CASK deletion in intestinal epithelia causes mislocalization of LIN7C and the DLG1/Scrib polarity complex without affecting cell polarity

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Running Head: CASK localizes DLG1/LIN7C in enterocytes

Abbreviations: MAGUK, EGFR, APC, MDCK, KID
Abstract

CASK is the mammalian ortholog of LIN2, a component of the LIN2/7/10 protein complex that targets epidermal growth factor receptor (EGFR) to basolateral membranes in *C. elegans*. A member of the MAGUK family of scaffolding proteins, CASK resides at basolateral membranes in polarized epithelia. Its interaction with LIN7 is evolutionarily conserved. In addition, CASK forms a complex with another MAGUK, the DLG1 tumor suppressor. Although complete knockout of *CASK* is lethal, the gene is X-linked, enabling us to generate heterozygous female adults that are mosaic for its expression. We also generated intestine-specific *CASK* knockout mice. Immunofluorescence analysis revealed that in intestine, CASK is not required for epithelial polarity or differentiation but is necessary for the basolateral localization of DLG1 and LIN7C. However, the subcellular distributions of DLG1 and LIN7C are independent of CASK in the stomach. Moreover, CASK and LIN7C show normal localization in *dlg1−/−* intestine. Despite the disappearance of basolateral LIN7C in CASK-deficient intestinal crypts, this epithelium retains normal localization of LIN7A/B, EGFR and ErbB-2. Finally, crypt to villus migration rates are unchanged in CASK-deficient intestinal epithelium. Thus, CASK expression and the appropriate localization of DLG1 are not essential for either epithelial polarity or intestinal homeostasis *in vivo*. 
Introduction

Membrane-associated guanylate kinases (MAGUKs) are cytoplasmic scaffolding proteins that organize macromolecular complexes at specialized regions of the plasma membrane (Funke et al., 2005). In recent years, it has been increasingly recognized that MAGUKs play critical roles in the establishment of intercellular junctions and the maintenance of cell polarity (Caruana, 2002). CASK is the mammalian ortholog of LIN2, a *C. elegans* MAGUK that was first identified as one of a group of three interacting proteins (LIN2, LIN7 and LIN10) required for basolateral localization of the epidermal growth factor receptor (EGFR) in certain polarized epithelial cells (Kaech et al., 1998). In the absence of the LIN2/7/10 protein complex, the *C. elegans* EGFR is mislocalized to the apical membrane, implying that this tripartite complex functions in basolateral protein targeting.

All three members of the LIN2/7/10 complex are evolutionarily conserved, and they are expressed by and associate within mammalian neurons (Borg et al., 1998; Butz et al., 1998). Although mammalian epithelial cells do not express the ortholog of LIN10, they do express both CASK and LIN7. (There are three closely related LIN7 isoforms in mammals, designated LIN7A,B,C/Velis-1,2,3/MALS-1,2,3; the one most widely expressed outside the nervous system is LIN7C (Butz et al., 1998; Irie et al., 1999; Jo et al., 1999).) The shared domain that mediates the CASK-LIN7 interaction is highly conserved (Doerks et al., 2000), and these two proteins can be co-immunoprecipitated from the Madin-Darby Canine Kidney (MDCK) epithelial cell line (Straight et al., 2000; Stetak et al., 2006). Evidence from multiple studies suggests that LIN7 functions in the basolateral targeting of membrane proteins in mammalian epithelial cells (Perego et al., 1999; Straight et al., 2001; Alewine et al., 2007). Furthermore, LIN7 appears to play a special role in regulating the mammalian EGFR family members (the ErbB receptors) by
promoting both their trafficking through the biosynthetic pathway and their stability at the basolateral plasma membrane (Shelly et al., 2003).

We showed previously that in human intestinal epithelial cells, CASK is basolaterally localized (Cohen et al., 1998) and complexed with another MAGUK, discs-large homolog 1 (DLG1) (Nix et al., 2000). Null mutations of the discs-large (Dlg) gene in Drosophila lead to loss of polarity, disorganization and marked overgrowth of imaginal disc epithelial cells during development (Stewart et al., 1972). Genetic studies have defined two other such “neoplastic tumor suppressor” genes with similar mutant phenotypes; these genes act in concert with Dlg to regulate embryonic epithelial polarity and proliferation (reviewed in (Bilder, 2004)). There is much evidence that mammalian DLG1 is also a tumor suppressor: it binds to the adenomatous polyposis coli (APC) protein to form a complex that regulates cell cycle progression (Ishidate et al., 2000), it is a critical target of several viral oncoproteins (Lee et al., 1997; Grassmann et al., 2005; Thomas et al., 2005), its decreased expression in human cervical dysplastic lesions correlates with malignant progression (Watson et al., 2002; Cavatorta et al., 2004), and it is often genetically altered in breast cancer (Fuja et al., 2004).

A murine CASK mutant generated by insertional mutagenesis (Laverty and Wilson, 1998) and CASK knockout mice produced via Cre-Lox technology (Atasoy et al., 2007) both exhibit cleft palate and die within twenty-four hours after birth. In order to study the function(s) of CASK in adult epithelia in vivo, we took advantage of the fact that the CASK gene is located on the X chromosome and is therefore transcriptionally silenced by X-inactivation during embryogenesis in females. We show here that female mice heterozygous for the null allele of CASK are viable and healthy but are mosaic for CASK expression in all tissues examined. CASK-deficient epithelial cells are histologically indistinguishable from their CASK-expressing
neighbors but show tissue-specific defects in the subcellular localization of LIN7C and DLG1. Surprisingly, the absence of CASK has no effect on the basolateral localization of either EGFR or ErbB-2 in the intestinal epithelium.
Materials and Methods

Mice. CASKflox mice were the generous gift of Dr. Thomas Südhof (Stanford University). Prm-Cre (129-Tg(Prm-cre)58Og/J, stock #003328) and Vil-Cre (B6.SJL-Tg(Vil-cre)997Gum/J, stock #004586) mouse strains were obtained from the Jackson Laboratory (Bar Harbor, Maine). Genotyping was performed by PCR amplification of CASK and Cre sequences from genomic DNA of tail samples, as described (Saam and Gordon, 1999; Atasoy et al., 2007). Murine epidermal growth factor (EGF; #354001) was obtained from BD Biosciences (Bedford, MA) and was reconstituted in sterile phosphate-buffered saline (PBS) at 100 μg/ml immediately before use. For EGF experiments, each mouse received 25μg of the growth factor or an equal volume of PBS by retro-orbital intravenous injection and was sacrificed 5 to 20 minutes later. For cell proliferation and migration studies, 1-1.25 mg bromodeoxyuridine (#550891, BD Pharmingen) per mouse was injected intraperitoneally, and animals were sacrificed at 1 or 24 hours thereafter. All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee at Yale University.

Antibodies. Rabbit polyclonal anti-CASK antibodies used in this study were those generated previously (Cohen et al., 1998) as well as those from Zymed/Invitrogen (#71-5000). Monoclonal anti-CASK antibodies were from Chemicon/Millipore (#MAB5230). Anti-DLG1 (#610874), anti-E-cadherin (#610181), anti-β-catenin (#610153), anti-BrdU (#555627) and anti-syndecan-1 (#553712) were from BD Biosciences Pharmingen; anti-LIN7 (recognizing all three isoforms; #184 003) was from Synaptic Systems; anti-LIN7C/Velis-3 (#51-5600), anti-ZO-1 (#33-9100), anti-occludin (#71-1500) and anti-claudin-5 (#34-1600) were from Zymed/Invitrogen; goat anti-scribble (#sc-11048), mouse anti-PKCζ (#sc-17781) and rabbit anti-integrin β1 (#sc-8978) were
from Santa Cruz Biotechnology, Inc.; rabbit anti-human chromogranin A and anti-human lysozyme were from Dako; anti-villin-1 (#2369), anti-phospho-EGFR (Tyr1173; #4407) and anti-ErbB-2 (#2242) were from Cell Signaling Technology, Inc.; monoclonal anti-actin was from Sigma (#A4700); the anti-EGFR used for immunoblotting was from Upstate/Millipore (#06-847). The anti-Na⁺,K⁺-ATPase used was mouse monoclonal antibody “α5” (Takeyasu et al., 1988), and rabbit anti-αII-spectrin was the affinity-purified “RAF-A” antibody (Harris et al., 1986). Secondary antibodies used in Western blots were horseradish peroxidase conjugates (Sigma and Chemicon). Secondary antibodies used for immunofluorescence were Alexa fluor 488 and 568 conjugates (Molecular Probes/Invitrogen).

**Immunofluorescence.** Tissues were fixed in 4% paraformaldehyde or periodate-lysine-paraformaldehyde (McLean and Nakane, 1974) overnight at 4°C before embedding in paraffin. Antigen retrieval was performed using a pressure-cooker containing citrate or EDTA buffer. Slides were pre-incubated with blocking buffer (5% goat serum, 1% bovine serum albumin in Tris-buffered saline (TBS)) for 1-2 hours at room temperature. Primary antibodies were diluted in blocking buffer and incubated with sections overnight in a humidified chamber at 4°C. Slides were then washed five times in TBS, once in TBS/0.01% Triton X-100, and once again in TBS (3 minutes per wash). Secondary antibodies were diluted in blocking buffer and incubated with sections for one hour at room temperature in the dark. After washing in TBS as before, slides were coverslipped with a 4,6-diamidino-2-phenylindole (DAPI)-containing, antifade mounting medium (ProLong Gold, Molecular Probes/Invitrogen). Sections were viewed on Zeiss Axioskop and Olympus IX70 fluorescence microscopes, and photomicrographs were obtained using
CytoVision (Applied Imaging/Genetix) and Spot (Diagnostic Instruments, Inc.) digital imaging systems.

**Intestinal mucosal preparation.** Wild type and Vil-Cre-CASK KO animals were sacrificed, and colons were immediately removed, placed on an ice-cold surface and opened longitudinally with dissecting scissors. Feces were discarded, and the mucosa was collected by scraping with a glass slide. Mucosal samples were homogenized in ten volumes of 2X protein sample buffer, using a glass homogenizer with a motor-driven Teflon pestle.
Results

Generation of CASK mosaic mice

Because the CASK gene is X-linked, females heterozygous for a null allele are predicted to be mosaic for CASK expression. We obtained transgenic mice bearing a CASK allele (CASKflox) in which the first coding exon is flanked by loxP recombination sites (Atasoy et al., 2007). These mice were mated with a transgenic strain (Prm-Cre) in which Cre recombinase is expressed exclusively in the male germline. Male progeny bearing both the CASKflox and Prm-Cre alleles were then crossed with wild type females to produce $\text{CASK}^{+/\text{-}}$ female progeny. These CASK heterozygotes are viable, healthy and phenotypically indistinguishable from wild type females. Genotypes were confirmed by PCR amplification from genomic DNA of the wild type and recombined (knockout) alleles of CASK. No morphologic abnormalities were found at necropsy, and microscopic surveys of all tissues revealed normal histology.

In order to demonstrate the mosaicism of CASK expression in $\text{CASK}^{+/\text{-}}$ mice, we examined multiple tissues by immunofluorescence microscopy. Female CASK heterozygotes show patchy CASK expression in epithelia of the stomach (Figure 1A), small and large intestine (Figure 1B and C), pancreas and kidney, in mosaic patterns consistent with X-inactivation (Griffiths et al., 1988; Novelli et al., 2003). The tight junction localization of ZO-1 appears intact in all of these tissues, both within CASK-expressing and CASK-deficient epithelial cells.

CASK deficiency does not alter intestinal epithelial cell polarity or differentiation

Since CASK is thought to function in the organization and maintenance of the basolateral membrane in polarized epithelial cells, we next sought to determine whether CASK deficiency is associated with an altered distribution of basolateral marker proteins. The adherens junction
components, β-catenin (Figure 1D) and E-cadherin (not shown) appear localized along the lateral plasma membranes of intestinal epithelial cells, whether or not CASK is present. Similarly, basolateral expression of the extracellular matrix receptor, integrin β1 (Figure 2E), the Na⁺,K⁺-ATPase (Figure S1A) and the heparan sulfate proteoglycan, syndecan-1 (Figure S1B) are intact in CASK-deficient intestinal epithelial cells. The cytoskeletal protein, αII-spectrin localizes to the cortical actin network at the basolateral membrane and to the terminal web underlying the apical brush border in small intestinal epithelia; this distribution is unaltered in CASK-deficient cells (Figure 2F).

In addition to the tight junction-associated MAGUK, ZO-1, the integral membrane tight junction components, occludin and claudin-5 were examined in CASK mosaic intestine and were found to show the same subcellular distribution in normal and CASK-deficient epithelial cells (Figure 2A, B). As expected, these proteins are concentrated at tight junctions, but they are also detected at lower levels all along the lateral plasma membrane. The atypical protein kinase C, PKCζ is associated with tight junctions as well as the apical plasma membrane (Figure 2C), and villin localizes exclusively to the apical brush border (Figure 2D); these localization patterns are preserved in CASK-deficient cells.

No abnormalities of differentiation were observed in CASK-deficient intestinal epithelium. Goblet cells and enterocytes, both identifiable by cell shape in immunofluorescently labeled sections, are present in roughly the same proportions in CASK-deficient epithelium as in the surrounding wild type epithelium. Paneth cells, labeled by anti-lysozyme immunofluorescence, are present at the bottoms of CASK-deficient crypts (Figure 1E), and enteroendocrine cells, identified by anti-chromogranin A immunostaining, are scattered throughout CASK-deficient, as well as normal villus epithelium (Figure 1F).
Basolateral polarity proteins are mislocalized in CASK-deficient intestinal epithelium

Immunofluorescence double-labeling of CASK mosaic small and large intestine revealed that while wild type epithelial cells show coincident basolateral membrane staining for CASK and DLG1, CASK-deficient cells have no detectable basolaterally-localized DLG1 (Figure 3A-F). To rule out an immunofluorescence artifact (e.g., “bleedthrough” of one color into another wavelength emission channel), we did single-labeling experiments and found that patches of epithelium lacking basolateral DLG1 were easily identifiable, even when no anti-CASK antibody had been applied to the tissue (Figure S1C). Moreover Scribble (Scrib), the mammalian ortholog of a Drosophila neoplastic tumor suppressor and a member of the highly conserved group of proteins (the Lgl/Dlg/Scrib polarity complex) that control membrane polarity, also appears absent from the basolateral membrane in CASK-deficient intestinal epithelia (Figure 3G-I).

The CASK-LIN7 interaction, originally identified in C. elegans, is conserved in mammalian neurons (Butz et al., 1998) and in renal epithelium (Straight et al., 2000). We examined the localization of LIN7C in CASK mosaic intestine and found that, like DLG1, LIN7C appears absent from the basolateral membranes of CASK-deficient epithelial cells (Figure 4). In wild type cells, two populations of LIN7C protein are observed, one basolateral and the other tight junctional; CASK-deficient cells show only junctional LIN7C staining (Figure 4B, D).

Immunostaining of CASK mosaic intestine with an antibody that recognizes all three isoforms of LIN7 showed somewhat different results. In fully mature cells of the small intestinal villi, basolateral accumulation of LIN7 is seen only in wild type cells and is absent in CASK-deficient cells (Figure 5A - C). However, in small and large intestinal crypts, LIN7 can be
detected at the basolateral membranes of both wild type and CASK-deficient cells, although its staining intensity is greatest in the former (Figure 5D - I). As CASK-deficient cells travel up the crypt-villus axis in the small bowel, their basolateral LIN7 staining intensity is progressively diminished until it becomes undetectable in the top halves of the villi. Tight junctional LIN7 staining remains intact throughout, even in the absence of CASK.

We next wanted to determine whether the disappearance of selected proteins from the basolateral membranes of CASK-deficient cells is associated with loss of those proteins or whether they are still present but in a diffuse intracellular distribution that is difficult to detect above background fluorescence levels. Because only a small percentage of the total intestinal epithelium is CASK-deficient in our mosaic animals, they are not suitable for quantitative analysis. We therefore generated intestine-specific CASK knockout mice in which the entire intestinal epithelium is genetically null for CASK. This was accomplished by crossing CASKflox mice with a transgenic strain that carries the Cre recombinase gene under the control of a villin promoter (Vil-Cre) (Madison et al., 2002). Male progeny that inherit both the CASKflox allele and the Vil-Cre transgene are viable and healthy but show a complete absence of CASK throughout the intestine on immunofluorescence analysis (Figure S2A, B). PCR performed on genomic DNA isolated from the intestinal mucosa of these mice confirmed the presence of the recombined CASK allele (CASK-ko). No basolateral DLG1 or LIN7C could be identified in the small or large intestinal epithelium by immunofluorescence (Figure S2).

Western blots of colonic mucosa from Vil-Cre CASK knockout mice and wild type littermate controls revealed that although there is no CASK protein in the knockout intestine, DLG1, Scribble and LIN7C are all easily detectable and are present at levels comparable to those in wild type mucosa (Figure 6). Thus, the disappearance of these proteins from the basolateral
membranes of CASK-deficient cells by immunofluorescence is not due to their degradation or down-regulated expression, but rather indicates mislocalization.

**CASK deficiency does not alter intestinal epithelial homeostasis**

The intestinal epithelium of adult mammals undergoes continuous self-renewal in which stem cells at the bottoms of the crypts of Lieberkühn give rise to progenitor cells that proliferate and differentiate as they move up the crypt-surface/villus axis, eventually to be shed into the intestinal lumen (van der Flier and Clevers, 2008). This homeostatic process takes place in a highly organized spatio-temporal pattern and offers an excellent system in which to study epithelial proliferation and migration.

In females, X chromosome inactivation occurs early in development, before morphogenesis of the crypt-villus architecture, and X-inactivation patches in the adult intestine typically contain multiple adjacent crypt-villus units (Griffiths *et al.*, 1988). As expected, crypts in CASK mosaic mice are almost invariably monophenotypic in terms of CASK expression, whereas villi are often biphenotypic, showing longitudinal stripes of wild type cells alongside stripes of CASK-deficient cells originating from different crypts at the base of one villus (for examples, see Figures 1, 4 and 7). However, we found fewer CASK-deficient crypts, overall, than anticipated. Because X chromosomes are chosen for inactivation at random in embryonic cells, we predicted that maternal X and paternal X-expressing patches in an adult tissue would each comprise approximately 50% of the epithelium. Immunofluorescence analysis of our mosaic animals, however, revealed that considerably fewer than half of their intestinal crypts are CASK-deficient. Quantitation in seven mosaic mice (age ≤ 4 weeks) showed an average of 15% CASK-deficient crypts (range 8-21%).
Proliferation of the intestinal epithelium in CASK<sup>+/−</sup> mosaic mice was examined first by immunolocalization of the proliferating cell nuclear antigen (PCNA) in small intestine (Figure S3). Cycling cells are restricted to crypts throughout, and there is no apparent difference between normal and CASK-deficient epithelium in either the location of the proliferative zone or the number of PCNA-positive cells per crypt. Next, the thymidine analog, bromodeoxyuridine (BrdU) was injected into CASK<sup>+/−</sup> mice in order to label dividing cells. One hour after BrdU injection, labeled epithelial nuclei are confined to crypts in the small intestine (Figure 7A) and to the bottom thirds of crypts in the colon (Figure S4A). Roughly equal numbers of BrdU-positive cells are seen in normal and CASK-deficient crypts, implying that the loss of CASK has no major effect on homeostatic epithelial proliferation rate. In mice that were sacrificed twenty-four hours after BrdU injection, labeled cells are seen extending into the mid-regions of the colonic crypts (Figure S4B), and BrdU-positive small intestinal epithelial cells are found throughout crypts as well as on the bases of villi (Figure 7B). Wild type and CASK-deficient columns of villus epithelium show BrdU-labeled cells extending to the same height, implying an equal crypt-to-villus migration rate.

**CASK and LIN7C show normal localization in DLG1-deficient intestinal epithelium**

Dlg1<sup>−/−</sup> mice, which are genetically null for DLG1, die soon after birth and show multiple craniofacial and urinary tract defects but no apparent gastrointestinal abnormalities (Mahoney et al., 2006). Immunofluorescence staining of embryonic day 16 intestine from these Dlg1 knockout mice and their wild type siblings revealed that CASK and LIN7C are properly localized in the absence of DLG1 (Figure 8). Thus, although CASK is necessary for DLG1
basolateral localization, there is no reciprocal requirement of DLG1 for CASK basolateral localization in intestinal epithelium.

**CASK does not influence DLG1 or LIN7C localization in gastric fundic glands**

In wild type animals, CASK and DLG1 are expressed in the stomach and show an overlapping basolateral membrane distribution in epithelial cells of the fundic (acid-producing) glands. Immunofluorescence staining of CASK mosaic stomach, however, revealed that DLG1 maintains its basolateral membrane distribution in this tissue, even in the absence of CASK (Figure 9A-C).

In wild type animals, the subcellular distribution of LIN7C in gastric glands differs from that in intestinal epithelium. Gastric epithelial cells (which include acid-producing parietal cells, zymogenic chief cells and surface mucous cells) exhibit LIN7C staining at tight junctions but not along basolateral membranes (Figure 9E). In parietal cells, there is an intracellular punctate staining pattern suggestive of overlap with the extensive internal tubulovesicular system that characterizes these unusual cells (Ogata, 1997). In mosaic animals, the localization pattern of LIN7C in CASK-negative gastric epithelium is the same as that in the adjacent, wild type gastric glands (Figure 9D-F).

**CASK is not required for basolateral expression of EGFR and ErbB-2 in intestinal crypts**

In order to test CASK-deficient colonic epithelia for the presence of EGFR on the basolateral cell surface, we injected EGF intravenously into CASK mosaic mice and assayed for EGFR activation by immunofluorescence using an antibody that specifically recognizes phosphorylated EGFR (pEGFR). As shown in Figure 10, pEGFR is found at epithelial basolateral membranes in
the bottom two-thirds of both wild type and CASK-negative crypts. Interestingly, the distribution of pEGFR along the lateral cell membrane differs from that of CASK. Whereas CASK is most highly concentrated at the basal aspect of the lateral membrane, the distribution of pEGFR appears uniform along most of the basolateral membrane, but shows a slight concentration at the apical border, at or near the apical junctional complex. The intensity and localization pattern of pEGFR staining in CASK-deficient crypts is identical to that in wild type crypts. Approximately equal amounts of total EGFR protein are detected in colonic mucosal lysates from wild type and Vil-Cre CASK knockout mice by immunoblot analysis (Figure S5).

Immunolocalization of ErbB-2 in these samples demonstrates that it, too, is basolaterally localized in both wild type and CASK-deficient epithelium (Figure 10D - F). The lateral membrane distribution of ErbB-2 is very similar to that of EGFR, with a slight accumulation at tight junctions. Unlike EGFR, however, ErbB-2 appears more evenly distributed along the crypt-surface axis, with only a subtle diminution of staining intensity in the colonic surface epithelium.

We could not detect pEGFR in the epithelium of small intestinal villi, although we found it to be present in both wild type and CASK-deficient small intestinal crypts. The distribution pattern of ErbB-2 in the small intestine parallels that of LIN7A/B; it is most abundant in crypts, weakly expressed at the bases of villi, and undetectable in the fully mature epithelium of the villus tips.
Discussion

Somatic mosaic analysis is a powerful tool for studying tissue morphogenesis; its usefulness in *Drosophila* research is well established (Blair, 2003). Although methods for mosaic analysis of mice are less developed, there is increasing interest in using this approach, especially in the study of carcinogenesis (Akyol *et al.*, 2008). The intestinal epithelium is particularly amenable to mosaic analysis because it undergoes continual, rapid self-renewal while maintaining a precise spatiotemporal organization. Mosaicism driven by X inactivation has been visualized in the murine intestine through histochemical detection of two X-linked enzymes in heterozygous females (Griffiths *et al.*, 1988; Shiojiri and Mori, 2003). In this previous work, X-linked gene expression was used as a marker to identify clonal patches of epithelium.

Our study has shown that CASK, too, can be used to identify clonal X-inactivation patches. In addition, it has revealed that although CASK-deficient intestinal epithelium is viable, differentiates normally and is able to self-renew, its growth is at a subtle disadvantage compared to wild type epithelium. This is evidenced by the finding that in adult *CASK*+/− mosaic mice CASK-deficient crypts comprise only about 15% of the total crypt number. In rodents, crypt morphogenesis occurs during the first postnatal week, and with respect to X inactivation, crypts rapidly become homogeneous (Shiojiri and Mori, 2003). During subsequent growth of the animal and in response to inflammation and/or injury, crypts replicate by a process termed crypt fission (Yen and Wright, 2006). The fact that CASK-deficient crypts constitute considerably less than half of the epithelium in female mosaics implies either that CASK-deficient cells are less likely than wild type to become established as crypt stem cells during development or that wild type crypts subsequently have a selective advantage over CASK-deficient crypts.
In CASK-negative intestinal epithelia of both mosaic mice and intestine-specific CASK knockout mice, DLG1 and LIN7C fail to adopt their normal basolateral membrane distribution. This finding of an *in vivo* requirement for CASK in the basolateral localization of DLG1 and LIN7C is consistent with the results of previous studies conducted on renal epithelial cells *in vitro*. Overexpression of a mutant, mislocalizing form of CASK in MDCK cells has been shown to partially disrupt the basolateral localization of endogenous DLG1 (Lee *et al.*, 2002) and LIN7 (Straight *et al.*, 2000). However, we find that CASK is not required for DLG1 or LIN7C localization in gastric fundic epithelium. Thus, its roles in epithelial basolateral membrane organization appear to be tissue-specific.

In intestinal and gastric epithelial cells, a fraction of LIN7 is colocalized with ZO-1 at the apical junctional complex. This pool of LIN7 is unaffected by the loss of CASK. A junctional localization of LIN7 is expected from its known association with the tight junction MAGUK, Pals1 (Kamberov *et al.*, 2000; Roh *et al.*, 2002). That LIN7 plays an important role in tight junction assembly has been demonstrated using small hairpin RNA-induced knockdown of its expression in MDCK cells (Straight *et al.*, 2006). Furthermore, LIN7C knockout mice display severe kidney defects resulting from tight junction abnormalities and disrupted polarity of a subset of renal epithelial cells (Olsen *et al.*, 2007). Although CASK-deficient intestinal epithelium shows displacement of LIN7C from basolateral membranes, the fact that its junctional pool of LIN7 remains intact probably explains the absence of the sort of overt polarity defects seen in LIN7C knockouts.

There is a good deal of evidence that LIN7 is involved in the basolateral targeting of certain transmembrane proteins. A relatively small protein, LIN7 contains an L27 domain that mediates interaction with CASK (or Pals proteins, (Doerks *et al.*, 2000)) and a PDZ domain that
mediates binding to a motif located at the C-terminus of some transmembrane proteins. Among the proteins with PDZ-binding C-terminal motifs recognized by LIN7 are: the epithelial $\gamma$-aminobutyric acid transporter, BGT-1 (Perego et al., 1999); $\beta$-catenin (Perego et al., 2000); inwardly-rectifying potassium channels of the Kir 2 family (Olsen et al., 2002; Leonoudakis et al., 2004); and two of the four mammalian ErbB receptors (Shelly et al., 2003). Several studies have analyzed the effects of LIN7 on the membrane targeting of these proteins when expressed in MDCK cells. In the cases of BGT-1, Kir 2.3, Kir 2.2 and ErbB-2, removal of the C-terminal motif, which leads to de-coupling from LIN7, causes destabilization of the channel/receptor at the basolateral membrane and internalization into endosomes (Perego et al., 1999; Olsen et al., 2002; Shelly et al., 2003; Leonoudakis et al., 2004). These and related observations have led to the model that LIN7 functions as an adaptor linking specific channel and receptor proteins to CASK, thereby stabilizing them in the basolateral plasma membrane by preventing their endocytosis (and possibly also by promoting their recycling to the plasma membrane from endosomes). In addition, LIN7 plays a role in regulating the trafficking of some proteins through the biosynthetic pathway: it is required for the translocation of newly synthesized ErbB-2 from the endoplasmic reticulum to the Golgi apparatus in MDCK cells (Shelly et al., 2003). This PDZ-independent function is mediated by binding of LIN7 to the tyrosine kinase domain of ErbB-2 through a region designated the “kinase interaction domain” (KID). That EGFR trafficking may be similarly regulated is suggested by the fact that it, too binds the KID domain of LIN7. Furthermore, the KID sequence is highly conserved, and all three LIN7 isoforms are capable of binding to EGFR (Shelly et al., 2003).

Our finding that EGFR and ErbB-2 are correctly localized at the basolateral plasma membranes of CASK-deficient intestinal crypt epithelial cells demonstrates that LIN7 does not
require the cooperation of CASK to promote the trafficking of these receptors through the biosynthetic pathway. The loss of basolateral membrane anchoring of LIN7C implies that CASK specifically tethers this isoform to the membrane. However, the LIN7A and/or B isoforms remain at basolateral membranes in the absence of CASK. Thus, they are available for PDZ-mediated binding of ErbB-2 and are presumably responsible for its stabilization in the plasma membrane. EGFR, which lacks a C-terminal PDZ-binding motif, is maintained in the membrane by other mechanisms.

Fully mature, CASK-deficient epithelial cells of small intestinal villi show a complete absence of basolateral membrane-associated LIN7 when stained with an antibody that recognizes all three isoforms. The most likely explanation is that LIN7C is the only isoform expressed in villi, whereas one or both of the other isoforms is/are expressed in crypts. This situation is reminiscent of that in the kidney, where there is differential expression of the three LIN7 isoforms in different segments of the nephron (Olsen et al., 2005). Neither EGFR nor ErbB-2 is detectable in the fully-differentiated villus epithelium, as expected for a non-proliferative cell population.

Syndecan is another basolateral receptor that is expressed by intestinal epithelia and associates with CASK (Cohen et al., 1998). Like ErbB-2, syndecan has a PDZ binding motif at its cytoplasmic C-terminus. Syndecan’s motif, however, does not bind LIN7 but rather binds directly to the PDZ domain of CASK. Although there is strong evidence that in MDCK cells a PDZ interaction controls the biosynthetic sorting of syndecan and thereby determines its basolateral localization, siRNA experiments have shown that this process is independent of CASK (Maday et al., 2008). Accordingly, we find that the loss of CASK in intestinal epithelia has no effect on the localization of syndecan.
MPP7, a recently characterized MAGUK, is structurally similar to CASK and is expressed in a variety of epithelial cell lines. Like CASK, it can interact with LIN7 and DLG1, and in vitro it competes with CASK for binding to those proteins (Bohl et al., 2007; Stucke et al., 2007). However, MPP7 does not play the same role as CASK in anchoring DLG1 to the basolateral membrane. In fact, siRNA knockdown experiments in a polarized breast epithelial cell line have shown that although DLG1 remains basolateral in the absence of MPP7, MPP7 loses its basolateral membrane distribution in the absence of DLG1 (Stucke et al., 2007).

Interestingly, siRNA knockdown of CASK in these cells has no effect on DLG1 localization (Stucke et al., 2007), which may be further evidence of the tissue specificity of CASK’s function.

Although the precise biochemical action(s) of DLG1 are poorly understood, its membrane localization appears to be critical for its effects on cell polarity, migration and proliferation. In *Drosophila*, Dlg is necessary for the formation of new plasma membranes during cellularization of syncitial embryos (Lee et al., 2003), and for the establishment of apico-basal polarity in developing epithelia (Bilder et al., 2003). In cultured mammalian epithelial cells, DLG1’s membrane recruitment is promoted by cell-cell contact (Reuver and Garner, 1998) and its abundance is regulated through the proteasome pathway (Mantovani et al., 2001). The transforming capacity of several viral oncoproteins depends on their ability to bind DLG1 and target it for degradation (Lee et al., 1997; Grassmann et al., 2005; Thomas et al., 2005). In migrating cells, DLG1 is associated with the basal plasma membrane and concentrates in the leading edge, at the attachment points of microtubule plus ends (Etienne-Manneville et al., 2005; Mimori-Kiyosue et al., 2007). At these sites, DLG1 and APC form a complex that is essential for directed cell migration. The DLG1-APC interaction is also reported to inhibit cell cycle
progression in fibroblasts, but the subcellular site(s) at which this occurs and its molecular mechanism are unknown (Ishidate et al., 2000). Interestingly, overexpression of truncated APC - which cannot bind DLG1 and is similar in this respect to mutant forms typically expressed by colon cancer cells - causes a loss of contact inhibition in epithelia (Mimori-Kiyosue et al., 2007). Together, these observations strongly implicate DLG1 in polarity and proliferation control and indicate that many of its functions depend on its association with the plasma membrane.

However, the results presented here demonstrate that de-coupling of DLG1 from the basolateral membranes of intestinal epithelial cells in vivo has no apparent effect on their polarity, self-renewal, differentiation or crypt-to-villus migration. In future studies, it will be interesting to examine the proliferative and migratory responses of such CASK-deficient cells in mouse models of intestinal mucosal injury and repair, as well as the effects of CASK gene deletion on intestinal carcinogenesis.
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References


Figure Legends

Figure 1. Indirect immunofluorescence microscopy of tissues from $CASK^{+/−}$ mice. Bars, 20 µm. Sections of stomach (A), small intestine (B) and colon (C) were doubly labeled with antibodies to CASK (red) and ZO-1 (green). (A) A longitudinal section through several gastric fundic glands reveals that while CASK is localized to epithelial basolateral membranes in most glands (upper left and lower right), there are patches in which CASK is not expressed (middle). ZO-1 is present at tight junctions throughout the tissue. (B) A longitudinal section through two small intestinal villi shows that CASK resides at basolateral membranes of enterocytes on the left side of one villus and is absent from the epithelium on the right side and on the adjacent villus. ZO-1 staining is intact in both villi. (C) A tangential section through colonic mucosa (with surface epithelium on the right) shows a CASK-negative patch of three crypts. Anti-ZO-1 stains tight junctions throughout. Nuclei are labeled blue by DAPI. (D-F) Small intestinal mucosa stained with anti-CASK antibodies (green). (D) Co-labeling with anti-β-catenin (red) reveals normal lateral membrane staining in CASK-negative enterocytes. (E) Co-labeling with anti-lysozyme (red) highlights Paneth cells at the bases of CASK-negative crypts. (F) Co-labeling with anti-chromogranin A (red) identifies scattered enteroendocrine cells within CASK-negative epithelium.

Figure 2. Immunofluorescence double labeling of CASK mosaic small intestinal villi. Sections of small intestine from $CASK^{+/−}$ mice were co-stained with antibodies to CASK (red) and antibodies (green) to occludin (A), claudin-5 (B), protein kinase Cζ (C), villin (D), β1-integrin (E) and αII-spectrin (F). The absence of CASK has no effect on the distribution patterns of these proteins.
Figure 3. Immunofluorescence double labeling of CASK mosaic intestine. Sections of colon (A-C) and small intestine (D-I) from CASK+/− mice were co-stained with anti-CASK (red, A, D and G) and anti-DLG1 (green, B and E) or anti-Scribble (green, H) antibodies. C, F and I are merged images. Bars, 20 μm.

Figure 4. Immunolocalization of LIN7C in CASK mosaic intestine. Bars, 20 μm. Small intestine from CASK+/− mice was doubly labeled with anti-CASK (green) and anti-LIN7C (red) antibodies (A-C) or labeled with anti-LIN7C alone (D, green). In B and D, LIN7C shows a basolateral membrane distribution in cells on only one side of the villus epithelium but is present at tight junctions (arrowheads) on both sides. (E) CASK+/− colon doubly labeled with anti-LIN7C (red) and anti-ZO-1 (green).

Figure 5. Immunolocalization of all three LIN7 isoforms in CASK mosaic intestine. Bars, 20 μm. Small intestine (A-F) and colon (G-I) from CASK+/− mice were doubly labeled with anti-CASK (red) and anti-LIN7A,B,C (green) antibodies.

Figure 6. Immunoblot analysis of colonic epithelium from wild type and villin-cre/CASK knockout mice. Mucosal homogenates were prepared from a pair of male, Vil-Cre transgenic littermates: one with wild type CASK (WT) and the other bearing the CASKflox allele (KO). Identical pairs of samples were separated by gel electrophoresis and blotted to nitrocellulose. Blots were probed with antibodies to the indicated proteins. (E-cad = E-cadherin)
Figure 7. Proliferation and migration studies of CASK+/− mosaic small intestine. Mice were injected with bromodeoxyuridine (BrdU) and sacrificed either 1 hour (A) or 24 hours (B) later. Sections were co-immunolabeled with antibodies to CASK (red) and BrdU (green), and nuclei were stained blue with DAPI. (A) and (B) are merged images; CASK staining alone is shown in the bottom panels. After one hour, BrdU-labeled cells are confined to crypts in CASK-deficient as well as normal epithelium (A). By 24 hours, BrdU-labeled cells have begun to migrate onto villi (B). Arrowheads indicate the farthest point reached on one villus and demonstrate that this distance is the same in CASK-deficient epithelium (right) as in normal epithelium (left).

Figure 8. Immunofluorescence characterization of Dlg1 knockout intestine. Sections of small intestine from embryonic day 16 (E16) Dlg1−/− mice and wildtype E16 controls (wt) were labeled with antibodies to CASK (A) and LIN7C (B). Bars, 20 μm.

Figure 9. Immunolocalization of DLG1 and LIN7C in CASK+/− mosaic stomach. Bars, 20 μm. Fundic mucosa doubly labeled with anti-CASK (green) and anti-DLG1 (red, B and C) or anti-LIN-7C (red, E and F) antibodies. Where CASK is expressed, it is found at basolateral membranes of epithelial cells (eg., parietal cells, arrows in A and D). DLG1 is basolaterally localized in both CASK-expressing and CASK-negative glands. LIN7C is not distributed basolaterally (compare arrows in D and E) but is seen at tight junctions (arrowheads in E) and within parietal cell cytoplasm. Its distribution, like that of DLG1, is independent of CASK expression.
Figure 10. Immunolocalization of EGFR and ErbB-2 in CASK\textsuperscript{+/-} mosaics colon. Mice were injected with EGF, and their colons were doubly labeled with anti-CASK (red) and either anti-phospho-EGFR (pEGFR, green, B) or anti-ErbB-2 (green, E) antibodies. Bars, 20 μm.