p120-catenin controls contractility along the vertical axis of epithelial lateral membranes

Huapeng H. Yu¹, Michael R. Dohn¹,², Nicholas O. Markham¹,³, and Robert J. Coffey³, Albert B. Reynolds¹

¹Department of Cancer Biology, Vanderbilt University, Nashville, Tennessee, USA, ²Department of Pharmacology, Vanderbilt University, Nashville, Tennessee, USA, ³School of Medicine, Vanderbilt University, Nashville, Tennessee, USA.

Corresponding Author:

Albert B. Reynolds
Vanderbilt University Medical Center
771 PRB 2220 Pierce Ave
Nashville, TN 37232
Phone: 615-343-9532 Fax: 615-936-6399
E-mail: al.reynolds@vanderbilt.edu

Competing Interests: The authors do not have any competing interests.
Summary

In vertebrate epithelia, p120-catenin mediates E-cadherin stability and suppression of RhoA. Genetic ablation of p120 in various epithelial tissues typically causes striking alterations in tissue function and morphology. Although these effects could very well involve p120's activity towards Rho, ascertaining the impact of this relationship has been complicated by the fact that p120 is also required for cell-cell adhesion. Here, we have molecularly uncoupled p120's cadherin stabilizing- and RhoA-suppressing activity. Unexpectedly, removing p120's Rho-suppressing activity dramatically disrupted the integrity of the apical surface, irrespective of E-cadherin stability. The physical defect was tracked to excessive actomyosin contractility along the vertical axis of lateral membranes. Thus, we suggest that p120's distinct activities toward E-cadherin and Rho are molecularly and functionally coupled, and this in turn enables the maintenance of cell shape in the larger context of an epithelial monolayer. Importantly, local suppression of contractility by cadherin-bound p120 appears to go beyond regulating cell shape, as loss of this activity also leads to major defects in epithelial lumenogenesis.

Introduction

A universal principle of organization for polarized epithelial cells is the physical and functional segregation of membranes into three distinctive domains. Basal membranes impart anchorage, lateral membranes organize adhesive contacts and apical membranes establish a free surface for exchange of materials (Mostov et al., 2003). On the other hand, epithelial tissues vary widely in size and shape to accommodate diverse epithelial functions (Gumbiner, 1996; Gumbiner, 2005). Although determinants of epithelial cell fate are well described, the molecular mechanisms controlling cell height and shape are poorly understood. In Drosophila melanogaster (Drosophila), recent evidence suggests that molecular gradients of the morphogen Dpp (Decapentaplegic) in the wing imaginal disc specify not just cell fate, but also position-specific control of epithelial architecture (Gibson, 2005; Shen and Dahmann, 2005). Although mechanistic details are still unclear, the Dpp pathway appears to modulate epithelial cell height, in part, by controlling compartmentalization of Rho1 (Drosophila homolog of RhoA) activity along the length of the lateral cell membrane (Gibson, 2005; Shen and Dahmann, 2005; Widmann and Dahmann, 2009). Whether (and how) Rho activity affects cell height in vertebrate epithelial systems is currently unknown.

A potentially important discrepancy between Drosophila and vertebrate systems is the relative function of p120-catenin (aka p120), which binds directly to the cytoplasmic juxtamembrane domain of E-cadherin in both systems. In C. elegans and Drosophila, p120 is considered dispensable, as genetically null adults are clearly viable (Fox et al., 2005; Myster et al., 2003; Pacquelet et al., 2003; Pettitt et al., 2003), albeit sensitive to
stress (Stefanatos et al., 2013). In vertebrates, on the other hand, p120 gene ablation is embryonic lethal. Downregulation in vertebrate epithelial tissues leads to a variety of morphologic defects and is observed frequently in most epithelial cancers (e.g., colon, lung, pancreas, breast, prostate) (Davis and Reynolds, 2006; Kurley et al., 2012; Smalley-Freed et al., 2010). Moreover, in contrast to its Drosophila and C. elegans counterpart, vertebrate p120 is essential for cadherin stability. Removal of p120 in most epithelial cell types causes rapid internalization of the cadherin complex, in vitro and in vivo (Davis, 2003; Davis and Reynolds, 2006; Kurley et al., 2012; Marciano et al., 2011; Smalley-Freed et al., 2010; Xiao, 2003). In Drosophila, the E-cadherin-containing adherens junction (AJ) is largely restricted to an apical compartment delimited by the septate junction. Compartmentalized suppression of Rho occurs along the lateral domain by a cadherin-independent mechanism and apparently translated into an increase in cell height (Widmann and Dahmann, 2009). Vertebrate E-cadherin (along with p120-, α- and β-catenins), on the other hand, is typically localized along the entire lateral membrane (Wu et al., 2014). Notably, vertebrate p120 is well established as an inhibitor of Rho (Fang, 2004; Noren et al., 2000; Ponik et al., 2013; Reynolds et al., 2000; Schackmann et al., 2011; Wildenberg et al., 2006; Zebda et al., 2013). In the cytoplasm, inhibition of Rho by p120 occurs by a RhoGDI-like mechanism and is mutually exclusive with binding to E-cadherin (Anastasiadis, in press). Membrane bound p120, on the other hand, can interact with a spectrum of Rho mediators (e.g., RhoGEFs, RhoGAPs, ROCK, Shroom3) depending on parameters such as cell and/or tissue type and sub-cellular localization (Lang et al., 2014; Noren et al., 2000; Ponik et al., 2013; Smith et al., 2011; Wildenberg et al., 2006). For example, in many polarized columnar epithelia, p120 interacts apically with specific RhoGEFs that modulate apical constriction (Lang et al., 2014) and basolaterally with RhoGAP family members (Klompstra et al., 2015; Zebda et al., 2013). These observations suggest that p120 acts as a coordinating hub for mediators of local Rho activity and raise the possibility that p120 in vertebrates may participate in regulating lateral cell height through local suppression of Rho.

Vertebrate p120 function has been extensively studied in conditional KO mice (Davis and Reynolds, 2006; Kurley et al., 2012 2012cs; Marciano et al., 2011; Perez-Moreno et al., 2006; Smalley-Freed et al., 2010). Phenotypes vary widely depending on the organ and tend to involve striking changes in tissue morphology. Although these phenotypes could potentially be linked to Rho (Ponik et al., 2013), distinguishing Rho-mediated effects from those caused by cadherin destabilization have been inherently difficult because p120’s effects on Rho are epistatic to and dependent on its cadherin stabilizing activity. Nonetheless, cadherin stability cannot by itself account for the wide spectrum of p120 KO phenotypes observed in vitro and in vivo (Davis and Reynolds, 2006; Dohn et al., 2009; Kurley et al., 2012; Perez-Moreno et al., 2006; Perez-Moreno et al., 2008; Ponik et al., 2013). Additionally, we and others have found that physiologically relevant results are often masked or blocked altogether when the cells are cultured on hard surfaces (Baker and Chen, 2012; Brugge, 2012; Dohn et al., 2009; Paszek et al., 2005; Töyli et al., 2010). Moreover, epithelial cells that are columnar in vivo adopt completely different shapes when cultured by conventional means on plastic. MDCK cells, for
example remodel into very flat disc-shaped cells featuring wide basal footprints and lateral domains that make strong cell-cell contacts but are otherwise almost nonexistent. We have therefore transitioned to 2D cultures on thick collagen pads (which enable cuboidal to columnar morphology) and/or 3D cell cultures in collagen. Here, using a vertebrate epithelial cell model (i.e., MDCK II cells), we separate p120’s cadherin stabilizing- and RhoA-suppressing functions under conditions that, for the first time, permit selective assessment of phenotypes caused by the impact of p120 on Rho. Unexpectedly, selectively removing p120’s Rho-suppressing activity dramatically disrupts the integrity of the apical surface by contracting lateral membranes, irrespective of E-cadherin stability. The physical defect stems from excessive actomyosin contractility along the vertical axis of lateral membranes, causing dramatic basal dislocation of the tight junction and expansion of the apical domain, leaving cell polarity intact. Moreover, the impact of this excess contractility goes beyond regulation of cell shape, as the effect is accompanied by major defects in epithelial lumenogenesis. Significantly, this defect is completely reversed by inhibition of ROCK or myosin, irrespective of E-cadherin stability. Thus, although most of p120 ablation phenotypes are attributed to adhesion defects, the phenotypes described here are rescued by suppression of Rho but not E-cadherin.

Results

p120 ablation disrupts the apical surface of MDCK cell monolayers leaving cell polarity intact

In many epithelial cell types, p120 ablation leads to complete loss of cell-cell adhesion (e.g., MCF10A, A431)(Kurley et al., 2012; Xiao, 2003), making it difficult to distinguish between direct consequences of p120-loss and collateral fallout associated with loss of all contact-dependent signaling. Moreover, p120 activity has important effects that manifest only in the context of adhesion-intact cell monolayers (e.g., lumen formation, collective migration) and are thus masked by loss of cell-cell contacts. MDCK cells circumvent many such issues because intercellular adhesion can be maintained by E-cadherin-independent junctions upon knockdown of p120, despite the near complete loss of AJs. Notably, tight junctions and desmosomes are unaffected(Dohn et al., 2009).

When cultured on plastic, the morphologies of WT and p120 KD MDCK cells are essentially identical (data not shown). When plated on collagen, however, the cells polarize and develop sufficient height to qualify as cuboidal or columnar cell monolayers, even when subconfluent. In this scenario, p120 KD induced dramatic changes in cell morphology. On the other hand, overexpression of p120 (isoform 1A or 3A) by at least two-fold has no overall impact cell shape (Figure S1C, D, E). Using Transmission Electron Microscopy (TEM), we observed large gaps between neighboring cells only in p120 KD cells (Figure S1F). Although the tight junction was retained, the apical surface at cell-cell contacts was significantly distorted (Figure S1F, white arrow). To further characterize this effect, the cells were immunostained for ezrin (apical marker) and the tight junction
marker cingulin. Normally, ezrin staining is confined to a thin zone (aka the apical section) across the top of the epithelial monolayer and highlights the perfectly flat apical surface of WT MDCK monolayers. Note that there is little or no detectable ezrin staining in other confocal planes (e.g., middle or basal sections)(Fig 1A,B). However, in the p120 KD cells, confocal cross sections show that ezrin staining clearly invaginates well into the middle section of the cell (Figure 1A, arrows). Figure 1C shows confocal cross sections of the same experiment. Note the aberrant presence of ezrin staining in the intercellular space across the middle section (Figure 1C, arrow). The lower panels show that p120 staining is confined to the lateral membranes in WT cells and substantially down-regulated in p120 KD cells. 3D reconstructions graphically illustrate the topography of ezrin stained WT and p120 KO surfaces viewed from the top (left panel) and bottom (right panel)(Figure 1D). Whereas the WT surface is relatively flat (see video 1), the KD surface is deeply invaginated, as illustrated dramatically by the bottom up view (Figure 1D, arrow, lower right panel, also see video 2).

Interestingly, the dome-shaped apical surfaces are reminiscent of apical expansion phenotypes induced by overexpression of members of the apical polarity complex (i.e., Par3, Par6)(Chalmers et al., 2005) or down-regulation of members of the lateral polarity complex (i.e., Lgl, Scrib, or Dlg)(Yamanaka, 2006). Overexpression of gp135 or down-regulation of KIBRA can also induce apical expansion(Nielsen et al., 2000; Yoshihama et al., 2011). For the polarity proteins, apical expansion is driven by mis-targeting of apical membrane proteins to lateral membranes, irrespective of the placement of tight junctions(Tanentzapf and Tepass, 2002). gp135 accumulation, on the other hand, physically expands the apical membrane, in part via recruitment of NHERF and Ezrin. Apical membrane also expands in 3D MDCK cysts upon KIBRA knockdown (KD), due to hyper-activation of aPKC. Notably, in the latter cases (gp135 and KIBRA KD), the mechanism was shown to not involve mis-targeting to apical or basolateral membranes. Instead, the tight junction is retained at the boundary between apical and lateral membranes but displaced basally by the expanding apical surface(Nielsen et al., 2000; Yoshihama et al., 2011). To determine whether either of these mechanisms was responsible for the p120 KD phenotype, we first examined the effect of p120 KD on the placement of tight junctions relative to the apical surface. Importantly, cingulin staining shows that tight junctions localize at the very tip of the surface invaginations, illustrating that the boundary between apical and lateral domains remain intact (Figure 1F). Maximal intensity projections of cingulin staining to the X-Y plane show that the p120 KD tight junctions remain circumferentially continuous, albeit basally dislocalized (Figure 1G). Being morphologically consistent with the gp135-NHERF-Ezrin or KIBRA KD mechanism, we measured Ezrin protein levels in the p120 WT and KD cells and found no differences (Figure 1I). Additionally, inhibition of aPKC activity via myristoylated PKCζ-PS inhibitor failed to rescue the apical membrane defect in p120 KD cells (Figure 1H). Thus, although morphologically similar to the apical expansion phenotypes associated with polarity genes, it appears that neither of the mechanisms accounts for the p120 ablation phenotypes here.
p120/E-cadherin interaction is essential for maintaining a flat apical membrane

In p120 rescue experiments, analysis of mosaic p120 rescue suggests that suppression of the apical membrane invagination is dependent on the p120/E-cadherin-mediated cell-cell contacts. For example, the cell in the crosshairs of Figure 1E, (right panel) makes E-cadherin based adhesions on three sides (Figure 1E, arrowhead). The fourth side (Figure 1E, arrowheads), on the other hand, does not, and exhibits apical defects (i.e. invagination) on that side only. Thus, although the phenotype could in theory be linked to a cytoplasmic or nuclear p120 function, the data strongly implies a mechanism involving the cadherin bound fraction of p120. Moreover, in exploratory studies we deleted each of the Arm repeats individually and conducted p120 KD/add-back experiments to identify domains in p120 required to suppress apical expansion. Interestingly, suppression was selectively mediated by repeats 1-6, exactly the same repeats that mediate E-cadherin binding to p120(Ireon, 2002) (Figure S1A,B).

To address mechanism, we used a single amino acid p120 mutant described recently (p120K401M)(Ishiyama et al., 2010a) to selectively uncouple its interaction with E-cadherin (Figure 2C, 3A,B). p120-1A and p120-3A isoforms containing the K401M mutation localized exclusively to the cytoplasm (Figure 2A) and failed to co-immunoprecipitate with or stabilize either E- or N-cadherin (Figure 2B, Figure S2A,B). Importantly, these mutants retain interaction with Kaiso, whose p120 interaction domain is known to overlap with that of E-cadherin (Figure 2B). p120 KD/add-back experiments with these p120 K401M mutants failed to rescue the apical invagination phenotype (Figure 2D), indicating that cytoplasmic p120 is inactive.

To determine whether direct interaction between p120 and E-cadherin is essential for the steady state suppression of apical invagination, we first asked whether simply targeting p120 to lateral membranes (irrespective of E-cadherin-binding) is sufficient. The p120 K401M mutants were fused at the C-terminus to a CAAX-box motif (Figure 3A,B), known to relocate cytoplasmic proteins to the plasma membrane(Seabra, 1998). Interestingly, localization of these p120 K401M-CAAX mutants is almost indistinguishable from that of endogenous p120, including exclusion from the apical surface. (Figure 3C,D). However, although p120 K401M-CAAX is abundantly expressed on lateral membranes, the unstable internalized pool of E-cadherin in the p120 KO cells is clearly not rescued by these mutants (Figure 3C). When plated on collagen, p120 KD cells expressing the p120 K401M-CAAX mutants showed no sign of apical invagination rescue (Figure 3D). Thus, while localization of p120 to lateral membranes is essential for suppression of apical invagination, it is clearly not sufficient.

Next, we asked whether E-cadherin is sufficient to suppress apical invagination, irrespective of p120-binding. Because E-cadherin is rapidly degraded if p120 is not bound, we first generated an endocytosis-resistant E-cadherin mutant (from human E-cadherin) by changing the classic di-leucine endocytosis motif to alanine...
residues (Miyashita and Ozawa, 2007; Nanes et al., 2012). To further rule out any contribution from bound p120, we also introduced the p120 uncoupling point mutation (i.e., Ecad E762A) (Ishiyama et al., 2010a), resulting in the construct termed Ecad-LAEa (see schematic, Figure 2C, 3B). When expressed in p120 KD MDCK cells, Ecad-LAEa localizes normally to lateral membranes (Figure 3E, 3rd column), whereas endogenous E-cadherin remains entirely cytoplasmic (Figure 3E, 1st column). Because the E-cadherin mAb RR1 used in column-1 is canine specific, it detects only the endogenous MDCK E-cadherin. Notably, the E-cadherin mAb used in column-3 (BD) recognizes both canine and human E-cadherin, permitting visualization of Ecad-LAEa. p120 staining in the Ecad-LAEa cell line, on the other hand, is comparable to that in the p120 KD cells and almost undetectable (Figure 3E). E-cad-LEAE is thus stably retained on lateral membranes despite almost undetectable p120-binding. Importantly, LAEA E-cadherin restored junctional localization of α-catenin that was internalized upon p120 KD in MDCK cells (Figure S3A, S3B) and cell-cell adhesion in the cadherin-deficient A431D cells (Figure S3C, S3D). To determine whether Ecad-LAEa can, in fact, suppress apical expansion, without assistance from p120, the cells were again plated on collagen and examined by Ezrin staining for apical membrane invagination. Unexpectedly, although Ecad-LAEa retention is in fact unaffected by p120 loss, it is nonetheless unable to reverse the apical invagination phenotype (Figure 3F). We further quantified the phenotype by measuring the length of apical invagination (LAI) as a proxy to the extent of apical expansion. As shown in Figure 3G, p120 KD induces a 3.2-fold increase of LAI (from 1.154 to 3.717 µm). Although Ecad-LAEa rescue did reduced the magnitude of the effect slightly (i.e. 2.7 fold vs 3.2 fold), these results show that stable surface retention of E-cadherin by itself was unable to appreciably rescue the p120 ablation phenotype. Moreover, to be described later, directly activating RhoA in WT cells leads to a comparable level of increase in LAI as Ecad-LAEa rescue (Figure 3G, 4D). Thus, instead of directly modulating RhoA activity, restoring the E-cadherin-actin linkage by Ecad-LAEa could potentially slightly limit the scale of apical membrane deformation by activated junctional contractility.

Apical invagination following loss of p120 is a function of excess contractility along lateral membranes

Another established consequence of p120 ablation is activation of the RhoA-ROCK-myosin pathway (Dohn et al., 2009; Perez-Moreno et al., 2006; Wildenberg et al., 2006), suggesting a potential role for unbalanced actomyosin contractility. To test whether the presence of collagen alters p120’s ability to inhibit this pathway, we blotted for downstream effectors of ROCK in WT, KD and RE cells cultured on collagen. As shown in Figure S4G, p120 KD induces a notable increase of phosphorylation of cofilin and p120 RE (Rescue, p120 KD + p120 1A) strongly suppresses this activity. Although the phospho-MLC antibodies we tried did not work well on Western blots, we did notice a dramatic increase of the protein level of MLC, and an upward band shift corresponding to the active phosphorylated form of MLC. (Figure S4G, MLC blot long exposure). These data all point to increased ROCK and myosin activity in p120 KD cells compared to WT or RE. To further test this hypothesis, we blocked the RhoA-ROCK-Myosin pathway by inhibition of either ROCK (Y27632) or Myosin II...
Indeed, even in the near complete absence of AJs (Figure 4C), both inhibitors completely reversed the apical defect associated with p120 KD (Figure 4A). Using the same quantification method described earlier, both Y27632 and Blebbstatin were found to reduce the length of apical invagination (LAI) of p120 KD cells to the same level as WT (Figure 4B). On the other hand, treatment with either DMSO or an aPKC inhibitor (PKCζ pseudo substrate) has no effect on LAI (Figure 4B). The defect, therefore, is not due to cadherin loss per se but is instead caused by excessive activation of Rho. It is worth noting that two Rho-uncoupled p120 mutants have been described previously, one involving deletion of an N-terminal region (ΔNTR), the other an intermediate sequence (ΔIns) of six amino acids located between ARM5 and ARM6 (Yanagisawa et al., 2008). However, this Rho-GDI-like activity is reported when p120 is over-expressed and apparently restricted to unbound p120 as it is not detected in E-cadherin-associated fractions (Anastasiadis and Reynolds, 2001; Reynolds et al., 2000; Yanagisawa et al., 2008). Further, these mutants (p120-ΔNTR, p120-ΔIns) completely rescued the apical defect in p120 KD cells (Figure S4A, S4B), indicating that the suppression of Rho by cadherin-bound p120 is in fact independent of the Rho GDI-like mechanism.

To better understand the impact of constitutively elevated Rho activity upon p120 loss, we next examined consequences of directly activating (DA-RhoA) or suppressing (DN-RhoA) RhoA, respectively, via expression of previously characterized (myc-tagged) dominant active (myc-RhoAG14V) and negative (myc-RhoAT19N) RhoA constructs (Hall, 1998). When introduced separately into wildtype MDCK cells, DA-RhoA effectively recapitulated the apical invagination defect (Figure 4D). Of particular interest is that the junctional presence of p120 is strongly maintained (Figure 4E), as is E-cadherin (Figure 4F), and yet the apical invagination defect is readily apparent. In contrast, cell morphology was unaffected by DN-RhoA (Figure 4D). Other variables, including the levels of members of E-cadherin complexes were unaffected by either construct (Figure 4G). Further, blocking ROCK activity effectively resolved the apical invagination defect induced by expression of DA-RhoA (Figure 4H). Thus, the apical defect is strongly associated with excessive contractility and fully dependent on ROCK.

To characterize the localization of active RhoA (RhoA-GTP form) upon p120 KD, a recently developed RhoA sensor, GFP- AHPH (kindly provided by Alpha Yap), was transiently transfected into both WT and p120 KD MDCK cells cultured on collagen gels. Low level GFP-expressing cells were imaged because high level expression causes the sensor to mis-localize diffusely to the cytoplasm. Interestingly, in collagen plated WT MDCK cells, RhoA-GTP was exclusively detected at the apical or basal (less frequent) domain. It was virtually never observed with p120 along the lateral membrane (Figure 5A, left panel). However, in p120 KD cells, RhoA-GTP relocalized to cell-cell contacts (Figure 5A, right panel) and notably, was no longer observed at the apical membrane. Confocal analysis placed the signal just under the the apical invaginations (Figure 5B, right panel). This data further supports the notion that p120 locally suppresses RhoA activity. We then examined the effect of p120 knockdown on Myosin II, a downstream effector of RhoA. Indeed, localization of the major
myosin isoform (NMMIIA) in MDCK cells reveals that it is normally excluded from lateral cell junctions (Figure 5A, arrow) (Yamada and Nelson, 2007; Yu et al., 2008). However, p120 KD induced a striking accumulation of NMMIIA at lateral membranes (Figure 5C arrow and D). Quantification of this phenomenon on a junction by junction basis reveals the robust presence of NMMIIA on 22.2 ± 4.3% (three independent experiments, 102/486 in total) of the p120 KD intercellular membranes, whereas NMMIIA is essentially never seen at WT cell junctions (i.e., 3/803, Figure 5E). Notably, the myosin bundles do not recapitulate the circumferential belt-like localization observed in some epithelial systems (Ebrahim et al., 2013; Smutny et al., 2010). Generation of cell contractility is mediated by conformational changes in the head domain of NMMIIA (Hall et al., 1982). Importantly, the recruitment of NMMIIA was almost invariably accompanied by apical membrane expansion (i.e., 98%, see Figure 5F), as evidenced by immuno-staining for Ezrin (Figure 5G). Strikingly, when present, NMMIIA was found at the very tip of the ezrin demarcated apical invagination (Figure 5G, lower-right panel), which was shown to terminate abruptly at the tight junction (Figure 1F). The data suggest an explanation for why the apical surface is so dramatically affected by loss of a lateral membrane protein. Apparently, the contractile force generated in the absence of p120 is a function of a locally activated RhoA and subsequent accumulation of myosin at the tight junction, which then transduces the force directly to the apical membrane, accounting for its invagination. We propose that this vertical suppression of contractility is a core function of p120, and ultimately essential for the characteristic rectangular morphology of individual epithelial cells and their collective ability to assemble a perfectly flat apical surface (Figure 5H, model).

**Suppression of junctional contractility is essential for lumen formation**

p120 KO often induces rather dramatic defects of epithelial morphogenesis in various organs. Among these, the more common irregularity is the impairment of generating of internal lumens. Whereas it was postulated to be simply a adhesion defect, we aim to use our established methodology here to examine whether p120’s activity toward RhoA plays a role in epithelial lumenogenesis. To mimic this morphogenetic process in vitro, we turned to two complementary assays that enable assessment of lumen formation under spontaneous or inducible conditions. The so-called “dome assay” takes advantage of the fact that cells attaching to the plate in the context of collagen spontaneously form a two-layered colony separated by multiple lumens (Figure 6A,B,C). Interestingly, in the absence of p120, the bilayer forms as in the WT scenario but no lumen is generated (Figure 6A,B). Note that lumens are clearly outlined by dense circular actin (Figure 6A) and appear as transparent bubble-like structures under bright field illumination (Figure 6B, black arrowheads). In contrast, the structures observed in p120-deficient bilayers are gaps, not lumens. Notably, under bright field microscopy, the transparent bubble-like structures, which are indicative of sealed lumens, are completely absent from the p120 KD bilayers (Figure 6B, compare upper and lower panels).

The second alternative, the so-called “collagen overlay assay”, was designed to be inducible, and more importantly, to form lumens independent of cell proliferation (Hall et al., 1982). Briefly, MDCK cells were
seeded at very high density on a layer of collagen and allowed to growth arrest as a confluent monolayer (Figure 6C). The cells were then overlaid with a second layer of collagen, invoking an intrinsic epithelial differentiation program that drives de novo lumen formation and regenerates the free apical surface (Figure 6C). Normally, this process involves relocation of apical proteins to intercellular junctions where the nascent lumen is formed (Hall et al., 1982), as illustrated by ezrin-stained circles (Figure 6D,E). However, in the absence of p120, ezrin localizes randomly across the entire cell membrane and lumen formation does not occur. Importantly, lumen formation is rescued by restoring p120 expression (Figure 6D,E). Thus, p120 is essential for lumen formation, apparently independent of its role in cell proliferation.

To distinguish potential Rho-mediated effects from those caused by cadherin destabilization, we used the same p120-CAAX and Ecad-LAEA mutants described above and tested their ability to rescue lumen formation in p120 KD cells. Indeed, neither p120 (Figure S4E) nor E-cadherin (Figure S4F) alone at the cell-cell junction is able to rescue this lumen formation defect (Figure S4, arrows showing where lumen form). Additionally, p120 mutants lacking the NTR or Ins regions faithfully rescued lumen formation (Figure S4C, S4D, arrows), consistent with a RhoGDI-independent mechanism. On the other hand, ROCK and myosin inhibitors efficiently reversed the effects of p120 knockdown, this time in the context of lumen formation. Using the same collagen overlay assay, we find that addition of either Y27632 or Blebbstatin, p120 KD cells are now able to target apical membrane to specified foci, restoring the ability to generate lumina (Figure 6F, arrows). The pictures were intentionally taken at low magnification to show restoration of the pattern of lumen formation in p120 KD cells in the presence of drug treatment. Although the effects of blebbstatin and Y27632 are not identical, both clearly rescue lumen formation, as exemplified by the distinct apical membrane foci surrounded by nucleus (Figure 6F, insert, arrows). Together, these observations indicate a pivotal role for cadherin-bound p120 in controlling junctional contractility during epithelial morphogenesis.

**Discussion**

Generation of an epithelial monolayer from individual cells is a coordinated process involving cell-cell adhesion and acquisition of shape (Figure 7B) (e.g., squamous, cuboidal, columnar). It is initiated by lateral cell-cell contacts, which then expand radially to form lateral membranes. The phenomenon is well characterized in two dimensional MDCK cell cultures and involves a zippering process along the X and Y axis (Yamada and Nelson, 2007). Notably, our model is similar in concept except that expansion also occurs in the z-axis to accommodate the vertical dimension induced by plating the cells on collagen. Normally, this process ends in the formation of a perfectly flat apical membrane (Figure 7A, the 3rd and 4th panel). In the absence of p120, the process apparently fails, resulting in a phenotype essentially identical to that exhibited by the WT cells at the earliest stage of epithelial maturation illustrated experimentally in Figure 7A (1 hr time point).
Whereas p120’s cadherin stabilizing activity is well-established and essential for adhesion (Step I, Figure 7B), the role of p120’s Rho-suppressing activity has been elusive. Here, we separate p120’s cadherin stabilizing- and RhoA-suppressing functions via a stabilized E-cadherin mutant that is retained on the cell surface irrespective of p120 binding. Surprisingly, the RhoA-suppressing activity is critical for suppressing contractility along the vertical axis of lateral epithelial membranes. Moreover, this function is essential for maintenance of individual cell shape in the overall context of collective epithelial architecture (Step II, Figure 7B). Although establishment of epithelial cell shape is generally attributed to tension-generating mechanisms such as apical constriction, here we demonstrate that along the lateral cell membranes, it is in fact suppression of contractility that is critical.

Importantly, the impact on cell shape (following p120 KD) was not rescued by forced E-cadherin stability (via the LAEA mutation, see Figure 3F) suggesting that the invagination phenotype is not primarily a function of cadherin stability. Although the stabilizing mechanism is well established, it is possible that cadherin signaling and/or other activities may nonetheless be compromised. On the other hand, the fact that the mutant interacts normally and restores α- and β-catenin to endogenous levels, indicates that the cytoskeletal linkage is largely intact. Moreover, in cadherin-deficient cell lines such as A431D, the behavior of the mutant is almost indistinguishable from that of WT E-cadherin. Further, inhibition of the Rho-ROCK-myosin pathway completely rescued the apical membrane defect, despite the fact that the entire E-cadherin complex was absent (Figure 4B). Additionally, ectopic expression of constitutively active Rho had no effect on E-cadherin stability and yet effectively recapitulated the apical defect associated with p120 ablation (Figure 4D, E). Remarkably, in WT cells, activated RhoA was detected almost exclusively at the apical membrane, whereas the signal shifted entirely to the lateral membrane (along with recruitment of myosin) upon depletion of p120. Thus, cadherin-bound p120 apparently maintains a low tension zone along the lateral membrane via suppression of RhoA. Notably, mutually exclusive presence of E-cadherin and Myosin II at cell-cell contacts is a relatively common phenomenon. For example, convergent extension in Drosophila is dependent on the segregation of E-cadherin and myosin II to D-V and A-P edges, respectively (Simões et al., 2010). Similarly, E-cadherin drives compaction in the early mouse embryo by redirecting myosin away from cell-cell contacts (Maitre et al., 2015).

Interestingly, although constitutive membrane targeting is frequently sufficient to activate receptor-associated cofactors, the CAAX-box targeting of cadherin-uncoupled p120 to basolateral membranes did not rescue p120 ablation. The experiment was remarkable in that the localization of E-cadherin-uncoupled CAAX-p120 and that of WT E-cadherin-bound p120 was essentially indistinguishable, and yet only the E-cadherin bound p120 was active. The crystal structure of the p120/E-cadherin complex shows the JMD core of E-cadherin embedded in a groove along one side of the Arm repeats, leaving most of the p120 surface still exposed and available for interaction with other proteins (Ishiyama et al., 2010b). Thus, one possibility is that suppression of Rho by p120 is enabled by (and perhaps dependent on) interaction with E-cadherin. Plausible mechanisms include an E-
cadherin triggered conformational change in p120, or alternatively, de-novo generation of a new “combinatorial” binding site consisting of polypeptides from both p120 and E-cadherin. A third possibility is that E-cadherin may simply hold p120 in an “active” orientation.

Notably, MDCK cells do express other cadherins (e.g., N-cadherin)(Stewart, 2000), which along with E-cadherin share a common cellular pool of p120(Carnahan et al., 2010). Apparently for that reason, E-cadherin knockdown alone in these cells does not noticeably alter p120 localization (Figure S2C,D), and removing just E-cadherin has no effect on either apical organization or lumen formation (Figure S2E,F). Thus, E-cadherin is not the only classical cadherin that can engage p120 to suppress Rho activity. For example, although E- and N-cadherin have clearly evolved disparate roles, as exemplified by their alternative usage in epithelial to mesenchymal transition (EMT), they (and probably other classical cadherins) are apparently redundant with respect to regulation of junctional tension.

An emerging paradigm in Drosophila development is the specification of cell height by compartmentalization of Rho activity along lateral epithelial membranes(Gibson, 2005; Shen and Dahmann, 2005; Widmann and Dahmann, 2009). For example, Dpp morphogen gradients specify the timing and amount of RhoGAP transcription during wing development. RhoGAP then accumulates along lateral membranes to suppress Rho. Removal of Dpp interrupts the pathway, causing unscheduled elevation of Rho-mediated contractility and shortening of the lateral membranes. Although acute p120-ablation is not directly comparable to the elegant spatiotemporal sculpting orchestrated by developmental programs, the end result is consistent with the drosophila paradigm and indicative of p120’s essential role in suppressing lateral contractility. Interestingly, although the RhoGAP involved has yet to be identified in our MDCK model, a very recent study in C.elegans has identified a previously uncharacterized linker, PICC-1 (PAC-1-Interacting Coiled-Coil Protein-1), that the bridges C. elegans p120 (JAC1) to PAC-1, a RhoGAP with specificity toward cdc42 and Rho(Anderson et al., 2008; Klompstra et al., 2015). Remarkably, PICC-1 turns out to be the worm homologue of vertebrate CCDC85B (aka DIPA), a direct p120 binding partner identified recently in our lab(Markham et al., 2014). This newly identified role for p120 seems rather important and unlikely to be confined to C. elegans, as virtually all of the C. elegans players in the story can be matched to highly conserved vertebrate homologs. Moreover, several clues support the notion that the scenario just described is quite likely to be conserved in the mammalian embryo. Thus, it appears that recruitment of various RhoGTPase modulators (such as the RhoGAP PAC-1) to control local GTPase activities may in fact be the paradigm for which p120 was originally intended in ancient metazoa.

Finally, suppression of contractility by p120 apparently goes well beyond the control of cell height and shape. Indeed, lumen formation is also dependent on appropriate regulation of tension at this level. Exactly how contractility controls these events is not yet clear, in part because NMMIIA activity impacts almost all of the
cellular processes that influence epithelial morphogenesis (Vicente-Manzanares et al., 2009). It is significant, however, that virtually all of the phenotypes induced by p120 ablation are effectively reversed by specific inhibition of ROCK. Although p120's cadherin-stabilizing function is clearly essential, the extent of rescue by ROCK inhibition across multiple phenotypes reinforces the notion that p120 is also a key regulator of cellular tension. Interestingly, E-cadherin is increasingly recognized as a mechanosensor of intercellular forces (Engl et al., 2014; le Duc et al., 2010; Smutny et al., 2010; Taguchi et al., 2011). The fact that p120 stabilizes E-cadherin on one hand and regulates contractility on the other, places p120 at the intersection between sensing and transducing mechanical forces at sites of cell-cell adhesion.
Methods

Antibodies and reagent

The primary antibodies of p120 (mAb pp120, 0.5 μg/ml, BD), (pAb F1aSH, 1:500) and (mAb 15D2, 2 μg/ml) were generated as described (Reynolds et al., 1994). 15D2 was used for immunoprecipitation, pp120 was used for western blotting, pp120 and F1aSh were used for immunofluorescence. Other antibodies include E-cadherin (1:1000, BD), E-cadherin (rr-1, 1:500), N-cadherin (1:500, BD), β-catenin (1:1000, Sigma-Aldrich), α-catenin (1:500), Ezrin (1:1000, BD), Cingulin (1:500, gifts from Dr. Sandra Citi), Tubulin (1:1000, VAPR), Cleaved-Caspase3 (1:500, Cell signaling), Kaiso (1:500, VAPR), Flag (1:1000, Sigma-Aldrich), Myc (9B11,1:500, Cell signalining), NMMIIA (1:500, Covance). Nucleus stained with Hoechst (1:1000), Actin stained with Alexa-fluor Phalloidin (594 or 488) (1:200, Invitrogen). Secondary antibodies for Western blotting were anti–mouse Alexa Fluor 680 (Invitrogen) and anti–rabbit IRdye 800 (Rockland Immunochemicals, Boyertown, PA). Secondary antibodies used for immunofluorescence analysis included anti–mouse IgG, anti–mouse IgG2a, anti–mouse IgG1, anti–mouse IgG2b, and anti–rabbit IgG conjugated to Alexa Fluor 488 or 594 (Invitrogen). Reagents used include ROCK inhibitor Y27632 (EMD/Millipore), (-) Blebbstatin (EMD/Millipore), myristoylated PKCζ pseudosubstrate (ENZO), DMSO (Fisher).

Plasmids

pRetroSuper retroviral vectors expressing shRNA directed against canine p120 was generated as previously described. LZRS-Neo-3XFlag-Gateway vector was used for exogenous expression of p120 (full-length and mutants). LZRS-Neo-MS was used for exogenous expression of E-cadherin (full-length and mutants), DN-RhoA-myc and DA-RhoA-myc. Point mutations were generated using SLIM (site-directed Ligase-Independent Mutagenesis), GFP-AHPH is a kind gift from Dr. Alpha Yap.

Virus production and transduction

Retrovirus was generated by transfecting the Phoenix 293 cells using calcium phosphate method. Retrovirus constructs used were all based on the LZRS-neo as described before. Virus was harvested 48 hrs post-transfection by passing the supernatant through a 0.45-μm filter. Target cells were infected by incubation with retrovirus-containing media containing 4 μg/ml Polybrene for overnight and replaced with normal culture medium. 48 hrs post-infection, cells were selected using either G418 (1 mg/ml) for 7 days. Lentiviral particles were generated by transfecting 293T cells with the petrosuper shRNA plasmid of interest, psPAX2 packaging plasmid, and pMD2.G envelope plasmid, using calcium phosphate method. Lentivirus was harvested 48 hrs post-transfection, and target cells were infected as described above. Approximately 48 hrs post-infection, infected cells were selected using puromycin (0.5 mg/ml) for 2 days.
Cell culture

MDCK II cells were cultured in DMEM (Life Technologies) supplemented with 10% fetal bovine serum (FBS; HyClone/Thermo Scientific) and 1% penicillin–streptomycin (Life Technologies/Invitrogen). Phoenix 293 and 293T cells were cultured in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin–streptomycin. Collagen solution per 1ml was made on ice by adding the following solutions sequentially: ddH_2O 75 μl, 10XDMEM 100 μl (D2429, Sigma), HEPES (200 mM) 100 μl, NaHCO3 (74 mg/ml) 50 μl, Collagen I (354236, BD) 670 μl, NaOH (40 mg/ml) 1 drop. For 2D collagen culture, 85 μl collagen solution was added into the chamber and allowed solidify in the incubator for 20 mins, then 10 μl resuspension of cells were mixed with 200 μl DMEM and added on top of the collagen gel. Culture for 3 days and then fixed and stained.

Collagen Overlay Assay

MDCK II cells were resuspended at 1.5X10⁶ cells/ml as described above. 55 μl collagen solution was added into each chamber and allowed solidify. 50 μl cells resuspension mixed with 200 μl DMEM were then added on top of the collagen gel. 24 hrs later, carefully remove the medium from the chamber, and add 85 μl collagen solution and allow solidify for 20 mins. 200μl DMEM was then added on top of the collagen gel and cultured for another 48 hrs before fixation and staining. Transparent lumen and tubules should be visible for wildtype MDCK II cells under bright-field microscope.

Immunofluorescence, Immunoblotting, Immunoprecipitation

Lysate preparation, western blot and immunoprecipitation procedures, immunostaining on 2D coverslips have been described previously. For immunostaining for cells on collagen, the entire collagen gel was transferred from the chamber slide to a 24-well plate. Fixed using 4% Paraformaldehyde in PBS+ (500 ml PBS, 1mM CaCl₂, 0.5 mM MgCl₂) for 30 mins. Washed with PBS+ and permeabilized with 0.025% Saponin in PBS+ for 1 hr. Washed with PBS+ and then incubated with quench solution (75 mM NH₄Cl, 20 mM Glycine in PBS+) for 1 hr. Washed and incubated with blocking buffer (1% BSA, 1% goat serum, 0.025% Saponin in PBS+) for 1 hr. Dilute primary antibody with blocking buffer and incubate at 4-C overnight. Washed for 4 hrs and incubated with secondary antibody at 4-C overnight. Washed for 4 hrs and stain the nucleus with Hoechst for 30 mins. Collagen gels were then transferred onto the slides, mount with prolong gold and store at 4-C before viewing. Images were collected using Zeiss LSM 510 confocal microscope at 40X or 63X magnification. For Z-stacks, 0.45 μm sections were taken. All images and movies were then processed with Image J with 3D view plugin or Volocity 6.3 demo.

Statistics

Statistical analyses were preformed using Prism (GraphPad La Jolla, CA, USA) with two-tailed Student’s t-tests or Mann-Whitney tests.
Acknowledgements

We wish to acknowledge the generous support of Vanderbilt’s Epithelial Biology Center and GI Special Program of Research Excellence, and outstanding assistance from the Vanderbilt Cell Imaging Shared Resource and Dr. Alissa Weaver with confocal microscopy. We thank Dr. Sandra Citi, Dr. W. James Nelson, Dr. Ian Macara and Dr. Alpha Yap for generously providing important antibodies and plasmids. This work was supported by NIH R01 CA111947 and NIH R01 CA55724 to A.B.R and the Vanderbilt GI SPORE (50 CA95103) to R.J.C.

Author Contributions

H.H.Y and A.B.R conceived the project; H.H.Y performed the experiments; M.R.D established the MDCK p120 KD stable cell line; N.O.M designed and made the LZRS-3XFlag-GW-Neo construct; R.J.C provided expert analysis, H.H.Y and A.B.R analyzed the data and wrote the paper.

Abbreviations list

p120, p120-catenin; Dpp, Decapentaplegic; AJ, Adherens Junction; Ecad, E-cadherin; NMMIIA, Non-Muscle Myosin Isoform IIA; EMT, Epithelial to Mesenchymal Transition;
References


Figures

Figure 1. p120 KD induces apical membrane expansion leaving apico-basal polarity intact

(A) p120 KD causes striking intercellular invaginations (lower panel, arrows) of the otherwise normally flat apical membrane (upper). X-Z-stacks are shown. Bar 10 µm.

(B) Schematics illustrates the apical membrane expansion phenotype. The dotted line shows the apical and middle confocal sections where images were taken in (c).

(C) Membranous Ezrin staining present at the middle section indicates deeply invaginated apical membranes. Bar 10 µm.

(D) 3D reconstruction of WT and p120 KD apical membranes (Ezrin staining). Top down (left) and bottom up (right) views are shown by Ezrin staining WT and p120 KD cells. Bottom up view (p120 KD) illustrates extensive and continuous apical expansion extending well into the lateral domain (white arrowheads). Also see video 1 and 2.

(E) Rescue of flat apical morphology by addback of p120. A mosaic area containing p120 KD cells (right) and the same cells rescued by expression of p120 (left) is shown. The white dashed box outlines the inset magnified on the right. The cell in the crosshairs shows junctional staining of p120 on three sides. The fourth side (white arrow) lacks a p120 positive neighbor and exhibits expansion of the apical membrane on that side only. Bar 10 µm.

(F) Co-staining with Ezrin and the tight junction marker Cingulin, shows that the boundary between the apical and lateral compartments are retained in both WT and p120 KD cells. Bar 10 µm.

(G) Maximum intensity projection of Cingulin staining confirms that tight junctions are intact and continuous in both WT and p120 KD cells. Bar 10 µm.

(H) Treatment of 20 μM of myristoylated PKCζ-PS inhibitor for 48 hrs failed to rescue the apical membrane defect in p120 KD cells.

(I) Immunoblotting shows p120 KD does not affect protein stability of Ezrin, irrespective of the culture condition used.
Figure 2. The E-cadherin-bound fraction of p120 is essential for suppression of apical expansion

(A) The E-cadherin-uncoupling point mutation K401M was introduced into p120 isoforms 1A and 3A. In contrast to WT p120, expression of the mutant p120 constructs in p120 KD cells reveals exclusively cytoplasmic staining. Bar 10 µm.

(B) Biochemical validation of selective uncoupling from cadherin binding of p120 K401M mutants. p120 immunoprecipitation confirms that K401M p120 mutants no longer bind E-cadherin or N-cadherin, but retain interaction with Kaiso.

(C) Schematic illustrating E-cadherin mutations that block E-cadherin endocytosis (LLAA) and uncouple p120-binding (E762A). The K401M mutation in p120 is also shown (lower panel).

(D) K401M mutants fail to rescue apical expansion of p120 KD cells. Z stacks are shown to illustrate the behavior of the apical membrane. White arrows mark the tight junction, as evidenced by cingulin staining. Bar 10 µm.
Figure 3. The p120-E-cadherin interaction is critical for suppression of the apical expansion phenotype

(A) Schematic of p120 constructs used. Note the CAAX motif is fused to K401M mutant p120, not WT.

(B) Schematic illustrating the cellular localization of E-cadherin and p120 mutants used in following experiments.

(C) Both WT and p120 KD cells were stably transfected with CAAX-box tagged K401M (KM) isoform 1A and 3A constructs. As expected, p120 KM-CAAX mutants did not stabilize E-cadherin at cell junctions. Notably, localization of the CAAX box constructs is essentially identical to that of endogenous p120.

(D) Lateral membrane association of p120 is not sufficient to suppress apical expansion in p120 KD cells. Notably, despite precisely co-localization with endogenous p120, the E-cadherin uncoupled CAAX-box p120 constructs do not rescue the apical expansion defect and show no sign of dominant active activity in WT cells. Bar 10 µm.

(E) p120 KD cells were stably transfected with an endocytosis-deficient human E-cadherin mutant (LAEA) containing the E762A p120 uncoupling mutation. In contrast to endogenous E-cadherin in the same cells, this LAEA mutant is retained at the cell surface in the absence of p120. Note that two distinct E-cadherin mAbs are used here, mAb r-1 specifically recognizes endogenous canine (e.g., MDCK), whereas the BD (610181) pan-E-cadherin mAb binds both canine and human E-cadherin. Bar 10 µm. For reasons that are unknown, mAb r-1 recognizes only E-cadherin on the cell surface: endocytosed pool is not recognized (e.g., the bright Golgi staining of endogenous E-cadherin staining in p120 KD cells is picked only by...
the BD E-cadherin mAb).

(F) Forced surface retention of E-cadherin in the absence of p120 did not rescue the apical expansion defect (bottom panel)
Bar 10 µm.

(G) Quantification of the apical expansion phenotype by measuring the LAI (Length of Apical Invagination). Note that p120
KD+LAEA has similar LAI as WT+DA-RhoA, and both are significantly larger than WT.
Figure 4. Activation of the RhoA-ROCK-Myosin pathway underpins both apical expansion and lumen formation defects caused by p120 KD.

(A) Inhibition of either ROCK or Myosin rescues wild type apical membrane organization in p120 KD cells. WT and KD cells were cultured overnight on collagen gels and then treated with either DMSO, Y27632 (10µM) or Blebbstatin (20µM) for another 48 hrs. Bar 10 µm.

(B) Quantification of the length of apical invagination (LAI) in WT and p120 KD cells upon treatment of either DMSO, PKCζ-PS inhibitor, Y27632 or Blebbstatin. Both Y27632 and Blebbstatin brought LAI down to base level, whereas DMSO or PKCζ-PS inhibitor had no effect.

(C) Inhibition of ROCK does not cause E-cadherin relocalization to cell junctions in p120 KD cells. Bar 10 µm.

(D) DA-RhoA expression by itself in WT cells effectively recapitulates the apical expansion defect. Note that overexpression of DN-RhoA has no effect on apical membrane organization. Bar 10 µm.


(F) E-cadherin localization is not affected by the overexpression of DN-RhoA or DA-RhoA.

(G) Immunoblotting confirms the expression of DN-RhoA or DA-RhoA and shows that the stability of cadherin complexes are not affected.

(H) Inhibition of ROCK blocks apical expansion induced by DA-RhoA. Bar 10 µm.
Figure 5. Aberrant RhoA-GTP and Myosin (NMMIIA) accumulation invariably marks the basal end of the apical invagination.

(A, B) GFP-AHPH was transiently transfected into either WT or p120 KD cells on collagen. Cells were then fixed and stained for p120 (A) or Ezrin (B). Only cells expressing low-levels of GFP-AHPH were imaged and 100 observations were made for both WT and p120 KD cells. Note that RhoA-GTP normally localizes to the apical membrane and excluded from the lateral membrane in WT cells. Upon p120 KD, RhoA-GTP became concentrated at the basal end of apical invagination. Bar 10 µm.

(C, D) NMMIIA is excluded from cell junctions in WT MDCK cells but are recruited to cell junctions upon p120 KD (arrows). Representative colonies are shown. Fluorescence line-scan analysis of NMMIIA and p120 staining are shown side by side. Bar 10 µm.

(E) Quantification of junctional NMMIIA accumulation on a junction by junction basis reveals robust its robust presence at 22.2±4.3% of p120 KD cell-cell contacts.

(F) Correlational analysis of apical expansion and junctional recruitment of NMMIIA in p120 KD cells. The first two columns show the percentage of Ezrin invaginations (Invag) with (+) and without (-) junctional staining of NMMIIA. The second set of columns show the percentage of the junctions containing junctional NMMIIA with (+) and without (-) Ezrin invaginations. 98% of the NMMIIA-containing junctions are accompanied by Ezrin-stained apical invaginations. Ezrin Invag was quantified on a junction-by-junction basis. A junction is defined as the interface between two cells and each cell normally has four to five such interfaces. Ezrin invag is defined by the presence of strong strand-like staining at these interfaces.

(G) When present, NMMIIA bundles (arrow) invariably localizes to the tip of the invaginating apical membrane (arrowhead). Representative images and magnified insets of X-Y and X-Z confocal stacks (white dashed boxes) are shown side by side. Bar 10 µm.

(H) Working model illustrating proposed role of p120 in maintenance of the epithelial phenotype and underlying mechanisms.
Figure 6. p120 is critical for lumen formation through suppression of contractility

(A,B) Confocal immunofluorescence (A) Bar 10 µm and bright field (B) Bar 20 µm imaging shows presence of lumens (white and black arrowheads, respectively) in WT but not p120 KD cultures. Images are day 9 MDCK “dome” structures (shown schematically in (C)). White dashed boxes designate insets shown at higher magnification to the right. Bright field imaging discriminates lumens (transparent bubble-like structures, black arrowheads) from intercellular spaces, the latter being visible by immunofluorescence (A), but not bright field (B).

(C) Schematic illustration of MDCK dome formation (left side) or inducible lumen formation (right sided) upon collagen overlay. Before collagen overlay, MDCK cells cultured on collagen form characteristic epithelial apical-basal polarity with apical membrane facing the medium. After collagen overlay, apical membranes are redistributed to cell junctions by transcytosis to initiate lumen formation, causing the monolayer to reorganize into a bilayer.

(D) p120 KD blocks the formation of lumens induced by collagen overlay. Effect is rescued by p120 addback. Tubular- and circular-lumens are visualized by Ezrin staining (arrows). Lumens are present in WT and p120 addback, but not p120 KD cells. Bar 20 µm.

(E) X-Y confocal and Z-stacks of WT, p120 KD and addback collagen overlay cultures. Z-stacks confirm in 3D the presence of sealed lumens when p120 is present. In contrast, actin staining in the absence of p120 is diffuse, indicating lack of lumens. Bar 20 µm. Schematic shows the distinction between lumens, which are sealed, and gaps, irregular spaces between cells.
(F) Inhibition of ROCK or Myosin rescues lumen formation in p120 KD cells. Each set of image is shown by a low magnification image taken at 20X on the left and two magnified insert view on the right. Note that lumen are recognized as the circular intensified actin staining (arrow) surrounded by nucleus. MDCK WT and KD cells cultured on collagen were treated with either DMSO, Y27632 (10µM) or Blebbstatin (20µM) for 24 hrs, overlaid with collagen, and cultured for another three days (with daily replacement of drug). Bar 20 µm.
Figure 7. p120’s activities toward E-cadherin and Rho are molecularly and functionally coupled to enable the maintenance of cell shape in the larger context of an epithelial monolayer. (A) Apical invagination is an inherent early feature and gradually retreats during epithelial monolayer formation. WT MDCK cells were trypsinized and plated at confluent density on 2D collagen gels. Cells were fixed at different time points and stained for Ezrin, p120. Confocal images were 3D reconstructed to generate the tilted view. Z-axis stacks of split channel and merged view are shown side by side for each time point. Bar 10 µm (B) Conceptually, epithelial maturation involves cell-cell adhesion (Step I) and acquisition of shape (Step II). p120’s cadherin stabilizing activity is well-established and essential for adhesion (Step I). On the other hand, by binding to E-cadherin, p120 locally suppresses RhoA-ROCK-Myosin pathway to establish a low-tension zone along the lateral membrane, which enables epithelial maturation into a geometrically organized monolayer (Step II). Finally, this local suppression of contractility by p120 is further required for various critical epithelial functions including cyst formation and lumenogenesis.