p120-CATENIN CONTROLS CONTRACTILITY ALONG THE VERTICAL AXIS OF EPITHELIAL LATERAL MEMBRANES

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To my loving, caring and unconditionally supportive wife, Hui

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LIST OF ABBREVIATIONS

AIG	anchorage independent growth
AJ	adherens junction
APC	adenomatous polyposis coli
ARM	Armadillo repeat
BTB	bric-a-brac, tramtrack, broad-complex
BMP	bone morphogenic protein
BSA	bovine serum albumin
CBC	crypt based columnar cell
ChIP	chromatin immunoprecipitation cKO conditional knockout
CRC	colorectal cancer
DN	dominant negative
DNA	deoxyribonucleic acid
E-cadherin	epithelial-cadherin
EGF	epidermal growth factor
EMT	epithelial to mesenchymal transition
GAP	GTPase associating/activating protein
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor GI gastrointestinal
GTP	guanosine triphosphate
IF	immunofluorescence
JMD	juxtamembrane domain
KD	knockdown
KO	knockout
mRNA	messenger RNA
N-cadherin	neuronal-cadherin

OE	overexpressing
PBS	phospho-buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
RTK	receptor tyrosine kinase
shRNA	short hairpin RNA
TGFβ	transforming growth factor beta
WT	wildtype
ZF	zinc finger

CHAPTER I

INTRODUCTION

Introduction to epithelial adhesion

Multicellularity and adhesion molecules

The rise of multicellularity marks a significant event across evolution, allowing biological systems to overcome the limit of surface-to-volume ratio of individual cells and greatly increase in size and complexity. (Carnahan et al., 2010; Oda and Takeichi, 2011) Whereas the origin of multicellularity is still under debate, an end result is two fundamental characteristics that distinguish multicellular organisms from either unicellular or pluricellular (colonial) species: intercellular adhesion and cross-cellular signaling. For example, Aliivibrio fischeri can live in a symbiotic form and achieve coordination of gene expression via luciferase signaling across multiple cells through a mechanism called quorum sensing. It does not, however, qualify as multicellular organism due to the lack of intercellular adhesion. In multicellular organisms, a number of gene families have evolved over time to serve this adhesive function. All have in common is a feature involving the molecular interaction between integral membrane proteins on apposing cells, and thus referred to as cell-adhesion molecules (CAMs). In vertebrates, there are four major types: 1) Occludins and Claudins; 2) Cadherins; 3) Ig-superfamily CAMs; 4) Selectins (Gumbiner, 2005a). CAMs can distribute loosely across the entire lateral membrane or cluster into distinct patches to form cell-cell junctions. Differing in the localization and function, intercellular junctions are further categorized as 1) Tight junctions (TJ); 2) Adherens Junctions (AJ); 3) Desmosomes 4) Gap Junctions (Gumbiner, 2005a).

Whereas all cellular junctions can generate adhesive force, their physical properties differ in terms of magnitude and half-life. Magnitude is largely determined by the quantity of adhesive molecules and the affinity of the protein-protein interactions. Half-life on the other hand, is more dependent upon CAM turnover ratio at the membrane. Technologies enabling quantitative measurement of the physical properties of these junctions are just now beginning to emerge. Intercellular adhesions differ markedly in different types of epithelium. For example, forces generated by normal movement of the gut result in constant wounding of the epithelial lining of GI tract. The epidermis on the other hand, withstands substantial mechanical stress and still maintains the integrity of intercellular adhesion. This flexibility in intercellular adhesions arises from selective usage of different CAM classes, different family members within a class, and different expression levels of CAMs. Consistently, the presence of tight junctions (TJ) or desomosomes (DM) varies across different tissue types, conferring different strength of intercellular adhesions. However, adherens junctions (AJ) on the other hand, are universally expressed across all tissue types. In other words, classic cadherin complex must sit at a unique position to integrate critical signaling pathways that are essential for all types of epithelial tissues.

Redundancy at Intercellular junctions

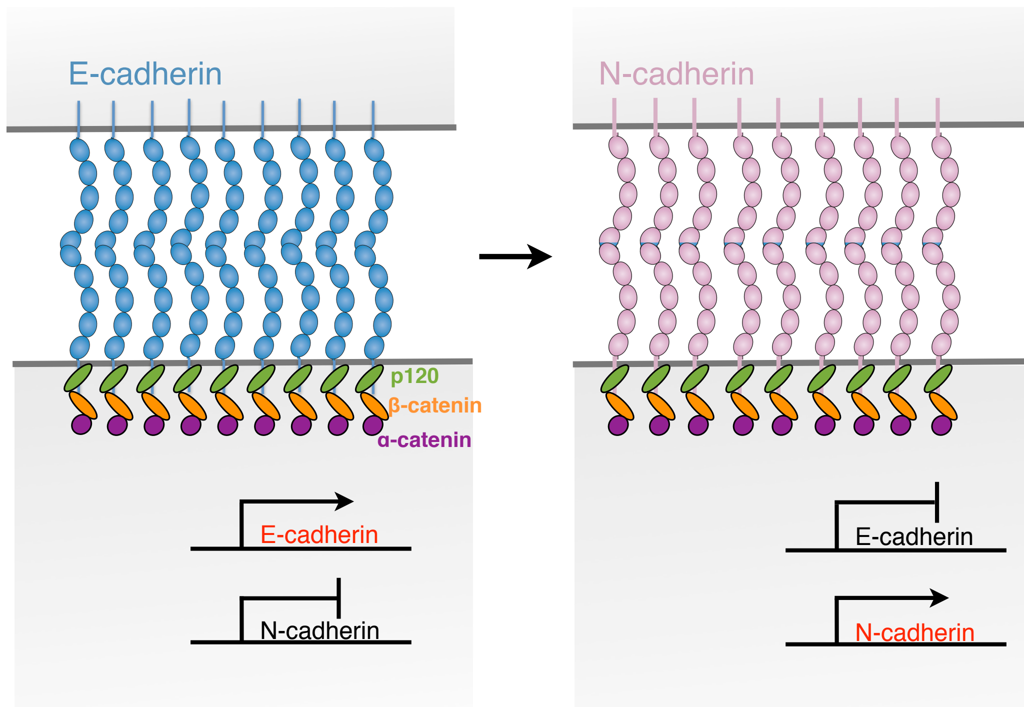
Vertebrates differ substantially from all other animals in that they have evolved 20 different classical cadherin family members. Each tissue type typically expresses two to four members (Gumbiner, 1996; Gumbiner, 2005b; Takeichi, 2014). The extracellular and transmembrane domains vary discretely as needed to confer tissue specificities. The intracellular domains on the other hand, are structurally and functionally conserved, as all of them invariably bind to p120-catenin (p120), β-catenin (or γ-catenin, functionally interchangeable at the cadherin complex, and for simplicity, will be refered to only as β-catenin afterwards) and α-catenin (indirectly through β-catenin). β-catenin mediates the linkage between cadherins and the actin cytoskeleton network via association with α-catenin, although it remains to be determined

whether this association is direct or indirect (mechano-sensitive)(Takeichi, 2014). p120 binds to the juxta-membrane domain and masks a canonical endocytic di-leucine motif to retain the complex at the cell surface. (DEE for VE-cadherin)(Miyashita and Ozawa, 2007; Nanes et al., 2012). Because p120 performs this function for all classical cadherins, it is widely viewed as a master regulator of cadherin stability. Indeed, if p120 is removed, the entire cadherin complex (including β, γ, α-catenin) is internalized and degraded (Davis et al., 2003; Ireton, 2002).

Since all classical cadherins share the same set of catenins, the very type of cadherins at AJs can switch from one to another (e.g. E-cadherin to N-cadherin) (Figure 1A), while normal localization of the catenins (p120, β-, α-catenin) is not affected. On the other hand, this design allows different types of cadherins to compensate for each other partly, which serves as a failsafe mechanism against catastrophe in cell-cell adhesion if one cadherin is mutated or down-regulated (Figure 1B). For the same reason, experimentally, depletion of one cadherin family member is often amorphic because of the compensation. To circumvent the redundancy between classical-cadherin family members, a very efficient method to deplete adherens junctions is directly decreasing the levels of p120-catenin. Depletion of p120 exposes this motif to endocytic machineries and leads to destabilization of most (if not all) classical cadherins across various epithelial types. In cell lines expressing multiple cadherin members, p120 depletion renders them all unstable. Thus, p120 KD or KO studies often lead to convincing results in testing whether AJs are critical for cell-cell adhesion. The MCF10A cells are particularly striking in that they rapidly disaggregate into single highly motile cells after treatment of siRNAs against p120, even though this cell type expresses multiple cadherins at high levels (Kurley et al., 2012). In mammary gland epithelium, p120 KO cells are sloughed off from the neighboring wildtype cells due to loss of adhesion. However, different results have been reported in some cell types. For example, in MDCK (Mardin-Darby Canine Kidney) cells, intercellular adhesion remains largely intact upon almost 90% percent down-regulation of p120 (Dohn et al., 2009). The colony formed by the p120-depleted cells is held together normally by forming tight junctions and desmosomes. Notably,

Cadherin Switching

A.



B.

Cadherin Sharing

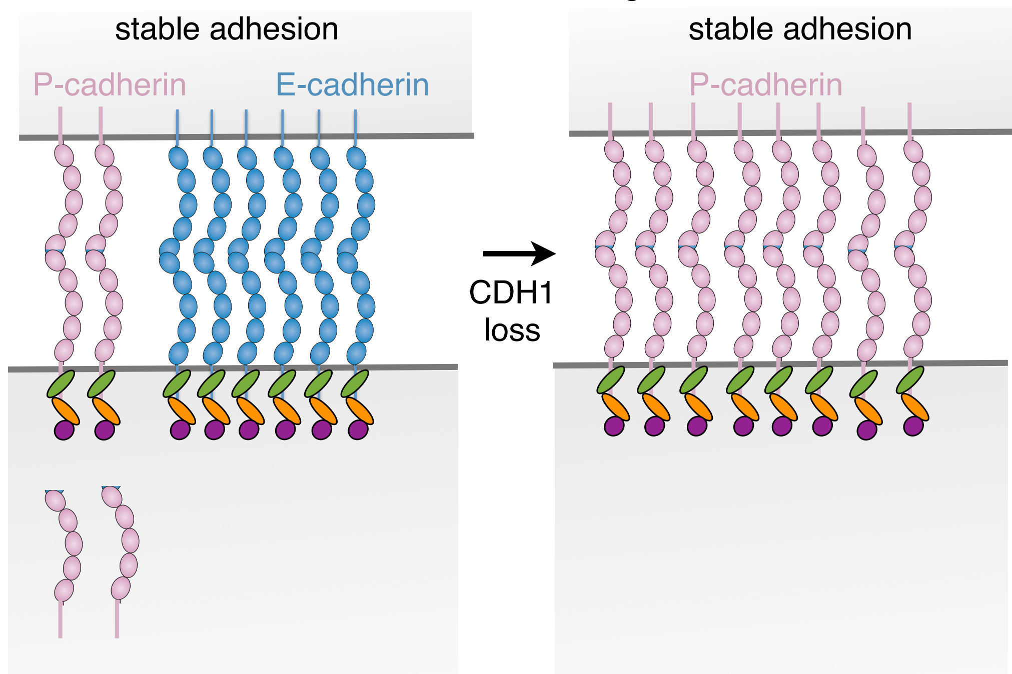


Figure 1. (A) Illustration of cadherin transition from E- to N-cadherin. Note that intracellular components (p120, beta-, alpha-catenin) are intact. **(B)** Illustration of cadherin sharing between E-cadherin and P-cadherin. In case of E-cadherin loss by mutation or deletion, P-cadherin is up-regulated at the adherens junction and maintains stable cell-cell adhesion.

upon p120 KO in the epidermis, no barrier defect is detected even though all CCC components are destabilized significantly (Perez-Moreno et al., 2006). These opposite results highlight an important but previously unresolved paradox: whereas AJs are indeed critical in providing physical attachment between cells, this requirement is nonetheless replaceable by other types of junctions (TJ, Desmosomes etc.) if they are available and expressed at sufficient levels. Thus, the next question is what is unique function of p120/cadherin complex that is not replaceable by other types of junctions.

Adhesion and signaling at adherens junctions

Mutual dependence between cell-cell adhesion and signaling at junctions

Cadherins mediate intercellular adhesion via linking neighboring cells to the cortical actin cytoskeleton network. In the mean time, cadherins also recruit actin modulators (directly or indirectly through catenins) to locally alter adhesion and/or cortical actin organization(Takeichi, 2014). Moreover, CCC has also been shown to crosstalk with other signaling pathways on the membrane to further regulate cell growth, motility, polarity and metabolism. Studying these roles, however, is inherently complicated by the interdependency between the adhesion and signaling activities of cadherin complex. Thus, it becomes increasingly necessary to separately investigate cadherins' role as an adhesion provider and a signaling organizer. The next section highlights serveral properties unique to p120-catenin, illustrating why separating these two activities is important, how a paradigm can be established and applied to investigate other cadherin-associated proteins.

A seminal study by Ozawa group in 2007 shed important insight on how to separate these two core acitivites of p120/E-cadherin. Mutation of a double-leucine motif upstream of the

p120-binding site renders Ecadherin resistant to endocytosis, irrespective of p120 binding. Thus, it is now possible to circumvent the requirement for p120 with respect to cadherin stability. Surprisingly, this analysis uncovered several novel adhesion-independent activities. For example, p120 KD in MDCK cells cultured on collagen gel were unable to maintain the cuboidal cell shape, with apical membranes abnormally invaginated into the intercellular space. This morphological defect cannot be rescued by expressing a stablized E-cadherin mutant (LAEA in this case, with less mutations), but was completely reversed by inhibition of the Rho-ROCK pathway. It is worth noting that the rescue by ROCK inhibition happens in the complete absence of E-cadherin complex at the junctions. Moreover, the Rho-suppressing activity of p120 appears to go well beyond regulation of cell shape, as loss of this activity also led to major defects in epithelial lumenogenesis, a common irregularity found in many p120 KO tissues. Applying this methodology in vivo should provide important new insights into roles of p120 beyond its cadherin stabilizing function. More importantly, the same mindset also applies to loss-of-function studies of other AJ components. For example, it remains to be determined whether the α-catenin KO phenotypes are consequences of impaired cadherin-linkage to the cytoskeleton or other signaling roles.

The role of p120/E-cadherin complex in epithelial architecture and morphogenesis

Epithelial monolayer is a highly organized structure consisting of individual polarized epithelial cells that are connected and aligned together on top of extracellular matrix. A universal principle of organization for polarized epithelial cells is the physical and functional segregation of membranes into three distinctive domains(Martin-Belmonte and Mostov, 2008). Basal membranes impart anchorage, lateral membranes organize adhesive contacts and apical membranes establish a free surface for exchange of materials(Brien and Zegers, 2002). The geometric organization of these three membrane compartments underlies the basis of cell shape (box2). Whereas apical and basal membranes are always one facet, the lateral membranes are

often segregated into four to six facets depending on the numbers of contacts with neighboring cells. The facet number of lateral membranes changes constantly during cell rearrangements, and can be directly modulated by myosin-mediated contractility. For example, during axis elongation in Drosophila, lateral membranes on the anterior-posterior (AP) side are contracted by the planar polarized myosin-II bundles, which lead to complete removal of the facets along the AP axis and so-called rosette formation(Kasza and Zallen, 2011) (Figure 2A). On the other hand, lateral membranes can expand along the vertical axis to accommodate increasing cell height. During wing imaginal disc development in Drosophila, cells start with the cuboidal cell shape, but quickly elongate and become highly columnar within a short time frame. The underlying mechanism was attributed to Dpp signaling and importantly, compartmentalized Rho1 and myosin-II activity along the length of lateral membranes(Gibson, 2005; Widmann and Dahmann, 2009). Thus it seems that the lateral membranes can be either stretched or shrunk by varying myosin activity, to accommodates changes in epithelial cell shapes. The when the area of lateral membranes change significantly, what happens to the cadherin complex that localizes within? During the onset of convergent extension, E-cadherin localization switches from a homogenous pattern (around all edges) to a planar polarized one with significant less along the AP edges. In the mean time, Rok and myosin-II become concentrated along AP edges and initiate contraction of the lateral membranes along this edge (Figure 2A). Importantly, this segregation between E-cadherin and Myosin-II is critical for convergent extension to proceed normally(Simões et al., 2010). There are two remaining issues to be resolved here. First, does increased activity of Myosin-II and contraction leads to increased endocytosis of E-cadherin along the AP edges? On the other hand, does the accumulation of E-cadherin along the DV edge actively suppress Myosin-II activity and contractility?

Whereas it is tempting to suggest the mutual exclusiveness between E-cadherin and myosin-II represents the underlying mechanism of symmetry breaking, there are conflicting evidences in different experimental systems. For example, at zonula adherens (ZA) in MCF10A

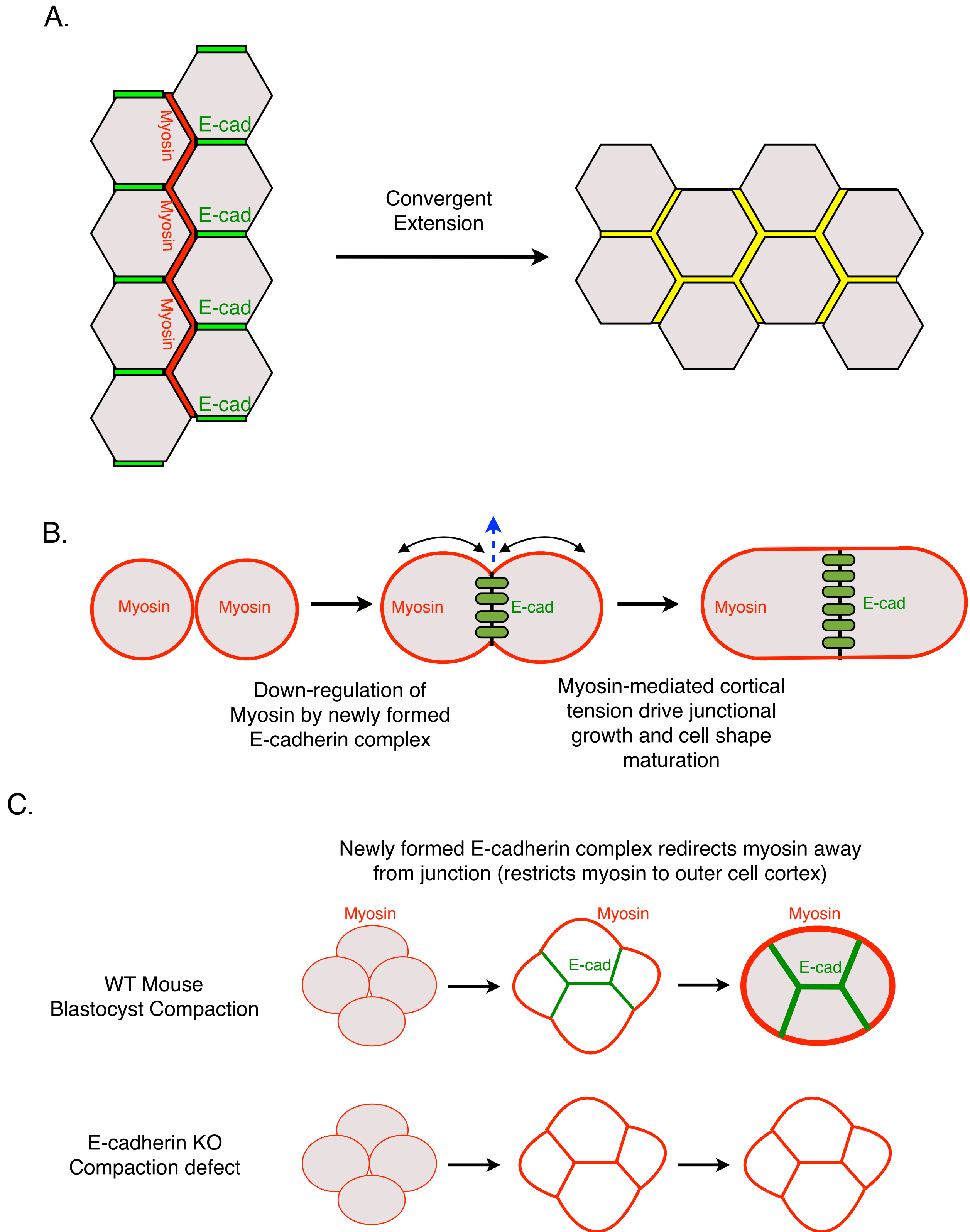


Figure 2. (A) Illustration of cell intercalation during convergent extension of drosophila germ band. Myosin concentrates along the D-V (Dorsal-Ventral) edge; E-cadherin accumulates along A-P (Anterior-Posterior) edge. **(B)** Illustration of nascent adherens junction formation. Note the down-regulation of myosin activity along the cell-cell contact by E-cadherin complex, which creates isotropic tensions (arrows) that drives cell shape maturation. **(C)** Illustration of mouse blastocyst compaction in WT and E-cadherin KO embryos.

or Caco2 cells, Myosin-II and E-cadherin co-localize, and seem to work together to promote the integrity of this apical specialization of cadherin-based junctions (Smutny et al., 2010). On the other hand, during de novo cell-cell adhesion, homophilic ligation of E-cadherin seems to actively exclude myosin-II from cell-cell contacts and restrict contractility to cell-medium contacts. For example, when a doublet of MDCK cells forms cell-cell contacts on plastic, RhoA activity is locally down-regulated by E-cadherin engagement (Figure 2B). In the mean time, myosin-II and phospho-MLC are excluded from the cell-cell contacts and concentrate at cell-medium contacts, enabling intercellular adhesion to expand along the X-Y axis(Yamada and Nelson, 2007). This pattern also applies to polarized epithelial cells during de novo adhesion formation. When cultured on collagen gels, MDCK cells establish a cuboidal cell shape without the prerequisite to reach confluence (compared to filters). In this case, the intercellular adhesion expands in both X-Y and Y-Z axis. It is worth noting that the expansion of cell-cell adhesion along Y-Z axis is actually equivalent to the establishment of cell height. During this process, myosin-II is again excluded from cell-cell contacts, where E-cadherin and p120-ctn localize. On the other hand, down-regulation of p120-ctn leads to loss of E-cadherin from cellular junctions and in the same time, accumulation of myosin-II bundles at cell-cell contacts, which in turn blocks expansion of cell-cell adhesion along the Z-axis. Thus, during de novo intercellular adhesion formation, E-cadherin complex actively suppress contractility across the entire lateral membrane, and enables enlargement of cell-cell contacts and establishment of epithelial shape. Recently, this mechanism is further confirmed in vivo. E-cadherin has long been suggested to be critical for early mouse embryo morphogenesis, specifically compaction, a process by which cells pack themselves into a tighter tissue. Whereas it was postulated that E-cadherin mediate this process solely by strengthening adhesion, this study nicely shows that in fact the mechanism is via redirecting contractility away from cell-cell contacts by E-cadherin (Figure 2C). Importantly, similar to the convergent extension in Drosophila, myosin-II and phospho-MLC become segregated from E-cadherin during 8-cell stage. It remains to be determined, how exactly E-cadherin suppresses myosin-II activity and excludes its localization from cell-cell contacts. One intuitive hypothesis is

that E-cadherin recruits a RhoGAP to cell-cell contacts and thus locally inhibits the RhoA-ROCK-Myosin pathway. There are multiple RhoGAPs shown to localize to cell-cell contacts, yet it remains unknown which RhoGAP is recruited to the E-cadherin complex in a spatiotemporal manner that coincides with de novo cell-cell adhesion formation and/or mouse embryo compaction. Regardless which RhoGAP is involved, an important paradigm seems to emerge: E-cadherin/p120 complex contributes to the maturation of cell-cell contacts via both mediating cell-cell adhesion and redistributing tension around the cell cortex.

p120 and RhoA: an ancient functional coupling critical in tumorigenesis

p120 is critical for cadherin stability in vertebrates by blocking endocytosis, but this mechanism does not apply to invertebrates such as C.elegans or Drosophila(Myster et al., 2003; Pettitt et al., 2003). Consistently, the double-leucine motif at the juxtamembrane domain of vertebrate classical cadherins is not conserved in either HMR-1 (C.elegans) or DE-cadherin (Drosophila). In these two organisms, p120 is considered dispensable, as genetically null adults are clearly viable and exhibit no observable defects in intercellular adhesion. On the other hand, a recently study shows that Drosophila p120 become necessary under stress and dp120ctn loss results in increased heat-shock sensitivity and reduced lifespan(Stefanatos et al., 2013). Interestingly, there are also alterations in the expression pattern of multiple *relish/NF-κB* target genes, although the underlying mechanism is unknown. This observation echoes a previous report for a role of p120 in NF-κB signaling in mice, which was attributed to p120's activity towards RhoA. In addition, another Drosophila study show that p120 and Rho1 (Drosophila homolog of RhoA) is genetically linked. In this case, *dCSK* loss in discrete patches led to epithelial exclusion, invasive migration and apoptotic death, which are mediated by dp120ctn, Rho1, JNK and MMP2(Vidal et al., 2006). Together, it appears that the activity of p120 towards RhoA is likely conserved in Drosophila, in contrary to its activity towards cadherins.

The dCSK study also reveals another potential evolutionary link between p120 and Src. dCSK is a direct inhibitor of Src, a non-receptor tyrosine kinase that is highly conserved across evolution(REYNOLDS, 2007). Thus the phenotypes upon dCSK loss are actually driven by the unscheduled activation of Src, and yet they all depend on the presence of p120 and Rho1. In vertebrates, p120 was originally identified as a Src phosphorylation substrate. Later, p120 was shown to be essential for Src-induced anchorage-independent growth of MDCK cells. The mechanism was then attributed to p120's activity towards RhoA (mammalian homolog of Rho1) since inhibition of ROCK bypassed this requirement of p120 and rescued growth of Src-transformed cells(Dohn et al., 2009). Thus the suppression of RhoA by p120 is necessary for Src-induced oncogenic growth.

Apparently, p120-RhoA-ROCK pathway is also controlled by Rac1, a major small GTPase involved in both actin dynamics and tumorigenesis. In fibroblasts, Rac1 is activated by PDGF receptor tyrosine kinase and initiates the so-called Bar-Sargi pathway that eventually leads to inhibition RhoA and formation of a distinct actin pattern, namely dorsal circular ruffles (DCR)(Wildenberg et al., 2006). p120 KD blocks this Rac1-induced RhoA suppression and leads instead to actin stress fibers (ASF) formation. The Rac1-RhoA antagonism seems to be also critical for epithelial morphogenesis. When culturing MDCK cells in 3D collagen, they spontaneously develop into a cyst-like structure with apical membrane facing a central located lumen. Inhibition of Rac1 activity through either the integrin-blocking antibody or dominant-negative Rac1 induces a so-called inverted polarity phenotype, with the apical pole facing cyst periphery instead and complete absence of lumen formation (O'Brien et al., 2001). This defect was then traced to activation of the RhoA-ROCK-Myosin pathway and treatment of either Y27632 or blebbstatin reversed the phenotype(Yu et al., 2005). Interestingly, p120 KD in MDCK cells also induced the inverted polarity defect. In this case, RhoA inhibition but not Rac1 activation rescued the phenotype, which put p120 downstream of Rac1 but upstream of RhoA in this pathway. Thus, p120 seems to mediate the Rac1-RhoA antagonism at both single and multicellular level.

Mechanical and molecular consequences of p120 modulation

Since the identification of p120 as an integral component of classical cadherin complex, numerous studies have been done to investigate consequences of modulating p120 levels in various cell lines and tissue types. However, the molecular and functional aftereffects are so diverse and interconnected that it remains difficult to pinpoint a pathway to accurately illustrate the biological activity of p120. At the molecular level, p120 has been shown to affect cadherin stability, the localization of α-catenin, p190RhoGAP, Epb41l5 (lulu), CCDC85 (DIPA), Rho-ROCK activity, Kaiso- , JNK-, NFkB- and YAP-mediated transcription. At the functional level, p120 has been involved in collective migration, lumenogenesis, oncogenic proliferation, Inflammation, stemness, differentiation, mitosis and stress response. A daunting but critical task would be to identify the interconnectivity and establish the hierarchy among these distinct molecular activities, and then connect them to the various functional manifestations. Additionally, recent progress on mechano-transduction revealed significant changes in cellular behaviors in response to mechanical cues. This adds another layer of complexity to the problem since p120 modulation is often accompanied by dramatic alterations in cell-cell adhesion, cell shape, actomyosin and microtubule networks(Wozniak and Chen, 2009). In other words, these physical consequences could indirectly affect the aforementioned phenotypes associated with p120 modulation. This section aims to address this vast interconnection and suggest a roadmap for future investigation of the biological activity of p120.

During contact inhibition of proliferation (CIP), epithelial cells within the monolayer stopped dividing when reaching the maximum space allotted. Whereas the process has been long linked to E-cadherin mediated cell-cell contact, Recent reports suggest instead that the mechanism is based on suppression of YAP activity(Aragona et al., 2013). There are at least two major pathways proposed through which E-cadherin engagement inhibits YAP. On one hand, CIP requires cadherin ligation and appears to trigger Hippo signaling through Merlin and

Kibra(Gumbiner and Kim, 2014). On the other hand, α-catenin can independently inhibit YAP activity through a sequestration mechanism via 14-3-3(Schlegelmilch et al., 2011). Since p120 KD or KO leads to destabilization of both cadherins and α-catenin, it is not surprising that down-regulation of p120 abrogates CIP and activates YAP. Additionally, whereas RhoA has been shown to activate YAP in many ways, elevated RhoA-ROCK activity upon p120 KD could contribute to YAP activation as well. While it worth finding out which downstream effect plays a major role, a more important question is how mechanical signals are sensed and transduced to YAP by the cadherin complex and whether or how p120 actively mediates this process. For example, does increased tension applied to the cadherin complex induce changes in p120 phosphorylation profile and modify the affinity between p120 and E-cadherin? Does E-cadherin/p120/β-catenin/α-catenin complex transit between a "open" and "closed" conformation in different tensional environments, sending an "on" or "off" switch signal to YAP? With advancing techniques in profiling components of cadherin complex aka. "cadhesome", it would be fascinating to compare the cadhesome under different tensional conditions and identify mechano-responsive interactions. Recent development of bioengineered devices that allow quantitatively controlled application of forces upon intercellular junctions should be ideal to test these hypotheses.

Hypothesis

For my thesis work, my hypothesis is that p120's activity toward E-cadherin and RhoA are molecularly separate but functionally dependent on one another. Whereas the cadherin-stabilizing activity is essential for maintaining strong cell-cell adhesion, the Rho-suppressing activity is required for apical membrane integrity and epithelial lumenogenesis. These two activities of p120 work together to promote the maintenance of cell shape in the larger context of an epithelial monolayer.

CHAPTER II

MATERIALS AND METHODS

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. For 3D cyst formation, 90% confluent MDCK II cells were trpsinized and diluted 1:10 into a fresh plate. Cultured overnight, cells were trpsinized again, dissociated using pipet and examined under microscope to make sure generation of single cell suspension. Cells were then counted using hemocytometer, centrifuged and resuspended to achieve the concentration of $1.5X10^6$ cells/ml. 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. Mix 20 µl cells resuspension with 1 ml collagen solution and distribute 100 µl to each well on the 16-well chamber slide (178599, Lab-Tek). Put into the incubator for approximately 20 mins and then add 200 µl DMEM on top of the collagen gel per well. 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.

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[19]. 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.

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(Wu et.al., 1998). 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 signaling), 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), DMSO (Fisher).

Plasmids

pRetroSuper retroviral vectors expressing shRNA directed against canine p120 was generated as previously described (Davis et al., 2003). 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 Ecadherin (full-length and mutants), DN-RhoA-myc and DA-RhoA-myc. Point mutations were generated using SLIM (site-directed Ligase-Independent Mutagenesis).

Collagen Overlay Assay

MDCK II cells were resuspended at $1.5X10^6$ 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[25]. For immunostaining for 3D cyst or 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 $CaCl_2$, 0.5 mM $MgCl_2$) 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 NH_4Cl, 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^oC overnight. Washed for 4 hrs and incubated with secondary antibody at 4^oC 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. For quantification of cyst volume, diameters from 60-80 cysts were measured and sphere volume was calculated accordingly. For cyst morphology distribution analysis, values are mean ± s.d. from three replicate experiments, with n>100 cysts per replicate.

CHAPTER III

P120-CATENIN CONTROLS CONTRACTILITY ALONG THE VERTICAL AXIS OF EPITHELIAL LATERAL MEMBRANES

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 plays an important role in modulating 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 (Reynolds et al., 2000). 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(Zebda et al., 2013; Ponik et al., 2013; Anastasiadis, 2015 in press). 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 any of 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 normally 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. Interestingly, the impact of this excess contractility is not restricted to 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

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 4C, D, E). Using Transmission Electron Microscopy (TEM), we observed large gaps between neighboring cells only in p120 KD cells (Figure 4F). Although the tight junction was retained, the apical surface at cell-cell contacts was significantly distorted (Figure 4F, white

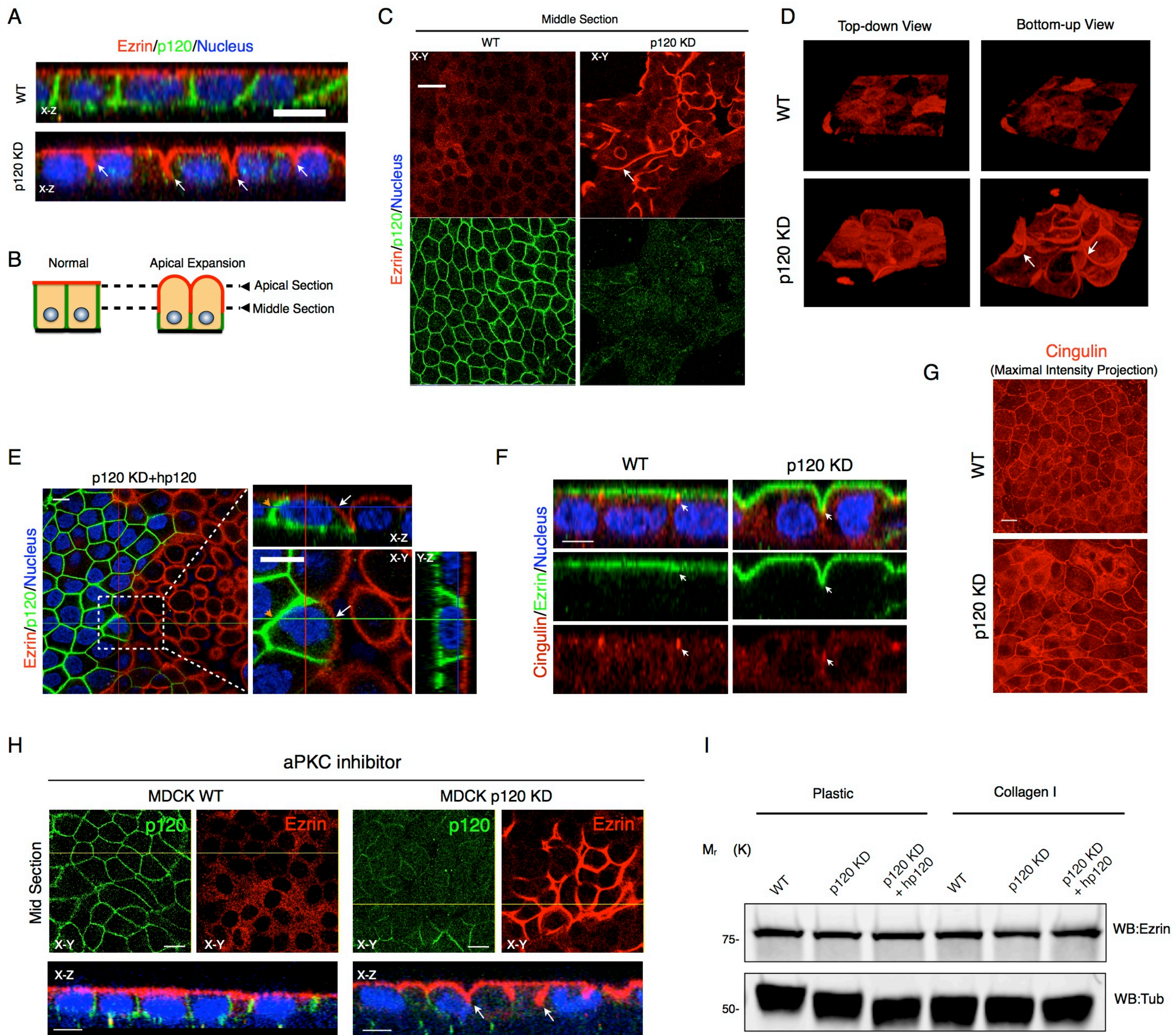


Figure 3. p120 KD induces apical membrane expansion leaving apico-basal polarity intact

(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 *μ*m.

(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 *μ*m.

(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 *μ*m.

(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 *μ*m.

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

(**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.

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 3A, arrows). Figure 3C shows confocal cross sections of the same experiment. Note the aberrant presence of ezrin staining in the intercellular space across the middle section (Figure 3C, 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 3D). 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 3D, arrow, lower right panel, also see video 2).

Interestingly, the dome shaped apical surfaces is 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

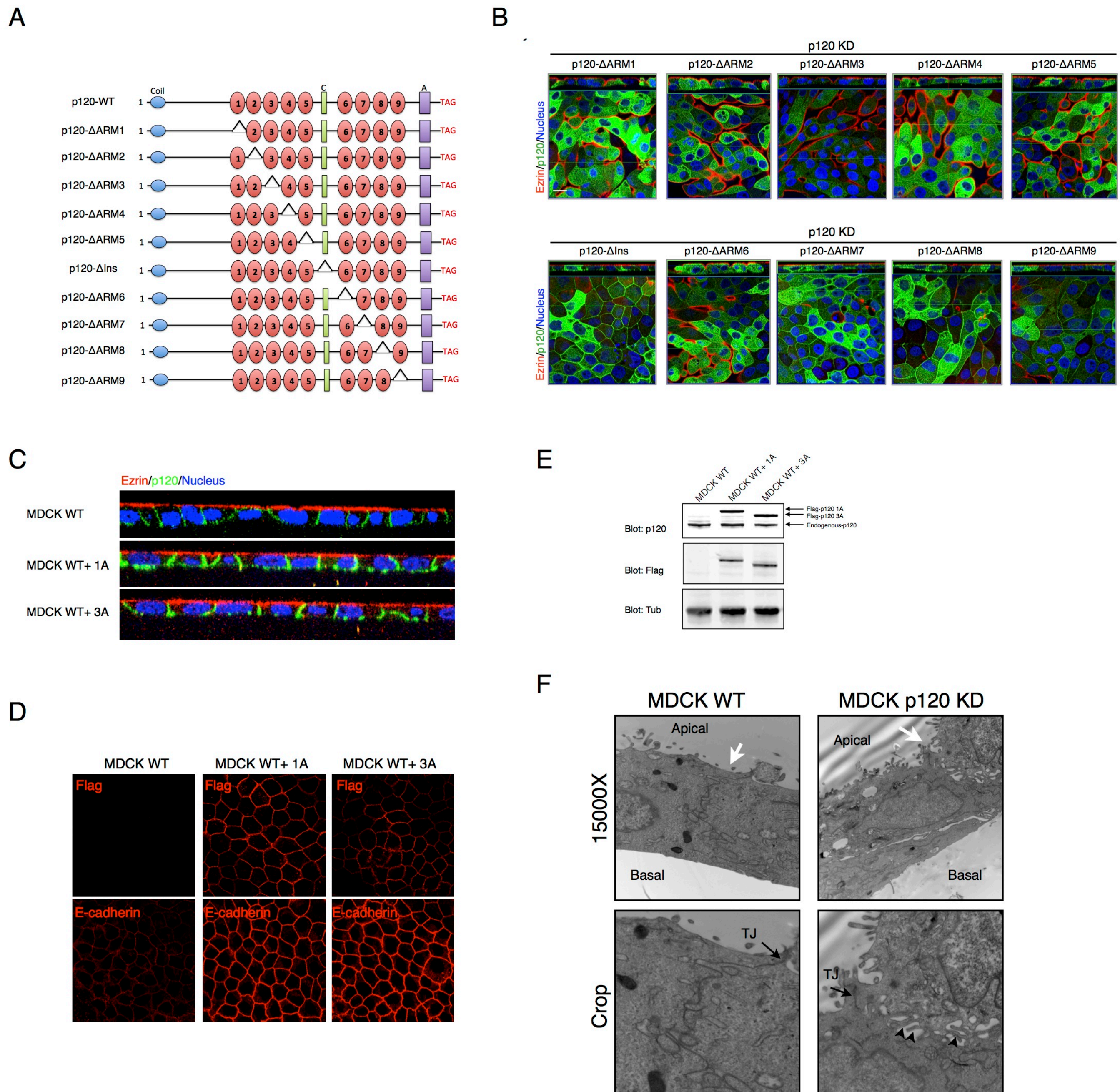


Figure 4. (A,B) ARM repeats 7, 8, 9 and Ins-domain of p120 is not required for maintaining apical-lateral membrane organization. **(A)** Schematics illustrating deletion mutants of p120. **(B)** p120 deletion mutants were stably expressed in the p120 KD cells using retroviral vector LZRS-neo. MDCK cells from each cell line were cultured on top of collagen for 3 days before fixed and stained with Ezrin, p120 and Hoechst. Confocal image from X-Y and X-Z view are shown. Note the flat apical membrane in p120-ΔIns, p120-ΔARM7, p120-ΔARM8, p120-ΔARM9 expression cells. Bar 10 μm. **(C)** Overexpression of p120 isoforms do not impact apical membrane organization. LZRS-Flag-p120-1A or LZRS-Flag-p120 3A was stably expressed in MDCK WT cells using retroviral vector. (**D**) Overexpression of p120 isoform 1A and 3A both significantly increased junctional concentration of E-cadherin. (**E**) Western blot suggest p120 level increased at least two fold due to overexpression of p120 isoforms. (**F**) Morphology of apical and lateral membrane in WT and p120 KD MDCK cells on collagen. Shown are the electron micrograph of thin section of Epon-embedded WT and p120 KD cells. 15000X Magnification was used. The zoom up images are shown below. Note the the differences of apical membrane at the cell-cell contacts (white arrow, upper panel), intact Tight Junction (TJ) marked by arrow (lower panel), long membrane extensions (arrowhead) develop at the loose contacts between neighboring lateral membranes in p120 KD cells, but not WT cells.

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 3F). 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 3G). 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 3I). Additionally, inhibition of aPKC activity via myristoylated PKCζ-PS inhibitor failed to rescue the apical membrane defect in p120 KD cells (Figure 3H). 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 3E, (right panel) makes E-cadherin based adhesions on three sides (Figure 3E, arrowhead). The fourth side (Figure 3E, 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(Ireton, 2002) (Figure 4A,B).

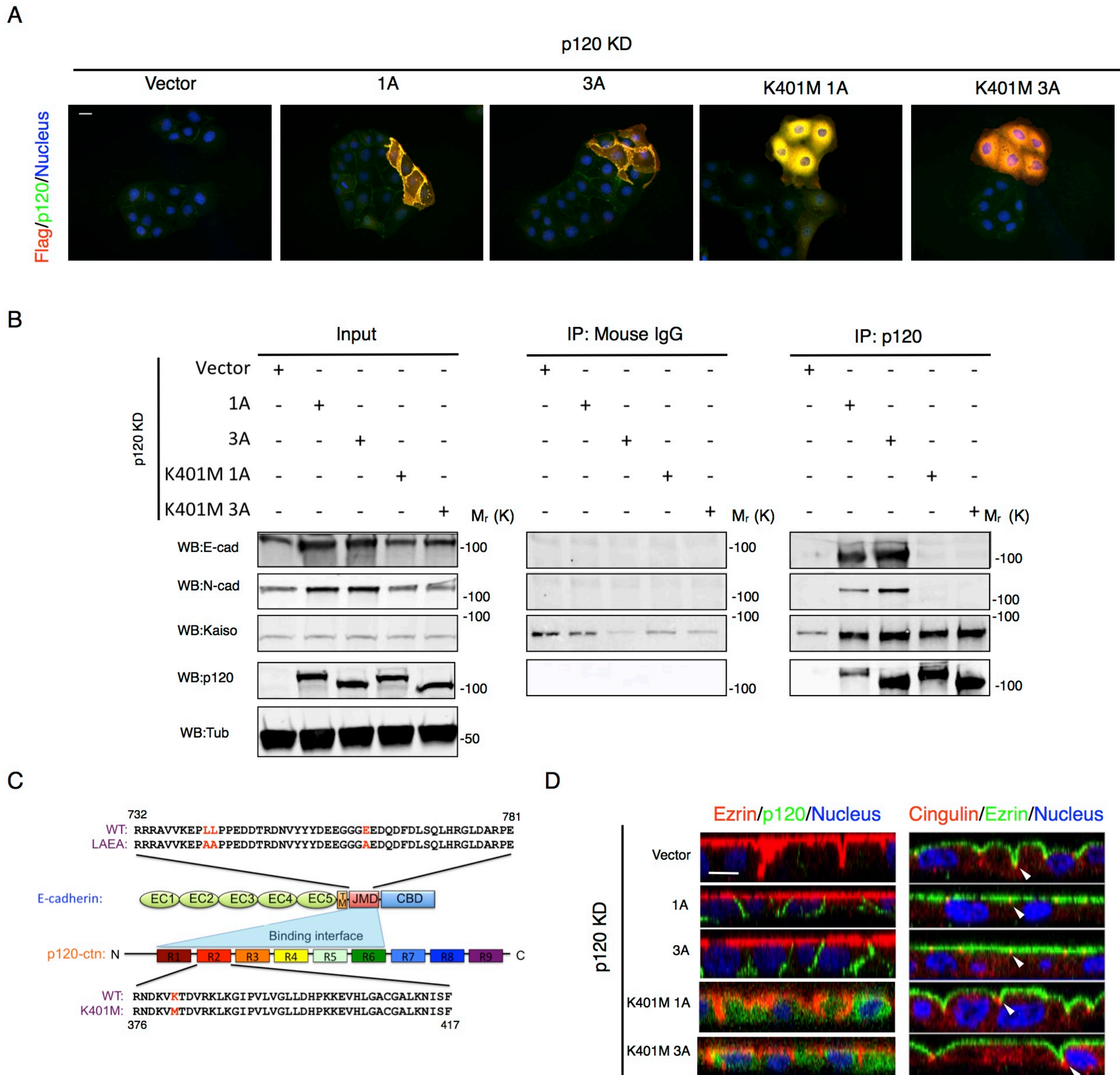


Figure 5. The E-cadherin-bound fraction of p120 is essential for suppression of apical expansion

(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.

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 5C, 5A,B). p120-1A and p120-3A isoforms containing the K401M mutation localized exclusively to the cytoplasm (Figure 5A) and failed to co-immunoprecipitate with or stabilize either E- or N-cadherin (Figure 5B, Figure 6A,B). Importantly, these mutants retain interaction with Kaiso, whose p120 interaction domain is known to overlap with that of E-cadherin (Figure 5B). p120 KD/add-back experiments with these p120 K401M mutants failed to rescue the apical invagination phenotype (Figure 5D), 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 7A,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 7C,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 7C). When plated on collagen, p120 KD cells expressing the p120 K401M-CAAX mutants showed no sign of apical invagination rescue (Figure 7D). 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

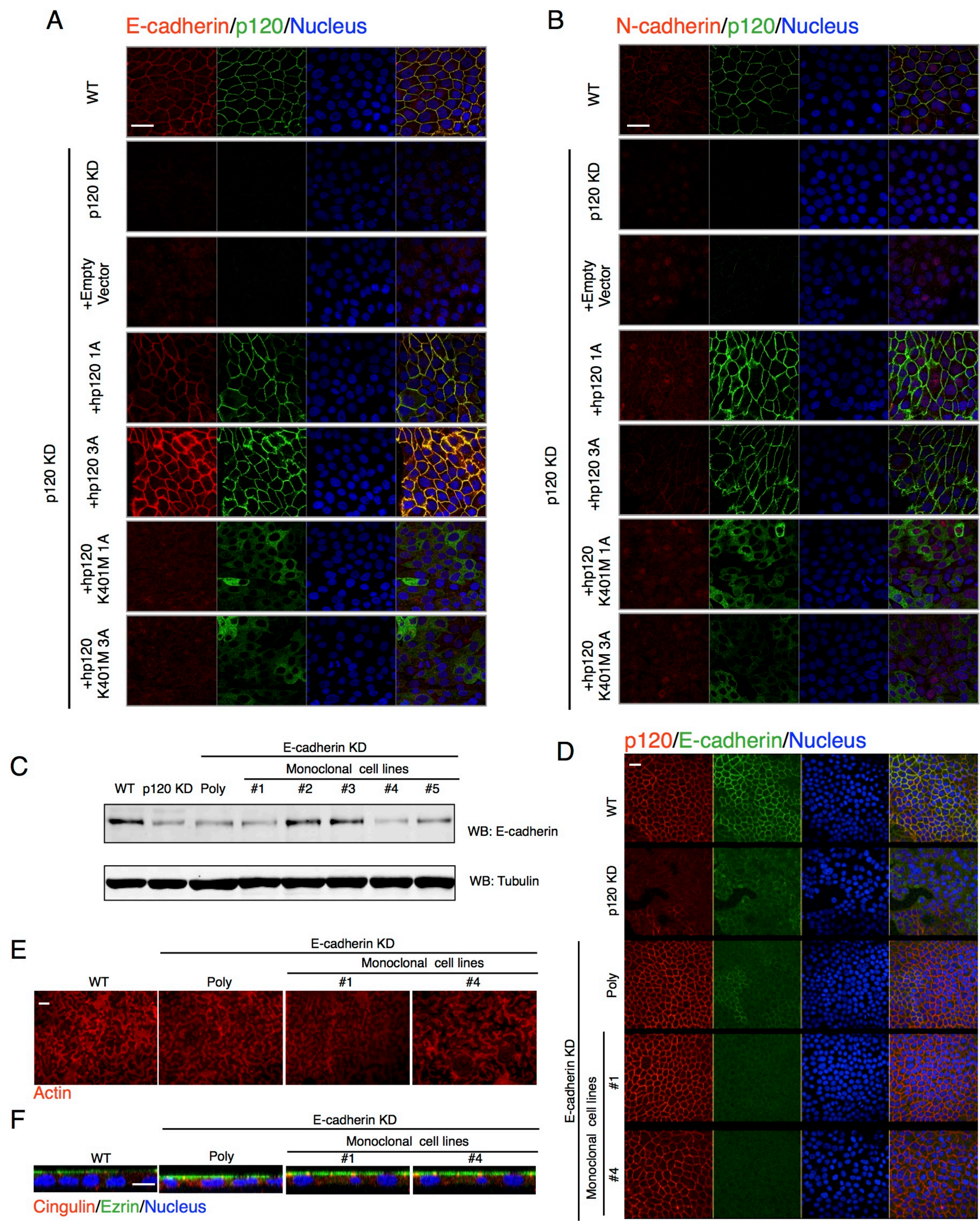
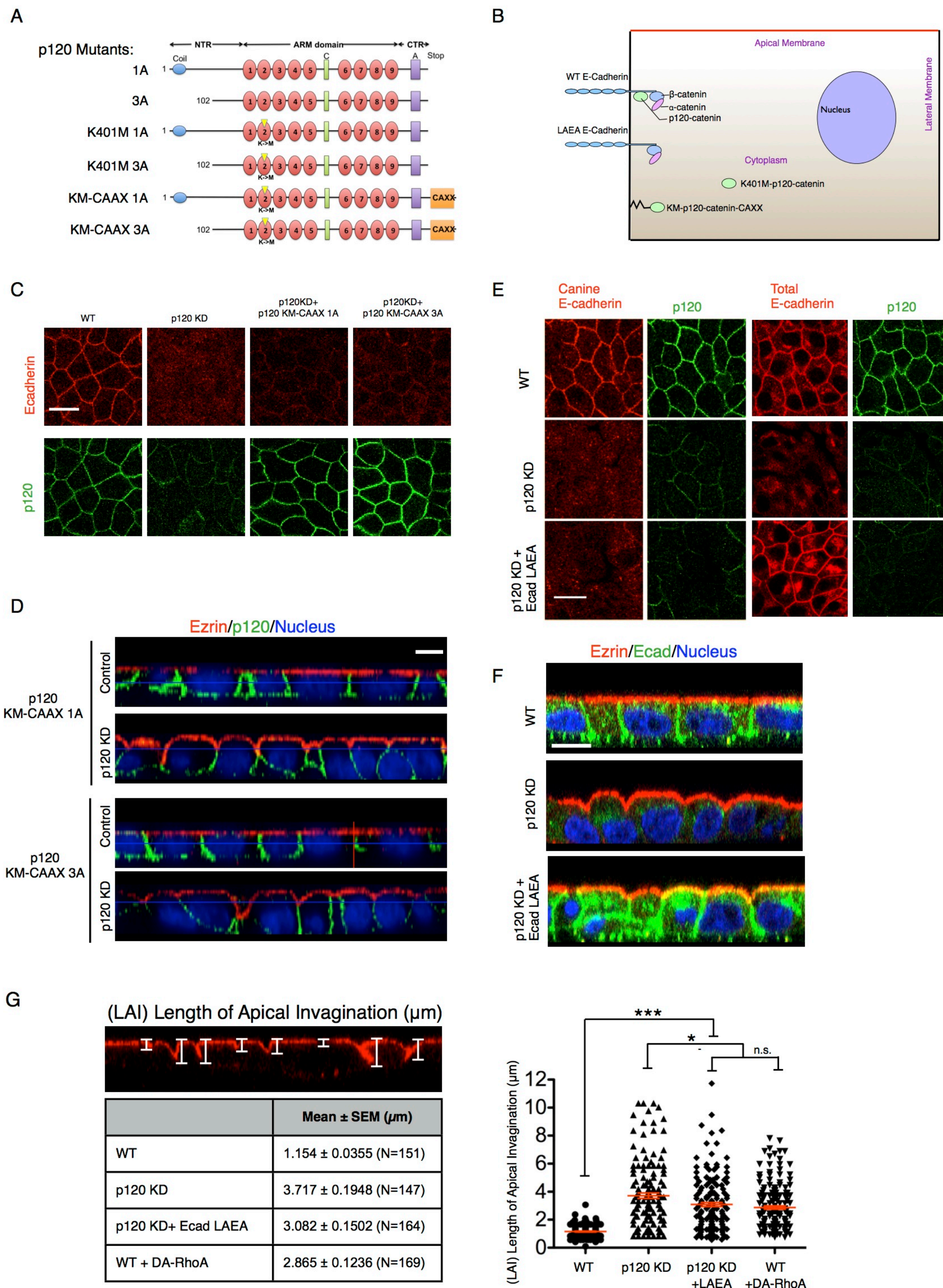


Figure 6 (A, B) Confirming p120 K401M mutants not stabilizing either E-cadherin or N-cadherin when they are expressed in the p120 KD cells. MDCK cells from each cell line were seeded on top of collagen gel for 3 days before fixed and stained with either (A) E-cadherin, p120 and Hoechst or (B) N-cadherin, p120 and Hoechst. Bar 10 μm. **(C)** Western blotting confirms the knockdown efficiency of shRNA against E-cadherin. Monoclonal cell lines from the polyclonal knockdown cell line were selected using cloning cylinder. Cell lysate were collected and blotted with E-cadherin and tubulin. Note that clone 1# and 4# have the lowest protein level of E-cadherin and thus the highest knockdown efficiency. **(D)** E-cadherin knockdown does not affect p120 junctional localization. MDCK cells from each cell line were seeded on top of collagen gel for 3 days before fixed and stained with p120, E-cadherin and Hoechst. Bar 10 μm. **(E)** E-cadherin knockdown does not affect normal lumen formation upon collagen overlay. Bar 20 μm. **(F)** E-cadherin knockdown does not affect apical membrane organization (i.e. remaining flat) or tight junction localization. Bar 10 μm.

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 5C, 7B). When expressed in p120 KD MDCK cells, Ecad-LAEA localizes normally to lateral membranes (Figure 7E, 3rd column), whereas endogenous E-cadherin remains entirely cytoplasmic (Figure 7E, 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 7E). 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 8A, 6B) and cell-cell adhesion in the cadherin-deficient A431D cells (Figure 8C, 6D). 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 7F). 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 7G, 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 7G, 7D). 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.



	Mean ± SEM (μm)
WT	1.154 ± 0.0355 (N=151)
p120 KD	3.717 ± 0.1948 (N=147)
p120 KD+ Ecad LAEA	3.082 ± 0.1502 (N=164)
WT + DA-RhoA	2.865 ± 0.1236 (N=169)

Figure 7. The p120-E-cadherin interaction is critical for suppression of the apical expansion phenotype

(A) Schematic of p120 constructs used. Note the CAXX 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 Ecadherin 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 CAXX-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 rr-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 rr-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.

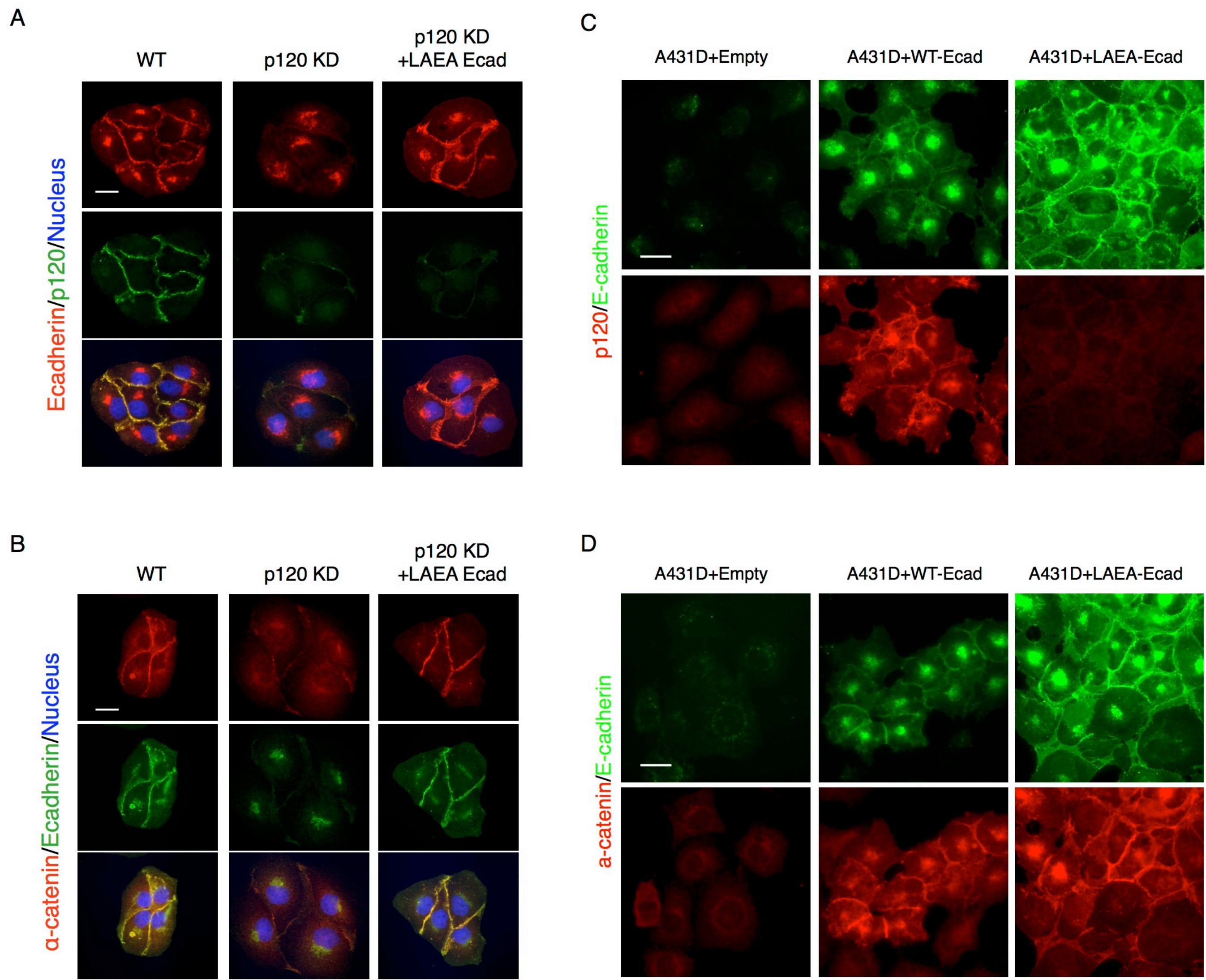


Figure 8 (A, B) LAEA E-cadherin restores actin cytoskeleton linkage via α-catenin in p120 KD MDCK cells. (A) LAEA E-cadherin stably localizes to cell-cell junctions in the absence of p120. **(B)** p120 KD leads to internalization of α-catenin but LAEA E-cadherin expression restores its junctional localization. Bar 10 μm. Both **(C, D) Both WT and LAEA E-cadherin re-epithelialize A431D cells.** A431D cells are deficient of endogenous E-cadherin, re-expression of WT-Ecadherin and LAEA-Ecadherin both reestablished stable cell-cell adhesion. Note that in **(C)** LAEA-Ecadherin failed to recruit p120 to junctions like WT-Ecadherin, and yet still re-establish stable cell-cell adhesions. **(D)**, both WT- and LAEA-Ecadherin recruited a-catenin to the junctions. Bar 10 μm.

Apical invagination 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 15G, 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 15G, 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 (Blebbstatin). Indeed, even in the near complete absence of AJs (Figure 9C), both inhibitors completely reversed the apical defect associated with p120 KD (Figure 9A). 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 9B). On the other hand, treatment with either DMSO or an aPKC inhibitor (PKCζ pseudo substrate) has no effect on LAI (Figure 9B). 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 15A, 13B), indicating that the

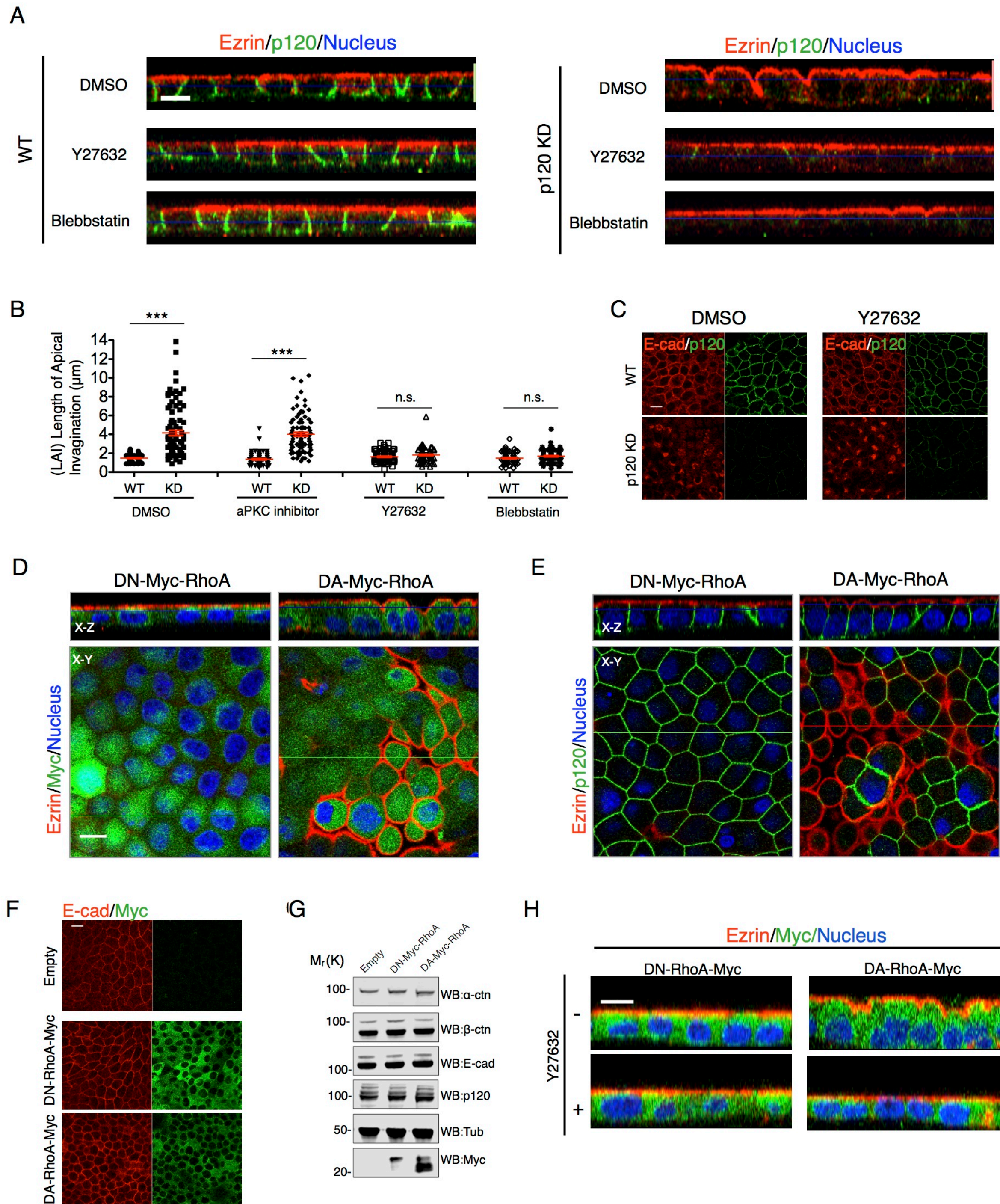


Figure 9. Activation of the RhoA-ROCK-Myosin pathway underpins both apical expansion and lumen formation defects caused by p120 KD.
(A) Inhibition of either ROCK or Myosin rescues wild type apical membrane organization in p120 KD cells. WT and KD cells were cultured overnight on collagen gels and then 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.
(E) Expression of dominant active RhoA induces apical expansion without affecting p120 localization. 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.

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 9D). Of particular interest is that the junctional presence of p120 is strongly maintained (Figure 9E), as is E-cadherin (Figure 9F), and yet the apical invagination defect is readily apparent. In contrast, cell morphology was unaffected by DN-RhoA (Figure 9D). Other variables, including the levels of members of E-cadherin complexes were unaffected by either construct (Figure 9G). Further, blocking ROCK activity effectively resolved the apical invagination defect induced by expression of DA-RhoA (Figure 9H). 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 10A, left panel). However, in p120 KD cells, RhoA-GTP relocalized to cell-cell contacts (Figure 10A, right panel) and notably, was no longer observed at the apical membrane. Confocal analysis placed the signal just under the the apical invaginations (Figure 10B, 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

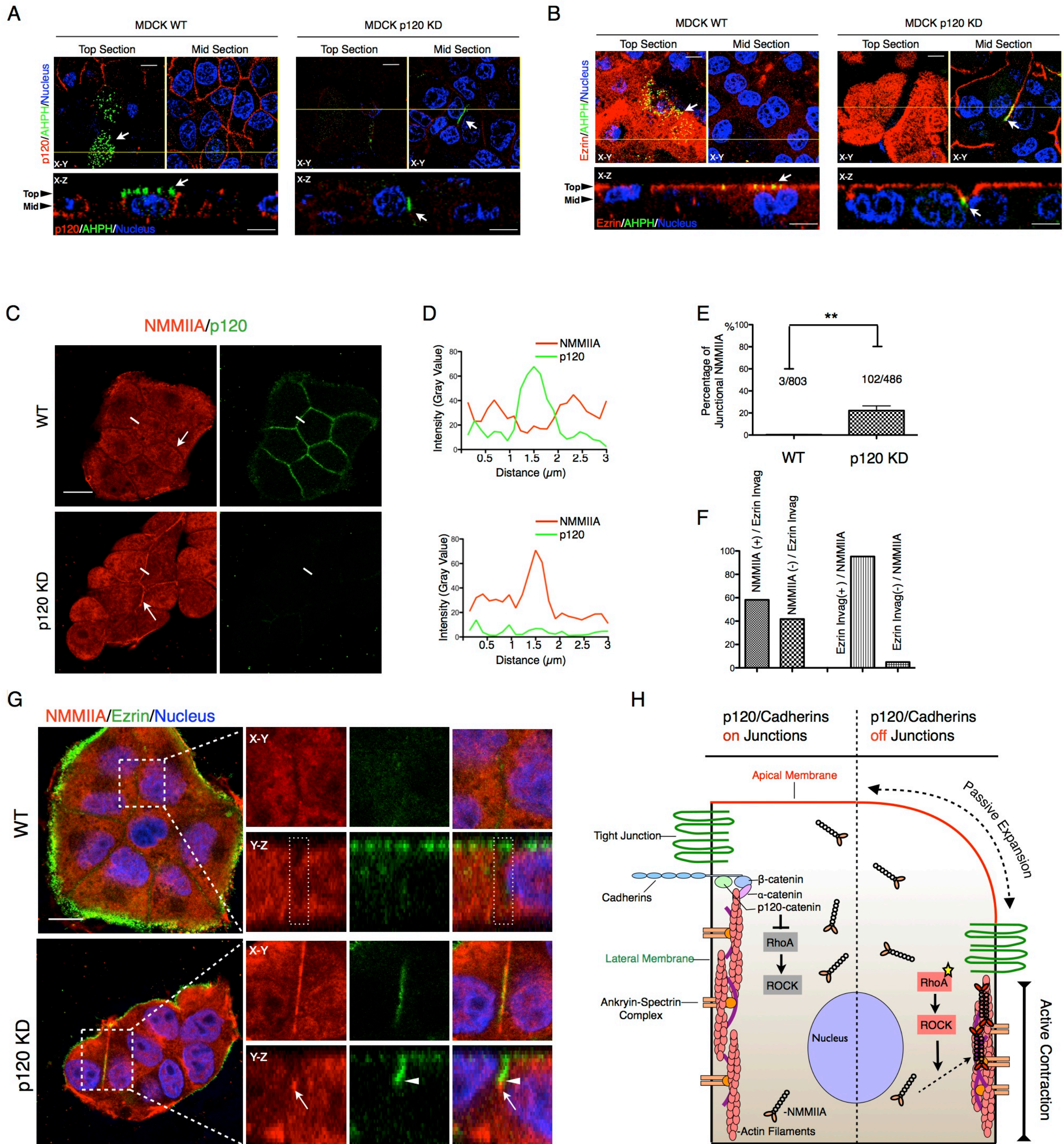


Figure 10. Aberrant RhoA-GTP and Myosin (NMMIIA) accumulation invariably marks the basal end of the apical invagination.

(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 (see a'). 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. Ezin 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.

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 10A, arrow)(Yamada and Nelson, 2007; Yu et al., 2008). However, p120 KD induced a striking accumulation of NMMIIA at lateral membranes (Figure 10C 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 10E). 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 10F), as evidenced by immuno-staining for Ezrin (Figure 10G). Strikingly, when present, NMMIIA was found at the very tip of the ezrin demarcated apical invagination (Figure 10G, lower-right panel), which was shown to terminate abruptly at the tight junction (Figure 3F). 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 10H, model).

Discussion

Generation of an epithelial monolayer from individual cells is a coordinated process involving cell-cell adhesion and acquisition of shape (Figure 11B)(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 11A, 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 11A (1 hr time point).

Whereas p120's cadherin stabilizing activity is well-established and essential for adhesion (Step I, Figure 11B), 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 11B). 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 7F) suggesting that the invagination

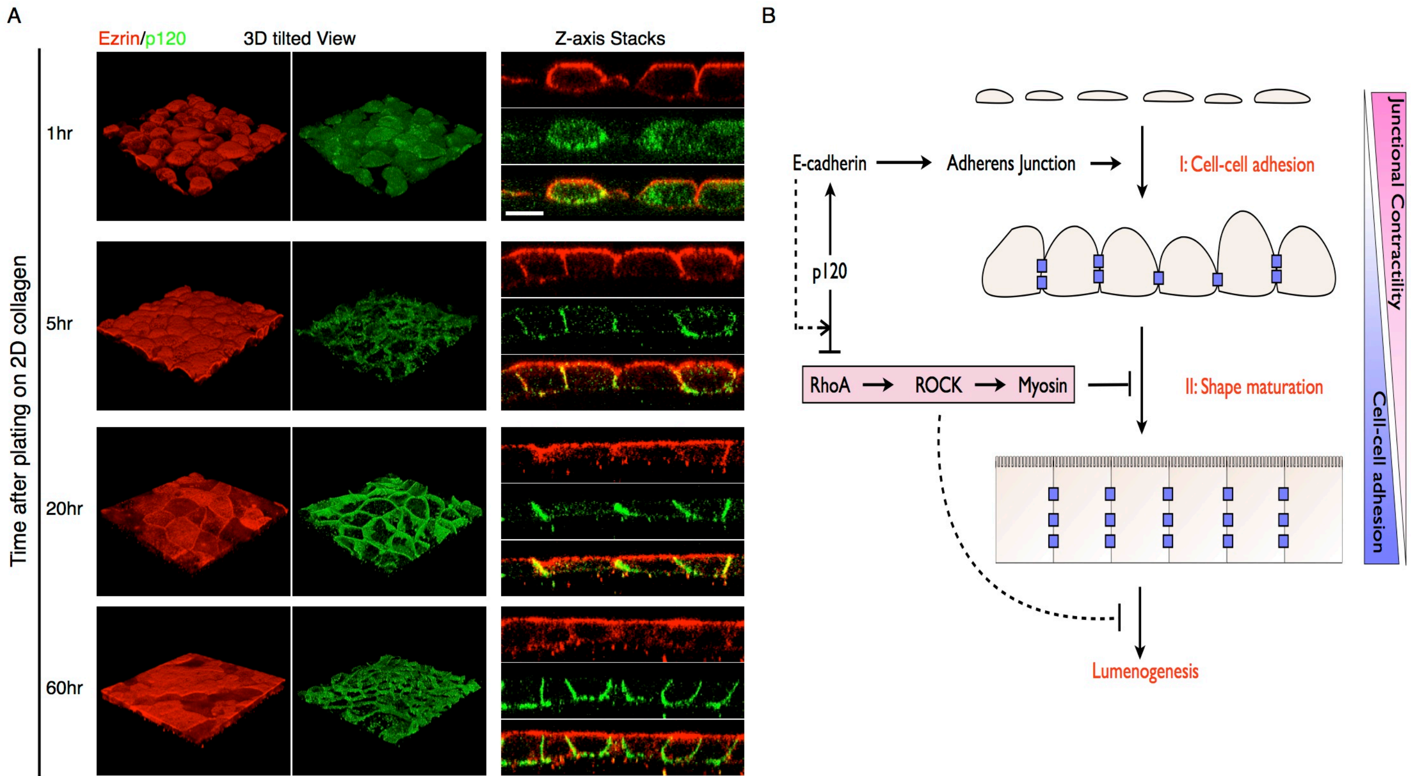


Figure 11. p120's activities toward E-cadherin and Rho are molecularly and functionally coupled to enable the maintenance of cell shape in the larger context of an epithelial monolayer. (A) Apical invagination is an inherent early feature and gradually retreats during epithelial monolayer formation. WT MDCK cells were trpsinized 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 suppress 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.

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 9B). 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 9D, 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 (Maître 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 8C,D), and removing just E-cadherin has no effect on either apical organization or lumen formation (Figure 8E,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.

CHAPTER IV

P120 IS CRITICAL FOR EPITHELIAL LUMENOGENSIS AND CYST FORMATION

Introduction

The embryonic lethality of p120 KO mice forces researchers to selectively knockout p120 in epithelial cells of various organs, using tissue-specific promoters. As mentioned earlier, depletion of p120-catenin is a very efficient method to remove all CCC from the membrane, which also means that both adhesive and signaling functions of cadherins are disrupted altogether. Thus presumably, phenotypes from p120 KD or KO are always a mixed bag of defective intercellular adhesion and cadherin-associated signaling. In most cases, it is very difficult to separate them apart. For example, p120 KO in the mammary gland leads to completely inhibition of branching morphogenesis(Kurley et al., 2012). One explanation could be that the mammary epithelial cells fail to form the tubular structure because they cannot efficiently adhere to each other. However, it also makes sense that the disruption in cadherin-associated signaling via p120-catenin to cortical actin network could leads to defects in collective cell migration, a process that is necessary for tubules formation. Consequently, although it is clear that p120-catenin being absolutely critical for mammary gland development, the underlying mechanism remains inconclusive. Interestingly, studies with results coming from a polar opposite direction shed lights on how to solve this inevitable problem. As noted earlier, p120 KO in mouse epidermis has no effect on intercellular adhesion due to compensation by the presence of extensive desmosomal junctions. And yet, a dramatic cell-autonomous inflammation develops within the epithelium, which is attributed to elevated NFkB activity(Perez-Moreno et al., 2006). In this case, it is obvious that cadherins' adhesive function is irrelevant here since the cells hold together nicely. As a result, the inflammatory phenotype can be exclusively contributed to the signaling role of p120-

catenin. An important clue to be drawn here is that depletion of p120-catenin in a context of intact intercellular adhesion is critical to define its signaling role. Whereas it is difficult to replicate the junctions profile of epidermis in other tissue types, a more generalizable approach would be restoring AJs directly after depletion of p120-catenin. A seminal study led by Ozawa group back in 2007 shed a critical insight on how to achieve this. Whereas p120-uncoupled Ecadherin is unstable, simultaneous mutating a double-leucine motif upstream of the p120 binding site renders Ecadherin resistant to endocytosis. In other words, the so-called EALA-Ecadherin mutants could mediate stable intercellular adhesions in the complete absence of p120. Based on this piece of observation, an experimental paradigm can be established: the result of whether p120 KD/KO phenotypes can be sufficiently rescued by EALA-Ecadherin would inform to what extent these phenotypes represent an adhesion or signaling defect. Thus applying this methodology in vivo should conclusively determine whether a certain p120 KO phenotype represents a defect in intercellular adhesion or impaired junctional signaling. Here we used a MDCK 3D cyst model to mimic epithelial morphogenesis in vivo, which in the mean time allowed easy genetic manipulation. It turns out that the Rho-suppressing activity is indeed required for both three-dimensional cell growth and epithelial lumenogenesis, both of which were previous masked by the apparent cell-cell adhesion defect.

Results

Cyst-growth is blocked by p120 KD and rescued by inhibition of ROCK or myosin

Although junctional tension has been examined extensively in 2D cell cultures (Maître et al., 2012; Liu et al., 2010; le Duc et al., 2010; Wu et al., 2014; Engl et al., 2014), cells grown in this manner lack 3D characteristics such as cell height and exhibit many other behaviors that are not observed *in vivo*. To interrogate p120 function under more complex conditions, we examined the effects of p120 KD on MDCK cell cyst morphogenesis in 3D collagen cultures. Normally,

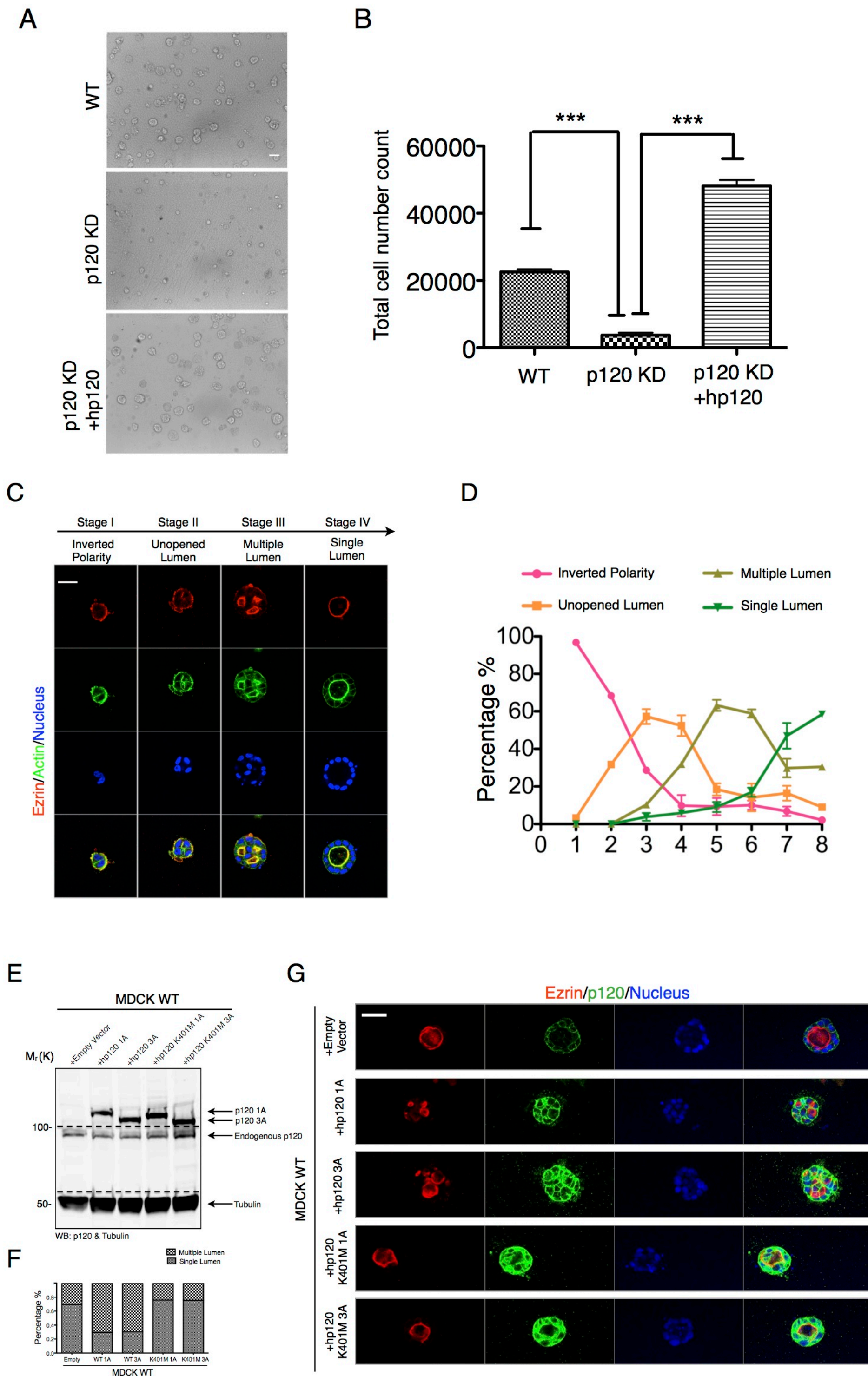


Figure 12 (A) Bright field images showing populations of WT, p120 KD, and add-back rescue cysts. Bar 50 μm. **(B)** MDCK p120 KD has 5-fold less total cell number per collagen gel compared to WT cells which suggests slower cell proliferation.**(C-D)** Staging criteria of cyst development and characterization of p120 KD phenotype. **(C)** Representative images of cyst morphology from each stage shown by Ezrin and Actin immunostaining. Bar 20 μm **(D)** Quantification of the distribution of each development stage over 8 days (60-80 cyst staining captured per time point, 3 independent experiments). **(E-F)** Overexpression of p120 induces multiple lumen formation. **(E)** Confirm expression of p120 mutants in the WT MDCK cells. Cell lysate were collected from each cell line and western blotted for p120 and tubulin. **(F)** MDCK cells from each cell line were embedded in 3D collagen gel for 9 days before fixed and stained with Ezrin, p120 and hoechst. Representative images are shown for each cell line. Bar 20 μm. **(G)** Histograms representing the relative distribution between single-lumen and multiple-lumen phenotypes in each cell line.

single cells embedded in collagen proliferate continuously and reach ~50 cells per cyst by day 7 (Figure 13E, top panel). In sharp contrast, we find that p120 KD induces growth arrest by day 4 at the 8-12 cell stage (Figure 13B, 11E, mid panel). Caspase 3 staining revealed that apoptosis actually decreases slightly in the p120 KD cells (Figure 13 C, D), suggesting that the size deficit is not linked to apoptosis but most likely growth arrest. Importantly, growth was restored by expression of shRNA-resistant p120 cDNA (Figure 13 E, bottom panel). As expected, E-cadherin was destabilized by p120 KD and rescued by expression of WT p120 (triple flag-tagged) from an shRNA-resistant cDNA (Figure 13 A). Total cell counts (Figure 12 B) and live cell imaging (Video 1 and 2) exclude the unlikely possibility that the reduced cyst size resulted from cell dissociation and/or migration.

The mechanism underlying cell growth arrest following p120 ablation is not yet clear. However, cell growth was effectively restored by addition of 2 µM or 4 µM Y27632 (ROCK inhibitor) to the p120 KD cultures, again implicating unscheduled activation of Rho. Interestingly, although the inhibitor is added at day zero, it is not until beyond day3 that the rescue effect is evident (Figure 13 G, H). Note that 4 µM Y27632 slowed cyst growth in general (Figure 13 H, both WT and KD), consistent with reported side effects in cytokinesis attributed to this drug when used at higher concentrations.

Inverted polarity caused by p120 knockdown is rescued by inhibition of ROCK

To facilitate quantitative measurements of specific morphogenetic events, the process of cyst morphogenesis was subdivided into four distinct stages using ezrin and actin as markers to track progression (Figure 12 C). Stage I (inverted polarity stage) is defined by inverted polarity, as indicated by localization of Ezrin externally at the cell-ECM interface, prior to initiation of lumenogenesis. Stage II (unopened lumen stage), is defined by the appearance of Ezrin stained pre-apical patches at cell-cell contacts marking the site of future lumen formation. Stage III (multiple lumen stage) is defined by the presence of two or more open lumens. Stage IV (single

lumen stage) is the mature cyst, a circular monolayer of cells with Ezrin-stained apical surfaces facing a centrally located lumen. Progression from one stage to the next is expressed as the percentage of cysts at each stage from day 1 to day 8 (Figure 12 C). Notably, on day 1, 96.76%±0.64% (Mean±SEM) of the cysts exhibit inverted polarity, indicating that they have yet to initiate the process of reorienting polarity. From day 2 to day 4, apical membrane components are efficiently relocated away from extracellular matrix and concentrated internally at cell-cell contacts, presumably by transcytosis (Apodaca et al., 2012).

p120 KD led to several morphogenetic defects in cyst development. After 8 days in collagen, 57.96%±4.94% of WT cysts had developed normally to stage 4. In contrast, the majority (68.51%±2.26%) of p120 KD cysts growth arrested in stage 1 (Figure 13 I, J). In the absence of p120, the normal internalization and relocation of apical membranes following exposure to collagen did not occur. Interestingly, restoring expression of p120 efficiently rescued the relocation defect but most of the rescued cysts (88.84%±1.84%) failed to form single lumens and instead arrested at the multiple-lumen stage 3 (Figure 13 I, J). To distinguish "partial rescue" (e.g., insufficient p120) from the possibility of an overexpression phenotype, we examined the effect of overexpressing p120 (isoforms 1A or 3A) in WT MDCK cells (i.e., retaining endogenous p120). These experiments show that p120 overexpression typically results in a multiple lumen phenotype (Figure 12, E-G), as also observed in the knockdown/add-back experiments. Given that the KD/AB cysts typically express higher than WT levels of p120, it appears that the multiple lumen effect we observe is due to p120 overexpression.

Interestingly, as with other p120 KD phenotypes, inhibition of ROCK (Y27632) effectively rescues inverted polarity, with the majority of cysts manifesting internal lumens (stages 2, 3, 4), as apposed to the inverted polarity (stage 1) observed in untreated controls (Figure 13 K, L). It is worth noting that Y27632 treatment does not rescue single-lumen formation of p120 KD cells

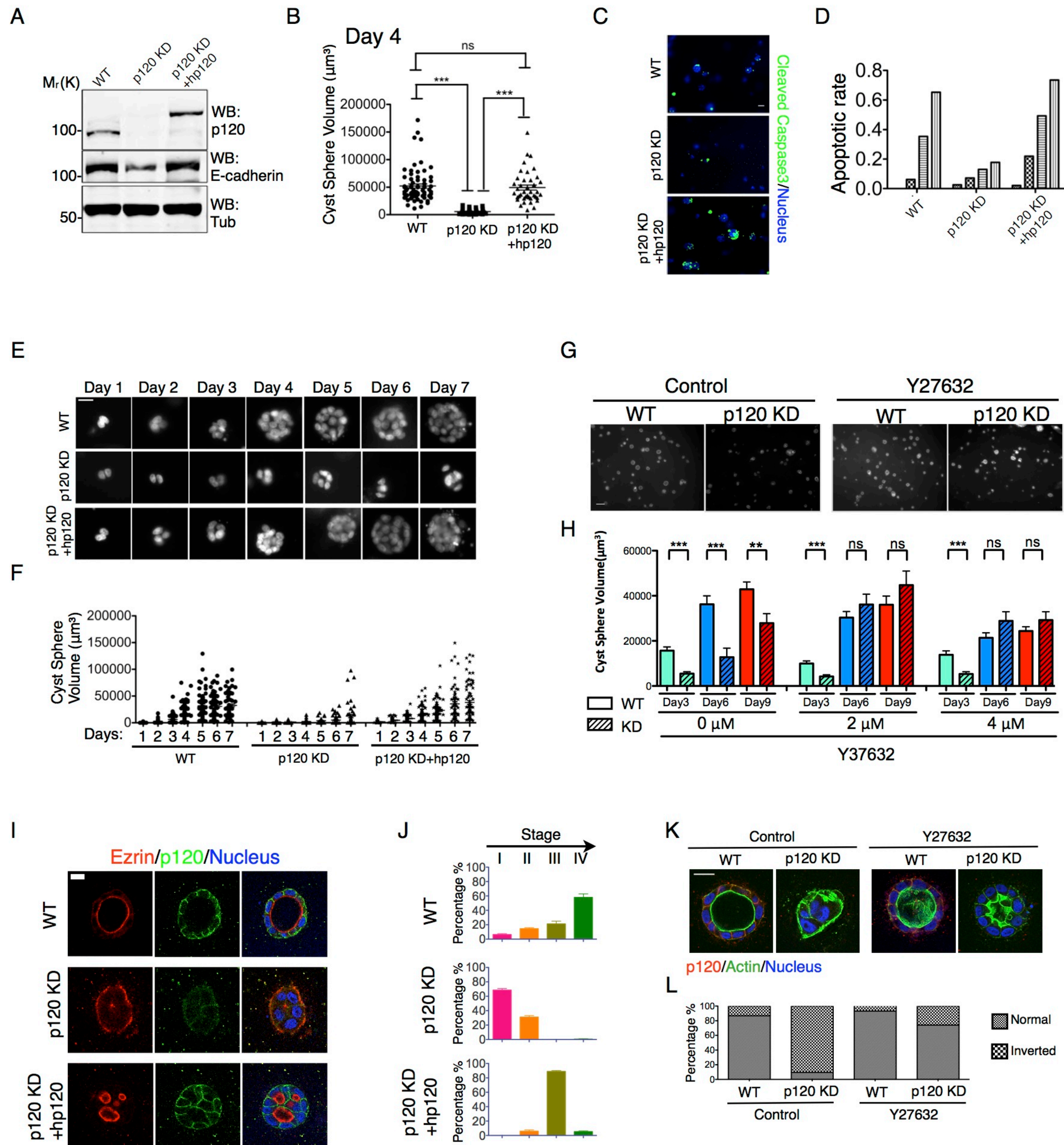


Figure 13. **Excessive junctional contractility induced by p120 KD leads to cyst formation defect**. MDCK cells were cultured in 3D collagen gels for up to 8 days and analyzed as indicated.

(A-F) Characterization of growth arrest induced by p120 KD. **(A)** Validation of p120 KD and rescue by immunoblotting. Note the E-cadherin down-regulation upon p120 KD and rescue by p120 add-back. Slower mobility of hp120 is due to the N-terminal triple flag-tag. **(B)** Quantification of cyst size on day 4 shows that p120 KD cysts are smaller than WT structures and rescued by shRNA resistant human p120-1A (hp120) cDNA (60-80 cysts measured per cell line). **(C,D)** Reduced apoptosis in p120 KD cultures evidenced by cleaved Caspase-3 immunostaining apoptotic rate measured as ratio of cleaved Caspase-3 positive to total cyst number.**(E)** Cyst development over 7 days in p120 KD and add-back cultures. p120 KD cells are growth arrested on Day4 (Nuclei imaged by hoechst staining). Bar 20 *µ*m **(F)** Quantification of (e). **(G,H)** There is no significant differences in cyst size between WT and p120 KD in presence of Y27632 after six days. MDCK WT and p120 KD cells are embedded in 3D collagen gel and treated with either DMSO or Y27632 (2*µ*M, 4*µ*M) for different time frames (3, 6, 9 days), Cyst Sphere volume from each group was quantified. Representative images from control and 2 *µ*M group at day 6 are shown in (g). Bar 50 *µ*m. **(I)** p120 KD cyst arrested at inverted polarity stage, p120 KD+hp120 cyst is arrested at multiple lumen stage. Images taken at day 8. Bar 10 *µ*m **(J)** Quantification of (i). **(K,L)** Majority of p120 KD cysts exhibit internal lumens in the presence of Y27632. Phenotypes are described as either normal (with apical membrane facing inside of the cyst) or inverted (with apical membrane facing the ECM). Percentage of inverted phenotype were quantified at Day9. Bar 20 *µ*m.

effectively, presumably because endogenous cadherins are required for this event (Jia et al., 2011; Troxell et al., 2001).

Suppression of junctional contractility is essential for lumen formation

During cyst development, cell proliferation and lumen formation are spatially and temporally linked. Deregulated junctional contractility upon p120 KD interrupts both cell growth and lumen formation, but whether these phenotypes are interdependent is unknown. To address this issue, we turned to two alternative assays that enable assessment of lumen formation under conditions where p120 KD does not suppress growth. 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 14 A,B,C). Interestingly, in the absence of p120, the bilayer forms as in the WT scenario but no lumen is generated (Figure 14 A,B). Note that lumens are clearly outlined by dense circular actin (Figure 14 A) and appear as transparent bubble-like structures under bright field illumination (Figure 14 B, 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 14 B, 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 14 C). 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 14 C). 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 14 D,E). However, in the absence of

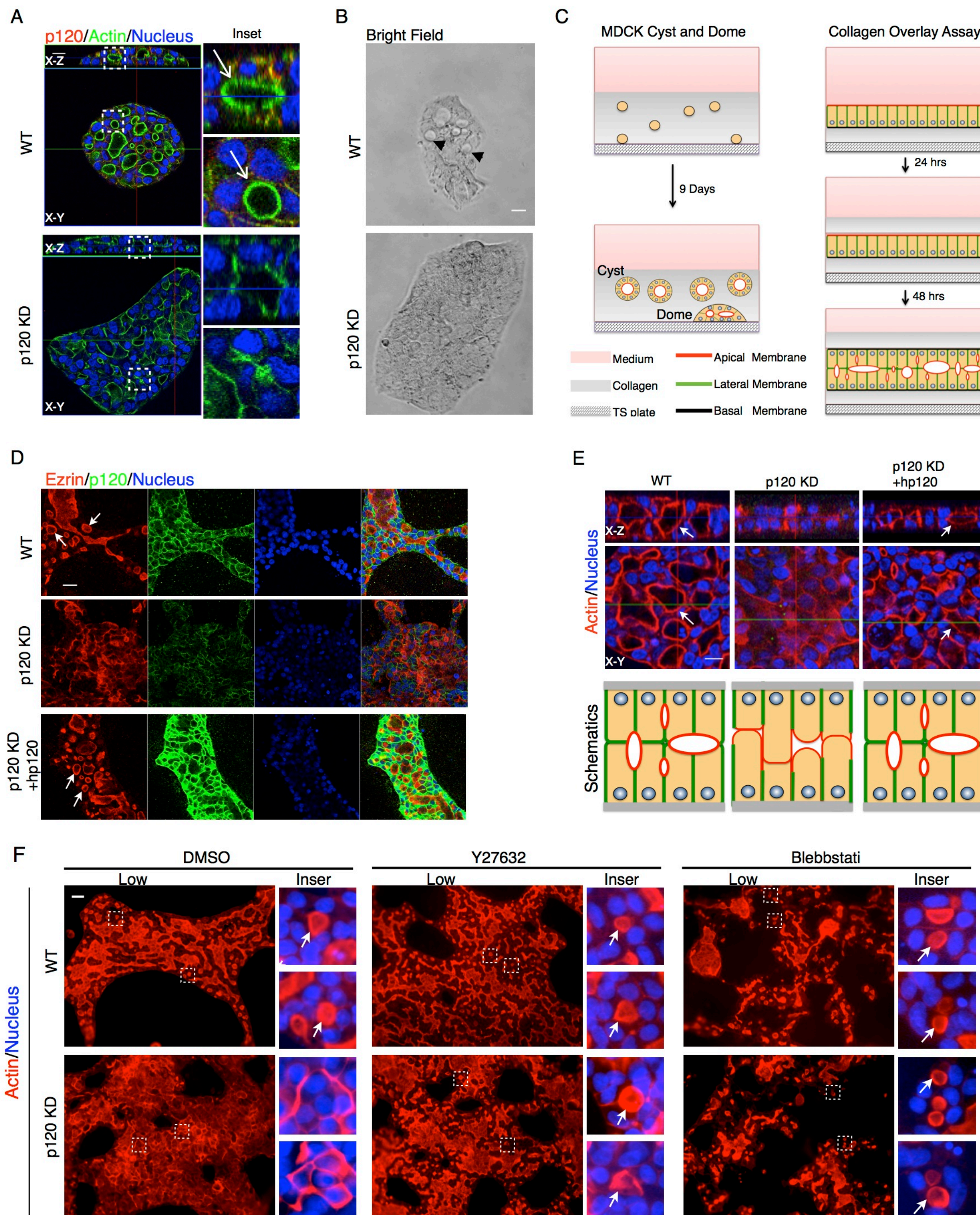


Figure 14. p120 is critical for lumen formation through suppression of contractility

(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 visualize 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.

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 14 D,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 15 E) nor E-cadherin (Figure 15 F) alone at the cell-cell junction is able to rescue this lumen formation defect (Figure 15, arrows showing where lumen form). Additionally, p120 mutants lacking the NTR or Ins regions faithfully rescued lumen formation (Figure 15 C, D, arrows), consistent with a RhoGDI-independent mechanism. Interestingly, ROCK and myosin inhibitors again rescue effects of p120 knockdown, this time in the context of lumen formation. Using the 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 14 F, 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 formation of lumen that is exemplified by the distinct apical membrane foci surrounded by nucleus (Figure 14 F, insert, arrows). Together, these observations indicate a pivotal role for cadherin-bound p120 in controlling junctional contractility during epithelial morphogenesis.

Discussion

The data indicate that suppression of contractility by p120 goes well beyond the control of cell height and shape. Lumen formation, orientation of cyst polarity and even cell growth are clearly dependent on appropriate regulation of tension at this level. Exactly how contractility controls these events is not yet established, in part because NMMIIA activity impacts nearly all

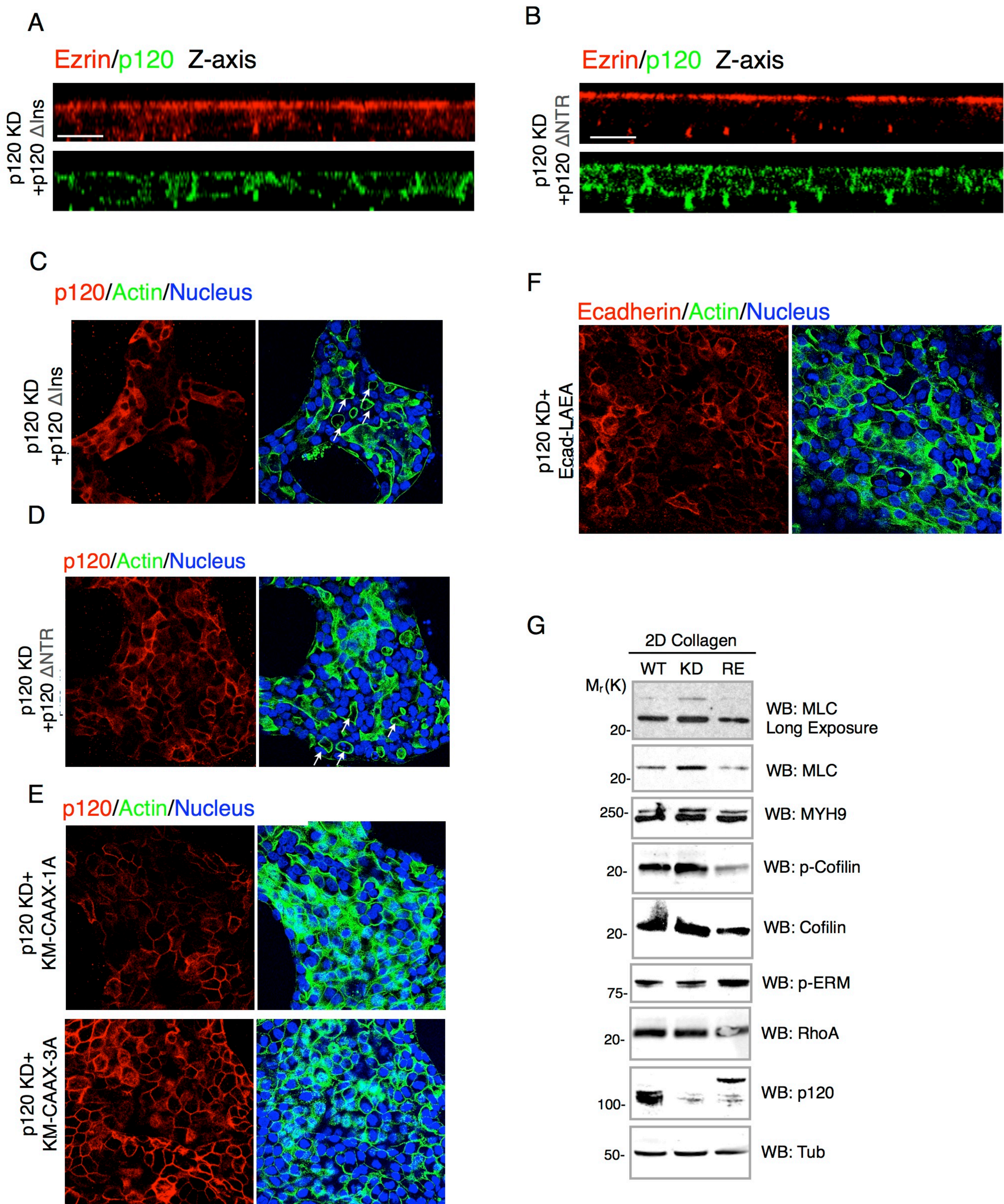


Figure 15: Suppression of Rho by cadherin-bound p120 is independent of the Rho GDI-like mechanism. (**A**) Deletion of intermediate region (Ins) of p120 (p120-ΔIns, illustrated in Supplemental Figure 1) or (**B**) NTR region (Figure 3A) does not impact its ability to rescue apical membrane morphology. Note that these two p120 mutants localize to both cell-cell junctions and cytoplasm. Junctional localization of these two mutants is shown by the arrows. Bar 10 μm. **(C,D)** RhoA-uncoupling p120 mutant faithfully rescued lumen formation when expressed in p120 KD cells. Note that p120-4A localizes almost exclusively to cell membrane whereas p120-Ins localizes to both cell membrane and cytoplasm, also seen in Figure 2F. Bar 20 μm. (**E**) E-cadherin uncoupled CAXX-box p120 constructs KM-CAAX 1A and 3A failed to rescue lumen formation upon expressing in p120 KD cells . Note the membrane location of these two mutants. Bar 20 μm **(F)** Forced surface retention of E-cadherin in the absence of p120 did not rescue lumen formation in p120 KD cells. Note the diffusive staining pattern of Actin with no formation of distinct circular foci as seen in WT cells. Bar 20 μm (**G**) p120 KD leads to increased ROCK activity as suggested by the increase of p-Cofilin levels. Cells from WT, KD, RE cell lines were grown on 2D collagen for 3 days before lysis and immunoblotting. Note the increase of MLC protein level and the upper band of MLC corresponding to p-MLC shown in the long exposure.

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 stabilizing function is clearly essential, the extent of rescue by ROCK inhibition 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; Smutny and Yap, 2010; le Duc 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.

It is worth noting that p120 KD seemed not to impact cyst polarity or lumenogenesis when cell were cultured in Matrigel. This apparent discrepancy could be explained by the differences between Collagen I (the model we use here) and Matrigel. The original report on cyst inverted polarity was performed in Collagen I. It turns out that Matrigel would actually suppress the inverted polarity phenotype because of the presence of laminin-1 (along with other ECM factors), which acts as a strong polarization signal to drive internal polarity orientation and lumenogenesis. For the same reason, MDCK cells in Matrigel generate internal lumens as early as the two-cell stage whereas it takes days for cells in 3D collagen to finish the transition from inverted to normal polarity. We suggest that p120 and RhoA play a direct role in this window of transition and p120 KD arrests the development at the inverted polarity stage. Laminin-1 (potentially with other ECM factors) bypasses the requirement of p120 in cells cultured in Matrigel, as shown in the Development paper and in our hands as well.

Previously, the role of RhoA in cyst formation was suggested to be downstream of integrin signaling. When cysts (grown in 3D collagen gel) were treated with beta1-integrin blocking antibody AIIB2, cysts show inverted polarity, and RhoA inhibition rescued the polarity. Potentially, we suggest two scenarios to explain this similarity. First, p120 may be required to mediate the inhibition of RhoA by Rac1 downstream of RTKs (i.e., the Bar-Sargi pathway).

Whether or not the exact mechanism applies to MDCK cells, the antagonism between Rac and Rho is clear, and p120-dependent. Interestingly, the same antagonism between Rac and RhoA controls cyst polarity orientation but is driven instead by beta1-integrin signaling. Under this hypothesis, p120 might actually play a downstream role of integrin-Rac1 signaling to mediate suppression of RhoA. Second, the crosstalk between integrin and cadherin signaling may actually represent two distinct steps during translocation of apical proteins from the outside to the inside during cyst formation. It is worth noting that the inverted polarity phenotype results from the failure of transcytosis of apical proteins through VACs (vesical apical compartment), which involves both endocytosis from the integrin localized outer membranes and exocytosis to the cadherin/p120 localized inner membranes. Disrupting either step would result in the failure to establish correct polarity orientation. While beyond the scope of this manuscript, investigating the interplay between p120/cadherin and VACs docking onto cell-cell junctions would be interesting and worthwhile.

CHAPTER IV

FUTURE DIRECTIONS

The controversy around the cross-regulation between cadherin complex and Myosin

p120-Ecadherin complexes represent a great tool for epithelium to build and maintain organized shapes. Actin-Myosin complexes on the other hand, serve as a two-edged sword: conferring resistance to deformation and in the mean time, providing reshaping forces as needed. Previous evidences center around mechanisms of “who controls who” or “friends or enemy”. In other words, does myosin-mediated contraction promote or disrupt the stability of cadherin-based junctions? On the other hand, does E-cadherin organize a signaling nexus to activate or deactivate actin-myosin activity? These are critical questions that definitely worth further investigations. However, it is worth noting that conclusions on either side might not be mutually exclusive. The epithelium varies significantly in shape, size and dynamics between different tissues, or even within the same tissue. The apparent opposite conclusions regarding how myosin and E-cadherin regulate each other may simply represent different requirements of the epithelium to acquire a particular shape or rearrange into a particular pattern. Thus, mechanisms aside, another potential future direction would be how to manipulate cell shapes or control cell rearrangements from an engineering perspective. First, separate tools need to be devised to tune up or down cadherin-based adhesion and myosin-mediated contractility, respectively. Second, combinatorial manipulations of both adhesion and contractility could be performed to investigate their impacts on individual cell shapes and intercellular rearrangements. Finally, observations from those manipulations should provide the basis for constructing theoretical models explaining mechanics of different epithelial structures and dynamics.

Adherens junctions as the sensor of mechanical forces

The classic outside-in and inside-out models nicely explain how integrin complexes "sense" and respond to mechanical forces that orginate from the extracellular matrix. On the other hand, only upon recently convincing evidences emerged to support that adherens junctions could also transduce forces in a similar manner. However, in comparison to the integrin-mediated mechano-transduction, three questions remain unanswered for cadherins. First, does the conformation of cadherins change in response to pulling or stretching forces? In other words, at what step are the physical forces translated into biochemical signals? Second, after the force being "sensed" by the cadherin complex, what intracellular components of the complex mediate(s) the signaling relay to cortical acto-myosin networks? In other words, how does the outside-in work here for adherens junctions? Finally, whereas it is well documented that individual epithelial cells change their shapes in response to intercellular tensions, how the epithelium coordinates the changes at short (among 5-6 neighboring cells) and long ranges (hundreds of cells)? It is worth noting that the "inside-out" step for adherens junctions would be actions upon neighboring cells, instead of extracellular matrix. Thus, the end of "inside-out" signaling of one particular cell might simply kickstart the "outside-in" signaling of the neighboring cells.

Experimental-wise, the foremost would be constructing devices that allow applying stretching, pulling or compressing forces upon epitheliums, ideally without provoking the integrin signaling. Second, proteomic assays of the "cadhesome" should be performed with or without the mechanical tensions applied. Proteins coming or leaving the cadherin complex under tensions would be the prime candidates for the tranducer of mechanical forces. Finally, live imaging or immunofluorescence on fixed cells against these candidates should reveal the temporal-spatial information on the dynamics of the mechano-transduction.

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