Vimentin and the K-Ras-induced actin-binding protein control inositol-
(1,4,5)-trisphosphate receptor redistribution during MDCK cell
differentiation.

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Abstract
Inositol-(1,4,5)-triphosphate receptors (InsP₃Rs) are ligand-gated Ca²⁺ channels that control Ca²⁺ release from intracellular stores and play a central role in a wide range of cellular responses. In most epithelial cells, InsP₃Rs are not uniformly distributed within the endoplasmic reticulum (ER) membrane with the consequence that agonist stimulation results in compartmentalized Ca²⁺ signals. Despite these observations, little is known about the mechanisms that regulate the intracellular localization of InsP₃Rs. Here, we report that exogenously expressed InsP₃R1-GFP and endogenous InsP₃R3 interact with the K-Ras-induced actin-binding protein (KRAP) in both differentiated and undifferentiated Madin-Darby canine kidney (MDCK) cells. KRAP mediates InsP₃R clustering in confluent MDCK cells and functions as an adapter, linking InsP₃Rs to vimentin intermediate filaments (IF). Upon epithelial differentiation, KRAP and vimentin are both required for InsP₃R accumulation at the periphery of MDCK cells. Finally, KRAP associates with vimentin in chicken B lymphocytes and with keratins in a breast cancer cell line devoid of vimentin. Collectively, our data suggest that IF in conjunction with KRAP may govern the localization of InsP₃Rs in a large number of cell types (including epithelial cells) and in various physiological or pathological contexts.
Introduction

In multicellular organisms, epithelial cells form continuous sheets covering external surfaces and internal cavities and organs. Their biological roles relate to their location at the interface between two dissimilar environments and include protection against noxious agents, restriction of paracellular diffusion, transcellular vectorial transport and secretion (Rodriguez-Boulan and Nelson, 1989). Epithelial cells display a cell surface organization that is well adapted to their specialized functions. Their plasma membrane can be divided into two distinct domains - the apical and basolateral membranes - each containing specific sets of proteins and lipids. The basolateral membrane, connected with adjacent cells or to the extracellular matrix, is separated from the apical surface, facing the lumen or the environment, by tight junctions (TJ). The latter form a semi-permeable diffusion barrier between individual cells as well as an intra-membrane diffusion fence that restricts the intermixing of apical and basolateral membrane components (Balda and Matter, 1998).

A wide range of epithelial functions such as fluid and electrolyte secretion (Kasai and Augustine, 1990; Ito et al., 1997), exocytosis (Maruyama et al., 1993; Ito et al., 1997) or TJ permeability (Nathanson et al., 1992) are regulated by cytosolic Ca\(^{2+}\). Videoimaging of Ca\(^{2+}\) indicators have demonstrated that, in a large number of epithelial cells, Ca\(^{2+}\) signals are remarkably organized in space and often polarized. In exocrine pancreatic cells, for example, secretagogue-evoked Ca\(^{2+}\) rises always start at the apical pole and either remain localized into this region (called the trigger zone) or rapidly propagate to the basal pole, as a Ca\(^{2+}\) wave. Apically confined Ca\(^{2+}\) increases can initiate apical exocytosis (Ito et al., 1997) or modulate paracellular permeability while apical-to-basal Ca\(^{2+}\) waves direct fluid and electrolyte secretion by sequential activation of apical and then basal ion channels (Kasai and Augustine, 1990).

Ca\(^{2+}\) mobilization is generally elicited in epithelial cells by stimulation of receptors linked to phospholipase C activation, leading to the subsequent production of inositol-(1,4,5)-trisphosphate (InsP\(_3\)) and release of Ca\(^{2+}\) from intracellular stores through InsP\(_3\) receptors (InsP\(_3\)Rs). Three InsP\(_3\)R isoforms that share a common structure consisting of an amino-terminal InsP\(_3\)-binding core, a C-terminal channel-forming domain and a central regulatory domain have been identified in mammals (Foskett et al., 2007). The three subtypes can assemble into homo- or heterotetramers (Joseph et al., 1995; Monkawa et al., 1995; Wojcikiewicz et al., 1995) to form InsP\(_3\)-gated Ca\(^{2+}\) channels, predominantly located on the membrane of the endoplasmic reticulum. Immunofluorescence studies have revealed that InsP\(_3\)R isoforms are diversely expressed and distributed in epithelia of various origins. In
pancreatic acinar cells, all three InP$_3$R isoforms are present and concentrated in the apical trigger zone in which Ca$^{2+}$ waves originate (Yule et al., 1997). In hepatocytes, InP$_3$R2 is exclusively found underneath the luminal membrane whereas InP$_3$R1 is distributed relatively uniformly throughout the cell (Hirata et al., 2002; Shibao et al., 2003; Cruz et al., 2010). In polarized Madin-Darby canine kidney (MDCK) cells, InP$_3$R3 accumulates in the vicinity of TJ (Colosetti et al., 2003, Hours and Mery, 2010). It has been hypothesized that the differential expression and subcellular localization of InP$_3$R subtypes largely contribute to the spatial patterning of Ca$^{2+}$ signals in epithelial cells (Vermassen et al., 2004).

Despite intensive research efforts, the molecular mechanisms involved in the recruitment of the different InP$_3$R isoforms to specific subdomains of the ER membrane remain elusive. In this study, we sought to identify the proteins responsible for the perijunctional accumulation of InP$_3$Rs in polarized MDCK cells. For that purpose, we stably expressed in MDCK cells the InP$_3$R1 isoform C-terminally fused to green fluorescent protein (referred to as InP$_3$R1-GFP) or a truncated version of this construct lacking the regulatory domain (named ΔRD-GFP). We established that, while InP$_3$R1-GFP behaves exactly like endogenous InP$_3$R3, ΔRD-GFP remains evenly distributed in the ER membrane whatever the differentiation state of the cells. Hypothesizing that deletion of the regulatory domain triggers loss of key protein interactions involved in the control of InP$_3$R subcellular distribution, we purified InP$_3$R1-GFP and ΔRD-GFP from confluent but incompletely differentiated MDCK cells and identified their binding partners by tandem mass spectrometry. In this report, we document an unexpected association of InP$_3$Rs with vimentin intermediate filaments (IF) through the K-Ras-induced actin-interacting protein (KRAP) and demonstrate that both KRAP and vimentin are required for the accumulation of InP$_3$Rs in the vicinity of TJ upon MDCK cell polarization.
Results

The regulatory domain controls the subcellular localization of InsP$_3$R1-GFP in MDCK cells

- We have previously shown that the recruitment of InsP$_3$R1-GFP near the tight junctions (TJ) in polarized MDCK cells depends on its N-terminal region (Hours and Mery, 2010). In order to more precisely define the domain which directs InsP$_3$R to the perijunctional area, a GFP-fused InsP$_3$R1 deletion mutant lacking most of the regulatory domain (amino acids 650 to 1949) and referred to as ΔRD-GFP was generated (Fig. S1A). MDCK cells were transfected with an expression vector encoding either InsP$_3$R1-GFP or ΔRD-GFP and cultivated for three weeks under selective growth medium including G418. GFP-positive cells were then sorted by flow cytometry, resulting in homogeneous populations clearly resolved from the non-transfected cells and with comparable median fluorescence intensities (Fig. S1B). The spatial distributions of InsP$_3$R1-GFP and ΔRD-GFP were then examined by confocal microscopy in sparse, confluent or fully polarized MDCK cells and compared to that of ER-resident proteins (PDI and InsP$_3$R3) or markers of epithelial differentiation (E-cadherin and ZO-1). In sparse cells (Fig. 1A), both InsP$_3$R1-GFP and ΔRD-GFP were found to be evenly distributed in a network of tubular structures spread throughout the cytosol and continuous with the nuclear envelope. This network was also stained by the anti-PDI antibody, indicating that both InsP$_3$R1-GFP and ΔRD-GFP were properly targeted to the ER.

In confluent but not yet polarized cells (Fig. 1B), ΔRD-GFP remained diffusely distributed in the ER, whereas InsP$_3$R1-GFP was found to redistribute into bright puncta. These puncta still resided in the ER membrane as they co-localized with PDI. Comparison of the staining patterns of PDI in InsP$_3$R1-GFP- and ΔRD-GFP-expressing cells did not reveal any obvious difference, suggesting that the accumulation of InsP$_3$R1-GFP into punctate structures was not due to morphological alterations of the ER. To rule out possible artifacts caused by the GFP-tag, we next examined the subcellular distribution of endogenous InsP$_3$R3 in confluent cells expressing either InsP$_3$R1-GFP or ΔRD-GFP. InsP$_3$R3 was found to accumulate into puncta similar in size and morphology in both cell lines and to co-localize extensively with InsP$_3$R1-GFP but only partially with ΔRD-GFP (Fig. 1C). Thus, InsP$_3$R3 forms clusters in confluent MDCK cells independently of InsP$_3$R1-GFP and the ability of InsP$_3$R1-GFP to produce a punctate pattern depends mainly on its regulatory domain.

In fully differentiated cells (Fig. 1D), InsP$_3$R1-GFP was predominantly detected near the apex of the E-cadherin-labeled lateral membrane and in proximity to the TJ marker ZO-1. The co-localization was extensive with InsP$_3$R3 but limited with PDI. In contrast, ΔRD-GFP pattern
poorly overlapped with that of InsP3R3 but was still clearly coincident with PDI staining. Taken together, these results suggest that deletion of the regulatory domain prevents both InsP3R1-GFP clustering in confluent MDCK cells and InsP3R1-GFP accumulation at the cell periphery upon epithelial cell differentiation.

**Identification of proteins that specifically bind to InsP3R1-GFP in confluent MDCK cells by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)** – To gain insight into the mechanism responsible for InsP3R1-GFP redistribution during the polarization process, we sought to identify proteins that interact with InsP3R1-GFP but not with the deletion mutant. Co-immunoprecipitation experiments were performed using GFP-trap beads and total cell lysates prepared from confluent but not fully polarized MDCK cells expressing InsP3R1-GFP or ΔRD-GFP. Bead-bound proteins were then eluted and size fractionated using SDS-PAGE. After colloidal blue staining, each lane was sliced and subsequently and processed for LC-MS/MS as explained in Materials and Methods. This led to the identification of two proteins that co-purify reproducibly with InsP3R1-GFP but not with ΔRD-GFP, namely InsP3R3 and the K-Ras-induced actin-binding protein (KRAP) (Table S1). The latter is a protein of 1,259 amino acids whose synthesis is stimulated by activated K-Ras (Inokuchi et al, 2004) and whose subcellular localization, interestingly, coincides with that of InsP3R isoforms 1, 2 and 3 in pancreatic acinar cells (Fujimoto et al., 2007).

**InsP3R1-GFP and endogenous InsP3R3 interact with KRAP independently of one another** – Western blot analysis was carried out to validate the results of the LC-MS/MS experiments and to examine the possible interaction of KRAP with endogenous InsP3R3. Total lysates were prepared from confluent MDCK cells expressing InsP3R1-GFP or ΔRD-GFP or from confluent non-transfected cells. Precipitations were performed using GFP-trap beads or anti-InsP3R3 antibodies coupled to protein G-sepharose beads. The input and precipitated proteins were then fractionated by SDS-PAGE, transferred to PVDF and probed with anti-GFP, anti-InsP3R3 or anti-KRAP antibodies. The results presented in figure 2 (panels A to C) confirm that InsP3R3 and KRAP physically associate with InsP3R1-GFP but fail to interact with ΔRD-GFP. They also indicate that endogenous InsP3R3 can bind KRAP independently of InsP3R1-GFP.

To determine whether InsP3R1-GFP interacts with KRAP directly or through InsP3R3 binding, the GFP-tagged construct was transiently expressed in DT40 triple InsP3R knockout (TKO) cells. The latter are pre-B-lymphocytes in which all three InsP3R genes have been disrupted (Sugawara et al., 1997). As previously described (Guillemette et al., 2005) and
illustrated in figure 2D, they are entirely devoid of InsP₃R1 but still contain, in the cytosol, a truncated form of InsP₃R3. They also express KRAP albeit at a lower level than MDCK cells (Fig. 2E). The plasmid encoding InsP₃R1-GFP was introduced into DT40 TKO cells by nucleofection. At 48 hours post-transfection, cells were lysed and InsP₃R1-GFP was precipitated using GFP-trap beads. As shown in figure 2F, InsP₃R1-GFP associates with KRAP independently of InsP₃R3 since KRAP but not the truncated form of InsP₃R3 was detected in the immunoprecipitate.

In agreement with the biochemical data, immunofluorescence staining of confluent MDCK cells expressing InsP₃R1-GFP showed extensive co-localization of KRAP and InsP₃R1-GFP in the punctate structures previously described and at the cell periphery (fig. S2A). Similarly, InsP₃R3 co-distributed with KRAP in both sparse and confluent wild-type MDCK cells (fig. S2B). Finally, InsP₃R1-GFP/KRAP co-localization was largely preserved in MDCK cells depleted of InsP₃R3 by RNA interference (fig. S2C). Taken together, these results indicate that the structural determinants required for KRAP binding are present in both InsP₃R3 and InsP₃R1-GFP but are lost in ΔRD-GFP.

**Down-regulation of KRAP impairs InsP₃R clustering and accumulation at the cell periphery** - To determine whether KRAP has any impact on InsP₃R localization in confluent MDCK cells, we next tried to down-regulate KRAP expression by RNA interference. MDCK cells expressing InsP₃R1-GFP were transfected before reaching confluence with siRNAs specific for canine KRAP (referred to as K1, K2 and K3) or with a nonsense duplex. Three days later, cells were lysed and subjected to western blotting to assess the expression of KRAP, InsP₃R1-GFP, InsP₃R3 and calnexin (which served as a loading control). Alternatively, cells were fixed and processed for dual fluorescence imaging of KRAP and InsP₃R localizations by confocal microscopy. As illustrated in figure 3A, the K2- and K3-siRNAs successfully reduced KRAP expression. KRAP silencing led to both decreases in InsP₃R levels (Fig. 3B) and dramatic changes in the subcellular localization of InsP₃R1-GFP (Fig. 3C). The latter no longer appeared to segregate into puncta or to accumulate at the cell periphery in confluent monolayers but remained evenly distributed in the ER membrane, like ΔRD-GFP, whatever the differentiation state of the cells. Similarly, in wild type MDCK cells treated with either the K2- or K3-siRNA duplex, InsP₃R3 protein level was reduced to approximately 60% of that of control cells and InsP₃R3 accumulation at the cell periphery upon epithelial differentiation was completely abrogated (Fig. S3). Taken together, these results suggest that KRAP modulates InsP₃R levels and controls InsP₃R distributions in polarized MDCK cells.
Association with KRAP is responsible for the detergent insolubility of InsP₃Rs in MDCK cells - KRAP has been reported to be an actin-binding protein (Inokuchi et al., 2004). If this is true, then KRAP should be resistant to mild detergent extraction and its binding partners as well. To verify this assumption, incompletely polarized MDCK monolayers expressing either InsP₃R1-GFP or ΔRD-GFP were homogenized in hypotonic buffer. Three fractions - hydrosoluble (HS), Triton X-100-soluble (TS) and Triton X-100 insoluble (TI) - were then sequentially collected by centrifugation and two of them (TS and TI) were analyzed by SDS-PAGE and western blotting. As evidenced by the representative immunoblots shown in figure 4A and by the densitometric analysis displayed in figure 4B, KRAP, InsP₃R1-GFP and InsP₃R3 were almost exclusively detected in the TI fraction, enriched in cytoskeletal components such as actin, vimentin and keratins. By contrast, ΔRD-GFP was predominantly found in the Triton X-100-soluble fraction.

To determine whether KRAP promotes the detergent insolubility of InsP₃Rs, the same fractionation protocol was then applied to confluent MDCK cells expressing InsP₃R1-GFP and transfected, 72 hours prior to lysis, with the K2- or K3-siRNA duplex or with a negative control (NC). As shown in figure 4 (panels C and D), down-regulation of KRAP expression with either of the two KRAP-targeting siRNAs led to a partial translocation of InsP₃R1-GFP and InsP₃R3 from the TI to the TS fraction without modifying the detergent solubility profiles of actin and vimentin. Taken together, these results indicate that association with KRAP is at least in part responsible for the Triton X-100 insolubility of InsP₃Rs in MDCK cells.

KRAP and InsP₃R1-GFP are linked to vimentin filaments in MDCK cells – To investigate whether KRAP links InsP₃Rs to F-actin in confluent MDCK cells, co-immunoprecipitation experiments were conducted using GFP-Trap beads and TI fractions prepared from wild-type or InsP₃R1-GFP-expressing MDCK cells. The Triton X-100 insoluble pellets were resuspended in a large volume of buffer containing three non-ionic and ionic detergents (1% Triton X-100, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate) and gently sonicated on ice for 10 seconds. The resulting material was then clarified by centrifugation and incubated sequentially with agarose-beads and GFP-Trap beads. Finally, the precipitated proteins were eluted and analyzed by western blot using antibodies recognizing GFP, KRAP, actin, vimentin or keratins. Surprisingly, InsP₃R1-GFP was found to associate with KRAP and vimentin but not with actin or keratins in the TI fraction (Fig. 5A). The same protocol was then applied to cells expressing the ΔRD-GFP mutant. As shown in figure 5B, the small fraction of ΔRD-GFP resistant to non-ionic detergent extraction did not
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cooprecipitate with either KRAP or vimentin. In contrast, the deletion mutant co-purified extensively with erlin-2 (Fig. S4), a protein which is localized in the Triton X-100-insoluble lipid rafts of the ER (Browman et al., 2006) and has been reported to mediate ER-associated degradation of activated InsP3Rs (Pearce et al., 2007; Pearce et al., 2009; Wang et al., 2009). Erlin-2 was also co-precipitated with InsP3R1-GFP but to a much lesser extent (Fig. S4). Taken together, these results indicate that the ΔRD-GFP mutant, which is unable to bind to KRAP, cannot associate with IF either. Incidentally, they also suggest that, in addition to KRAP, erlin-2 may contribute (though marginally) to the detergent insolubility of InsP3R1-GFP.

To further characterize the association of KRAP with vimentin, we took advantage of the DT40 TKO cells which express vimentin and KRAP but are devoid of functional InsP3Rs. DT40 TKO cells were lysed in a buffer containing 1% Triton X-100, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate. Whole-cell extracts were then immunoprecipitated with anti-KRAP antibodies coupled to protein G-sepharose beads. Western blot analysis revealed that KRAP and vimentin but not the truncated cytoplasmic form of InsP3R3 were present in the immunoprecipitates (Fig. 5C). Thus, KRAP and vimentin can interact with each other in the absence of InsP3Rs, suggesting that KRAP may connect InsP3Rs to vimentin IF in MDCK cells.

Association with vimentin is required for the accumulation of InsP3Rs at the cell periphery – To explore the functional significance of the interaction between KRAP, InsP3Rs and vimentin, we used RNA interference to reduce the vimentin protein level in MDCK cells expressing InsP3R1-GFP. Seventy two hours after transfection, KRAP and InsP3R1-GFP localizations in cells treated with either the control siRNA or one of the duplexes targeting canine vimentin (referred to as V1, V2 and V3) were analyzed by immunofluorescence and confocal microscopy. In parallel, proteins were harvested from the cells and subjected to immunoblot analysis to monitor the expression of vimentin, KRAP, InsP3Rs and calnexin. At last, cell fractionation experiments were conducted as described before on the siRNA-transfected cells to determine whether vimentin knockdown increases the solubility of KRAP and InsP3Rs in cold non-ionic detergent. The V1- and V2-siRNA duplexes were quite effective in reducing vimentin protein content (by 60% on whole cell population) (Fig. 6A) but they did not significantly affect InsP3R or KRAP expression (Fig. 6B). As illustrated by the x-y projections of entire z-stacks displayed in panels 6C and as evidenced by the intensity profiles obtained from individual focal planes shown in panels 6D, in cells exhibiting more than 80% depletion of vimentin, KRAP and InsP3R1-GFP still co-localized extensively into
punctate structures distributed throughout the cytosol. However, the accumulation of both proteins at the cell periphery appeared severely compromised. Finally, panels 6E and 6F reveals that the distributions of KRAP and InsP3R1-GFP between the TS and the TI fractions were not significantly modified by vimentin knockdown.

Taken together, these results indicate that vimentin is not required for the formation of KRAP/InsP3R1-GFP complexes but may control KRAP and InsP3R1-GFP accumulation at the cell periphery upon epithelial cell differentiation. They also suggest that KRAP-mediated clustering of InsP3Rs may lead to the formation of large Triton X-100-insoluble aggregates which sediment quickly when spun at high speed whether or not they are linked to cytoskeletal elements. Alternatively, in V1- and V2-siRNA-treated cells, other proteins may substitute for vimentin in promoting the detergent insolubility of KRAP/InsP3R complexes.

Stable expression of a truncated form of vimentin in MDCK cells alters InsP3R3 and KRAP localization in polarized cells – To further confirm that an organized vimentin IF network is required for the proper localization of InsP3R/KRAP complexes in polarized MDCK cells, we generated stable cell lines expressing either GFP or a GFP-tagged truncated form of vimentin. The dominant-negative vimentin mutant (VimDN-GFP) consists of the first 138 amino acids of hamster vimentin, C-terminally fused to GFP and has been shown to disrupt vimentin IF organization when expressed in various cell types (Kural et al., 2007; Mendez et al., 2010; Schoumacher et al., 2010; Helfand et al., 2011). Isolation of the stably transfected cell populations was performed by combining antibiotic selection and cell sorting of GFP-positive cells.

The effects of VimDN-GFP expression on the subcellular distribution of InsP3R3, KRAP and vimentin were then examined by immunofluorescence and confocal microscopy. Two different monoclonal antibodies were used to stain the cells for vimentin. Neither of them was supposed to react with the deletion mutant. Indeed, on the one hand, the rabbit antibody (clone EPR3776) was raised against a synthetic peptide corresponding to the C-terminus of human vimentin that is missing in VimDN-GFP; on the other hand, the mouse antibody (clone V9) has been reported to detect a truncated form of vimentin, lacking the first 138 amino acids (Huet et al., 2006), suggesting that the recognized epitope is also located in the C-terminal part of the molecule. Cells were processed for immunofluorescence microscopy 7 (like in the siRNAs experiments) or 10 days after plating (to exclude incomplete differentiation). As shown in figure 7A, expression of the VimDN-GFP mutant in MDCK cells did not seem to disrupt vimentin IF organization. Insufficient expression and partial nuclear sequestration of the deletion mutant may account for these results. In spite of that, the
peripheral accumulation of InsP3Rs (Fig. 7A) and KRAP (data not shown) was reduced as well as 10 days after plating in cells expressing VimDN-GFP as compared to control cells expressing GFP alone.

Subcellular fractionation and co-immunoprecipitation experiments were then performed and revealed that, in contrast to GFP, the VimDN-GFP mutant co-fractionate with membrane and cytoskeletal proteins (Fig. 7B) and associate with KRAP and InsP3R3 (Fig. 7C). Thus, association of KRAP/InsP3R complexes with an assembly-incompetent vimentin mutant that cannot be incorporated into filaments and disruption of vimentin IF using the siRNA interference technique have very similar effects on the subcellular distribution of InsP3Rs.

**KRAP/InsP3R complexes interact with keratins in cells devoid of vimentin** – Unlike MDCK cells which contains distinct vimentin IF and keratin IF networks, most mature epithelial cells do not naturally express vimentin. KRAP/InsP3R complexes were not found to associate with keratins in confluent MDCK cells. However, we wondered what would happen in cells devoid of vimentin. To answer this question, we used MCF-7 breast adenocarcinoma cells. The latter, as previously reported (Lahat et al., 2010) and as confirmed by the immunoblots and immuofluorescence stainings shown in figure 8 (panels A and B), exhibit no vimentin protein expression. In contrast, in MCF-7 cells, the keratin 8 level was significantly higher than in MDCK cells. Membrane and cytoskeletal proteins were isolated from MCF-7 cells and subjected to immunoprecipitations with anti-KRAP antibodies coupled to protein G-sepharose beads. Immunoprecipitates were then analyzed by SDS-PAGE and immunoblotting. As shown in figure 8C, keratin 8 readily co-purified with KRAP/InsP3R complexes, suggesting that keratins can substitute for vimentin and bind to KRAP in MCF-7 cells.
Discussion

MDCK cells, derived from canine kidney, have been extensively used to study the establishment and maintenance of epithelial cell polarity (Dukes et al., 2011). After reaching confluence, they start to undergo a morphological conversion from a fibroblast-like to a well polarized-epithelial phenotype. This process is accompanied by a redistribution of InsP3R3 within the ER membrane, from a diffuse pattern to large isolated clusters which finally accumulate in the vicinity of TJ in fully differentiated cells (Colosetti et al., 2003). To gain insight into the molecular mechanisms underlying InsP3R trafficking in MDCK cells, we have generated stable cell lines expressing GFP-tagged versions of full-length or truncated InsP3R1 and we have characterized their phenotypes. We have demonstrated that both the clustering and perijunctional accumulation of InsP3R1-GFP, occurring during MDCK cell polarization, are critically dependent on an intact regulatory domain. Using co-immunoprecipitation assays combined with mass spectrometry, we have identified KRAP as a protein that co-purifies with InsP3R1-GFP but not with its regulatory domain-deleted mutant. Then, using DT40 cells expressing single InsP3R subtype, we have established that both InsP3R1 and InsP3R3 contain the molecular determinants required for KRAP binding.

While this work was in progress, it was shown that KRAP can be co-immunoprecipitated with multiple InsP3R subtypes from various normal mouse tissues (Fujimoto et al., 2011a), indicating that KRAP/InsP3R complexes also exist in vivo. The KRAP-binding site was mapped, by deletion analysis, to residues 1-610, and was conserved among all members of the InsP3R family. Intriguingly, despite the fact that it contains this domain, the ΔRD-GFP mutant does not interact with KRAP in MDCK cells. This discrepancy may be due to the fact that the binding site located in the N-terminal part of the InsP3R has in our construct either a modified conformation or accessibility to KRAP. However, as the host cells used by Fujimoto and colleagues for truncated receptor-expression were not intrinsically deficient in InsP3R, one cannot exclude that the published data reflect the ability of the deletion mutants to form heterotetramers with endogenous InsP3Rs rather than their direct interaction with KRAP.

Using the RNA interference technique, we have now demonstrated that KRAP is required for the peripheral accumulation of both InsP3R1-GFP and InsP3R3 in polarized MDCK cells. By contrast, in mouse pancreatic acinar cells, the localization patterns of InsP3R3 and InsP3R1 were found to be differentially sensitive to KRAP depletion since the pericanalicular accumulation of InsP3R3 was lost but that of InsP3R1 was preserved in pancreatic acinar cells from KRAP knockout mice (Fujimoto et al., 2011a). In addition, KRAP and InsP3R1 do not
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co-localize in all cell types. For instance, in mouse hepatocytes, InsP3R1 is relatively
uniformly distributed throughout the cytosol (Hirata et al., 2002; Shibao et al., 2003; Cruz et
al., 2010) whereas KRAP is exclusively detected in the subapical area (Fujimoto et al.,
2011a). Taken together, these results suggest that, in vivo, unknown factors may modulate
InsP3R1 localization by preventing its binding to KRAP. Interestingly, by searching the
Swiss-Prot data base using the blast algorithms, we have identified a protein called CCDC129
for “coiled coil domain containing-protein 129” which bears marked sequence homology to
KRAP. The regions corresponding to residues 171 to 256 (encompassing the putative InsP3R
binding site) and 953 to 1038 (forming the coiled coil domain) of KRAP (Fujimoto et al.,
2011b) are particularly well conserved in CCDC129, strongly suggesting that the two proteins
may fulfill similar functions. Further studies will be needed to determine whether KRAP and
CCDC129 exhibit a preference for one InsP3R subtype over another.

Using co-fractionation and co-immunoprecipitation experiments, we have shown that
KRAP/InsP3R complexes are linked to IF in different cell lines. The two proteins co-purify
with keratins in MCF-7 cells which are devoid of vimentin. In contrast, in MDCK cells,
which contain separate vimentin and keratin IF networks, KRAP and InsP3Rs bind vimentin
rather than keratins. Vimentin is a type III IF protein normally expressed in cells of
mesenchymal origin and absent from mature epithelial tissues (Thiery, 2002). However,
vimentin is detected during early stages of development in almost all primitive cell types
(including epithelial progenitors) and can reappear in epithelial cells involved in various
processes which require cell migration such as wound healing, tissue regeneration or tumor
invasion (Thiery, 2002; Savagner, 2010). The fact that KRAP/InsP3R complexes are able to
bind both vimentin and keratins may guarantee their attachment to IF whatever the
differentiation and physiological status of epithelial cells.

Finally, several lines of evidence support the notion that KRAP controls the subcellular
localization of InsP3Rs by connecting them to the IF network. First, knockdown of KRAP
expression in MDCK cells increases the Triton X-100-solubility of InsP3Rs, suggesting that
the latter are not linked to IF in the absence of KRAP. Second, KRAP still associates with
vimentin in DT40 TKO cells devoid of InsP3R. Third, the peripheral accumulation of InsP3Rs
in polarized MDCK cells requires intact vimentin IF and is impaired in cells expressing a
vimentin mutant still able to bind to KRAP but unable to assemble into filaments. Vimentin
IF are known to gradually redistribute during establishment of apical-basal polarity in MDCK
cells (Oriolo et al., 2007, Phua et al., 2009). Whereas they mainly emanate from the
perinuclear region and extend toward the cell periphery in subconfluent cells, vimentin IF
InsP$_3$R-KRAP interactions in MDCK cells

concentrate underneath the apical membrane and just below the apical cell-cell junctions in fully polarized cells. We propose that KRAP functions as an adapter which connects InsP$_3$Rs to adjacent vimentin filaments and that remodeling of the IF network, occurring during cell polarization, causes redistribution of InsP$_3$R/KRAP complexes to the perijunctional area. This model is consistent with a growing body of evidence suggesting that IF are not purely mechanical components of the cytoskeleton but also provide dynamic scaffolds for localization and long distance transport of signaling molecules within cells. For instance, vimentin has been shown to trigger the retrograde transport of the phosphorylated Erk mitogen-activated protein kinase in injured nerves (Perlson et al., 2005) whereas, in epithelial cells, keratins are required for ezrin delivery to the apical membrane (Wald et al., 2005) and proper localization of Albatross to the apical junctional complex (Sugimoto et al., 2008).

In conclusion, our data shed new light on the molecular mechanisms underlying the scaffolding properties of KRAP and enlarge its activity spectrum. Indeed, KRAP, in conjunction with IF, probably controls the subcellular localization of InsP$_3$Rs in differentiating, fully polarized or dedifferentiated epithelial cells but also in other cell types such as B lymphocytes. Further studies are required to determine whether the interaction between KRAP and IF is direct and how it is regulated in time and space.
Materials and Methods

Materials - Dulbecco's modified Eagle's medium (DMEM), opti-MEM medium, fetal calf serum (FCS), penicillin/streptomycin/fungizone, trypsin-EDTA, Geneticin (G418) and the ProLong Gold Antifade reagent were purchased from Invitrogen. RPMI medium 1640 was obtained from LONZA AG. Restriction endonucleases were purchased from Promega. GFP-trap (coupled to agarose beads) was purchased from ChromoTek. All other chemicals were of the highest grade available and were obtained from Sigma. Wild-type DT40 cells and DT40 triple InsP3R-knockout (TKO) cells were obtained through the courtesy of Tomohiro Kurosaki (Kansai Medical University, Kansai, Japan).

Antibodies - Primary monoclonal antibodies used in this study were mouse anti-cadherin (BD Biosciences), mouse anti-caveolin (BD biosciences), mouse anti-InsP3R3 (BD Biosciences), mouse anti-Protein Disulfide Isomerase (PDI) (Stressgen), mouse anti-GFP (Roche Applied Science), mouse anti-α-tubulin (Sigma-Aldrich), mouse anti-vimentin (clone V9, Sigma-Aldrich), mouse anti-pan keratins (clone PCK-26, Sigma-Aldrich), rabbit anti-vimentin (EP73776, Epitomics) and Rat anti-ZO-1 (kindly provided by Bruce Stevenson, University of Alberta, Edmonton, Canada). The polyclonal antibodies used were all raised in rabbit and directed against actin (Sigma-Aldrich), calnexin (Sigma-Aldrich), erlin-2 (Sigma-Aldrich), KRAP (Proteintech) and InsP3R1 (Parys et al., 1995). The rabbit polyclonal antibody recognizing the three InsP3R isoforms with equal affinity was developed and purified as previously described (Bultynck et al., 2004). Normal rabbit IgG were purchased from Sigma-Aldrich. Secondary antibodies used for immunofluorescence labeling were goat antibodies to rabbit, mouse or rat IgG conjugated to Alexa Fluor 488, 568 or 633 (Invitrogen). Secondary antibodies used for western blots were horseradish peroxidase (HRP)-conjugated goat antibodies to mouse or rabbit IgG (Amersham).

Plasmid constructs and Small Interfering RNAs (siRNAs) - The plasmid referred to as pInsP3R1-GFP and encoding the mouse type 1 InsP3R fused to the N-terminus of GFP was kindly provided by Richard E. Tunwell (Department of Physiology, University College London, London, United Kingdom). The plasmid encoding ΔRD-GFP was obtained as follows: the 2.1 kb restriction fragment obtained by digestion of pInsP3R1-GFP with SacI was subcloned into a pBluescript KS plasmid. The resulting construct was then cut by CelII and EcoICRI and the 1.7 kb digestion product was ligated to the 9 kb CelII/Eco47III restriction fragment excised from pInsP3R1-GFP. The integrity of the open reading frame was verified by restriction analysis and sequencing. The dominant-negative GFP-vimentin(1–138) construct was a generous gift from Eva Kotula (Institut Curie, Orsay, France).
The siRNAs duplexes targeted to canine KRAP, canine InsP₃R3 and canine vimentin were designed based on NCBI accession XM_535986, XM_538867.2 and XM_535175.2 respectively (Table S2) and chemically synthesized by Eurogentec. Non-targeting control siRNA was obtained from the same vendor.

**Cell culture and transfections** - MDCK cells were cultured in DMEM supplemented with 5% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin sulfate and 0.25 µg/ml fungizone at 37°C in a humidity-controlled incubator with 7% CO₂. Transfection of plasmid DNA into MDCK cells was performed by the calcium phosphate coprecipitation method as previously described (Hours and Mery, 2010). Approximately 16 hours after transfection, cells were washed twice with Ca²⁺/Mg²⁺-free phosphate-buffered saline (PBS) and placed in fresh growth medium containing 600 µg/ml of active G418. Three weeks later, G418-resistant cells were harvested by trypsinisation and those exhibiting a marked green fluorescence were selected by flow cytometry (FACS Vantage, Becton Dickinson) and expanded. Transient transfections of siRNA duplexes were carried out by using the Lipofectamine™ RNAiMAX reagent (Invitrogen) according to manufacturer’s protocol. For western blots and immunofluorescence assays, cells (10⁴/well) were plated in 24-well plates containing glass coverslips and grown for 4 days in culture medium without antibiotics before transfection. For membrane preparation, trypsinized cells were resuspended in growth medium without antibiotics and seeded at 2.5x10⁵/well into 6-well plates 24 hours before transfection. The siRNA duplex-Lipofectamine™ RNAiMAX complexes were prepared in optiMEM medium (Invitrogen). siRNA duplexes were used at a final concentration of 20 nM and 1.5 µl or 7.5 µl (depending on well size) of Lipofectamine™ RNAiMAX were added per well. The culture medium was changed 24 hours post transfection and cells were used for experiments 48 hours later.

DT40 TKO cells were grown in RPMI medium 1640 supplemented with 10% FCS, 1% chicken serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin sulfate, 0.25 µg/ml fungizone and 50 µM β-mercaptoethanol in a 5% CO₂ incubator at 37°C. Plasmid DNA (5 µg) was transiently transfected into 2x10⁶ DT40-TKO cells using an Amaxa Nucleofector (Solution T, program B-23) according to the manufacturer's protocol. The human breast adenocarcinoma cell line MCF-7 was cultured in DMEM supplemented with 10% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin sulfate, 0.1 mM non essential amino acids and 1 mM sodium pyruvate, in a 5% CO₂ incubator at 37°C.
**Immunofluorescence** - Cells, attached to glass coverslips, were rinsed twice with PBS pH 7.4, fixed at room temperature with 4% paraformaldehyde in PBS for 20 minutes and then permeabilized using 0.5% Triton X-100 in PBS for 5 minutes. For staining with anti-vimentin or anti-keratins antibodies, adherent cells were first incubated for 2 minutes at 4°C with 4% formaldehyde in PBS (to prevent cell shrinkage) and then fixed/permeabilized in ice-cold methanol for 10 minutes. Blockade of non-specific binding sites was performed by incubating the cells with PBS containing 0.2% gelatin for 30 minutes. Fixed cells were stained for 1 hour with the primary antibody, washed three times with the blocking solution and then incubated for 1 hour with a 1:300 dilution of the appropriate secondary antibody. All antibody dilutions were prepared in PBS supplemented with 0.2% gelatin and incubations were carried out at room temperature. After extensive washing with PBS, coverslips were mounted using ProLong Gold Antifade reagent and examined with a confocal microscope (Eclipse TE-2000-Nikon-C1, France) equipped with a 63X plan-apochromatic oil-immersion objective (NA = 1.4) and air-cooled Argon and He–Ne lasers. Optical sections were collected at 0.2 μm intervals and images were processed using Photoshop 7 (Adobe) software. Image analysis was performed with ImageJ.

**Cell fractionation** – The Triton X-100-soluble and insoluble fractions were prepared as previously described (Hours and Mery, 2010). The Triton X-100-insoluble material was solubilized in ice-cold buffer A (PBS supplemented with 0.5% SDS, 2 mM EDTA and protease inhibitors) or buffer B (containing 50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% Sodium deoxycholate, 0.1% SDS and protease inhibitors) and then gently sonicated on ice for 10 s. Protein concentrations were determined with the Bio Rad DC kit using bovine serum albumin as standard. Proteins were distributed between the three fractions approximately as follows – hydro-soluble (HS) 70%, Triton X-100-soluble (TS) 10%, Triton X-100-insoluble (TI) 20%. Polyacrylamide gels were loaded with equal amounts (30 μg) of protein.

The membrane and cytoskeletal (M+C) fraction was obtained as follow: cells were washed twice with PBS at 4°C and then harvested in ice-cold hypotonic buffer containing: 50 mM Tris.HCl pH 7.4, 10 mM KCl, 2 mM EDTA and complete protease inhibitors (Roche). The resulting mixture was subjected to three cycles of rapid freezing and thawing to lyse the cells and passed 10 times through a 21-gauge needle to shear DNA. Normal osmolarity was then restored by adding 150 mM NaCl. Cell lysates were centrifuged for 1 hour at 100,000 g and 4°C in a SW65 rotor (Beckman Coulter). The supernatant, representing the hydro-soluble
fraction, was removed and stored at -20°C. The pellet was resuspended in ice-cold buffer B and the resulting mixture was passed 10 times through a 25-gauge needle.

**Immunoprecipitation assays** - MDCK cells were rinsed twice with ice-cold PBS pH 7.4 and then harvested in a buffer consisting of 20 mM Tris.Cl pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100 and complete protease inhibitors. After removal of cellular debris by centrifugation for 10 minutes at 10,000 g and 4°C, the cell extracts were pre-incubated with protein G-sepharose beads for 2h. Appropriate antibodies were applied to pre-cleared lysates for 16h and immune complexes were then precipitated with protein G-sepharose beads for 2 hours. Alternatively, cell lysates or membrane fractions solubilized in buffer B were pre-cleared with agarose beads for 16h at 4°C and then GFP-trap coupled to agarose beads was added for 90 minutes. All the incubations were performed at 4°C on a rotating wheel. The beads were then recovered by centrifugation at 600 g for 3 min and washed five times with either the lysis buffer or buffer B. The immunoprecipitates were eluted by boiling the samples in 100 μl 2X Laemmli buffer for 5 minutes and then fractionated by SDS-PAGE.

**SDS-PAGE and immunoblotting** - Proteins were fractionated on a SDS-PAGE polyacrylamide gel and transferred to Immobilon-P membranes (Millipore) in 25 mM Tris.HCl, 0.19 M glycine and 20% ethanol. The polyvinylidene difluoride membranes were blocked overnight at 4°C in PBS containing 5% non fat dry milk and 0.05% Tween 20, rinsed twice with water and then incubated for 2 hours with the primary antibody. After three washes with PBS/0.05% Tween 20, the membrane was allowed to react for 1 h with a 1:4000 dilution of the appropriate HRP-conjugated secondary antibody. The blots were washed three times with PBS/0.05% Tween 20 and the immune complexes were visualized by chemiluminescence (ECL Western blotting analysis system, Amersham). All antibody dilutions were prepared in PBS supplemented with 0.05% Tween 20 and incubations were carried out at room temperature.

**MS Analysis.** GFP-trap precipitates prepared from confluent, incompletely polarized MDCK cells were resolved on 4–12% SDS/polyacrylamide gels (Invitrogen). After staining with colloidal Coomassie blue (G250, Bio-Rad), each lane was excised into 10 to 12 equivalent gel slices (regardless of the Coomassie staining profile) in order to analyze the entire protein constituents of the sample. Each slice was then cut into 1 x 1 mm pieces, reduced and alkylated by using DTT and iodoacetamide, respectively, and subjected to digestion with trypsin (Sigma) as previously described (Fevrier et al., 2004). Extracted peptides were dried and solubilized in solvent A (5% acetonitrile, 0.1% formic acid).
Liquid chromatography-MS/MS analysis - Samples were loaded on a C18 precolumn (Dionex) and after 3 min of desalting, the precolumn was switched on line with the analytical C18 column (C18 PepMap™, Dionex) equilibrated in 95% solvent A and 5% solvent B (80% acetonitrile, 0.085% formic acid). Bound peptides were eluted using a 5 to 50% gradient of solvent B during 60 min at a 200 nl/min flow rate. Data-dependent acquisition was performed on the LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific) in the positive ion mode. Survey MS scans were acquired in the orbitrap on the 475-1200 m/z range with the resolution set to a value of 60 000. Each scan was recalibrated in real time by co-injecting an internal standard from ambient air into the C-trap. The five most intense ions per survey scan were selected for CID fragmentation and the resulting fragments were analyzed in the linear trap (LTQ). Target ions already selected for MS/MS were dynamically excluded for 180 s. Data were acquired using the Xcalibur software (version 2.0.7) and the resulting spectra were then analyzed via the Mascot™ Software using the NCBI nr *Canis lupus familiaris* database. All data were manually validated.
Acknowledgments:

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References


**Figure legends**

**Figure 1:** Subcellular localizations of InsP$_3$R1-GFP and ΔRD-GFP in MDCK cells of increasing confluence. 3, 5 or 10 days after plating, MDCK cells expressing either InsP$_3$R1-GFP or ΔRD-GFP were fixed, permeabilized, stained for PDI, InsP$_3$R3, E-Cadherin or ZO-1 (as indicated) and imaged by confocal microscopy. Top panels show representative individual x-y confocal sections. Bottom panels represent either enlarged views of the regions covered by squares (A-C) or x-z projections of confocal stacks (D). Scale bar, 10 μm. (x-z), 7.5 μm.

**Figure 2:** KRAP binds to InsP$_3$R1-GFP and InsP$_3$R3 but not to the deletion mutant. (A,B) Total cell lysates prepared from confluent MDCK cells expressing InsP$_3$R1-GFP or ΔRD-GFP were incubated with GFP-Trap beads. The immunoprecipitated proteins were fractionated by SDS-PAGE, blotted and analyzed for GFP, InsP$_3$R3 or KRAP. (C) Confluent or sparse wild-type MDCK cells were lysed. InsP$_3$R3 was immunoprecipitated and associated KRAP was detected by western blot. (D,E) Protein extracts, prepared from wild-type (wt) DT40 cells or from DT40 triple InsP$_3$ receptor-knockout (TKO) cells, were subjected to SDS-PAGE and immunoblotted using anti-InsP$_3$R1, anti-InsP$_3$R3 or anti-KRAP antibodies. Bands corresponding to full length or truncated forms of InsP$_3$Rs are identified. (F) InsP$_3$R1-GFP was expressed in DT40 TKO cells and precipitated from total cell extracts using GFP-trap beads. The input and bead-bound proteins were separated by SDS-PAGE and analyzed by western blot. The blot membrane displayed in the middle panel was probed with anti-GFP antibodies and then, after stripping, with anti-InsP$_3$R3 antibodies. Non-transfected DT40 TKO cells were used as negative controls. The results shown are representative of two to four independent experiments. IP: immunoprecipitation; WB: western blot.

**Figure 3:** siRNA-mediated knockdown of KRAP and its effect on InsP$_3$R protein level and localization. MDCK cells expressing InsP$_3$R1-GFP were transfected, using lipofectamine, with different KRAP-targeting siRNAs (referred to as K1, K2 and K3) or with a nonsense duplex (negative control). Cells were processed for immunoblot or immunofluorescence analysis 72h post-transfection. (A) Representative immunoblots showing expression of KRAP, InsP$_3$R1-GFP, InsP$_3$R3 or calnexin (which served as a loading control) in total cell extracts prepared from the siRNA-treated cells. (B) Densitometric analysis of western blots performed with NIH image J software. Data are presented as the means ± s.d of results from three independent experiments. (C) Individual x-y confocal sections showing the distributions of KRAP and InsP$_3$R1-GFP in the siRNA-treated cells.
“Zooms” are magnifications of the boxed areas in the adjacent images. Scale bar, 10 μm. The data shown in C are representative of three independent experiments.

**Figure 4: KRAP-binding renders InsP₃Rs insoluble in Triton X-100.** (A,B) Confluent MDCK cells expressing InsP₃R1-GFP or ΔRD-GFP were lysed by osmotic shock and sequentially extracted with Triton X-100 and SDS to partition cellular proteins into hydro-soluble (HS), Triton X-100-soluble (TS) and Triton X-100-insoluble (TI) fractions. Aliquots of the TS and TI fractions (30 μg of protein) were then resolved by SDS-PAGE, blotted and probed with antibodies recognizing GFP, InsP₃R3, KRAP, actin, vimentin or keratins. (A) Immunoblots representative of the relative distribution of full length and truncated InsP₃Rs between the TS and the TI fractions in confluent cells. (B) Quantitative densitometric analysis of western blots from two independent experiments performed with NIH image J software. The results shown are mean ± s.d. and take into account both the densitometric value obtained from the immunostaining and the percentage of each fraction loaded. (C,D) TS and TI fractions were prepared from InsP₃R1-GFP-expressing MDCK cells transfected, 72h prior to lysis, with a KRAP-specific siRNA (K2 or K3) or with a control duplex (NC). Samples representing equivalent amounts of proteins from each fraction were subjected to SDS-PAGE, blotted and analyzed for GFP, InsP₃R3, KRAP, actin and vimentin. (C) Immunoblots showing the effect of KRAP depletion on the distribution of InsP₃Rs between the TS and TI fractions. (D) Quantitative densitometric analysis of western blots from two independent experiments carried out with NIH image J software. The results shown are mean ± s.d. WB: western blot.

**Figure 5: InsP₃R/KRAP complexes are linked to vimentin intermediate filaments.** (A,B) Triton X-100-insoluble fractions, isolated from confluent parental MDCK cells (NT) or from cells expressing either InsP₃R1-GFP or ΔRD-GFP, were resuspended in buffer B (containing 1% Triton X-100, 0.5% sodium deoxycholate and 0.1% SDS) and incubated with GFP-Trap beads. The input and precipitated proteins were then fractionated by SDS-PAGE, blotted and analyzed with anti-GFP, anti-KRAP, anti-actin, anti-vimentin or anti-keratins antibodies. (C) DT40 TKO cells were lysed in buffer B. Whole-cell lysates were immunoprecipitated with anti-KRAP or control (rabbit IgG) antibodies coupled to protein G-sepharose beads. Western blot analysis was performed using anti-KRAP, anti-vimentin and anti-InsP₃R3 antibodies. All the immunoblots shown are representative of three independent experiments. WB: western blot.

**Figure 6: Effects of vimentin knockdown on InsP₃R expression, localization and detergent-solubility.** MDCK cells expressing InsP₃R1-GFP were transfected, using
lipofectamine, with different vimentin-targeting siRNAs (referred to as V1, V2 and V3) or with a nonsense duplex (negative control). Cells were processed for immunoblot analysis, immunofluorescence microscopy or subcellular fractionation 72 h post-transfection. (A) Representative immunoblots showing expression of vimentin, actin (which served as a loading control), InsP₃R1-GFP, InsP₃R3 or KRAP in total cell extracts prepared from the siRNA-treated cells. (B) Densitometric analysis of western blots performed with NIH image J software. Data are presented as the means ± s.d. of results from three independent experiments. (C) Representative x-y projections of stacks of 40 images collected at 0.2 μm intervals along the z-axis, showing the distribution of InsP₃R1-GFP (green), KRAP (grey) and vimentin (red) in cells treated with the indicated siRNAs. scale bar, 10 μm (n=3). (D) Fluorescence intensity profiles of InsP₃R1-GFP, KRAP and vimentin along the lines indicated in C and obtained from individual x-y sections. (E) Immunoblots representative of the relative distribution of KRAP, InsP₃R1-GFP and InsP₃R3 between the Triton X-100-soluble (TS) and the Triton X-100-insoluble (TI) fractions in the siRNA-treated cells. (F) Quantitative densitometric analysis of western blots from two to four independent experiments performed with NIH image J software. The results shown are mean ± s.d. WB: western blot.

**Figure 7: Stable expression of a dominant negative vimentin mutant decreases peripheral accumulation InsP₃R3 in polarized MDCK cells.** (A) 7 or 10 days after plating, MDCK cells expressing GFP or the VimDN-GFP mutant were fixed, permeabilized, doubled-stained for vimentin and InsP₃R3 and then imaged by confocal microscopy. The rabbit anti-vimentin monoclonal antibody (clone EPR3776) that was used recognizes canine vimentin but does not react with the VimDN-GFP mutant. Representative x-y projections and x-z of stacks of 33 images collected at 0.2 μm intervals along the z-axis are presented. Dotted lines highlight the basal membrane. Scale bar, 10 μm. (C) Confluent MDCK cells expressing GFP or VimDN-GFP were lysed by osmotic shock followed by three cycles of rapid freezing and thawing. Membrane and cytoskeletal proteins (M+C) were pelleted by centrifugation at 100,000 g and solubilized in buffer B containing 1% Triton X-100, 0.5% sodium deoxycholate and 0.1% SDS. Hydro-soluble (HS) proteins were recovered in the 100,000 g supernatant. Aliquots of the HS and M+C fractions (30 μg/lane) were analyzed by immunoblotting using anti-GFP antibodies. The data presented are representative of two independent experiments. (D) (M+C) fractions, isolated from confluent MDCK cells expressing GFP or VimDN-GFP were solubilized in buffer B and incubated with GFP-Trap beads. The input and precipitated proteins were then fractionated by SDS-PAGE, blotted and
analyzed with anti-GFP, anti-KRAP and anti-InsP₃R3 antibodies. The immunoblots shown are representative of three independent experiments. WB: western blot.

**Figure 8: KRAP and InsP₃Rs co-purify with keratin 8 in MCF-7 cells.** (A) Representative immunoblots showing expression of vimentin and keratin 8 in (M+C) fractions isolated, as described in the legend to figure 7, from either confluent MDCK cells or MCF-7 cells. (B) Nomarski and corresponding confocal images of MCF-7 cells immunostained for vimentin or keratins are shown. (C) (M+C) fractions, isolated from MCF-7 cells, were solubilized in a buffer containing 1% Triton X-100, 0.5% sodium deoxycholate and 0.1% SDS and incubated with anti-KRAP or control antibodies coupled to protein G-sepharose. The input and precipitated proteins were then fractionated by SDS-PAGE, blotted and analyzed with anti-KRAP, anti-pan InsP₃R and anti-pan keratin antibodies. The immunoblots shown are representative of two independent experiments.
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