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Identification of adherens junction-associated GTPase activating proteins by the fluorescence localization-based expression cloning.

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This paper is dedicated to the memory of the late Shoichiro Tsukita.

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Abstract

The junctional complex, including tight junctions (TJs), adherens junctions (AJs), and desmosomes, plays crucial roles in the structure and functions of epithelial cellular sheets. In this study, we evaluated the fluorescence localization-based expression cloning (FL-REX) method as an approach to identify novel molecular components of TJs and AJs. Using an expression library of cDNA-GFP-fusions derived from mRNA of a mouse epithelial cell line, we confirmed that cDNAs for various known TJ- and AJ-components could be cloned in the FL-REX. Furthermore, cDNAs for ARHGAP12 and SPAL3, two putative GTPase activating proteins (GAPs) for small G proteins, were cloned as novel components of the junctional complex. Immunofluorescence staining using antibodies generated in-house demonstrated that these GAPs were localized at epithelial cell-cell junctions in various mouse tissues, and were specific to AJs when observed under confocal laser-scanning microscopy. These data suggest that FL-REX is a powerful tool to identify novel proteins localized at TJs and AJs.

Keywords

Junctional complex; Adherens junctions; Tight junctions; FL-REX; GFP; GAP; ARHGAP12; SPAL3; Rap1

Introduction

The junctional complex, which consists of tight junctions (TJs), adherens junctions (AJs), and desmosomes (DSs), is a hallmark of epithelial cell types in vertebrates [1]. The junctional complex plays crucial roles in mechanical adhesion between epithelial cells to form cellular sheets (AJs and DSs), as well as regulation of paracellular transport to maintain ionic homeostasis between different body compartments (TJs) [2,3,4,31]. Previous studies have identified a number of molecular constituents for each junction type in the junctional complex, enabling molecular characterization of the structure and function of each junction. One of the common features of these cell-cell junctions is the connection of adhesion molecules in the plasma membrane with cytoskeletons via cytoplasmic plaque proteins [2,3,4,31]. In addition to these structural proteins, a number of signaling molecules localized at the cytoplasmic region of the junctional complex are thought to be involved in the functional regulation of each cell-cell junction, cell growth, and morphogenesis of epithelial polarity [2,3,4,31].

An optimal approach to identify molecular constituents of each cell-cell junction is the purification of these structures followed by protein analyses of their components. Among the elements of the junctional complex, the purification of DSs from calf muzzles has been demonstrated, and various DS-associated proteins, including desmogleins and desmoplakins, have been identified from this DS fraction in one-dimensional SDS-PAGE

[5,6]. In contrast, the purification of TJs and AJs was less successful [7,8], although alternatively, the production of monoclonal antibodies is a promising technique to identify the molecular constituents of these structures [9,10]. For example, several TJ-associated proteins including ZO-1 and occludin were discovered by the localization-based screening of monoclonal antibodies generated against a TJ-enriched plasma membrane fraction isolated from rodent or avian tissues [9,10]. However, a limitation of this approach is that it depends on the antigenicity, which cannot be controlled. In addition to biochemical and immunological approaches, searches for binding partners of cell-cell junction-associated proteins by co-precipitation and yeast two-hybrid screening have also identified many novel cell-cell junction-related molecules. Nevertheless, there are likely to be yet unidentified proteins localized at the junctional complex. Identification and characterization of these novel molecular components is important for further understanding of the structure and functional regulation of each junction at the molecular level.

To complement these biochemical and immunological approaches, the localization-based expression cloning would be useful to identify cDNAs for novel components of the junctional complex. In this 'visual screening' method, green fluorescent protein (GFP)-fused cDNA or genomic library is initially introduced into the cells. The cells in which exogenous GFP-fusion proteins are localized at certain cellular

structures are then selected by fluorescence microscopy, and their cDNAs are cloned. This localization-based screening was first described in *Schizosaccharomyces pombe* [32], and then in mammalian cultured cells to identify a new type of nuclear envelope membrane protein [33]. Furthermore, Kitamura and colleagues developed the fluorescence localization-based expression cloning (FL-REX) by the use of a retrovirus vector, which allows controllable introduction of cDNA libraries into cells with high-efficiency. However, as of yet, reports on further applications of this method are limited [12].

In this study, we evaluated the FL-REX in obtaining cDNAs of novel molecular constituents localized at TJs and AJs in epithelial cells. We have confirmed that various cDNAs for known TJ- or AJ-associated proteins could be cloned in the FL-REX. Further, using this method we have identified two putative GTPase activating proteins (GAPs) for small G-proteins as novel components of the junctional complex. Using in-house generated polyclonal antibodies (pAbs) for these GAPs, they were found to be localized at AJs in various epithelial tissues. These data suggest that FL-REX is a powerful tool to identify novel molecular constituents of the junctional complex.

Materials and Methods

Cell culture and transfection

Epithelial cell lines, MDCK II, CSG120/7, and EpH4 were kindly provided by Dr. M. Murata (The University of Tokyo, Tokyo, Japan), Dr. W. Birchmeier (Max-Delbruck-Center for Molecular Medicine, Berlin, Germany), and Dr. E. Reichmann (University Children's Hospital Zurich, Zurich, Switzerland). A potent retrovirus packaging cell line, Plat-E [13], was kindly gifted by Dr. T. Kitamura (The University of Tokyo, Tokyo, Japan). All cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. To establish the MDCK II cells competent to mouse retrovirus infection, the ecotropic mouse retrovirus receptor (EcoVR) cDNA (provided by Dr. T. Kitamura) was subcloned into pCAGGS-neodelEcoRI [14] and transfected to MDCK II cells with LipofectAMINE with Plus reagent (Invitrogen, Carlsbad, California, USA). One G418-resistant clone, MDCKIIVR20, was used for the expression cloning.

Construction of the cDNA library for FL-REX

A mouse retrovirus vector, pMX, was kindly provided by Dr. T. Kitamura. To avoid the background fluorescence of native GFP from the vector without cDNA insert in the cDNA-GFP fusion library, we initially generated pMX-EGFPN-Met(-), a pMX-derived vector containing the EGFP sequence (CLONTCH) in which the ATG codon for the first

methionine was deleted, and the EcoRI site with the DNA sequence encoding four glycines were inserted instead. For the construction of the cDNA library, poly(A)+ RNA was isolated from CSG120/7 epithelial cells [15] using FastTrack (Invitrogen). The cDNA was synthesized from the poly(A)+ RNA with random primers using Time Saver^{TM} cDNA Synthesis Kit (Amersham, Mountain View, California, USA). The cDNA fragments longer than 1kb were size-fractionated from the agarose gel using GFX^{TM} PCR DNA and Gel Band Purification Kit (GE Healthcare, Little Chalfont, Buckinghamshire, England) and were inserted into the EcoRI site of pMX-EGFPN-Met(-), upstream of EGFP cDNA. The ligated DNA was electroporated into DH5α competent cells (Electromax DH5α; Invitrogen) by Gene Pulser (BioRad, Hercules, California, USA). Plasmid DNA was purified after 12 h culture in 200 ml of LB medium. The resulting library contained 4.9 x 10^6 independent clones with an average insert size of 1.3 kb. The cDNA library was then converted to the high titer retroviruses in Plat-E packaging cells as described previously [11].

Localization-based expression screening

MDCKIIVR20 cells were infected with the retrovirus library at ~20% infection efficiency as described previously [12]. After two days, infected cells were trypsinized, and the GFP-positive cells collected by fluorescence-activating cell sorting (FACS) and sparsely plated on glass-based dishes (IWAKI, Tokyo, Japan). 48-72 h after plating the

cells were scanned under an Olympus IX71 fluorescence microscope (Olympus, Tokyo, Japan), and cell colonies that contain cells with GFP-signals of cell-cell junctions were marked. At the same time the surrounding colonies were scraped with needles under a phase contrast microscope and removed by aspiration. After 4-5 days expansion, the positive colonies were picked up, trypsinized, and replated on the glass-based dishes. Cell clones showing the junctional staining were then selected under a fluorescent microscope, picked up in the same way, and expanded to prepare their genomic DNAs which were used as a template for PCR to recover the integrated cDNA with two primers (GGTGGACCATCCTCTAGACT and GTCGCCGTCCAGCTCGAC). By the use of cDNA databases, full-length cDNAs were cloned by RT-PCR from total RNA of EpH4 cells. In six experiments ~1.2 x 10^6 cells in total with GFP fluorescence were collected by FACS and subjected to the screening.

Antibodies

Bacterial expression constructs of the GST fusion proteins containing aa 72-226 of ARHGAP12 and aa 1040-1412 of SPAL3 were generated by subcloning the corresponding DNA fragments amplified by PCR into pGEX4T-1 (Amersham). Each fusion protein was then produced in Escherichia coli, purified with glutathione-Sepharose 4B beads (Amersham), and injected subcutaneously into rabbits to raise polyclonal antibodies (pAbs). The identical antigen regions of ARHGAP12 and

SPAL3 were also subcloned into pMAL-c2 (New England Laboratory, Wobum, Massachusetts, USA) to produce maltose-binding protein fusions. For affinity purification of the pAbs, bacterial lysates of maltose binding protein-ARHGAP12 and –SPAL3 fusion proteins were subjected to SDS-PAGE and blotted to nitrocellulose membranes. Membrane pieces blotted with the fusion proteins were incubated with the rabbit sera. After washing with PBS, the bound pAbs were collected by treatment of membranes with 0.2 M citrate buffer (pH 2.3). Anti-E-cadherin mAb (ECCD-2) was kindly provided from Dr. M. Takeichi (Riken Center for Developmental Biology, Kobe, Japan). Anti-nectin-2 mAb was kindly provided from Dr. Y. Takai (Osaka University, Osaka, Japan). Anti-occludin mAb MOC37 was generated and characterized as described previously [35]. Anti-ZO-1 mAb R26.4 developed by Dr. D. Goodenough was obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242.

Immunofluorescence microscopy

Specimens for immunofluorescence microscopy were prepared as described previously [10]. To analyze the distribution of proteins in cultured cells, cells grown on glass coverslips were fixed with 1% formaldehyde in PBS for 15min at room temperature, permeabilized with 0.2% triton X-100 in PBS for 15min, and then washed three times

with PBS. After blocking with 1% bovine serum albumin in PBS for 15min, samples were treated with primary antibodies and washed with PBS followed by treatment of secondary antibodies. For immmunlocalization of proteins in mouse tissues, frozen sections (~5 μm thick) of various mouse tissues embedded in O.C.T compound were cut in a cryostat, mounted on glass coverslips, air-dried, and fixed with 95% ethanol at –20°C for 30 min followed by 100% acetone at room temperature for 1min. After being washed with PBS, sections were blocked with 1% bovine serum albumin for 15 min, treated with primary antibodies, and washed with PBS followed by treatment with secondary antibodies. After being washed with PBS, samples were mounted in 30% MOWIOL (CALBIOCHEM, La Jolla, California, USA). Specimens were observed using an Olympus IX70 fluorescence microscope (Olympus), a Zeiss Axio-phot photomicroscope, or Zeiss LSM510 confocal laser-scanning microscope (Carl Zeiss, Jena, Germany).

Results

Cloning of known cDNAs for TJ- and AJ-associated proteins in the FL-REX

In the present study, to improve the efficiency of the FL-REX for the screening and identification of novel components localized at the epithelial junctional complex, we adopted several modifications to reported methods. First, we created a cDNA-GFP fusion library from the transcripts of CSG120/7 epithelial cells, a cell line derived from the mouse salivary gland. Secondly, to construct the retrovirus expression library for cDNA-GFP-fusions, the ATG codon of the first methionine of GFP in the vector was deleted in advance to avoid the background signal of native GFP from vectors without cDNAs, which are located upstream of GFP. This appeared to be effective as the ratio of cells with GFP signals in total cells infected with the retrovirus cDNA-GFP library was ~0.4%, compared with 4% in the study by Nishimura et al., who preserved the initiation ATG of GFP [12]; infection efficiency approximately 20% in both studies. Next, cells were grown on glass-based culture plates instead of plastic ones to increase the detection sensitivity of GFP signal under the visual screening with an inverted fluorescence microscope.

Since TJs and AJs circumscribe the cells at the most apical part of the lateral plasma membrane, cells showing concentration of GFP-fusions into the apical cell-cell junction could be easily identified in the FL-REX. From ~1.2 x 10^6 GFP-positive cells collected

by FACS in six experiments, we obtained a number of GFP-fusions with known TJ- and AJ-associated proteins (Table 1). The typical localization of these GFP-fusions in MDCK cells are shown in Fig. 1. The most abundant TJ proteins found by our screening were claudins, including claudin-2, -3, -4, and -7. cDNAs of claudins were obtained in every ~10^4 GFP-positive cells; this frequency was much higher than that of other junctional molecules (data not shown). cDNAs for other TJ-associated proteins CAR, ZO-1, and ZO-2 were also cloned in this screening. As cDNAs of known AJ-associated proteins, partial sequences of alpha-catenin, beta-catenin, plakoglobin, alpha-actinin-4, and shroom2 were obtained.

Among these GFP fusions, non-cell-cell junctional localizations were also observed. For example, CAR (aa. 1-287)-GFP fusion was often detected as patches at the basolateral membrane of MDCK cells (Fig. 1B). In addition, ZO-1 (aa. 1-309)-GFP fusion was localized at the nucleus and cytoplasm, and its concentration at tricellular contacts was remarkable (Fig. 1C). These localizations are likely to be artifacts as both proteins are known to be highly concentrated at tight junctions [7,34]. In contrast, strengthened localization of GFP-fusions into cell-cell junctions was also observed. α-catenin (aa. 634-875) showed a high concentration at the apical cell-cell junction (Fig. 1E), although endogenous α-catenin is distributed throughout the lateral plasma membrane including the apical cell-cell junction (data not shown).

Identification of ARHGAP12 and SPAL3, putative GTPase activating proteins for small G proteins, as components of epithelial cell-cell junctions

In the FL-REX screening based on the junctional localization, we have cloned GFP-fusions containing partial cDNAs for two putative GTPase activating proteins (GAPs) for small G-proteins, neither of which have been reported to be localized at epithelial cell-cell junctions. One GFP-fusion contained aa. 1-535 of ARHGAP12 (GenBank accession no. NM029277), a putative Rho GTPase activating protein, whose cloning in human has been reported already (Fig. 2A) [16]. ARHGAP12 contains Src homology 3 (SH3) domain, two conserved tryptophans (WW) domain, pleckstrin homology (PH) domain, and Rho GAP domains in this order (Fig. 2E), and this overall domain structure is shared by other potential Rho GAPs including ARHGAP9 and ARHGAP15 [17]. Another GFP-fusion contained aa. 1050-1439 of SPAL3 (GenBank accession no. NM001081028) (Fig. 2C), a putative Rap GTPase activating protein. SPAL3 is closely related to SPAL (SPA-1 like-protein), which was originally identified as a binding protein of PSD-95/SAP90, a scaffold protein of the post-synaptic density [18]. Similar to SPAL, SPAL3 contains Rap GAP, PDZ, and coiled-coil domains in this order (Fig. 2F). We then cloned full-length cDNAs of ARHGAP12 and SPAL3 in mice, and confirmed that their full-length constructs with GFP-tags were recruited to cell-cell contact sites when overexpressed in MDCK cells (Fig. 2B, D).

Since GFP-fusuion with aa. 1-535 of ARHGAP12 containing a SH3 domain and WW domains localize at cell-cell junctions, we attempted to identify which domain is required for this localization. We constructed ARHGAP12-GFP mutants in which the SH3 domain (aa.13-71) or two WW domains (aa.263-388) were deleted, and produced MDCK cells stably expressing these deletion mutants. In fluorescence microscopy, however, neither of these constructs concentrated into cell-cell junctions (Fig. S1).

Generation of antibodies for ARHGAP12 and SPAL3

To examine the tissue expression and the subcellular localization of ARHGAP12 and SPAL3, we raised rabbit polyclonal antibodies for these proteins. In Western blotting of the membrane on which cell lysates of GFP-tagged ARHGAP12-overexpressing HEK293 cells were loaded, anti-ARHGAP12 antibody recognized a band of ~120 kD, which is consistent with the mass of molecular weights of GFP (27 kD) and ARHGAP12 (92 kD) (Fig. 3A). Similarly, in the lysates of HEK293 cells overexpressing GFP-tagged SPAL3 aa.341-1776, anti-SPAL3 antibody recognized a band of ~180 kD, which is consistent with the mass of the putative molecular weights of GFP (27 kD) and SPAL3 aa.341-1776 (158 kD) (Fig. 3B). Both antibodies could not detect endogenous proteins in the lysate of parent HEK293. To evaluate the ability of these antibodies for immunostaining, mouse L fibroblasts transiently transfected with

expression vectors for GFP-tagged mouse ARHGAP12 or GFP-tagged SPAL3 were labeled with these antibodies. As shown in Fig. 3C-D', anti-ARHGAP12 antibody and anti-SPAL3 antibody intensively recognized only cells with GFP signals from GFP-ARHGAP12 and GFP-SPAL3 respectively. Moreover, when EpH4 cells were immunolabeled with these antibodies, the staining patterns of cell-cell junctions were observed around the cells (Fig. 3E, G). No staining was observed following pretreatment of primary antibodies with excess recombinant antigens, indicating that endogenous ARHGAP12 and SPAL3 are localized at cell-cell junctions in EpH4 epithelial cells (Fig. 3F, H). Neither anti-ARHGAP12 nor anti-SPAL3 antibodies we generated could detect endogenous ARHGAP12 or SPAL3 protein in EpH4 cells in Western blotting probably because of their weak reactivities to blotted proteins. However, the transcripts of both proteins were detected by RT-PCR in various mouse cultured cell lines including epithelial cells, fibroblasts, and teratocarcinoma cells such as EpH4, CSG120/7, L, NIH3T3, and F9 cells (data not shown).

Subcellular localization of ARHGAP12 and SPAL3

Localization of ARHGAP12 and SPAL3 in mouse tissues was analyzed by immunofluorescence microscopy. Tissue sections were double-stained with either pAb of these GAPs and a mAb of ZO-1. ZO-1 is localized at TJs in epithelial cells, but is often used as a good marker for the junctional complex including TJs and AJs at the

conventional fluorescence microscopy since its concentration into cell-cell contacts is remarkably clear compared with those of AJ components such as E-cadherin and nectins, which are also localized at the basolateral membrane domain. As shown in Fig. 4, ARHGAP12 was localized at the junctional complex of tissues including the small intestine, the kidney, the salivary gland, and the liver. SPAL3 was detected at the junctional complex of various tissues including the lung, the small intestine, the kidney, and the salivary gland. In addition to the junctional complex, staining signals of ARHGAP12 and SPAL3 were detected in the cytoplasm and the basolateral membrane respectively, each of which appeared to be specific; pretreatment of the antibody with excess recombinant antigen of each protein abolished staining (data not shown). We observed no detectable ARHGAP12 or SPAL3 staining in blood vessels, suggesting that both GAPs are localized at cell-cell junctions in epithelial cells, but not in endothelial cells (data not shown).

To determine the precise localization of ARHGAP12 and SPAL3 within the junctional complex, double immunofluorescence staining of mouse small intestine was performed, and expression of these GAPs were compared with that of occludin [10], a marker for TJs, and E-cadherin and nectin-2 [19], markers for AJs, by confocal laser-scanning microscopy. As shown in Fig. 5, junctional expression of ARHGAP12 was more basal than that of occludin, and mostly overlapped with E-cadherin and

nectin-2. SPAL3 showed the same distribution (Fig. 5). These results indicate that ARHGAP12 and SPAL3 are localized at AJs in the junctional complex of epithelial cells.

Localization of these GAPs at AJs suggested the possibility that these proteins are recruited to AJs by interacting with components of cadherin-mediated cell-cell adhesion. Thus, we examined the localization of these proteins in EL cells in immunofluorescence microscopy. EL cells were established from mouse L cells, which lack cadherin-mediated adhesion, by the stable introduction of E-cadherin [38]. EL cells exhibit E-cadherin-dependent cell-cell adhesion, and it has been reported that basic components of cadherin-mediated cell-cell adhesion such as E-cadherin, α-catenin, β-catenin are concentrated into cell-cell contact sites of EL cells [39]. In immunofluorescnece staining, interestingly, endogenous SPAL3 was clearly colocalized with E-cadherin at cell-cell contact sites in EL cells whereas it was distributed in the cytoplasm in L cells (Fig. 6), suggesting the interaction of SPAL3 with basic components of cadherin-mediated cell-cell adhesion. The staining signal of EL cells with anti-ARHGAP12 pAb was very faint and its concentration at cell-cell contact sites was not detected (data not shown). Although this might be due to low expression level of ARHGAP12 in EL cells, exogenous ARHGAP12-GFP expressed by transient transfection was not concentrated at cell-cell contact sites, neither (data not shown).

Discussion

There is only one prior report of FL-REX used for the cDNA cloning of cell-cell junction-associated proteins [12]. In that study fluorescence-activating cell sorting (FACS) was introduced into the original FL-REX method, and the tight junction-associated protein identified JEAP was identified using a cDNA library generated from an endothelial cell line. However, further application of this method has not been reported. In this study, we adopted several modifications to previous methods to improve the efficiency of the screening, and suggest that the FL-REX is a useful tool to identify novel components of TJs and AJs as a complement to biochemical and immunological approaches.

The FL-REX has several advantages compared to biochemical approaches in the identification of novel proteins localizing at intercellular junctions, and also at various cellular structures. First, novel proteins can be obtained without purification of the cellular structure, which is often very difficult. Thus, the FL-REX is useful as far as cDNA libraries are available. Usually, only limited sources can be used for biochemical purifications of certain cellular structures. In contrast, the FL-REX is applicable to various cDNA sources, including most tissues and cell lines, as the established method for the cDNA library production is common to all sources. Secondly, since screening is performed in the native condition in living cells, proteins can be identified that are

typically lost during biochemical isolation of cellular structures due to their low affinity with them. Previously reported isolation methods for intercellular junctions often expose the junctional membranes to unphysiological conditions, such as hypotonic buffer solution [7,8], in which some protein-protein interactions might be disrupted. These problems can be excluded in the FL-REX.

Conversely, there are also some problems in FL–REX. When expression cloning is performed with conventional cDNA libraries prepared from cellular poly(A)+ RNAs, the efficiency of the cloning for given cDNAs is dependent on their expression levels, implying that cloning of cDNAs with low expression is difficult. Furthermore, localization domains should have correct conformations in GFP-fusions to show the correct subcellular localization. These limitations may account for our results demonstrating that claudins were cloned in our screening in much higher frequency than other junctional proteins. The construction of averaged cDNA libraries might overcome these problems to clone different set of cDNAs. We have already confirmed that the use of cDNA libraries generated from cDNAs with different average sizes, or from different sources, provides different spectra of obtained cDNAs for AJ- and TJ- associated proteins (unpublished data), and further screening is ongoing in our laboratory.

In the present study, using the FL-REX method we cloned two putative GAPs for small G proteins, ARHGAP12 and SPAL3, as novel components of AJs. AJs

circumscribe epithelial cells at their apical margins with actin filaments closely apposed to the membranes on the cytoplasmic side [1,2]. One of the important regulators of the actin cytoskeleton is the Rho family GTPase, which includes Rho, Rac, and Cdc42. Accumulating evidence suggests that this family are also involved in the formation and function of AJs and TJs, probably through rearrangement of actin filaments [20,21]. The activities of Rho family GTPases are spatially and temporally controlled by guanine nucleotide exchange factor (GEF), which exchanges GDP for GTP, and GTPase activating protein (GAP), which accelerates the GTPase activity. To date, several GEFs and GAPs of Rho family GTPases have been demonstrated to be involved in the formation and configuration of TJs and AJs in epithelial cells [22, 23, 24].

ARHGAP12 is a member of closely related Rho GAPs including ARHGAP9 and ARHGAP15 [17]. ARHGAP9 demonstrated substantial GAP activity toward Cdc42Hs and Rac1, and its overexpression in human leukemia cells resulted in the repression of adhesion of cells to the extracellular matrix [25]. ARHGAP15 showed Rac1-specific GAP activity, and its overexpression in Hela cells resulted in an increase in actin stress fibers and cell contraction [17]. Since ARHGAP12 is closely related to ARHGAP9 and 15, one possibility is that ARHGAP12 is involved in the regulation of AJ formation by spatially suppressing the activity of Rac1 and/or Cdc42 at AJs, although the specificity of GAPs has to be determined *in vivo* in each specific cell type. Alternatively ARHGAP12

may be involved in some other functions triggered by junction assembly. Since ARHGAP12 mutants, which lack either SH3 domain or WW domains, did not concentrate into cell-cell contacts in epithelial cells, both domains seems to be required for the localization of ARHGAP12 at AJs. It has been reported that SH3 domains of vinexin bind to vinculin [36] and lp-dlg [37], both of which are localized at AJs. Therefore, the SH3 domain of ARHGAP12 may also interact with these proteins. To date, the interaction of the WW domain with AJ-associated proteins has not been reported.

The sequence similarity of SPAL3 with those of SPA-1 and SPAL [18] [26], both of which are Rap-GAPs, suggests that SPAL3 is also a Rap-specific GAP. Although Rap1 has been implicated in the control of integrin-mediated cell adhesion, recent evidence indicates that Rap1 also plays an important role in cadherin-mediated cell adhesion, including adherens junction formation [27]. Rap1 activity rescued Ras-induced disruption of cell-cell adhesion in MDCK cells and hepatocyte growth factor-induced cell scattering, suggesting that Rap1 positively regulates cadherin-based cell-cell adhesion [28]. Further, Hogan et al. reported that C3G, a Rap1GEF, interacts with the cytoplasmic domain of E-cadherin, and that ligation of the extracellular domain of E-cadherin enhances Rap1 activity, which in turn is necessary for the proper targeting of E-cadherin molecules to maturing cell-cell contacts [29]. Takai and

colleagues have recently shown that Rap1 is also involved in adherens junction formation through nectin-afadin system, another adhesion system of adherens junctions [30]. These observations suggest that SPAL3 may function in adherens junction formation by balancing the role of Rap1 by counteracting RapGEFs. The aa. 1050-1439 of SPAL3, which was required for localization at cell-cell contact sites in epithelial cells, does not contain any known domain structure (Fig. 2B,F). However, the fact that SPAL3 colocalized with E-cadherin at cell-cell contact sites in EL cells suggests that this region of SPAL3 interacts with the basic components of cadherin-mediated cell-cell adhesion, such as E-cadherin, α-catenin, β-catenin, and their binding proteins.

Although, so far, simple overexpression of ARHGAP12 and SPAL3 in EpH4 mouse epithelial cells seems not to affect cell morphology at the confluent condition (data not shown), these GAPs may be involved in the maturation of newly formed cell-cell contacts, the organization of actin stress fibers, or cell adhesion activity. Not only analyses along this line but also studies by knock down, overexpression of their dominant negative forms, and identification of binding partners will lead to better understanding their functions in epithelial AJs.

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Figure legends

Figure 1. **Fluorescent images of GFP-fusions localizing at cell-cell junctions in MDCK cells.** In the FL-REX using a retrovirus expression library generated from CSG120/7 cells, various GFP-fusions with known TJ- and AJ-associated proteins were obtained. Six examples are shown. (A) claudin-7 (aa. 1-197), (B) CAR (aa. 1-287), (C) ZO-1 (aa. 1-309), (D) a-actinin-4 (aa. 125-624), (E) a-catenin (aa. 634-875), and (F) Shroom2 (aa. 1-348). A GFP-fusion with CAR (aa. 1-287) localized at the lateral plasma membrane of MDCK cells as well as at cell-cell junctions as patches (arrowheads in B). A GFP-fusion with ZO-1 (aa. 1-309) was localized at nuclei and tricellular contacts (asterisks and arrowheads in C, respectively) in addition to cell-cell junctions. Scale bars; 10 μm.

Figure 2. ***Identification of ARHGAP12 and SPAL3 as novel components of epithelial cell-cell junctions by the FL-REX***. GFP-fusions with ARHGAP12 aa. 1-535 (A) and with SPAL3 aa. 1050-1439 (B) were concentrated at cell-cell junctions of MDCK cells in the FL-REX. Full-length ARHGAP12 (C) and SPAL3 (D) fused with GFP were also localized at cell-cell junctions. Domain structures of ARHGAP12 and SPAL3 were

predicted by Pfam and SMART programs, and shown in (E) and (F) respectively. Scale bars; 10 μm.

Figure 3. Evaluation of the specificity of anti-ARHGAP12 pAb and anti-SPAL3 pAb. (A), (B) Western blotting. Cell lysates of HEK293 cells expressing GFP-tagged full-length ARHGAP12 (ARHGAP12-GFP) or GFP-tagged SPAL3 aa.341-1776 (SPAL3-GFP) were immunoblotted with anti-ARHGAP12 pAb (A) or anti-SPAL3 pAb (B). Asterisks indicate specific bands of ARHGAP12-GFP and SPAL3 aa.341-1776-GFP respectively. Both antibodies did not recognize endogenous proteins in the lysate of parent HEK293 loaded on the same membranes (mock). (C-D') Immunostaining. ARHGAP12-GFP-expressing L cells (ARHGAP12-GFP/L) (C and C') and SPAL3-GFP-expressing L cells (SPAL3-GFP/L) (D and D') grown on coverslips were immunostained with anti-ARHGAP12 pAb (C') and anti-SPAL3 pAb (D'). The GFP signals from ARHGAP12-GFP (C) and SPAL3-GFP (D) completely overlapped with intensive signals of antibody staining. (E) Immunofluorescent image of EpH4 cells stained with anti-ARHGAP12 pAb. The junctional staining was undetectable after pretreatment of the primary antibody solution with an excess amount of the recombinant MBP-ARHGAP12 (aa.72-226) protein (F). (G) The immunofluorescence image of EpH4 cells stained with anti-SPAL3 pAb. The junctional staining was undetectable after

pretreatment of the primary antibody solution with an excess amount of the recombinant MBP-SPAL3 (aa.1040-1412) fusion proteins (H). Scale bars; 10 μm.

Figure 4. Immunolocalization of ARHGAP12 and SPAL3 in mouse tissues. Upper panel: Frozen sections of mouse kidney, liver, and pancreas were double stained with anti-ARHGAP12 pAb (A-C) and anti-ZO-1 mAb (A'-C'). Fluorescent signals from ARHGAP12 and ZO-1 were mostly overlapped at cell-cell junctions of renal epithelial cells in the kidney (A and A'), hepatocytes in the liver (B and B'), and exocrine cells in the pancreas (C and C'). Lower panel: Frozen sections of mouse lung, small intestine, and pancreas were double stained with anti-SPAL3 pAb (D-F) and anti-ZO-1 mAb (D'-F'). Fluorescent signals from SPAL3 and ZO-1 were mostly overlapped at cell-cell junctions of bronchial epithelial cells in the lung (D and D'), intestinal epithelial cells (E and E'), and exocrine cells in the pancreas (F and F'). Scale bars; 10 μm.

Figure 5. Distribution of ARHGAP12 and SPAL3 within the junctional complex. Upper panel: Double immunofluorescence staining of mouse small intestine with anti-ARHGAP12 pAb and anti-occludin mAb (A-A'''), with anti-ARHGAP12 pAb and anti-E-cadherin mAb (B-B'''), or with anti-ARHGAP12 pAb and anti-nectin-2 mAb (C-C'''). In the merged image, ARHGAP12 (an arrow) was distributed more on the basal

side of intestinal epithelial cells than occludin (an arrowhead) (A'' and A'''), whereas mostly colocalized with E-cadherin (B'' and B''') and nectin-2 (C'' and C''') at the junctional complex. Lower panel: Double immunofluorescence staining of mouse small intestine with anti-SPAL3 pAb and anti-occludin mAb (D-D'''), with anti-SPAL3 pAb and anti-E-cadherin mAb (E-E'''), or with anti-SPAL3 pAb and anti-nectin-2 mAb (F-F'''). In the merged image, SPAL3 (an arrow) was distributed more on the basal side of intestinal epithelial cells than occludin (an arrowhead) (D'' and D'''), whereas colocalized with nectin-2 (E'' and E''') and E-cadherin (F'' and F'''). Occludin was used as a marker of TJs, wheareas nectin-2 and E-cadherin were used as markers of AJs. Scale bars; 10 μm.

Figure 6. Localization of ARHGAP12 and SPAL3 in L cells expressing E-cadherin. Double immunofluorescence staining of EL cells and L cells with anti-SPAL3 pAb (A, B) and anti-Ecadherin mAb (A', B'). In EL cells, SPAL3 and E-cadherin were colocalized at cell-cell contact sites (A, A') whereas SPAL3 was distributed in the cytoplasm in L cells (B). Scale bars; 10 μm.

Figure S1. Localization of deletion mutants of ARHGAP12-GFP in MDCK cells. Two deletion mutants of ARHGAP12-GFP were stably introduced into MDCK cells and their

subcellular localizations were analyzed by fluorescent signal of GFP. ARHGAP12-GFP/SH3(-) and ARHGAP12-GFP/WW(-) lacks the SH3 domain (aa.13-71) and two WW domains (aa.263-388), respectively. The deletion constructs were generated by PCR and both of these deleted domains were replaced with glutamine and isoleucine. Scale bars; 10 μm.

Table1

Table 1. Known TJ- and AJ-associated proteins identified in the FL-REX in this study.

Protein	Encoded (total) amino acids	Reported localization
Claudin-2*	1-207 (230)	TJ
Claudin-3*	2-211 (219)	TJ
Claudin-4*	1-189 (210)	TJ
Claudin-7*	1-197 (211)	TJ
CAR*	1-287 (365)	TJ
ZO-1	1-309 (1745)	TJ
ZO-2	1-486 (1167)	TJ
α-catenin	634-875 (906)	AJ
β-catenin	1-719 (781)	AJ
Plakoglobin*	82-472 (745)	AJ
α-actinin-4*	125-624 (912)	AJ
Shroom2	1-348 (1487)	AJ

* Multiple clones were obtained and their representative data presented.

Figure.1
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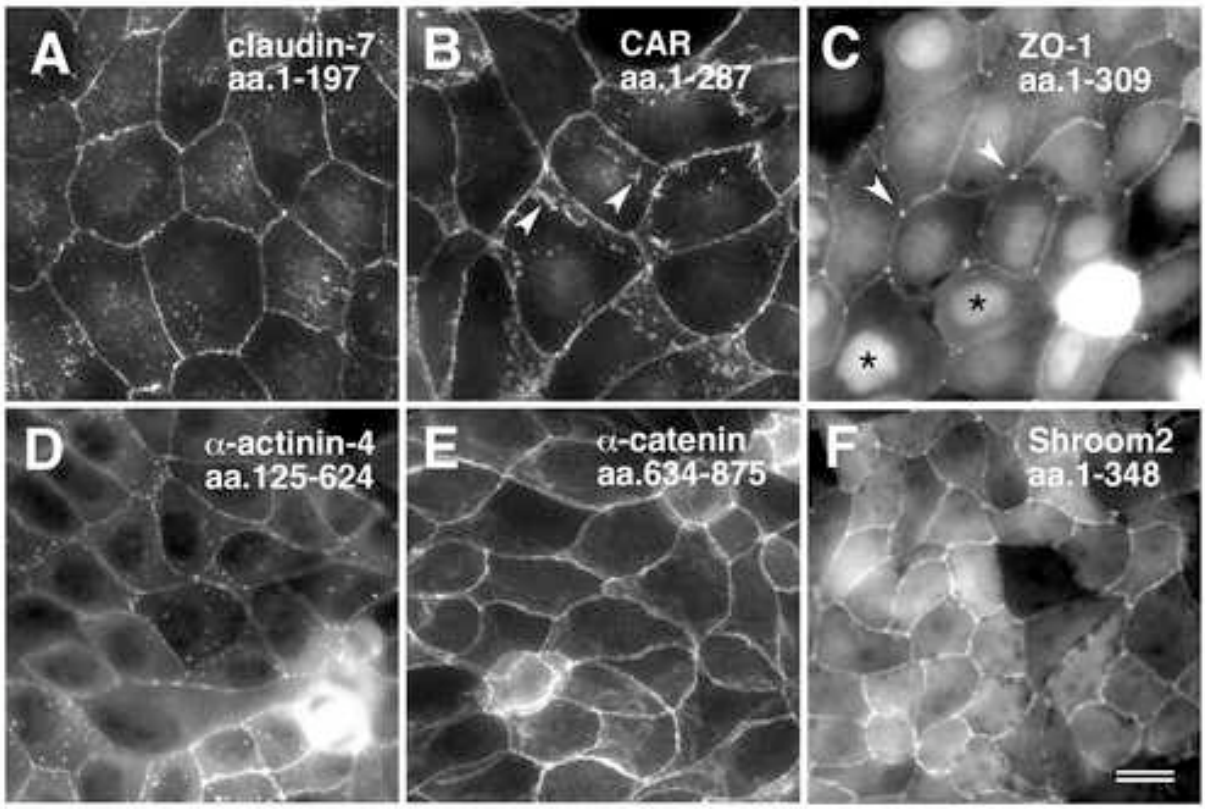

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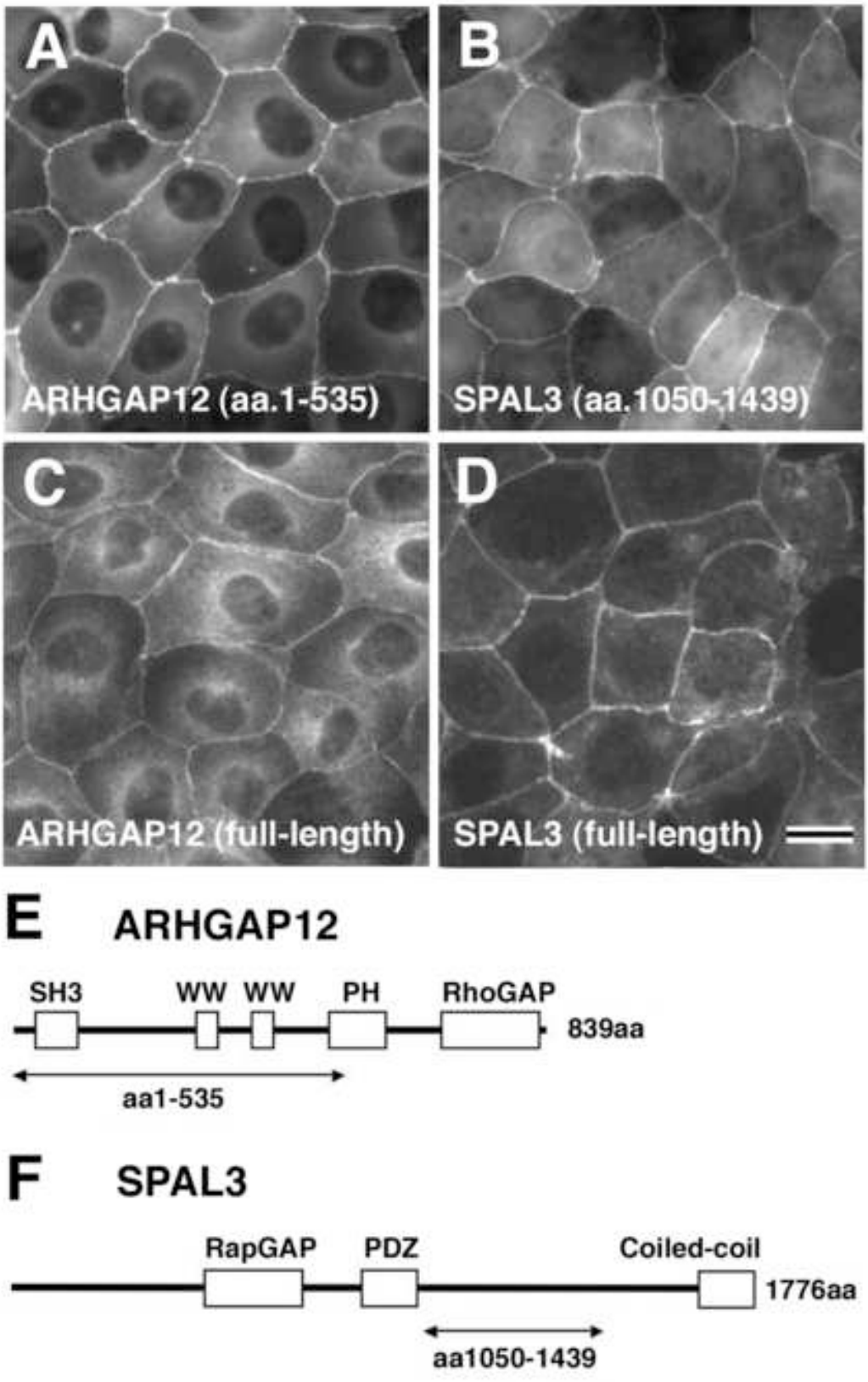

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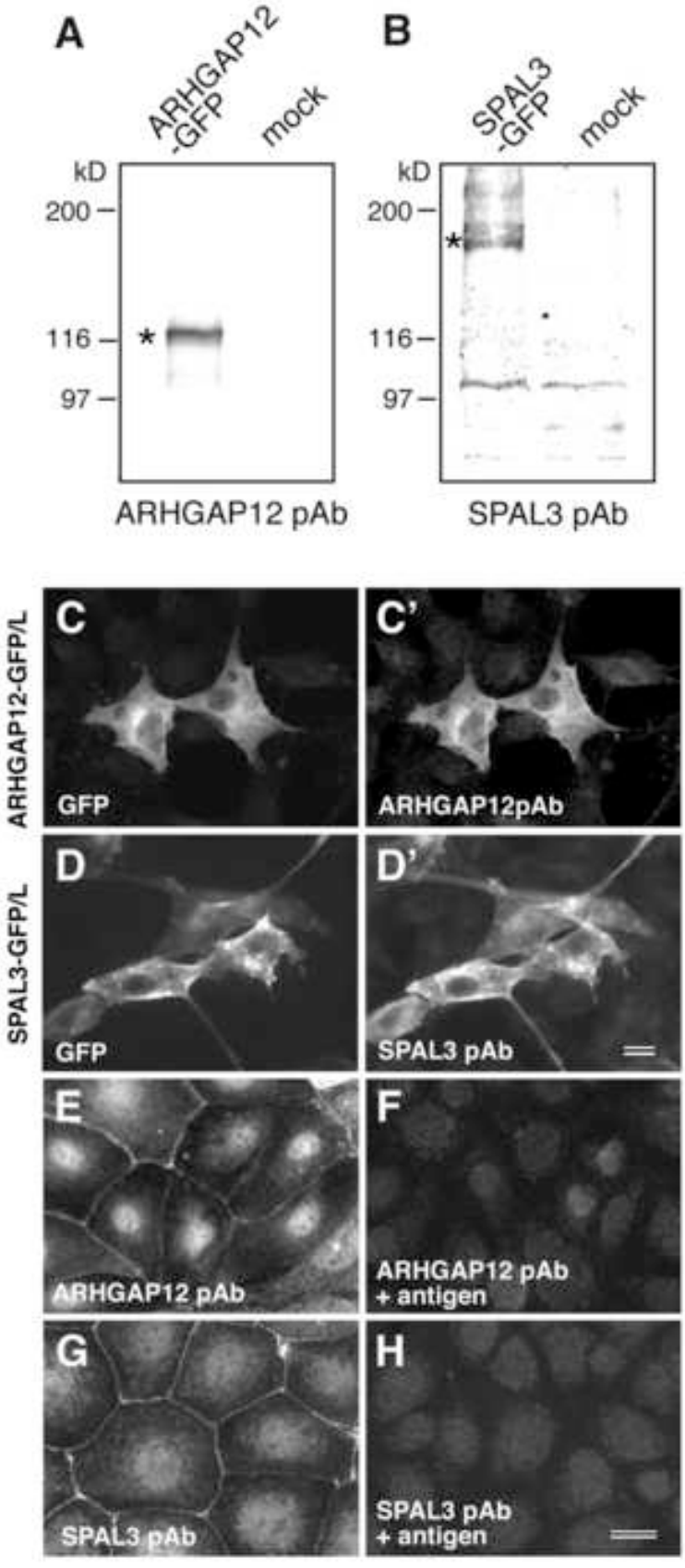

Figure 4

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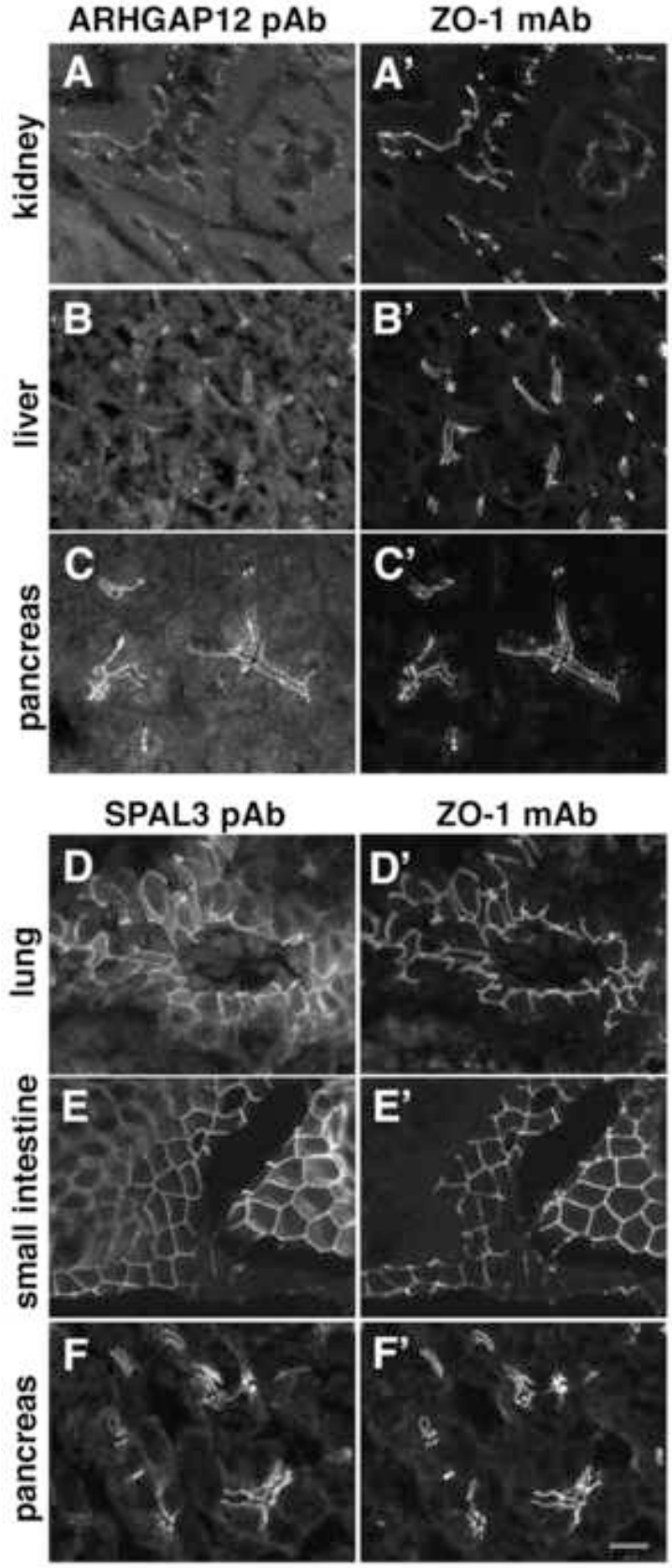

Figure 5

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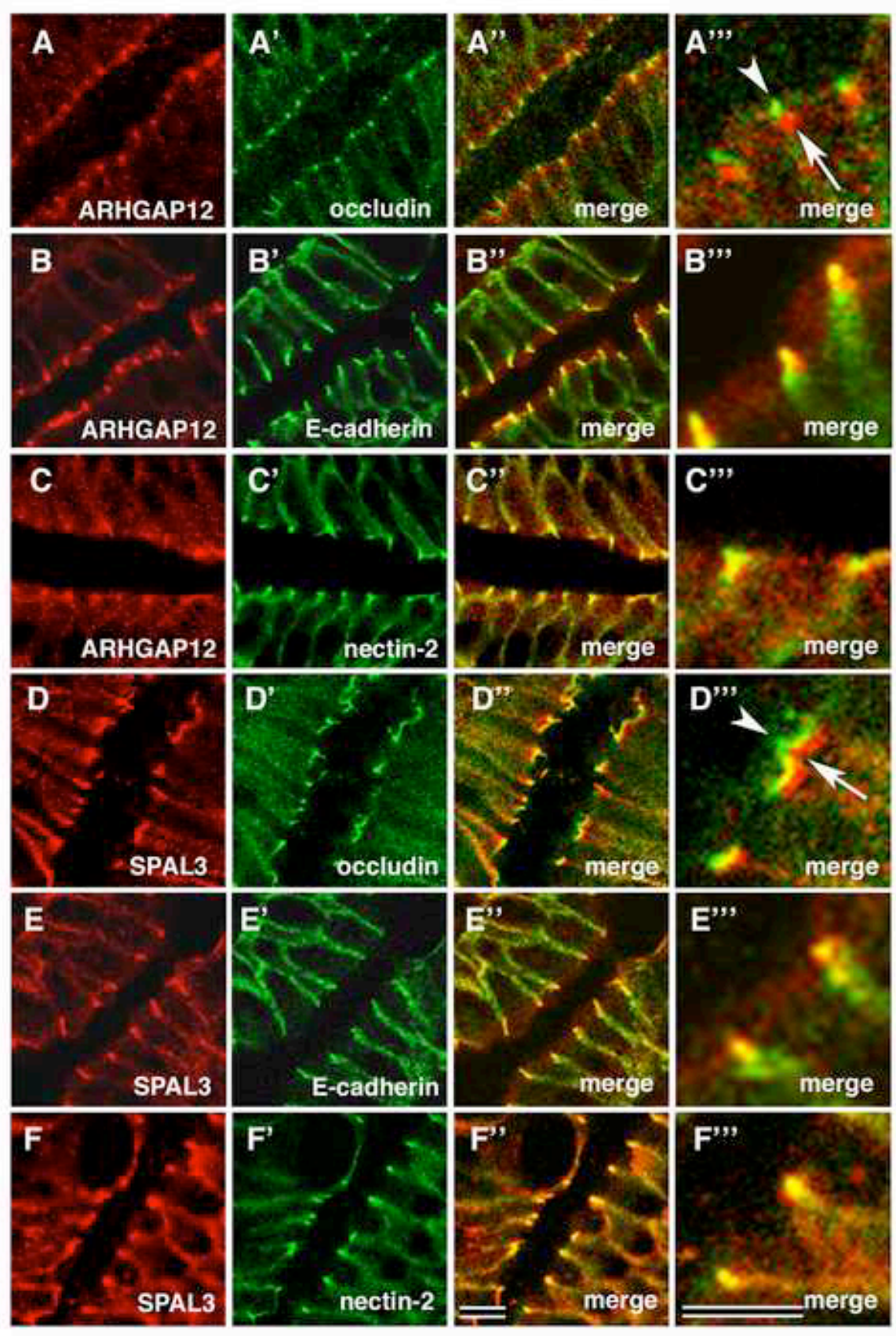

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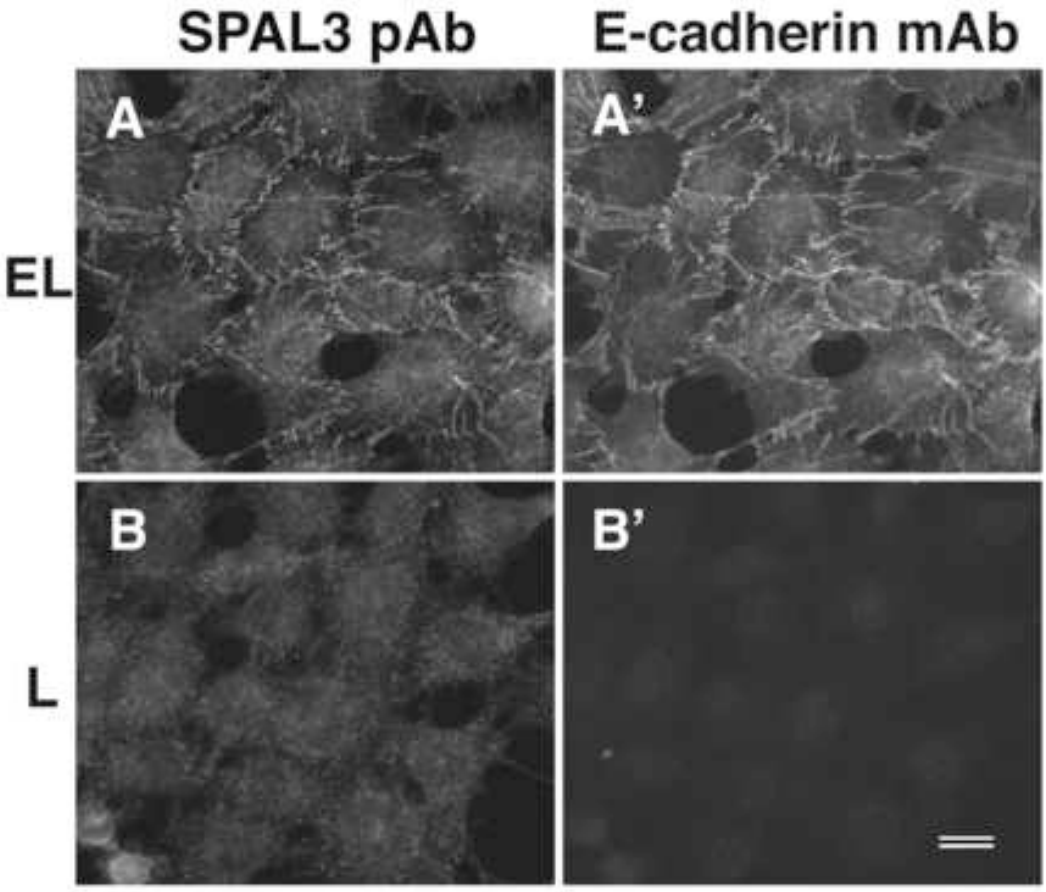

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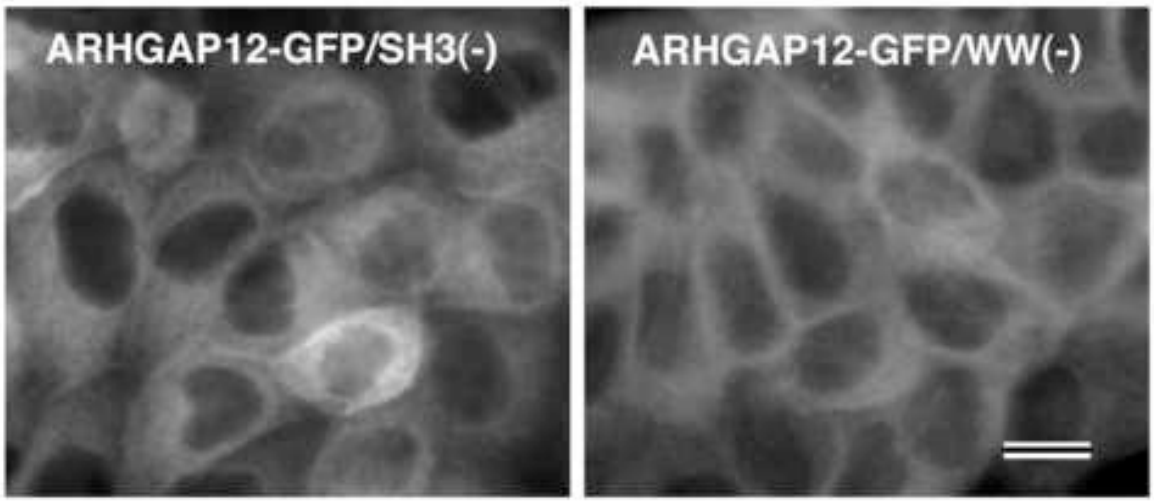

Figure S1