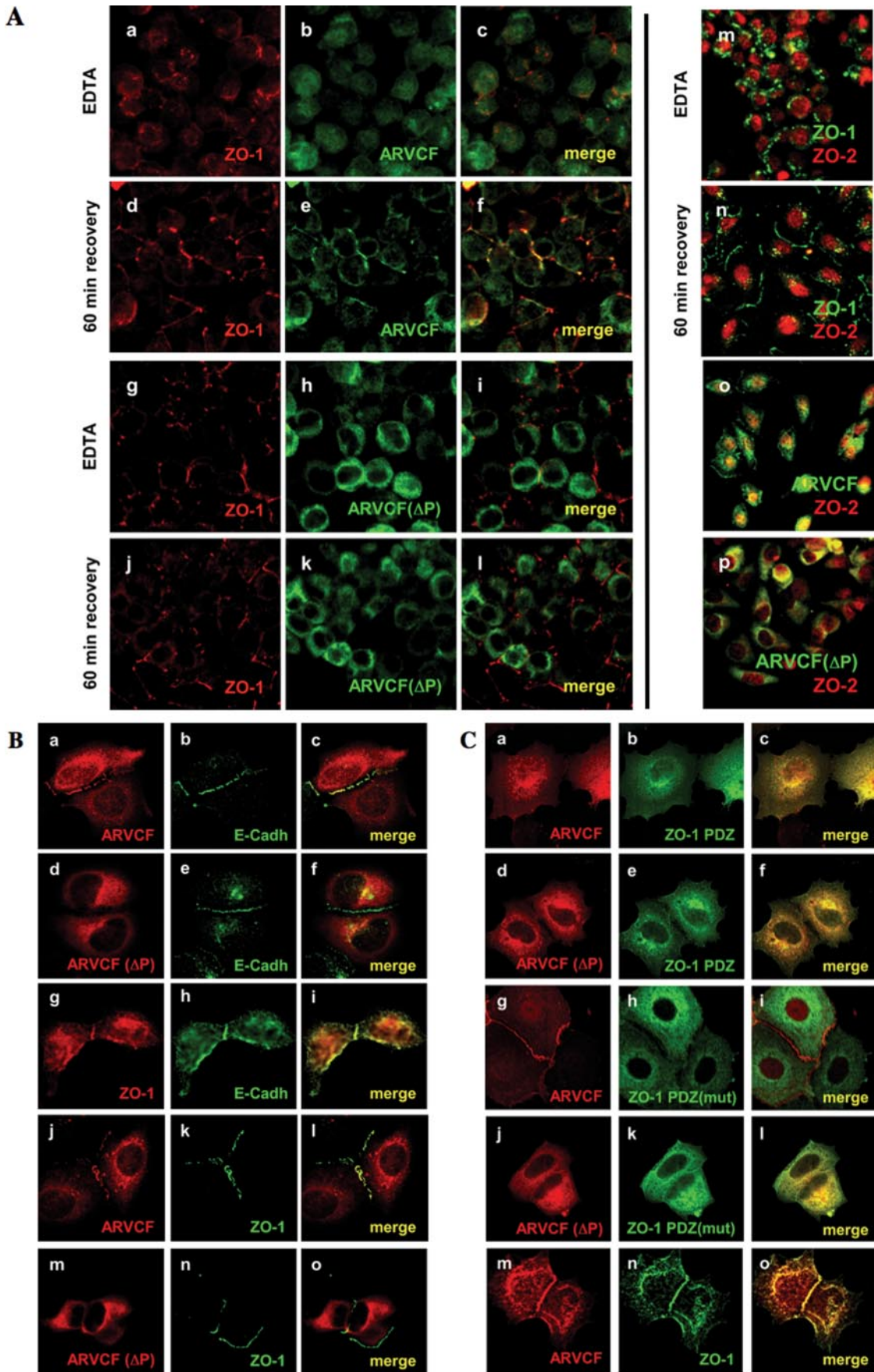


Figure 7.



expressing ARVCF compared with either control cells or cells expressing ARVCF( $\Delta$ P) (Figure 6D), suggesting that ARVCF can link ZO-1 to E-cadherin. Basal levels of E-cadherin present in the ZO-1 precipitates from control cells or cells expressing ARVCF( $\Delta$ P) likely reflect the known association of ZO-1 with E-cadherin via  $\alpha$ -catenin (Itoh *et al.*, 1993, 1997; Rajasekaran *et al.*, 1996; Ando-Akatsuka *et al.*, 1999).

To confirm complex formation between E-cadherin, ARVCF, and ZO-1 in vitro, ARVCF and the cytosolic domain of E-cadherin were translated in vitro and incubated with immobilized GST-ZO-1 PDZ. ARVCF and the E-cadherin tail bound to the ZO-1 PDZ domains (Figure 6E), and binding of either protein required an intact PDZ-binding motif in ARVCF. This experiment confirms the ability of ARVCF to link E-cadherin to ZO-1 and shows that a complex containing the three proteins can assemble in vitro.

In conclusion, ARVCF can link ZO-1 to E-cadherin, and in vivo, the armadillo domains of ARVCF as well as its interaction with PDZ domain proteins contribute to the association of ARVCF with E-cadherin.

#### **Plasma Membrane and Nuclear Localization of ARVCF Require the Interaction with PDZ-Domain Proteins and Are Regulated by Cell-Cell Adhesion**

Variable degrees of nuclear localization have been reported for ARVCF in different cell types (Kaufmann *et al.*, 2000; Mariner *et al.*, 2000; Waibler *et al.*, 2001). To determine whether nuclear localization of ARVCF was regulated by cell-cell adhesion, a "calcium-switch" protocol was used to reversibly disassemble and reseal AJs and TJs (Lawrence *et al.*, 2002). Briefly, filter-grown MDCK cells expressing ARVCF were treated with EDTA to break down cell-cell adhesion and the subcellular distribution of ARVCF and ZO proteins was analyzed, either immediately after the EDTA treatment or after recovery to allow the cells to reestablish cell-cell contact.

**Figure 7 (facing page).** Plasma membrane recruitment and nuclear localization of ARVCF require the PDZ-binding motif and are regulated by cell-cell adhesion. (A) Calcium switch experiments. MDCK cells grown on filters and expressing either ARVCF (a–f) or ARVCF( $\Delta$ P) (g–l) were incubated with EDTA to deplete calcium and disrupt cell-cell adhesion. The distribution of ARVCF (green) and ZO-1 (red) was analyzed after EDTA-treatment or after the incubation for 60 min in calcium-containing media to allow reestablishment of cell-cell adhesion, by staining with suitable antibodies and visualization by confocal microscopy. In panels where staining for ARVCF and ZO-1 was merged, yellow indicates colocalization of the two proteins. In m–p, ZO-2 (red) was colocalized with ZO-1 (green, m and n), or ARVCF or ARVCF( $\Delta$ P) (green, o and p, respectively), either after calcium depletion or a 60-min recovery. (B) Recruitment of ARVCF to sites of cell-cell contact in MCF7 cells. Sparsely plated MCF7 cells either transfected with cDNAs for ARVCF (a–c and j–l), ARVCF( $\Delta$ P) (d–f and m–o), or not transfected (g–i) were processed for immunofluorescence staining, and the localization of ARVCF, ARVCF( $\Delta$ P), and endogenous E-cadherin and ZO-1 visualized by confocal microscopy. Colocalization of the respective proteins is yellow in the merged images. (C) Effect of ZO-1 mutants on ARVCF localization in MCF7 cells. Sparse MCF7 cells coexpressing ARVCF or ARVCF( $\Delta$ P) (a–c, g–i and d–f, j–l, respectively) and either ZO-1 PDZ domains (a–f), a ZO-1 PDZ carrying inactivated PDZ-domains [PDZ(mut); g–l] or a full-length ZO-1 construct were analyzed by immunofluorescence staining and confocal microscopy. ZO-1 constructs carried an N-terminal myc tag. Colocalization of the respective proteins is yellow in the merged images.

Calcium depletion resulted in the loss of ARVCF and ZO-1 from the plasma membrane (Figure 7A, a–c). Although ARVCF was observed in both the cytoplasm and the nucleus, ARVCF( $\Delta$ P) was excluded from the nucleus (g–i). ZO-1 remained in the cytosol showing a punctuate staining similar to that previously reported under these conditions (Ivanov *et al.*, 2004). After a 60-min recovery, ARVCF and ZO-1 were recruited to sites of initial cell-cell contact, where they extensively colocalized (d–f). The amount of ARVCF present in the nucleus was notably reduced after recovery. Although ZO-1 relocated to sites of cell-cell adhesion in cells expressing ARVCF( $\Delta$ P), the latter remained cytosolic (j–i).

ZO-1 released from the plasma membrane was excluded from the nucleus after calcium depletion, whereas ZO-2 efficiently accumulated in the nucleus (m). Redistribution of ZO-2 from the nucleus back to sites of cell-cell contact lagged behind that of ZO-1 or ARVCF (n–p) and required a longer recovery (our unpublished data; Islas *et al.*, 2002).

Thus, disruption of cell-cell adhesion results in the release of ARVCF from the plasma membrane and increased appearance in the nucleus. Furthermore, nuclear localization of ARVCF apparently requires the interaction with a PDZ-domain protein.

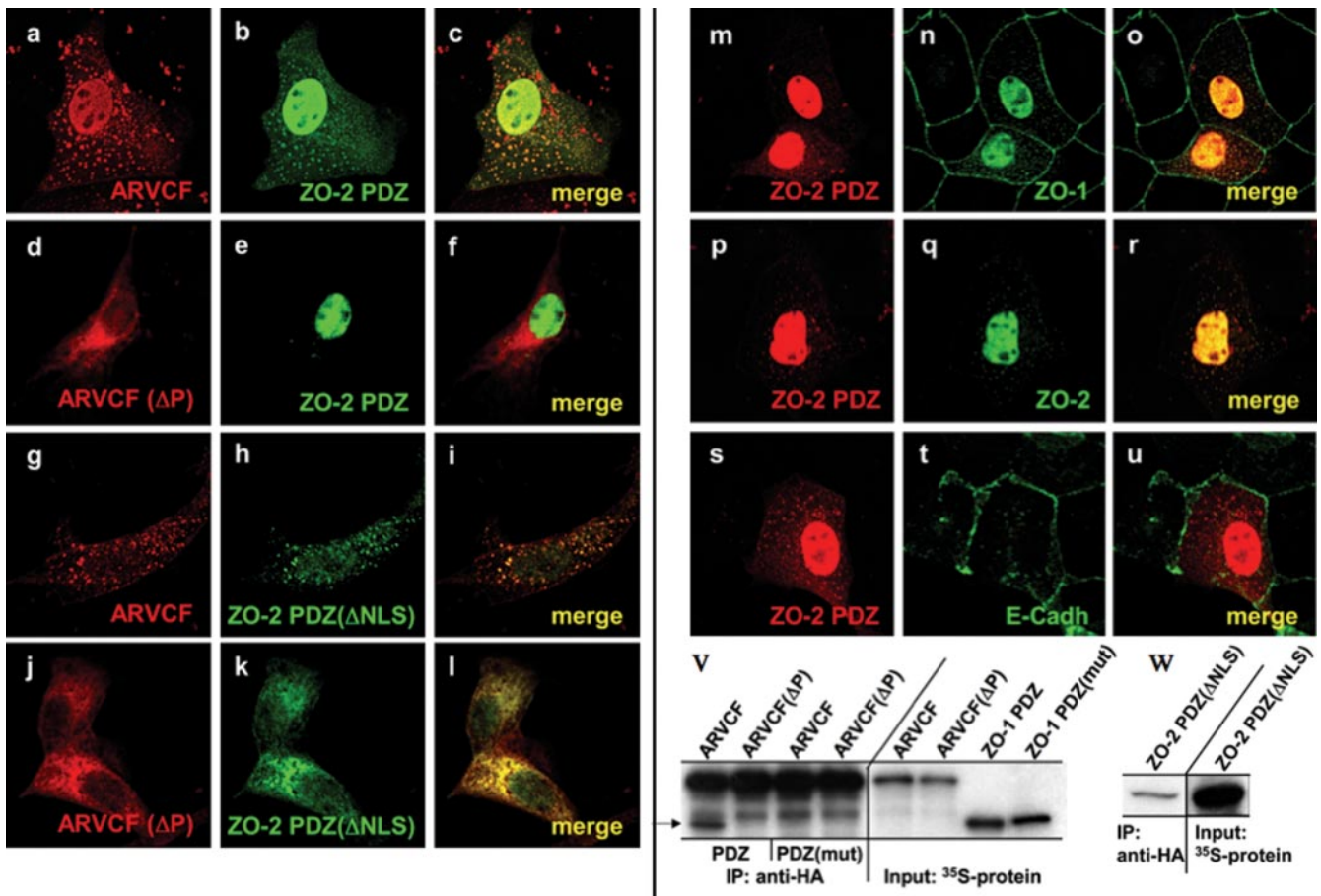
#### **ARVCF, ZO-1, and E-Cadherin Are Recruited to Sites of Initial Cell-Cell Contact**

Given the presence of ARVCF at sites of cell-cell contact after recovery from calcium depletion, we further characterized the recruitment of ARVCF, ZO-1, and E-cadherin to sites of initial cell-cell adhesion in sparse cultures of transiently transfected MCF7 cells.

As shown in Figure 7B and similar to MDCK cells, ARVCF and E-cadherin (a–c), ZO-1 and E-cadherin (g and h), as well as ARVCF and ZO-1 extensively colocalized to sites of cell-cell contact. In cells expressing ARVCF( $\Delta$ P), which remained cytoplasmic, E-cadherin (d–f) and ZO-1 (m–o) were still recruited to contact sites.

Because plasma membrane localization of ARVCF required an intact PDZ-binding motif and thus the ability to interact with ZO-1 (or other PDZ-domain proteins), we analyzed whether overexpressing ZO-1 mutants affects the recruitment of ARVCF to regions of cell-cell adhesion. Overexpressing an N-terminal portion of ZO-1 encoding the three PDZ domains (Figure 1B, ZO-1 PDZ) interfered with the recruitment of ARVCF to sites of cell-cell contact (Figure 7C, a–c). As observed in MDCKI cells (Reichert *et al.*, 2000b), the ZO1 PDZ domains remained mostly cytoplasmic and presumably prevented recruitment of ARVCF to the plasma membrane by competing for its interaction with wild-type ZO-1 and/or other PDZ-domain proteins. In contrast, overexpression of a ZO-1 PDZ construct in which the ability of the PDZ domains to interact with ligand had been obliterated [Figure 1B, ZO-1 PDZ(mut)], did not interfere with plasma membrane recruitment of ARVCF (g–i). As expected, neither ZO-1 PDZ nor ZO-1 PDZ(mut) altered the cytoplasmic distribution of ARVCF( $\Delta$ P) (d–f and j–l) nor was membrane localization of ARVCF affected by overexpressing wild-type ZO-1 (m–o). Coimmunoprecipitation experiments confirmed that in vitro-translated [<sup>35</sup>S]methionine-labeled ARVCF and ZO-1 PDZ domains associated with each other and that the binding was abolished by mutation of either the PDZ-binding motif in ARVCF (ARVCF( $\Delta$ P) or the PDZ domains in ZO-1 PDZ [ZO-1 PDZ(mut)] (Figure 8, v).

Thus, E-cadherin, ZO-1, and ARVCF are recruited to sites of initial cell-cell contact. Binding of the ZO-1 PDZ domains per se does not facilitate membrane recruitment of ARVCF,



**Figure 8.** Role of ZO-2 in nuclear localization of ARVCF. MDCK cells expressing ARVCF (a–c and g–i) or ARVCF(ΔP) (d–f and j–l) were transfected with cDNAs encoding the ZO-2 PDZ domains (a–f) or ZO-2 PDZ domains with an inactivated NLS [ZO-2 PDZ(ΔNLS), g–l] and analyzed by immunofluorescence staining and confocal microscopy. Colocalization of the respective proteins is yellow in the merged images. In m–u, the distribution of endogenous ZO-1, ZO-2, and E-cadherin was analyzed in MDCK cells transfected with a plasmid encoding the ZO-2 PDZ domains. ZO-2 constructs carried an N-terminal Xpress tag. Colocalization of the respective proteins is yellow in the merged images. v, mutation of the PDZ domains in ZO-1 PDZ abolishes the interaction with ARVCF. ARVCF or ARVCF(ΔP) and ZO-1 PDZ or ZO-1 PDZ(mut) were in vitro cotranslated in the presence of [<sup>35</sup>S]methionine. ARVCF or ARVCF(ΔP) was then immunoprecipitated, the precipitates fractionated by SDS-PAGE, and the dried gels used for autoradiography. w, deletion of the NLS in ZO-2 PDZ(ΔNLS) does not affect the interaction with ARVCF. ARVCF and ZO-2 PDZ(ΔNLS) were in vitro cotranslated in the presence of [<sup>35</sup>S]methionine, ARVCF was then immunoprecipitated, the precipitates fractionated by SDS-PAGE, and the dried gels used for autoradiography. An aliquot of the in vitro translation reaction was directly analyzed to monitor the amount of protein added to the reaction. The arrow shows the location of ZO-1 PDZ (v).

indicating a requirement for the intact ZO-1 and possibly its association with membrane proteins and/or the cytoskeleton for this process.

#### The PDZ Domains of ZO-2 Mediate Efficient Nuclear Localization of ARVCF

Given the interaction between the ZO-2 PDZ-domains and ARVCF (Table 1) and the nuclear localization of the two proteins after disruption of cell-cell adhesion (Figure 7A), we explored the possible role for ZO-2 in nuclear localization of ARVCF. ZO-2 has several nuclear localization signals (NLSs) within its N-terminal part (Jaramillo *et al.*, 2004), and we therefore analyzed the subcellular distribution of ARVCF in MDCK cells cotransfected with cDNAs for ARVCF and ZO-2 PDZ (Figure 1B) or a mutant with impaired nuclear localization capacity [ZO-2 PDZ(ΔNLS); Figure 1B].

Overexpression of ZO-2 PDZ displaced ARVCF from the plasma membrane into punctate cytoplasmic structures and

strongly enhanced nuclear accumulation of ARVCF (Figure 8, a–c). ARVCF and ZO-2 PDZ colocalized not only in the nucleus but also in the cytoplasmic structures. The ZO-2 PDZ induced displacement of ARVCF from the plasma membrane as well as the colocalization support the interaction between the two proteins. ARVCF(ΔP) was excluded from the nucleus (d and e), showing that nuclear localization of ARVCF required its ability to interact with ZO-2 PDZ.

ZO-2 PDZ(ΔNLS) was still able to relocalize ARVCF from the plasma membrane to the cytoplasmic structures (g–l), indicating that deletion of the NLS did not affect the interaction with ARVCF (see below). ARVCF, however, was effectively excluded from the nucleus in cells expressing ZO-2 PDZ(ΔNLS), directly implicating ZO-2 PDZ in nuclear translocation and/or retention of ARVCF.

Interestingly, the ZO-2 PDZ domains also partially relocalized endogenous ZO-1 from sites of cell-cell contact to the nucleus (m–o), likely reflecting association between ZO-1 and ZO-2 through PDZ domain interactions (Wittchen *et al.*, 1999). Consistent with previous observations (Jaramillo *et*

*al.*, 2004), endogenous ZO-2 accumulated in the nucleus of sparse cell cultures (p-r). The plasma membrane localization of E-cadherin, in contrast, was not altered by the expression of ZO-2 PDZ (s-u). As expected from the functional effect of ZO-2 PDZ( $\Delta$ NLS) on ARVCF localization and its codistribution with ARVCF, coimmunoprecipitation experiments using *in vitro*-translated [<sup>35</sup>S]methionine-labeled ARVCF and ZO-2 PDZ( $\Delta$ NLS) confirmed the interaction between the two proteins (Figure 8, w).

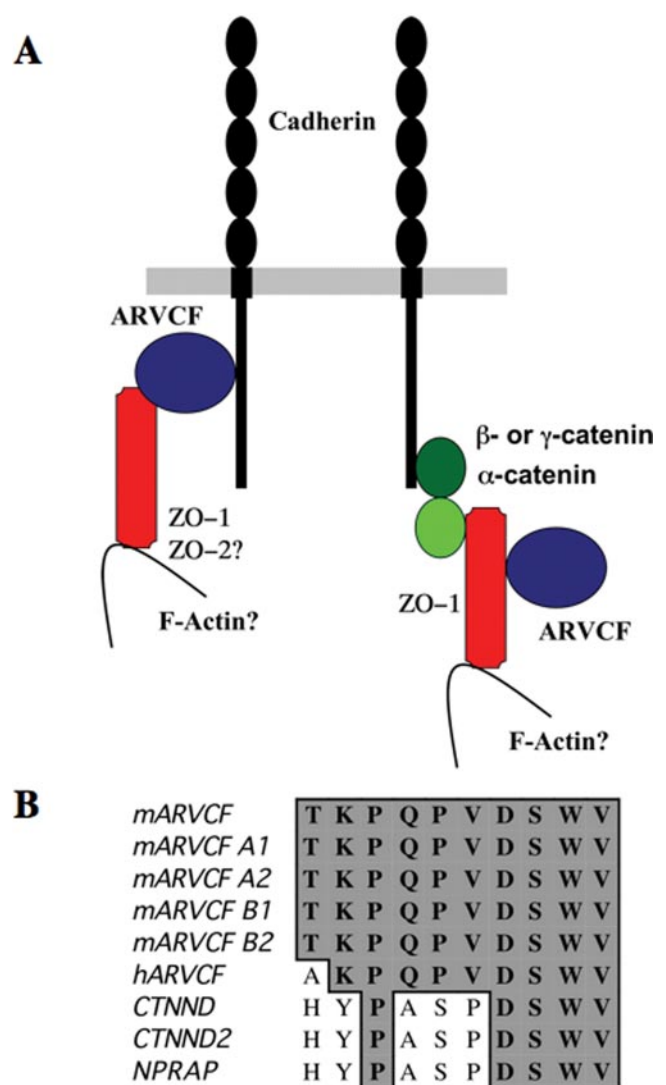
In conclusion, the ZO-2 PDZ domains mediate nuclear accumulation of ARVCF in a process that requires both a PDZ-based interaction and the NLS in ZO-2, consistent with a role for ZO-2 in nuclear translocation and/or retention of ARVCF.

## DISCUSSION

We identified ARVCF as a binding partner of ZO-1 and ZO-2 and characterized the role of PDZ-domain proteins in plasma membrane and nuclear localization of ARVCF. Based on several assays, including yeast two-hybrid, pull-down, and ligand overlay, ARVCF was shown to bind directly to ZO-1 through the interaction of a C-terminal type 1 PDZ-binding motif (SWV) in ARVCF and PDZ domains in ZO-1. ARVCF also interacts with PDZ domains of ZO-2, but not ZO-3, confirming the notion that different ZO proteins recruit distinct partners to TJs.

Coprecipitation and colocalization experiments showed that ARVCF and ZO-1 also interact *in vivo*. Interestingly, the two proteins only colocalized in the apical most region of the lateral plasma membrane of fully polarized MDCK cells, thus defining two distinct pools of plasma membrane-associated ARVCF, one in the region of the apical junctional complex, the second along the length of the lateral domain. Because inactivation of the PDZ-binding motif led to the loss of the bulk of ARVCF from the plasma membrane, PDZ-domain proteins other than ZO-1 such as Erbin (Laura *et al.*, 2002) are likely to be involved in localizing ARVCF to the lateral plasma membrane. Cell type-specific expression of different PDZ-domain proteins promoting the interaction of ARVCF with cadherins or other membrane-associated proteins may thus explain why colocalization of ARVCF and cadherins is often dependent on the cellular context (Waibler *et al.*, 2001). Interestingly, overexpression of ARVCF, but not ARVCF( $\Delta$ P), resulted in a more lateral distribution of ZO-1, consistent with the two proteins interacting. Because endogenous ARVCF levels are too low to be detected in MDCK cells (see also Mariner *et al.*, 1999), it is not clear whether the endogenous protein preferentially localizes to the more apical or basal region, or whether it is distributed along the length of the lateral plasma membrane. In any case, because the bulk of the overexpressed ARVCF localized to the plasma membrane, the mechanism(s) that recruit ARVCF were not saturated in the transfected MDCK cells.

ARVCF can directly interact through its armadillo repeats with cadherins (Kaufmann *et al.*, 2000; Waibler *et al.*, 2001), and it has been proposed that membrane localization of ARVCF is mediated through this association. It was therefore surprising that inactivation of the PDZ-binding motif resulted in the loss of ARVCF from the plasma membrane, in particular because cadherins do not contain PDZ domains. We confirmed that *in vitro*, the armadillo repeats are indeed required for binding ARVCF to the cytosolic tail of E-cadherin. Under these conditions, inactivation of the PDZ-binding motif does not affect the association of the two proteins. *In vivo*, however, ARVCF lacking the armadillo domains was still present at the plasma membrane, albeit at



**Figure 9.** Putative E-cadherin/ARVCF/ZO-1 associations and C-terminal PDZ binding motifs in members of the p120<sup>ctn</sup> protein family. (A) Schematic model of possible E-cadherin complexes. Cadherins may associate directly with the armadillo repeats of ARVCF, which in turn may bind to the ZO-1 PDZ domains via its PDZ-binding motif. Alternatively, cadherins may bind ARVCF indirectly via their known interaction with  $\beta$ - or  $\gamma$ -catenin,  $\alpha$ -catenin, and ZO-1. ZO-2 also may associate with ARVCF. (B) Alignment of the C terminus of different members of the p120<sup>ctn</sup> protein family. The C-terminal 10 amino acids of ARVCF splice variants and the different members of the p120<sup>ctn</sup> family are aligned. Identical amino acids are boxed and shaded. With the exception of p120<sup>ctn</sup> itself, the C-terminal PDZ-binding SWV motif identified in ARVCF is conserved, indicating that other members of the p120<sup>ctn</sup> family also may bind to ZO-1, ZO-2, and possibly other PDZ-domain proteins.

reduced levels. A fraction of the mutant lacking the armadillo repeats also coprecipitated with E-cadherin and only the inactivation of the PDZ-binding motif in ARVCF( $\Delta$ A) led to the complete loss of membrane localization and E-cadherin coprecipitation. This suggests that ARVCF can bind to E-cadherin via different mechanisms (Figure 9A). On one hand, ARVCF may associate with cadherins via the armadillo repeats and with ZO-1 via the PDZ-binding motif, thereby linking ZO-1 to E-cadherin. Indeed, a complex between ZO-1 PDZ domains, E-cadherin and ARVCF could

form in vitro and overexpression of ARVCF increased the amount of ZO-1 that coprecipitated with E-cadherin. Such a complex also may explain the redistribution of ZO-1 from TJs to a more lateral location. Alternatively, based on the known association of ZO-1 with the cadherin complex via  $\alpha$ -catenin (Itoh *et al.*, 1993, 1997; Rajasekaran *et al.*, 1996; Ando-Akatsuka *et al.*, 1999), ZO-1 could link ARVCF indirectly to E-cadherin. Such an interaction could explain the displacement of ARVCF from the plasma membrane after overexpression of the ZO-1 (or ZO-2) PDZ domains. In addition, it also is possible that a fraction of ARVCF is recruited to tight junctions by directly binding to ZO-1 or ZO-2.

Plasma membrane localization of ARVCF not only required a functional PDZ-binding motif but also cell-cell adhesion. Dissociation of cell-cell adhesion or sparse culture conditions led to the release of ARVCF from the plasma membrane into the cytoplasm and the nucleus. Although a presence of ARVCF in the nucleus has been reported (Mariner *et al.*, 2000; Waibler *et al.*, 2001), a cell-cell adhesion-dependent shuttling between plasma membrane and nuclear localizations has not been documented. Even without affecting cell-cell adhesion, nuclear localization of ARVCF( $\Delta$ ) was increased, correlating with the less efficient membrane localization of ARVCF after deletion of the armadillo repeats. Under any condition, mutation of the PDZ-binding motif resulted in the effective exclusion of ARVCF from the nucleus, showing that nuclear translocation and/or retention requires a functional PDZ-binding motif and hence the interaction with a PDZ-domain protein.

ZO-1 remained cytoplasmic in sparse cell cultures or after disruption of cell-cell adhesion, suggesting that it does not participate in nuclear localization of ARVCF. ZO-2, in contrast, relocated to the nucleus with ARVCF, and, given the interaction between the ZO-2 PDZ domains and ARVCF, raised the possibility that ZO-2 may play a role in nuclear localization of ARVCF. Such a role for ZO-2 is indeed supported by the ability of the ZO-2 PDZ domain to efficiently relocate ARVCF from the plasma membrane to the nucleus in a process that required the ability of the two proteins to interact and the presence of a functional NLS in the ZO-2 PDZ domains. Thus, ZO-2 could be involved in nuclear translocation and/or retention of ARVCF and play a role in regulating postulated functions of ARVCF in gene expression. Interestingly, in addition to the nuclear relocalization, the PDZ domains of ZO-2 induced cytosolic patches that contained ARVCF. Although the nature of these patches remains to be elucidated, they did not contain E-cadherin nor did they colocalize with a lysosomal marker (our unpublished data).

Little is known about the role of ARVCF in cell-cell adhesion. Members of the p120<sup>ctn</sup> family bind laterally to cadherins and have been postulated to regulate, positively or negatively, the lateral clustering of adhesion complexes (Ozawa and Kemler, 1998) and thus adhesive strength (Ozawa and Kemler, 1998; Yap *et al.*, 1998). More recently, several p120<sup>ctn</sup> family members, including ARVCF, have been implicated in cadherin turnover (Davis *et al.*, 2003). Despite a common role in cadherin turnover, ARVCF lacks the cellular branching activity associated with the expression of p120<sup>ctn</sup> in fibroblasts (Reynolds *et al.*, 1996; Mariner *et al.*, 2000), or the induction of a fibroblastoid phenotype observed for  $\delta$ -catenin in MDCK cells (Lu *et al.*, 1999). Consistent with these studies, we did not observe morphological changes by overexpressing wild-type or mutant ARVCF in several cell lines, including 3T3 adipocytes, where *Xenopus laevis* ARVCF has been reported to induce branching (Fang

*et al.*, 2004). This apparently contradictory result may reflect the fact that *Xenopus* ARVCF is structurally more closely related to mammalian p120<sup>ctn</sup> than to mammalian ARVCF. Although E-cadherin, ZO-1, and ARVCF may play a role in initial stages of cell-cell adhesion, assembly of junctional components was not visibly altered by the overexpression of wild-type or mutant ARVCF. Also other functional parameters, such as transepithelial resistance, cyst formation in Matrigel, and wound healing were not significantly altered, possibly reflecting functional redundancy between ARVCF and the more abundant p120<sup>ctn</sup> (Davis *et al.*, 2003; Fang *et al.*, 2004). Because ZO-1 links to the actin cytoskeleton (Fanning *et al.*, 1998, 2002; Wittchen *et al.*, 1999), one function of the interaction of ARVCF with ZO-1 may be to anchor cadherins, possibly in the vicinity of the apical adhesion complex, to F-actin.

In conclusion, although the precise cellular functions of ARVCF remain to be elucidated, its recruitment to either the plasma membrane or the nucleus, and hence postulated functions in adhesion and transcription, respectively, seem to be tightly regulated by interactions with PDZ-domain proteins. The C-terminal PDZ-binding motif in ARVCF is conserved among the different alternative splice variants and, with the exception of p120<sup>ctn</sup> itself, is also found in the other p120<sup>ctn</sup> family members (Figure 9B), suggesting that they may also bind ZO-1, ZO-2, or other PDZ-domain proteins. Whether these interactions regulate the subcellular localization of other members of the p120<sup>ctn</sup> family remains to be determined.

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## REFERENCES

- Adams, C.L., Chen, Y.T., Smith, S.J., and Nelson, W.J. (1998). Mechanisms of epithelial cell-cell adhesion and cell compaction revealed by high-resolution tracking of E-cadherin-green fluorescent protein. *J. Cell Biol.* 142, 1105–1119.
- Ando-Akatsuka, Y., Yonemura, S., Itoh, M., Furuse, M., and Tsukita, S. (1999). Differential behavior of E-cadherin and occludin in their colocalization with ZO-1 during the establishment of epithelial cell polarity. *J. Cell. Physiol.* 179, 115–125.
- Angst, B.D., Marcozzi, C., and Magee, A.I. (2001). The cadherin superfamily: diversity in form and function. *J. Cell Sci.* 114, 629–641.
- Barker, R.J., Price, R.L., and Gourdie, R.G. (2002). Increased association of ZO-1 with connexin43 during remodeling of cardiac gap junctions. *Circ. Res.* 90, 317–324.
- Borrmann, C.M., Mertens, C., Schmidt, A., Langbein, L., Kuhn, C., and Franke, W.W. (2000). Molecular diversity of plaques of epithelial-adhering junctions. *Ann. N.Y. Acad. Sci.* 915, 144–150.
- Cerejido, M., Shoshani, L., and Contreras, R.G. (2000). Molecular physiology and pathophysiology of tight junctions. I. Biogenesis of tight junctions and epithelial polarity. *Am. J. Physiol.* 279, G477–G482.
- Davis, M.A., Ireton, R.C., and Reynolds, A.B. (2003). A core function for p-120-catenin in cadherin turnover. *J. Cell Biol.* 163, 525–534.
- Doyle, D.A., Lee, A., Lewis, J., Kim, E., Sheng, M., and MacKinnon, R. (1996). Crystal structures of a complexed and peptide-free membrane protein-binding domain: molecular basis of peptide recognition by PDZ. *Cell* 85, 1067–1076.
- Fang, X., Ji, H., Kim, S.W., Park, J.I., Vaught, T.G., Anastasiadis, P.Z., Ciesiolka, M., and McCrea, P.D. (2004). Vertebrate development requires ARVCF and p120 catenins and their interplay with RhoA and Rac. *J. Cell Biol.* 165, 87–98.

- Fanning, A.S., Jameson, B.J., Jesaitis, L.A., and Anderson, J.M. (1998). The tight junction protein ZO-1 establishes a link between the transmembrane protein occludin and the actin cytoskeleton. *J. Biol. Chem.* *273*, 29745–29753.
- Fanning, A.S., Ma, T.Y., and Anderson, J.M. (2002). Isolation and functional characterization of the actin binding region in the tight junction protein ZO-1. *FASEB J.* *16*, 1835–1837.
- Gonzalez-Mariscal, L., Betanzos, A., and Avila-Flores, A. (2000). MAGUK proteins: structure and role in the tight junction. *Semin. Cell Dev. Biol.* *11*, 315–324.
- Honing, S., and Hunziker, W. (1995). Cytoplasmic determinants involved in direct lysosomal sorting, endocytosis, and basolateral targeting of rat Igp120 (lamp-I) in MDCK cells. *J. Cell Biol.* *128*, 321–332.
- Islas, S., Vega, J., Ponce, L., and Gonzalez-Mariscal, L. (2002). Nuclear localization of the tight junction protein ZO-2 in epithelial cells. *Exp. Cell Res.* *274*, 138–148.
- Itoh, M., Nagafuchi, A., Moroi, S., and Tsukita, S. (1997). Involvement of ZO-1 in cadherin-based cell adhesion through its direct binding to alpha catenin and actin filaments. *J. Cell Biol.* *138*, 181–192.
- Itoh, M., Nagafuchi, A., Yonemura, S., Kitani-Yasuda, T., and Tsukita, S. (1993). The 220-kD protein colocalizing with cadherins in non-epithelial cells is identical to ZO-1, a tight junction-associated protein in epithelial cells: cDNA cloning and immunoelectron microscopy. *J. Cell Biol.* *121*, 491–502.
- Itoh, M., Yonemura, S., Nagafuchi, A., and Tsukita, S. (1991). A 220-kD undercoat-constitutive protein: its specific localization at cadherin-based cell-cell adhesion sites. *J. Cell Biol.* *115*, 1449–1462.
- Ivanov, A.I., Nusrat, A., and Parkos, C.A. (2004). Endocytosis of epithelial apical junctional proteins by a clathrin-mediated pathway into a unique storage compartment. *Mol. Biol. Cell* *15*, 176–188.
- Jaramillo, B.E., Ponce, A., Moreno, J., Betanzos, A., Huerta, M., Lopez-Bayghen, E., and Gonzalez-Mariscal, L. (2004). Characterization of the tight junction protein ZO-2 localized at the nucleus of epithelial cells. *Exp. Cell Res.* *297*, 247–258.
- Kaufmann, U., Zuppinger, C., Waibler, Z., Rudiger, M., Urbich, C., Martin, B., Jockusch, B.M., Eppenberger, H., and Starzinski-Powitz, A. (2000). The armadillo repeat region targets ARVCF to cadherin-based cellular junctions. *J. Cell Sci.* *113*, 4121–4135.
- Kausalya, P.J., Reichert, M., and Hunziker, W. (2001). Connexin45 directly binds to ZO-1 and localizes to the tight junction region in epithelial MDCK cells. *FEBS Lett.* *505*, 92–96.
- Laura, R.P., Witt, A.S., Held, H.A., Gerstner, R., Deshayes, K., Koehler, M.F., Kosik, K.S., Sidhu, S.S., and Lasky, L.A. (2002). The Erbin PDZ domain binds with high affinity and specificity to the carboxyl termini of delta-catenin and ARVCF. *J. Biol. Chem.* *277*, 12906–12914.
- Lawrence, D.W., Comerford, K.M., and Colgan, S.P. (2002). Role of VASP in reestablishment of epithelial tight junction assembly after Ca<sup>2+</sup> switch. *Am. J. Physiol.* *282*, C1235–C1245.
- Lu, Q., Paredes, M., Medina, M., Zhou, J., Cavallo, R., Peifer, M., Orecchio, L., and Kosik, K.S. (1999). delta-catenin, an adhesive junction-associated protein which promotes cell scattering. *J. Cell Biol.* *144*, 519–532.
- Mariner, D.J., Sirotkin, H., Daniel, J.M., Lindman, B.R., Mernaugh, R.L., Patten, A.K., Thoreson, M.A., and Reynolds, A.B. (1999). Production and characterization of monoclonal antibodies to ARVCF. *Hybridoma* *18*, 343–349.
- Mariner, D.J., Wang, J., and Reynolds, A.B. (2000). ARVCF localizes to the nucleus and adherens junction and is mutually exclusive with p120(ctn) in E-cadherin complexes. *J. Cell Sci.* *113*, 1481–1490.
- Marrs, J.A., and Nelson, W.J. (1996). Cadherin cell adhesion molecules in differentiation and embryogenesis. *Int. Rev. Cytol.* *165*, 159–205.
- Mathur, M., Goodwin, L., and Cowin, P. (1994). Interactions of the cytoplasmic domain of the desmosomal cadherin Dsg1 with plakoglobin. *J. Biol. Chem.* *269*, 14075–14080.
- Mauro, L., Bartucci, M., Morelli, C., Ando, S., and Surmacz, E. (2001). IGF-I receptor-induced cell-cell adhesion of MCF-7 breast cancer cells requires the expression of junction protein ZO-1. *J. Biol. Chem.* *276*, 39892–39897.
- Müller, T., Choidas, A., Reichmann, E., and Ullrich, A. (1999). Phosphorylation and free pool of beta-catenin are regulated by tyrosine kinases and tyrosine phosphatases during epithelial cell migration. *J. Biol. Chem.* *274*, 10173–10183.
- Nagafuchi, A. (2001). Molecular architecture of adherens junctions. *Curr. Opin. Cell Biol.* *13*, 600–603.
- Nieset, J.E., Redfield, A.R., Jin, F., Knudsen, K.A., Johnson, K.R., and Wheelock, M.J. (1997). Characterization of the interactions of alpha-catenin with alpha-actinin and beta-catenin/plakoglobin. *J. Cell Sci.* *110*, 1013–1022.
- Ozawa, M., and Kemler, R. (1998). The membrane-proximal region of the E-cadherin cytoplasmic domain prevents dimerization and negatively regulates adhesion activity. *J. Cell Biol.* *142*, 1605–1613.
- Paffenholz, R., and Franke, W.W. (1997). Identification and localization of a neurally expressed member of the plakoglobin/armadillo multigene family. *Differentiation* *61*, 293–304.
- Peifer, M., Berg, S., and Reynolds, A.B. (1994). A repeating amino acid motif shared by proteins with diverse cellular roles. *Cell* *76*, 789–791.
- Rajasekaran, A.K., Hojo, M., Huima, T., and Rodriguez-Boulan, E. (1996). Catenins and zonula occludens-1 form a complex during early stages in the assembly of tight junctions. *J. Cell Biol.* *132*, 451–463.
- Reichert, M., Muller, T., and Hunziker, W. (2000a). The PDZ domains of zonula occludens-1 induce an epithelial to mesenchymal transition of Madin-Darby canine kidney I cells - Evidence for a role of beta-catenin/Tcf/Lef signaling. *J. Biol. Chem.* *275*, 9492–9500.
- Reichert, M., Muller, T., and Hunziker, W. (2000b). The PDZ domains of zonula occludens-1 induce an epithelial to mesenchymal transition of Madin-Darby canine kidney I cells. Evidence for a role of beta-catenin/Tcf/Lef signaling. *J. Biol. Chem.* *275*, 9492–9500.
- Reynolds, A.B., Daniel, J.M., Mo, Y.Y., Wu, J., and Zhang, Z. (1996). The novel catenin p120cas binds classical cadherins and induces an unusual morphological phenotype in NIH3T3 fibroblasts. *Exp. Cell Res.* *225*, 328–337.
- Reynolds, A.B., Herbert, L., Cleveland, J.L., Berg, S.T., and Gaut, J.R. (1992). p120, a novel substrate of protein tyrosine kinase receptors and of p60v-src, is related to cadherin-binding factors beta-catenin, plakoglobin and armadillo. *Oncogene* *7*, 2439–2445.
- Rimm, D.L., Koslov, E.R., Kebriaei, P., Cianci, C.D., and Morrow, J.S. (1995). Alpha 1(E)-catenin is an actin-binding and -bundling protein mediating the attachment of F-actin to the membrane adhesion complex. *Proc. Natl. Acad. Sci. USA* *92*, 8813–8817.
- Sheng, M., and Sala, C. (2001). PDZ domains and the organization of supramolecular complexes. *Annu. Rev. Neurosci.* *24*, 1–29.
- Sirotkin, H., *et al.* (1997). Identification of a new human catenin gene family member (ARVCF) from the region deleted in velo-cardio-facial syndrome. *Genomics* *41*, 75–83.
- Staddon, J.M., Smales, C., Schulze, C., Esch, F.S., and Rubin, L.L. (1995). p120, a p120-related protein (p100), and the cadherin/catenin complex. *J. Cell Biol.* *130*, 369–381.
- Stevenson, B.R., and Keon, B.H. (1998). The tight junction: morphology to molecules. *Annu. Rev. Cell Dev. Biol.* *14*, 89–109.
- Toyofuku, T., Yabuki, M., Otsu, K., Kuzuya, T., Hori, M., and Tada, M. (1998). Direct association of the gap junction protein connexin-43 with ZO-1 in cardiac myocytes. *J. Biol. Chem.* *273*, 12725–12731.
- Vasioukhin, V., Bauer, C., Yin, M., and Fuchs, E. (2000). Directed actin polymerization is the driving force for epithelial cell-cell adhesion. *Cell* *100*, 209–219.
- Waibler, Z., Schafer, A., and Starzinski-Powitz, A. (2001). mARVCF cellular localisation and binding to cadherins is influenced by the cellular context but not by alternative splicing. *J. Cell Sci.* *114*, 3873–3884.
- Weiss, E.E., Kroemker, M., Rudiger, A.H., Jockusch, B.M., and Rudiger, M. (1998). Vinculin is part of the cadherin-catenin junctional complex: complex formation between alpha-catenin and vinculin. *J. Cell Biol.* *141*, 755–764.
- Wittchen, E.S., Haskins, J., and Stevenson, B.R. (1999). Protein interactions at the tight junction. Actin has multiple binding partners, and ZO-1 forms independent complexes with ZO-2 and ZO-3. *J. Biol. Chem.* *274*, 35179–35185.
- Yap, A.S., Niessen, C.M., and Gumbiner, B.M. (1998). The juxtamembrane region of the cadherin cytoplasmic tail supports lateral clustering, adhesive strengthening, and interaction with p120ctn. *J. Cell Biol.* *141*, 779–789.
- Yonemura, S., Itoh, M., Nagafuchi, A., and Tsukita, S. (1995). Cell-to-cell adherens junction formation and actin filament organization: similarities and differences between non-polarized fibroblasts and polarized epithelial cells. *J. Cell Sci.* *108*, 127–142.
- Zahraoui, A., Louvard, D., and Galli, T. (2000). Tight junction, a platform for trafficking and signaling protein complexes. *J. Cell Biol.* *151*, F31–36.
- Zhou, J., Liyanage, U., Medina, M., Ho, C., Simmons, A.D., Lovett, M., and Kosik, K.S. (1997). Presenilin 1 interaction in the brain with a novel member of the Armadillo family. *Neuroreport* *8*, 2085–2090.