An apical MRCK-driven morphogenetic pathway controls epithelial polarity

Ceniz Zihni1, Evi Vlassak2,7, Stephen Terry1,7, Jeremy Carlton3, Thomas King Chor Leung4,5, Michael Olson6, Franck Pichaud1, Maria Susana Balda1,8 and Karl Matter1,8

Polarized epithelia develop distinct cell surface domains, with the apical membrane acquiring characteristic morphological features such as microvilli. Cell polarization is driven by polarity determinants including the evolutionarily conserved partitioning-defective (PAR) proteins that are separated into distinct cortical domains. PAR protein segregation is thought to be a consequence of asymmetric actomyosin contractions. The mechanism of activation of apically polarized actomyosin contractility is unknown. Here we show that the Cdc42 effector MRCK activates myosin-II at the apical pole to segregate aPKC–Par6 from junctional Par3, defining the apical domain. Apically polarized MRCK-activated actomyosin contractility is reinforced by cooperation with aPKC–Par6 downregulating antagonistic RhoA-driven junctional actomyosin contractility, and drives polarization of cytosolic brush border determinants and apical morphogenesis. MRCK-activated polarized actomyosin contractility is required for apical differentiation and morphogenesis in vertebrate epithelia and Drosophila photoreceptors. Our results identify an apical origin of actomyosin-driven morphogenesis that couples cytoskeletal reorganization to PAR polarity signalling.

Epithelial cells polarize and form distinct cell surface domains that have different biochemical compositions, reflecting their different functions. The apical domain often undergoes a morphogenetic process leading to the development of actin-rich structures that support specific apical functions, such as the brush border membrane of absorptive epithelia or the light-harvesting domain of Drosophila photoreceptors. Formation of such apical specializations relies on the recruitment of specific cytosolic factors that determine apical morphogenesis and, hence, requires asymmetric distribution of cytosolic components. Epithelial polarization is regulated by apical and basolateral polarity determinants. Among these are the evolutionarily conserved PAR proteins that segregate into two distinct cortical domains. In epithelia, the boundary between the two domains, the apical/lateral border (tight junctions in vertebrates, adherens junctions in flies), is marked by Par3, which is recruited to the cell surface bound to the Par6–aPKC complex. In response to apical Cdc42 activation, Par3 dissociates, demarking the apical/lateral border, and the Par6–aPKC complex segregates into the differentiating apical domain. Studies in Caenorhabditis elegans one-cell stage embryos suggest that PAR protein segregation relies on asymmetric actomyosin activity, generating movement of anterior PAR complexes to the anterior pole, which results in the formation of two cortical domains that harbour distinct PAR proteins. Anterior PAR proteins correspond to apical PARs in epithelia. The functional importance of actomyosin and, if relevant, how and where asymmetric myosin-II activity is generated to drive apical accumulation of PAR proteins in epithelia is not clear. Identifying such mechanisms, however, is essential to understand how the interplay between mechanical forces generated by actomyosin contractility and biochemical signalling guide epithelial polarization and morphogenesis.

In epithelia, RhoA is known to generate contractile forces driving junction formation and remodelling, a mechanism important during epithelial sheet movement and elongation. In contrast, apical Cdc42 activation not only drives apical differentiation but also promotes apical expansion at the cost of the basolateral domain, counteracting junctional actomyosin-generated apical constriction. In analogy to the C. elegans embryo model, one would expect a mechanism of myosin-II activation at the apical pole to create an actomyosin activity gradient that favours apical polarization if apical segregation of Par6–aPKC is indeed driven by actomyosin contractions. Therefore, we asked whether and how apical Cdc42 signalling activates asymmetric ac-
myosin contractility to stimulate apical polarization and plasma membrane morphogenesis, and how such a mechanism interacts with countering junctional RhoA signalling. Here, we show that the Cdc42 effector MRCK activates apical actomyosin contractility, initiating a pathway regulating apical morphogenesis, and cooperates with the aPKC–Par6 complex, which downregulates RhoA-driven junctional actomyosin contractility, to drive apical polarization.

RESULTS

MRCK-activated myosin-II drives apical morphogenesis

As epithelial cells polarize and develop a specialized apical membrane domain, myosin-II polarizes apically at distinct sites along the apical membrane domain including the junctional circumferential actomyosin belt\textsuperscript{18,19}. In cultured canine kidney epithelial MDCK cells that spontaneously differentiate, we found that phosphorylated MLC (myosin regulatory light chain), demarking active myosin-II, is localized basolaterally in non-polarized cells and becomes increasingly enriched along the apical membrane domain, forming caps that define the apical cellular cortex, as epithelial cells polarize and differentiate over a period of a few days (Fig. 1a). Since apical polarization of PAR proteins and morphogenesis depends on apically polarized Cdc42 activation\textsuperscript{3,20}, we asked whether a Cdc42-dependent mechanism driving polarized myosin-II activation is at the origin of apical polarization and morphogenesis.

MRCKs are Cdc42 effector kinases that activate myosin-II by phosphorylating MLC\textsuperscript{21}. Using spontaneously differentiating MDCK cells, we asked whether short interfering RNA (siRNA)-mediated MRCK knockdown affected formation of apical actomyosin caps. Indeed, MRCK-depleted cells failed to form differentiated apical membranes as microvilli did not assemble, apical accumulation of F-actin and active myosin-II did not occur, and the cells had a reduced height (Fig. 1b–h and Supplementary Fig. 1a–c). All of these MRCK siRNA-induced effects were rescued by expression of siRNA-resistant MRCK\textsubscript{β}-Flag, supporting specificity. MRCK was also required for the differentiation of human intestinal epithelial Caco-2 cells that also spontaneously polarize and differentiate (Supplementary Fig. 1c,d). Hence, MRCK is essential for the differentiation of vertebrate epithelial cells from different tissues.

To determine whether MRCK-generated apical myosin-II activation is a conserved mechanism, we turned to Drosophila melanogaster: their differentiating photoreceptors represent a well-characterized invertebrate model system to study epithelial polarity, PAR polarity signalling, and apical differentiation\textsuperscript{5,7,22–24}. Drosophila photoreceptors undergo epithelial polarity remodelling during retinal development and evolve a characteristic, F-actin-rich apical membrane domain (Fig. 2a). Strikingly, differentiating photoreceptors displayed active myosin caps at the apical cortex, which were not formed in cells mutant for gek, the orthologue of MRCK\textsubscript{β} (Fig. 2b). Loss of gek function also resulted in attenuation of F-actin signal at the apical membrane domain and gross defects in the morphology of the F-actin-rich apical organelle known as the rhabdomere (Fig. 2c–f), which consists of a stack of approximately 60,000 microvilli, indicative of defective actomyosin contractility\textsuperscript{22}. Introduction of a phosphomimetic MLC transgene, spaghetti squash-EE (sqh\textsuperscript{EE}), rescued the phenotype of gek mutant cells, demonstrating that myosin-II activation acts downstream of gek/MRCK (Fig. 2g,h). MRCK is thus a conserved driver of apical myosin activation and morphogenesis in vertebrate epithelia and Drosophila photoreceptors.

Apical determinant Dbl3, a Cdc42 guanine nucleotide exchange factor, controls MRCK activity

We have previously shown that the guanine nucleotide exchange factor Dbl3 activates Cdc42 at the apical pole to drive epithelial polarization and morphogenesis\textsuperscript{17}. To determine whether Dbl3 signalling stimulates MRCK-mediated myosin activation, we conditionally expressed Dbl3-myc in MDCK cells. This resulted in increased apical phosphorylation of MLC along with enhanced apical F-actin accumulation and microvilli induction; both inhibited by knockdown of MRCK (Fig. 3a–e). Inhibition of ROCK\textsubscript{II} in polarizing MDCK cells did not prevent apical actomyosin activation and morphogenesis, indicating that apical myosin activation is not Rho-dependent (Supplementary Fig. 2). Moreover, our results indicate that Dbl3 is a major activator of MRCK in mammalian cells since constitutive overexpression of MRCK\textsubscript{β}-Flag could not rescue Dbl3 knockdown effects (Supplementary Fig. 3a–d). Only catalytically active, but not inactive Dbl3 that is unable to activate Cdc42 (ref. 17), activated MLC phosphorylation (Fig. 3f). Thus, the activation of apical myosin caps requires Cdc42 and MRCK activation.

To determine the temporal nature of apical MLC activation, we generated a conditional Dbl3-Myc MDCK cell line that constitutively expresses EGFP-MLC; the conditional Dbl3-myc transgene was then induced by the addition of tetracycline. Time-lapse fluorescence microscopy revealed that concurrent to induction of Dbl3-myc protein, increased apical EGFP-MLC activity was detected from 2.5 h of tetracycline treatment (Supplementary Video 1 and Supplementary Fig. 3e). Analysis in fixed cells revealed that conditional expression of Dbl3-myc resulted in active myosin cap formation 2.5 h from the onset of tetracycline addition along with apical accumulation of MRCK\textsubscript{β}, increasing further at 6 h, along the expanding apical membrane domain (Fig. 4a and Supplementary Fig. 3f,g). Thus, apical MRCK recruitment and MLC phosphorylation follow the Dbl3-myc protein expression profile. Apical actomyosin cap formation correlated with apical polarization of Par6–aPKC, the brush border component ezrin, and microvilli induction (Fig. 4a–c and Supplementary Fig. 3g). Constitutive expression of MRCK\textsubscript{β}-Flag, which is autoinhibited until bound to Cdc42–GTP, in conditional Dbl3-expressing cells accelerated the formation of apically activated myosin caps, which became enriched with MRCK\textsubscript{β}-Flag, during polarization and brush border induction (Fig. 4b,c and Supplementary Fig. 3f,g). MRCK is thus recruited to the apical membrane by Dbl3-mediated Cdc42 activation to drive apical polarization and differentiation of epithelial cells. Apical localization of MRCK\textsubscript{β} was also observed in vivo in mouse renal tubules and intestine (Fig. 4d), and GEK was found at the apical photoreceptor cortex (Fig. 4e). Hence, apical MRCK polarization occurs in vitro and in vivo, and in vertebrates and invertebrates.

Apical Cdc42 activates a dual effector mechanism that antagonizes junctional myosin activity

RhoA and ROCK regulate actomyosin activity along cell–cell junctions, generating a circumferential actomyosin belt that is required for junction assembly and remodelling, and apical constriction\textsuperscript{15,18,19}. Activation of apical Cdc42 by Dbl3 promotes apical expansion of apical myosin activation and morphogenesis in vertebrate epithelia and Drosophila photoreceptors.
process opposing apical constriction. Apical Cdc42 signalling may thus not only promote apical MRCK-driven myosin activation but also downregulate junctional actomyosin contractility to create an actomyosin activity gradient that favours apical expansion and apical PAR protein segregation. In agreement with apical activation of myosin-II by MRCK, conditional expression of Dbl3-myc stimulated apical Cdc42 activity, as revealed by a fluorescence resonance energy transfer (FRET) biosensor, confirming previously published loss-of-function experiments (Fig. 5a,b). Addition of a Cdc42 inhibitor during Dbl3 induction blocked the formation of apical active myosin caps (Fig. 5c,d). Cdc42 activation is thus required for Dbl3 to induce MRCK-dependent, apically polarized actomyosin caps. Dbl3 stimulates apical Cdc42-dependent aPKC–PAR6 effector complex activation and, hence, enrichment of aPKC at the cell cortex (Fig. 5e,f and Supplementary Fig. 4a). As aPKC is an inhibitor of the RhoA activator LULU-2 (ref. 16), we asked whether Cdc42 activates a dual effector mechanism that downregulates junctional myosin-II activity via aPKC whilst activating apical actomyosin contractility via MRCK (Fig. 5g).

To determine whether Dbl3-induced Cdc42 activation is indeed an inhibitor of RhoA at junctions, we first monitored junctional localization of LULU-2 and p114RhoGEF. The latter protein is a major junctional guanine nucleotide exchange factor for RhoA that is activated...
Figure 2 MRCK/gek regulates apical myosin activation and morphogenesis in differentiating Drosophila photoreceptors.  

(a) Schematic illustrating the similarities and corresponding plasma membrane domains of vertebrate epithelial cells and Drosophila photoreceptors. Indicated are apical microvilli and the apical zone enriched in active myosin-II.  
(b,c) Confocal sections of Drosophila pupal mosaic retinas showing wild-type (WT) cells (blue nuclei) and cells mutant for gek stained for F-actin (c) or p-MLC (b). Mutant cells are labelled with asterisks.  
(d) Quantification of apical actin enrichment shows analysis of wild-type and mutant cells (paired within sections; n represents 11 animals, a t-test was used to calculate the P value). (e,f) Analysis of rhabdomere integrity in wild-type and gek mutant cells using two different gek alleles (gekNP5192 or gek1080) demonstrates severe defects including splitting.  
(g,h) Confocal sections of Drosophila eyes showing wild-type cells and cells mutant for gekNP5192 but expressing sqh-SqhEE (blue nuclei; labelled with asterisks; the quantification represents pairs within sections and is based on n=8 animals, a t-test was used to calculate the P value). NS, not significant.

by binding to LULU-2, a FERM (4.1 protein, ezrin, radixin, moesin) domain-containing protein homologous to Drosophila Yurt. Concomitant with increased aPKC recruitment, conditional expression of Dbl3-myc promoted LULU-2 dissociation from junctions (Fig. 6a,b and Supplementary Fig. 4d). Junctional p114RhoGEF levels also decreased following Dbl3 induction (Fig. 6a,b and Supplementary Fig. 4d). Dbl3 induction did not affect expression levels of LULU-2 and p114RhoGEF but resulted in enhanced ezrin phosphorylation, a marker of apical differentiation (Supplementary Fig. 4b,c). Dbl3-myc induction thus leads to reduced junctional association of proteins that activate RhoA signalling. To confirm that junctional recruitment of LULU-2 is regulated by Dbl3-activated aPKC, we knocked down Dbl3...
Figure 3 MRCK functions as an effector of Dbl3–Cdc42 signalling. (a) Scanning electron microscopy analysis of microvilli induction by MDCK cells with tetracycline-inducible Dbl3-myc expression transfected with MRCK siRNA determined by measuring areas of microvilli clusters from scanning electron microscopy scans. (b–d) Induction of active myosin at cortical caps (A) or basal membrane (B), and enrichment of F-actin at the apical cortex during polarization and differentiation, stimulated by conditional expression of Dbl3-myc with or without MRCK siRNA knockdown. The white arrowheads highlight the apical membrane cortex labelled for F-actin. (e) Immunoblot analysis of total active myosin levels following conditional expression of Dbl3-myc and siRNA knockdown of MRCK. (f) Immunoblot analysis of total active myosin-II levels following conditional expression of Dbl3-myc or GEF-inactive Dbl3Y645A-myc. The schematic illustrates the formation of apical caps formed by activated myosin-II and F-actin. Unprocessed original scans of blots are shown in Supplementary Fig. 8. For all quantifications, n = 3 independent experiments and shown are the data points, means ± 1 s.d. (in black), the total number of cells analysed for each type of sample across all experiments, and P values derived from t-tests. NS, not significant. Scale bars, a, 1 μm; b, 10 μm.

or inhibited aPKCζ activity, which resulted in an increase in LULU-2 localization at cell junctions (Supplementary Fig. 4e–h). These results indicate that apical activation of Cdc42 antagonizes the molecular machinery that activates RhoA at cell junctions.

We next asked whether RhoA activity and junctional actomyosin activation is indeed affected by Dbl3 signalling. Analysis of RhoA activity at the apical domain using a FRET biosensor revealed enriched RhoA activity at cell–cell contacts as previously reported15.
Figure 4 Cdc42 activates MRCK apically, stimulating apical myosin-II activation and differentiation. (a–c) Analysis and quantification of apical p-MLC, aPKC_ζ and p-ezrinT567 at the apical membrane domain using confocal z-section analysis and of brush border induction using scanning electron microscopy of MDCK cells with tetracycline-inducible Dbl3-myc expression with or without constitutive expression of MRCKβ-Flag. The white arrowheads highlight the apical membrane cortex labelled for F-actin. (d) Localization of MRCKβ in mouse kidney and small intestine. (e) Localization of GEK in polarizing pupal Drosophila photoreceptors. For all quantifications, n=3 independent experiments and shown are the data points, means ± 1 s.d., the total number of cells analysed for each type of sample across all experiments, and P values derived from t-tests (red/blue values refer to comparisons within categories and black values to comparisons between categories after 2.5 h). In d and e, the arrows point to apical domains positive for MRCK/GEK. Scale bars, electron micrographs, 1 μm; confocal immunofluorescence images, 10 μm.

Induction of Dbl3 expression resulted in a 50% decrease in RhoA activity at cell–cell contacts (Fig. 6c,d). Similarly, a GFP fusion protein containing the RhoA-binding domain of rhotekin, another sensor to monitor the cellular distribution of active RhoA_28, was enriched at cell–cell contacts prior to Dbl3 induction but diffusely distributed when expression of the exchange factor had been induced, supporting the conclusion that enhanced Dbl3 signalling inhibits junctional RhoA activity (Fig. 6e). Active p114RhoGEF forms a stable complex with ROCKII and myosin-II, stimulating junctional double phosphorylation of MLC (pp-MLC)_15. Conditional expression of Dbl3 strongly reduced junctional pp-MLC staining (Fig. 6f,g), further supporting an inactivation of the p114RhoGEF/RhoA pathway that promotes...
Cortical polarization of PAR proteins is thought to rely on both biochemical signals and mechanical forces generated by actomyosin contractions\(^6\)\(^{-13}\). Cdc42 activation promotes apical polarization by stimulating activation of Par6–aPKC, leading to recruitment to junctions, Par3 phosphorylation and biochemical destabilization of the initial Par6–aPKC/Par3 complex\(^6\)\(^,\)\(^7\). Since activation of apical actomyosin contractility by MRCK stimulates apical differentiation, we asked whether MRCK-activated myosin-II mediates aPKC–Par6 separation from Par3 at junctions. To determine first whether MRCK activity itself is required, we used an inhibitor that blocks the catalytic activity of MRCK\(^20\). Induction of Dbll3-myc expression led to a shift in the position of tight junctions, demarked by Par3, from the apical end of the lateral membrane towards the basal membrane, and an extended apical domain, labelled by aPKC, and a reduced basolateral domain, stained with anti-scribble antibody, as previously described (Fig. 6a)\(^\text{fig:6a}\). Inhibition of MRCK blocked separation of Par3 from aPKC as well as the junctional shift in control and Dbll3-overexpressing cells, indicating that MRCK activity was required even when apical Cdc42 activation and, hence, aPKC signalling\(^21\) was increased by enhanced expression of the guanine nucleotide exchange factor. If the same experiments were repeated with blebbistatin, an inhibitor of myosin-II activity that blocks actomyosin contractions\(^3\), separation of aPKC from Par3 and junctional shifting was also inhibited, and led to aPKC from Par3 and junctional shifting was also inhibited, and led to...
to increased stable complex assembly (Fig. 7b,c). Blebbistatin also strongly attenuated brush border induction (Fig. 7d,e) comparable to what we had observed in response to depletion of MRCK (Fig. 3a). Thus, these results indicate that MRCK-activated myosin-II motor activity is required for separation of Par6–aPKC from Par3 following apical Cdc42 activation but not for the initial recruitment.
Figure 7 MRCK-stimulated myosin-II activation drives PAR separation at tight junctions. (a,b) Confocal analysis of aPKCζ localization at tight junctions in medium/advanced polarizing/differentiating MDCK cells with tetracycline-inducible Dbl3-myc expression without or with BDP5290, a MRCK inhibitor (a), or blebbistatin, a myosin-II inhibitor (b). The position of tight junctions, demarked by Par3, is indicated by the white arrowheads. Note, tight junctions move towards the basal membrane in response to apical expansion17. The larger magnifications show examples of cells in which aPKC segregates from Par3 (all controls and enhanced by Dbl3-myc expression) or in which aPKC and Par3 co-localize (cells treated with MRCK or myosin-II inhibitor). aPKCζ segregation from Par3 at tight junctions is highlighted by 'Seg'. (c) Immunoprecipitation analysis of aPKCζ–Par3 complex formation in cells conditionally expressing Dbl3-myc with or without blebbistatin. Note, increased Par3–aPKC complex formation in Dbl3-myc-induced cells without blebbistatin is due to increased transient interactions that promote apical expansion17. (d,e) Quantification of microvilli induction in MDCK cells conditionally expressing Dbl3-myc with or without blebbistatin. The quantification in d is based on n=3 independent experiments and shown are the data points, means ±1 s.d. (in black), the total number of cells analysed for each type of sample across all experiments, and P values derived from t-tests. Unprocessed original scans of blots are shown in Supplementary Fig. 8. Scale bars, electron micrographs, 1 µm; confocal immunofluorescence images, 10 µm.

of Par6–aPKC to form the tight-junction-associated complex with Par3. This is in agreement with the reported diffusive properties of PAR proteins in C. elegans, where PAR protein segregation, but not cortical recruitment, is actomyosin-dependent32. Since Dbl3-mediated Cdc42 activation also stimulates aPKC to phosphorylate Par3 (ref. 17) and to downregulate junctional RhoA signalling (Fig. 6), our results demonstrate that both effector mechanisms stimulated by active apical Cdc42 are required for apical PAR protein separation and segregation: the biochemical activity of aPKC–Par6 that destabilizes the PAR complex and downregulates junctional myosin contractility, and the activation of apical myosin contractility by MRCK that drives apical PAR protein separation and domain morphogenesis.

Dbl3/MRCK-stimulated actomyosin contractility drives apical segregation of PAR proteins and polarization of cytosolic brush border determinants

Cdc42-activated aPKC–Par6 recruitment to junctions is a transient process and the complex then proceeds to expand along the apical membrane, segregating from Par3 and tight junctions, to define the apical cortex33. Hence, we next asked whether
Figure 8  MRCK-activated actomyosin contractility drives apical PAR segregation and polarization of cytosolic factors. (a,b) Levels of EGFP-Lifeact (EGFP-LA), Par polarity and brush border proteins localized at the apical membrane domain in MDCK cells conditionally expressing Dbil3-myc, treated with blebbistatin and followed by washout (WO). AM, apical membrane; AM/C, apical membrane divided by cytosolic. (c,d) Levels of EGFP-Lifeact (EGFP-LA), Par polarity and brush border proteins localized at the apical membrane domain in MDCK cells conditionally expressing Dbil3-myc following blebbistatin treatment and washout in the absence or presence of BDP5290 to inhibit catalytic activity of MRCK. (e–i) Measurements of co-localization coefficients of PAR polarity and brush border proteins at the apical membrane domain in MDCK cells conditionally expressing Dbil3-myc transiently inhibited with blebbistatin followed by washout for 2 h (green indicates co-localization, see also Supplementary Figs 5 and 6; the arrowheads point to apical membrane). Expansion along the apical membrane is powered by MRCK-dependent myosin-II motor activity. Polarization of aPKC–Par6 along the apical cell cortex, which formed a stable complex, was inhibited in conditional Dbil3-myc-expressing cells treated with blebbistatin and rapidly reversed following washout (Fig. 8a,b and Supplementary Fig. 5). The reversal of myosin motor inhibition
required MRCK kinase activity, confirming the requirement of MRCK-dependent myosin-II activation for apical polarization of the aPKC–Par6 complex (Fig. 8c,d). It is thought that a rapid apical phosphorylation/dephosphorylation cycle of ezrin is required to confine ezrin and microvilli to the apical membrane domain in polarized epithelial cells, which is facilitated by apical localization of cytosolic factors such as the kinase SLK\(^2\)\(^3\)\(^5\). Similarly, NHERF-1, also known as EBP-50, engages in a transient association with ezrin to regulate microvillar structure\(^1\(^4\). Myosin motor-dependant cortical polarization was concomitant with an apical enrichment of such cytosolic regulators of brush border formation and was required for their co-localization with ezrin at the apical pole, indicating that MRCK-induced apical domain formation included polarization of cytosolic factors required for plasma membrane specialization and morphogenesis (Fig. 8e–i and Supplementary Fig. 6). Our data thus support a model in which epithelial cortical polarization and cytosolic polarization are linked\(^7\). Hence, MRCK signalling unifies the mechanisms for asymmetric segregation of cytosolic components to the vicinity of the apical membrane with cortical polarization (Fig. 8j). Apical membrane staining of aPKC and moesin in Drosophila gek mutant photoreceptors was also attenuated (Fig. 8k,l and Supplementary Fig. 7). These effects were rescued by co-expression of sqh\(^2\), a phosphomimetic MLC construct, in mutant gek cells (Fig. 8m,n), demonstrating the importance of MRCK for activation of myosin-II during apical differentiation of Drosophila photoreceptors.

**DISCUSSION**

We have identified a distinct, evolutionarily conserved actomyosin-powered morphogenetic pathway at the apical domain that is activated by Cdc42-mediated stimulation of MRCK and drives epithelial morphogenesis and the development of epithelial polarity. Cdc42 and MRCK signalling serves to coordinate biochemical signalling and actomyosin-generated mechanical forces. Unlike the mechanism of action of Cdc42, the spatial activation of Cdc42 is likely to vary across species since far less GEF diversity exists in Drosophila, and the closest orthologue of Db\(l3\) in insects lacks a Sec14 domain, which is crucial for Db\(l3\) function in vertebrates\(^1\(^7\).

Cell polarization is thought to be induced by mechanical forces generated by an actomyosin activity gradient\(^1\(^1\)-\(^1\(^3\)-\(^3\). Our results indicate that apical Cdc42-stimulated MRCK signalling functions in the culminating stages of epithelial polarization leading to the accumulation of Par6 and aPKC at the apical domain by promoting the actomyosin-driven segregation of the Par6–aPKC complex from the junctional complex along the differentiating apical domain and thus promoting apical identity and morphogenesis. Moreover, cytosolic factors that regulate apical membrane specialization such as SLK and NHERF-1, which mediate localized ezrin activation and brush border assembly, are concentrated at the apical pole, linking cortical and cytosolic polarization. As active ezrin also promotes apical Db\(l3\) recruitment and Cdc42 activation\(^1\(^7\), this process represents a positive feedback mechanism that enhances apical differentiation and reinforces the polarized epithelial phenotype.

Apical Cdc42 activation stimulates two cooperating effector pathways that induce the formation of apically polarized actomyosin contractility to drive PAR protein polarization: MRCK-mediated apical myosin-II activation and Par6–aPKC-mediated inhibition of junctional RhoA activation via negative regulation of LULU-2 and p114RhoGEF and, hence, reduced junctional actomyosin contractility. The biochemical activity of aPKC–Par6 also supports apical PAR segregation by phosphorylating Par3 and, thereby, destabilizing its interaction with the aPKC–Par6 complex\(^6\)-\(^7\). Hence, activation of the two Cdc42 effector pathways integrates biochemical and mechanical force-generating pathways stimulating apical segregation of PAR proteins, polarization and domain morphogenesis. The downregulation of junctional RhoA signalling by apical Cdc42 activation further suggests a sequential model for the function of ROCK and MRCK in myosin-II activation: RhoA/ROCK signalling drives early steps of junction formation\(^1\(^5\) and remodelling such as in the early eye disc\(^2\(^9\)-\(^3\(^6\)-\(^3\(^7\); subsequent Cdc42/MRCK activation then leads to a reorientation of the actomyosin contractility gradient and, hence, apical polarization and differentiation. The antagonism between junctional RhoA and apical Cdc42 signalling may be bidirectional. In vertebrates, junctional RhoA activity is generated by p114RhoGEF, a pathway that is stimulated in cells that undergo apical constriction due to increased levels of LULU-2 (ref. 15,16). The fly homologue of LULU-2, Yurt, regulates apical domain size negatively by inhibiting the activity of Crumbs, which is positively regulated by aPKC\(^2\(^7\), suggesting that LULU-2 signalling may indeed antagonize apical Cdc42-driven mechanisms. Hence, mechanisms of mutual antagonism between Cdc42-stimulated MRCK- and RhoA-activated ROCK-driven actomyosin contractility may exist that create a cellular code for dynamic polarized morphogenetic processes underlying the generation of tissue- and organ-specific cell surface fates during differentiation of various cell types.■

**METHODS**

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of this paper.

**ACKNOWLEDGEMENTS**

This work was supported by the BBSRC (BB/L007584/1 and BB/N014855/1) and the Wellcome Trust (0999173/Z/12/Z). Work in the E.P. laboratory, including support to E.V., was funded by an MRC grant (MC\_UU\_120183/8). The N2 A\(^T\) monoclonal antibodies, developed by E. Wieschaus, were obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, Iowa 52242. Stocks obtained from the Bloomington Drosophila Stock Center (NIH P400D1018537) were used in this study.

**AUTHOR CONTRIBUTIONS**

C.Z. performed most of the vertebrate and E.V. the Drosophila experiments. All other authors performed particular subsets of experiments. C.Z., M.S.B. and K.M. designed the project and drafted the manuscript. All authors read and contributed to the final version of the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

Published online at http://dx.doi.org/10.1038/ncb3592

Reprints and permissions information is available online at [www.nature.com/reprints](http://www.nature.com/reprints). Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

1. Meilman, I. & Nelso, W. J. Coordinated protein sorting, targeting and distribution in polarized cells. _Nat. Rev. Mol. Cell Biol._ **9**, 833–845 (2008).
2. Sauvanet, C., Wayt, J., Pelaseyed, T. & Bretscher, A. Structure, regulation, and functional diversity of microvilli on the apical domain of epithelial cells. _Annu. Rev. Cell Dev. Biol._ **31**, 593–621 (2015).
Transfection. Cells were cultured and transfected using Interferin transfection cell lines were grown on a maintenance concentration of 0.5 μg/ml. Selected clones were then transfected with pCDNA4TO-Dbl3-myc and selected in DMEM with 10% fetal bovine serum with Blasticidin (0.5 μg/ml); Zeocin (200 μg/ml); Cdc42 inhibitor ML141 (10μM), and the ROCKII inhibitor GSK 269692 (10 nM) were purchased from Tocris Bioscience. The apPKCζ-myr stabilized inhibitor was purchased from Merck Millipore and used at a concentration of 40μM. For conditional expression of Dbl3-myc, the Dbl3 pcDNA-myc expression vector was constructed as described in ref. 17. MDCK cells were transfected with a plasmid encoding the tetacycline repressor (pCDNA-TK) using the calcium phosphate method18, cultured in DMEM with 10% fetal bovine serum and selected with Blasticidin (0.5 μg/ml)19. Selected clones were then transfected with pCDNA4TO-Dbl3-myc and selected in DMEM with 10% fetal bovine serum with Blasticidin (0.5 μg/ml)19; PAA Laboratories) and Zeocin (200 μg/ml); Thermo Fisher Scientific). The MDCK pcDNA4TO-Dbl3-myc/MRCKζ-Flag cell line was generated by transfecting rat MRCKζ-Flag into the MDCK pcDNA4TO-Dbl3-myc TET-R cell line and selecting in DMEM with 10% fetal bovine serum with Blasticidin (0.5 μg/ml)19; Zeocin (200 μg/ml)19 and G418/Geneticin (600 μg/ml); Thermo Fisher Scientific. MDCK and Caco-2 cells constitutively expressing MRCKζ-Flag were transfected analogously and cultured in DMEM with either 10% or 20% fetal bovine serum and G418 (600 μg/ml)19; G418). To generate EGFP-LA or EGFP-MLC pcDNA4TO-Dbl3-myc MDCK TET-R cell lines retroviral transduction constructs EGFP-Lifeact were cloned at EcoRI and XhoI sites using the following primers: 5′-AGAGAUCGCCUUCUUGUC-3′; 5′-CTCAAGAGACTTTGAGATAT-3′; and 5′-GAGA-UCCCGGAG-3′. Constructs were co-transfected with pVSVG into GP2-293 cells (Clontech). Supernatants were collected, clarified and concentrated using a CFI Aparochromat Nano-Crystal 60× oil lens (NA 1.2). Crossover between CFP and YFP filters was calibrated by imaging CFP and YFP expressed alone using all four emission/excitation filter combinations. FRET efficiency maps were then produced with the Nikon software and quantified with ImageJ. The plasmid encoding the GFP-RhoA-gBD construct was obtained from Addgene and was described previously20.

Mammalian mammalian microscopy. Cells were fixed and mounted for scanning electron microscopy as previously described21. Samples were analysed in a Sigma Field Emission scanning electron microscope (Carl Zeiss) operating at 5 kV. Digital images were recorded using Carl Zeiss (SmartSEM) software.

Drosophila strains and genetics. Files were maintained at 25 °C on standard food. To generate whole mutant eyes, the EGF/GMRhid system was used22. An affinity-purified and affinity-purified MRCK inhibitor BDP-5290 was synthesized by the Cancer Research UK Beatson Institute Drug Discovery Group and used at a concentration of 10μM. Blebbistatin (10 μM final concentration), the Cdc42 inhibitor ML141 (10μM), and the ROCKII inhibitor GSK 269692 (10 nM) were purchased from Tocris Bioscience. The apPKCζ-myr stabilized inhibitor was purchased from Merck Millipore and used at a concentration of 40μM. For conditional expression of Dbl3-myc, the Dbl3 pcDNA-myc expression vector was constructed as described in ref. 17. MDCK cells were transfected with a plasmid encoding the tetacycline repressor (pCDNA-TK) using the calcium phosphate method18, cultured in DMEM with 10% fetal bovine serum and selected with Blasticidin (0.5 μg/ml)19. Selected clones were then transfected with pCDNA4TO-Dbl3-myc and selected in DMEM with 10% fetal bovine serum with Blasticidin (0.5 μg/ml)19; PAA Laboratories) and Zeocin (200 μg/ml); Thermo Fisher Scientific). The MDCK pcDNA4TO-Dbl3-myc/MRCKζ-Flag cell line was generated by transfecting rat MRCKζ-Flag into the MDCK pcDNA4TO-Dbl3-myc TET-R cell line and selecting in DMEM with 10% fetal bovine serum with Blasticidin (0.5 μg/ml)19; Zeocin (200 μg/ml)19 and G418/Geneticin (600 μg/ml); Thermo Fisher Scientific. MDCK and Caco-2 cells constitutively expressing MRCKζ-Flag were transfected analogously and cultured in DMEM with either 10% or 20% fetal bovine serum and G418 (600 μg/ml)19; G418). To generate EGFP-LA or EGFP-MLC pcDNA4TO-Dbl3-myc MDCK TET-R cell lines retroviral transduction constructs EGFP-Lifeact were cloned at EcoRI and XhoI sites using the following primers: 5′-GAA TTC ATG AGT GTC GCA GAT TTT AAC AAA TTT CAA GAA GCA TTC GAG-3′ and 5′-CTC GAG TTC TCC TTC CTG TTA GAT GCT TAA TTT TTT GAT CAT TTC GAC ACC CAT GAA TTC GTC-3′ and MLC by PCR amplification using the following primers 5′-GAA TAA GAA TTC ATG AGT GTC GCA GAT TTT AAC AAA TTT CAA GAA GCA TTC GAG-3′ and 5′-GAG TCT CTC GAG CTA GTC GTC TTC TCC ACC TTC GCT GGA-3′ into retroviral packing vector pMSC28 EGFP IRES Puro, a MLV-based retroviral system as described previously22. Constructs were co-transfected with pVSVG into GP2-293 cells (Clontech). Supernatants were collected, clarified by centrifugation (200g, 5 min), filtered (0.45μm) and used to infect MDCK Dbl3 Tet-R cells in the presence of 8 μg/ml Polybrene (Millipore) at (MOI) <1. Cell lines were selected for either puromycin or G418 (Thermo Fisher Scientific) at a final concentration of 2.0 μg/ml or 800 μg/ml respectively. Selected clonal cell lines were grown on a maintenance concentration of 0.5 μg/ml puromycin or 400 μg/ml G418.

Transfection. Cells were transfected and selected using interferon transfection reagent (Polytransfection) using the method described in ref. 17 using siRNAs targeting mammalian human 14-3-3ζ and mouse 14-3-3ζ as previously described and were repeated at least three times. Transfection. Cells were transfected and selected using interferon transfection reagent (Polytransfection) using the method described in ref. 17 using siRNAs targeting mammalian human 14-3-3ζ and mouse 14-3-3ζ as previously described and were repeated at least three times.
accommodate for variation of height along individual cells, and values were then averaged. p-MLC (Ser19), F-actin, EFP-LA, polarity and brush border protein pixel intensity was measured using ImageJ software. For each cell, background was measured and subtracted from the sample value. Co-localization coefficients were measured in Zeiss Confocal XY or Z-scans using Zeiss Zen 2000 co-localization software that applies the principal of the Pearson's coefficient, using the cross-hairs facility to subtract background from each channel. Drosophila. Pixel intensity/membrane length measurements of apical membrane and Arm domains were determined by analysing confocal images of gek mosaic retinas at 40% APF using ImageJ.

Statistics and reproducibility. For the quantifications shown, the provided n values refer to independent experiments and the numbers in the graphs refer to the total of analysed cells per type of sample across all experiments. Statistical significance was tested in most experiments using two-tailed Student's t-tests, pairing values derived from the same experiment. The FRET experiments are shown as standard box plots (25th to 75th percentiles, with a line at the median; whiskers extend to the maximum/minimum data points) and were analysed with a non-parametric Mann–Whitney–Wilcoxon test as the data were not normally distributed. The experiments were not quantified in Figs 1b, 3e,f and 7c, as well as Supplementary Figs 3e, 4; Fig. 6j, 11; Fig. 2e,f, 8; Fig. 8l, 8; Fig. 4e, 8; Fig. 8m, 7; and Supplementary Fig. 7d, n = 7.

Data availability. All data supporting the conclusions here are available from the authors on reasonable request.

38. Matter, K., Brauchbar, M., Bucher, K. & Hauri, H. P. Sorting of endogenous plasma membrane proteins occurs from two sites in cultured human intestinal epithelial cells (Caco-2). Cell 60, 429–437 (1990).

39. Matter, K., Hunziker, W. & Mellman, I. Basolateral sorting of LDL receptor in MDCK cells: the cytoplasmic domain contains two tyrosine-dependent targeting determinants. Cell 71, 745–753 (1992).

40. Carlton, J. G. & Martin-Serrano, J. Parallels between cytokinesis and retroviral budding: a role for the ESCRT machinery. Science 316, 1908–1912 (2007).

41. Benais-Pont, G. et al. Identification of a tight junction-associated guanine nucleotide exchange factor that activates Rho and regulates paracellular permeability. J. Cell Biol. 160, 729–740 (2003).

42. Baldy, M. S., Garrett, M. D. & Matter, K. The ZO-1-associated Y-box factor ZONAB regulates epithelial cell proliferation and cell density. J. Cell Biol. 160, 423–432 (2003).

43. Sourisseau, T. et al. Regulation of PCNA and cyclin D1 expression and epithelial morphogenesis by the ZO-1-regulated transcription factor ZONAB/DbpA. Mol. Cell Biol. 26, 2387–2398 (2006).

44. Yoshizaki, H. et al. Activity of Rho-family GTPases during cell division as visualized with FRET-based probes. J. Cell Biol. 162, 223–232 (2003).

45. Slowers, R. S. & Schwarz, T. L. A genetic method for generating Drosophila eyes composed exclusively of mitotic clones of a single genotype. Genetics 152, 1631–1639 (1999).

46. Lee, T. & Luo, L. Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. Neuron 22, 451–461 (1999).

47. Winter, C. G. et al. Drosophila Rho-associated kinase (Drok) links Frizzled-mediated planar cell polarity signaling to the actin cytoskeleton. Cell 105, 81–91 (2001).

48. Waithier, R. F. & Pichaud, F. Immunofluorescent staining and imaging of the pupal and adult Drosophila visual system. Nat. Protoc. 1, 2635–2642 (2006).

49. Karagiosis, S. A. & Ready, D. F. Moesin contributes an essential structural role in Drosophila photoreceptor morphogenesis. Development 131, 725–732 (2004).

50. Pinal, N. et al. Regulated and polarized PtdIns(3,4,5)P3 accumulation is essential for apical membrane morphogenesis in photoreceptor epithelial cells. Curr. Biol. 16, 140–149 (2006).

51. Crawley, S. W., Morek, M. S. & Tyska, M. J. Shaping the intestinal brush border. J. Cell Biol. 207, 441–451 (2014).
Supplementary Figure 1  Quantification of microvilli induction and role of MRCK in apical morphogenesis of Caco-2 cells. (a) Broad view of apical surface of MDCK cells by scanning electron microscopy after MRCK knockdown and after complementation with MRCKβ-flag. (b) Measurement of apical membrane brush border cluster induction using threshold function of Nikon imaging software. (c,d) Levels of microvilli induction in human Caco-2 intestinal epithelial cells following MRCK knockdown without or with complementation with MRCKβ-flag. Panel d is based on n=3 independent experiments and shows the data points, means ± 1 SD (in black), the total number of cells analysed for each type of sample across all experiments, and p-values derived from t-tests.
Supplementary Figure 2  Inhibition of ROCK does not affect Dbl3-induced apical actomyosin activation. Confocal analysis of apical pMLC activity and F-actin in MDCK cells upon induction of Dbl3 expression by tetracycline in a conditional cell line in the absence or presence of the ROCK inhibitor GSK269962. Arrowheads point to apical cortex. Panel b is based on n=3 independent experiments and shows the data points, means ± 1 SD (in black), the total number of cells analysed for each type of sample across all experiments, and p-values derived from t-tests. Scale bars: 10 µm.
Supplementary Figure 3  MRCKβ expression does not rescue loss of apical Myosin-II activation and differentiation in Dbl3-depleted MDCK cells: (a-c) Levels of Myosin phosphorylation at the apical membrane domain (A) and basal membrane (B) and F-actin during polarization and differentiation of MDCK cells following Dbl3 siRNA transfection and with constitutive expression of MRCKβ-flag. Quantifications are based on n=3 independent experiments and show the data points, means ± 1 SD (in black), the total number of cells analysed for each type of sample across all experiments, and p-values derived from t-tests show means ± 1 SD, n=3. (d) SEM images of the effect of MRCK knock down on microvilli induction and conditional expression of Dbl3-myc. Note, MRCKβ-flag does not rescue the Dbl3 depletion-induced phenotype. (e) Time course of tetracycline-induced Dbl3-myc expression in MDCK cells. Asterisks highlight time points with clear induction of Dbl3-myc expression that were used for the functional assays. Unprocessed original scans of blots are shown in Supplementary Figure 8. (f) Confocal z-sections showing representative images of either endogenous or constitutively expressed MRCKβ in MDCK cells following conditional tetracycline-inducible expression of Dbl3-myc. Constitutive expression of MRCKβ-flag accelerates activation of Myosin-II at the apical membrane, highlighted by white arrows. Asterisks highlight basolateral MRCK localization at earlier stages of polarization. (g) Confocal Z-sections showing representative images of pEzrinT567 localization in MDCK cells following conditional tetracycline-inducible expression of Dbl3-myc without or with constitutive expression of MRCKβ-flag. Constitutive expression of MRCKβ-flag accelerates enrichment of active Ezrin at the apical membrane domain, indicated by white arrowheads. Scale bars: 10 µm.
Supplementary Figure 4 Cdc42 stimulates aPKC recruitment and antagonizes Rho-signalling. (a) Conditional tetracycline-induced expression of Dbl3 in MDCK cells stimulates increased recruitment of aPKCζ. Shown are confocal xy. (b) Levels of protein expression during tetracycline-induced Dbl3-myc expression. Unprocessed original scans of blots are shown in Supplementary Figure 8. (c) Quantification of levels of pEzrinT567 following tetracycline-induced Dbl3-myc expression. (d) Conditional tetracycline-induced expression of Dbl3 in MDCK cells stimulates loss of junctional p114RhoGEF and LULU-2. Shown are confocal z-sections. Junctions are indicated with white arrowheads. (e,f) Spontaneously polarizing MDCK cells were treated with control or Dbl3 siRNAs, or a myristoylated aPKCζ inhibitor. LULU-2 localization at tight junctions was then analysed by confocal microscopy using ZO-1 as a marker for tight junctions (indicated by arrowheads). Quantifications are based on n=3 independent experiments and show the data points, means ± 1 SD (in black), the total number of cells analysed for each type of sample across all experiments, and p-values derived from t-tests. Scale bars: 10 µm.
Supplementary Figure 5. MRCK-activated Myosin-II motor activity drives apical polarization of Par6-aPKC complex. (a) MDCK cells conditionally expressing tetracycline-inducible Dbl3-myc were analysed by confocal microscopy using co-localization software based on the Pearson’s correlation coefficient. Co-localization is calculated in grid 3 of the scatter charts. Bright green represents co-localization of aPKC\(\zeta\) and Par6\(\beta\), which increases and is confined to the apical cortical membrane following polarization stimulated by conditional tetracycline inducible Dbl3-myc expression in MDCK cells and is dependent on Myosin-II motor activity. Coloured outlines of apical membrane domains represent calculation areas of co-localization coefficients. Scale bars: 10\(\mu\)m. (b) Co-immunoprecipitation of Par6 and aPKC indicating formation of stable complexes that are independent of Myosin-II motor activity and Dbl3 expression. Unprocessed original scans of blots are shown in Supplementary Figure 8.
Supplementary Figure 6 Dbl3-activated Myosin-II motor activity drives apical polarization and co-localization of brush border regulators. (a-d) MDCK cells conditionally expressing tetracycline-inducible Dbl3-myc were analysed by confocal microscopy using co-localization software based on the Pearson’s correlation coefficient. Co-localization is calculated in grid 3 of the scatter charts. Bright green represents co-localization of Ezrin and SLK or NHERF-1 and SLK, which increases and is confined to the apical membrane domain following polarization stimulated by conditional tetracycline inducible Dbl3-myc expression in MDCK cells and is dependent on Myosin-II motor activity. Coloured outlines of apical membrane domains represent calculation areas of co-localization coefficients. Scale bars: 10µm. (e) Expression levels of differentiation markers upon Dbl3 and Myosin manipulation. Unprocessed original scans of blots are shown in Supplementary Figure 8.
Supplementary Figure 7 Myosin-II activation mediates gek function in pupal photoreceptors. (a-c) Confocal sections of a pupal retinas showing wild type cells (blue nuclei) and gek mutant cells (labelled with asterisks) stained for the apical markers aPKC and Moesin, or the junctional marker Arm. (d) Quantification of the effect of mutant gek on junctional levels of Arm (measurements from wild type and neighbouring mutant cells, paired within sections; based on n=7 animals; p value was calculated with a t-test).
Supplementary Figure 8 Unprocessed original scans of blots.
Supplementary Figure 8 Continued
Figure 7c Cont’d

Supplementary Figure 3e

Supplementary Figure 8 Continued
Supplementary Figure 8 Continued
Supplementary Figure 8 Continued
Supplementary Table Legend

Supplementary Table 1 Details of antibodies used. Information about antibody sources and specificity.

Supplementary Video Legend

Supplementary Video 1 Induction of polarized myosin activation by Cdc42. Apical Cdc42 activation by conditional expression of Dbl3-myc in MDCK cells constitutively expressing EGFP-MLC was induced by adding tetracycline followed by recording image stacks every 5 minutes. Shown are projections of 4 images derived from the apical domain. The experiment was performed five times.
Experimental design

1. Sample size

Describe how sample size was determined.

The sample size was chosen based on previous experience with similar experimental designs. Averaging three independent experiments is a traditional approach in the type of experiments reported here. A statistical section is included in the Method section.

2. Data exclusions

Describe any data exclusions.

No data were excluded

3. Replication

Describe whether the experimental findings were reliably reproduced.

All experiments were independently reproduced. Almost all images shown are examples of images that represent either independent repeats of multiple animals that were analysed. In some cases, images are shown in the extended data and the quantifications are part of a main figure; such connections are all pointed out when the data are described. Data are reported with information about repeats in the corresponding figure legends. Additionally, the Methods section contains information about statistics that also includes numbers of repeats and animals analysed.

The exception are some of the immunoblots if they were not quantified (confirmation of knockdowns and transgene expression). Such blots are representative for 3 or more repetitions.

4. Randomization

Describe how samples/organisms/participants were allocated into experimental groups.

No randomization was applied. The animal studies were performed by inducing mutant clones within individual animals; hence, sections were analysed that simultaneously contain both mutant and wild type cells.

5. Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

The investigators were not blinded to allocation during experiments and outcome assessment as analysis was based on induction of mutant clones.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.
6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

- The exact sample size \( (n) \) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
- A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- A statement indicating how many times each experiment was replicated
- The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
- A description of any assumptions or corrections, such as an adjustment for multiple comparisons
- The test results (e.g. \( P \) values) given as exact values whenever possible and with confidence intervals noted
- A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
- Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.

Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

Images were quantified with ImageJ, Zeiss ZEN2000, Photoshop 7 and CS6, and NIS Nikon Imaging Software. Co-localisation coefficients were calculated with the Zeiss 700 microscope software. For statistics, MS Excel (V15) and JMP (V12) were used.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

All materials are either commercially available as described in the Method section or, if not, are available from the authors.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

This information is available in Supplementary Table 1.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

Original cell stocks were obtained from Ira Mellman (MDCK cells) and Hans-Peter Hauri (Caco-2 cells)

b. Describe the method of cell line authentication used.

Staining of specific markers for kidney (MDCK) and intestine (Caco-2), sequencing to confirm species identity.

c. Report whether the cell lines were tested for mycoplasma contamination.

All cell lines tested negative for mycoplasma.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

No commonly misidentified cell lines were used.
Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

Drosophila - To generate whole mutant eyes, the EGUF/GMRhid system was used in combination with the gekNP5192 and gekomb1080 alleles. Canton S flies were used as control.

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

The study did not involve human research participants.