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ROCK1-directed basement membrane positioning coordinates epithelial tissue polarity

William P. Daley[1,2,*], Elise M. Gervais[1,2], Samuel W. Centanni[2,‡], Kathryn M. Gulfo[2,§], Deirdre A. Nelson[2] and Melinda Larsen[2,¶]

SUMMARY

The basement membrane is crucial for epithelial tissue organization and function. However, the mechanisms by which basement membrane is restricted to the basal periphery of epithelial tissues and the basement membrane-mediated signals that regulate coordinated tissue organization are not well defined. Here, we report that Rho kinase (ROCK) controls coordinated tissue organization by restricting basement membrane to the epithelial basal periphery in developing mouse submandibular salivary glands, and that ROCK inhibition results in accumulation of ectopic basement membrane throughout the epithelial compartment. ROCK-regulated restriction of PAR-1b (MARK2) localization in the outer basal epithelial cell layer is required for basement membrane positioning at the tissue periphery. PAR-1b is specifically required for basement membrane deposition, as inhibition of PAR-1b kinase activity prevents basement membrane deposition and disrupts overall tissue organization, and suppression of PAR-1b together with ROCK inhibition prevents interior accumulations of basement membrane. Conversely, ectopic overexpression of wild-type PAR-1b results in ectopic interior basement membrane deposition. Significantly, culture of salivary epithelial cells on exogenous basement membrane rescues epithelial organization in the presence of ROCK1 or PAR-1b inhibition, and this basement membrane-mediated rescue requires functional integrin β1 to maintain epithelial cell-cell adhesions. Taken together, these studies indicate that ROCK1/PAR-1b-dependent regulation of basement membrane placement is required for the coordination of tissue polarity and the elaboration of tissue structure in the developing submandibular salivary gland.

KEY WORDS: PAR-1b (MARK2), ROCK, Basement membrane, Morphogenesis, Polarity, Salivary gland, Mouse

INTRODUCTION

Tissue function emerges from tissue structure and depends upon precise organization of its specialized cell types. Loss of normal tissue organization is a defining characteristic of many diseases and can be a prerequisite for the development of disease (Bissell et al., 2003; Chapin and Caplan, 2010; Collins and Bonnemann, 2010). Epithelial tissue organization is established during development when cells acquire apicobasal polarity (Bryant and Mostov, 2008; Nelson, 2003). The polarity of individual cells must additionally be coordinated at the tissue level, yet the molecular mechanisms governing tissue-level coordination of polarity are not well understood.

Epithelial cell organization and function require positional information from the surrounding microenvironment (Bryant and Mostov, 2008; Kass et al., 2007); such cues include contacts with both adjacent cells and the basement membrane. A prominent role for cell-cell junctions in the establishment of apicobasal polarity has been identified (Vega-Salas et al., 1987a; Vega-Salas et al., 1987b; Wheelock and Johnson, 2003a; Wheelock and Johnson, 2003b), and basement membrane-mediated signaling is a prerequisite for apical domain establishment and cell-cell junction formation in many developmental and 3D cell-based model systems (Li et al., 2003; Miner and Yurchenco, 2004; Plachot et al., 2009; Weir et al., 2006). The basement membrane underlies epithelial cells at their basal surface; it provides structural support, forms a scaffold for cell adhesion (Sequeira et al., 2010; Yurchenco and Wadsworth, 2004), separates epithelial cells from the surrounding stroma and regulates cell behavior (Kalluri, 2003; Yurchenco et al., 2004). Active remodeling of the basement membrane mediates changes in tissue shape during epithelial morphogenesis (Bernfield and Banerjee, 1982; Bernfield et al., 1984; Nakanishi and Ishii, 1989) and is essential for tissue homeostasis (Larsen et al., 2006a; Xu et al., 2009). Thus, defining the molecular mechanisms by which basement membrane is localized and impacts epithelial tissue organization is essential to understand epithelial organ development and disease.

Studies using mammalian cell lines have revealed complex regulation of cell polarity (Goldstein and Macara, 2007; Suzuki and Ohno, 2006). The apical PAR complex and the basolateral PAR-1b (also known as MARK2 and EMK1) kinase are required for the establishment of epithelial polarity (Etemad-Moghadam et al., 1995; Guo and Kemphues, 1995; Hung and Kemphues, 1999; Suzuki and Ohno, 2006; Tabuse et al., 1998), and their complementary localization is controlled, in part, through a process of mutual exclusion (Benton and St Johnston, 2003; Suzuki et al., 2004). Although the apical PAR complex is required for the establishment of epithelial cell-cell junctions (Hirose et al., 2002; Suzuki et al., 2001), the mechanism by which PAR-1b defines the basolateral domain is not well understood. However, a recent study demonstrated that PAR-1b

[1]Graduate program in Molecular, Cellular, Developmental, and Neural Biology, University at Albany, State University of New York, Albany, NY 12208, USA.
[2]Department of Biological Sciences, University at Albany, State University of New York, Albany, NY 12208, USA.

*Present address: NIH, NIDCR, Laboratory of Cell and Developmental Biology, Bethesda, MD 20892, USA
‡Present address: Department of Biomedical Sciences, Medical University of South Carolina, Charleston, SC 29403, USA
§Present address: College of Medicine, SUNY Downstate Medical Center, Brooklyn, NY 11203, USA
¶Author for correspondence (mlarsen@albany.edu)

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promotes laminin deposition within the basement membrane in cultured cells by restricting laminin receptor localization (Masuda-Hirata et al., 2009).

To determine how basement membrane and cell polarity control epithelial tissue architecture, we utilized ex vivo organ culture of mouse embryonic submandibular salivary glands (Grobstein, 1953; Patel et al., 2006), the development of which requires interactions with the basement membrane (Bernfield and Banerjee, 1982; Larsen et al., 2006b; Rebustini et al., 2009; Sakai et al., 2003). Rho-associated coiled-coil containing kinase (ROCK) regulates epithelial morphogenesis in the lung (Moore et al., 2005), kidney (Meyer et al., 2006; Michael et al., 2005) and salivary gland (Daley et al., 2009). During salivary gland and lung development, ROCK promotes basement membrane remodeling to mediate changes in tissue shape through a myosin II-dependent pathway that requires cellular contractility (Daley et al., 2009; Moore et al., 2005). Recent studies have also implicated ROCK in the control of cell polarity via interactions with the apical PAR complex (Ishiuchi and Takeichi, 2011; Nakayama et al., 2008; Simoes Sde et al., 2010). Here, we identify a novel function for ROCK1 in the regulation of PAR-1b function and resultant basement membrane positioning to control tissue polarity in developing salivary glands, a role that is biochemically distinct from the previously reported myosin-dependent pathway by which ROCK regulates epithelial morphogenesis. We demonstrate that ROCK aligns cell polarity via restriction of the basement membrane to the epithelial periphery to create an ordered tissue architecture. We also demonstrate that a basement membrane-mediated outside-in signal downstream of ROCK and PAR-1b that requires functional integrin β1 promotes the maintenance of epithelial cell morphology and cell-cell adhesions. These studies provide new insight into the mechanisms by which basement membrane controls epithelial integrity and organization.

MATERIALS AND METHODS

Ex vivo organ culture

Mouse submandibular salivary glands were dissected from timed-pregnant female mice (strain CD-1, Charles River Laboratories) at embryonic day (E) 13 (day of plug defined as E0), following protocols approved by the University at Albany IACUC Committee, and cultured as previously described (Daley et al., 2009; Rebustini and Hoffman, 2009; Sakai and Onodera, 2008). The reaggregation assays were performed as described (Wei et al., 2007). For virus infection, epithelial rudiments were infected for 1 hour with a 1:100 dilution of kinase-dead PAR-1b or a 1:20 dilution of wild-type PAR-1b adenovirus (Bohm et al., 1997; Cohen et al., 2004) and were recombined with four E13 mesenchymes or cultured in Matrigel (BD Biosciences; diluted 1:1 with media). The pharmacological inhibitors Y27632 and (–)-blebbistatin (Calbiochem) and ROCK1 and ROCK2 siRNAs were used as described (Daley et al., 2009). PAR-1b siRNA was used similarly (s65472 and s65473, Applied Biosystems). Morphometric analysis was performed as described (Daley et al., 2009).

Immunocytochemistry and immunoblotting

Immunostaining and confocal microscopy were performed essentially as described (Daley et al., 2009; Larsen et al., 2003) with all antibody incubations performed overnight at 4°C. Protein assays and immunoblotting were performed as described (Daley et al., 2009; Larsen et al., 2003). For antibodies and dilutions see Table 1.

Cell culture assays

Mouse SIMS cells, an adult submandibular ductal epithelial cell line (Laoide et al., 1996; Laoide et al., 1999), were cultured in DMEM containing 10% FBS and antibiotics (Invitrogen). SIMS cells were treated with either 20 μM Y27632 for up to 18 hours or transfected for 48 hours with 1 μg PAR-1b siRNA (s65472 and s65473). In some experiments, cells were treated with Y27632 or PAR-1b siRNA for 18 hours on glass, trypsinized, transferred to microwell dishes coated with a thin layer (20 μl) of Matrigel diluted 1:1 with DMEM/F12 media, and cultured for an additional 18 hours in the continued presence of inhibitor.

For hanging drop cultures (Foty, 2011; Foty and Steinberg, 2005), 2×10^3 SIMS cells were resuspended in 20 μl drops on tissue culture dish lids, inverted over PBS, and cultured for up to 48 hours. For siRNA treatment, cells were transfected on glass for 18 hours, transferred to hanging drops, and cultured for an additional 18 hours. Cells were fixed and processed for immunocytochemistry as described (Cukierman et al., 2001; Daley et al., 2009) and imaged with a Zeiss Cell Observer Z1. See Table 1 for antibodies and dilutions.

For cell migration assays, SIMS cells were cultured with vehicle control or 20 μM Y27632 on glass or 1:1 Matrigel. Time-lapse microscopy was performed as described using a Zeiss Cell Observer Z1 (Daley et al., 2009) and quantified as described (Larsen et al., 2006b) using Volocity (Improvision). Graphs were displayed and two-way analysis of variance (ANOVA) with a Bonferroni post-test was performed using GraphPad Prism.

Table 1. Antibodies

Antigen	Source	Application	Dilution
E-cadherin	Clone 36, BD Transduction	ICCc, ICCwm	1/250
Na^+/K^+-ATPase	EP1845Y, Epitomics	ICCwm	1/100
Collagen IV	AB769, Millipore	ICCc, ICCwm	1/100
	AB769P, Millipore	ICCc, ICCwm	1/100
Collagen IV α2	T-15, Santa Cruz	IB	1/1000
Perlecan	Clone A7L6, Millipore	ICCwm	1/100
Laminin-111	Dr H. Kleinmann*	ICCwm	1/100
Fibronectin	R5386, Dr K. M. Yamada*	ICCwm	1/100
PAR-1b (MARK2)	HPA038790, Sigma	ICCc, ICCwm	1/100
	C-16, Santa Cruz	IB	1/500
ZO-1	Clone 1A12, Invitrogen	ICCc, ICCwm	1/100
ROCK1	EP786Y, Abcam	ICCc	1/250
ROCK2	H-85, Santa Cruz	ICCwm	1/100
c-Myc	9E10, Santa Cruz	ICCwm	1/250
GFP	ab290, Abcam	ICCwm	1/250
GAPDH	Fitzgerald	IB	1/10,000
Rhodamine phalloidin	Invitrogen	ICCc, ICCwm	1/350
SYBR Green I	Invitrogen	ICCc, ICCwm	1/100,000

ICCc, immunocytochemistry in cells; ICCwm, immunocytochemistry in whole-mounts; IB, immunoblot.
*NIH, NIDCR, Laboratory of Cell and Developmental Biology, Bethesda, MD 20892, USA.

RESULTS

ROCK1 restricts basement membrane to the basal periphery of developing salivary gland epithelium

The mouse E13 submandibular salivary gland epithelium consists of epithelial buds with a single layer of organized outer columnar cells (OCCs) surrounding a less organized cluster of central, or inner, polymorphic cells (IPCs) (Kadoya and Yamashina, 2010; Walker et al., 2008). When cultured with the ROCK inhibitor Y27632, the columnar morphology of the OCCs was lost, and they took on a less organized appearance (Fig. 1A). Since the OCCs directly contact the basement membrane, we questioned whether this ROCK inhibitor-induced loss of cellular organization was associated with misregulated basement membrane. In control cultures, the basement membrane proteins fibronectin, perlecan, collagen type IV and laminin-111 (α1, β1, γ1) were present at the basal periphery of epithelial buds, whereas treatment with Y27632 or ROCK1 siRNAs, but not ROCK2 or non-targeting siRNAs, induced accumulations of basement membrane within epithelial buds (Fig. 1B, supplementary material Fig. S1A). *xz* projections of confocal stacks revealed that such accumulations occurred throughout the epithelium (Fig. 1B), are largely colocalized (supplementary material Fig. S1A,B) and are extracellular (supplementary material Fig. S1C,D). Myosin light chain 2 is a downstream effector of ROCK (Totsukawa et al., 2000) that is required for the assembly of fibronectin and subsequent branching morphogenesis in the developing salivary gland (Daley et al., 2009); yet glands treated with the myosin inhibitor blebbistatin showed virtually no aberrant interior basement membrane accumulations (Fig. 1C). Thus, non-muscle myosin type II activity is not required for basement membrane positioning, suggesting that another pathway downstream of ROCK is required for this function.

Since the OCCs directly contact the basement membrane, we reasoned that these cells might be responsible for its production. To test this hypothesis, we used a primary cell aggregation assay, in which salivary glands are dissociated into a single-cell suspension that can reaggregate and undergo branching morphogenesis (Wei et al., 2007). In the presence of Y27632, the tissue aggregates were less organized than in controls, suggesting that although ROCK inhibition does not prevent the reassociation of primary cells, it does affect their organization (Fig. 2A). In control aggregates 8 hours post-dissociation, a subset of cells at the basal periphery showed cytoplasmic and basal localization of collagen IV and, by 72 hours, collagen IV showed continuous peripheral basal localization in each aggregate, indicating that the basement membrane had been reassembled (Fig. 2B). In Y27632-treated

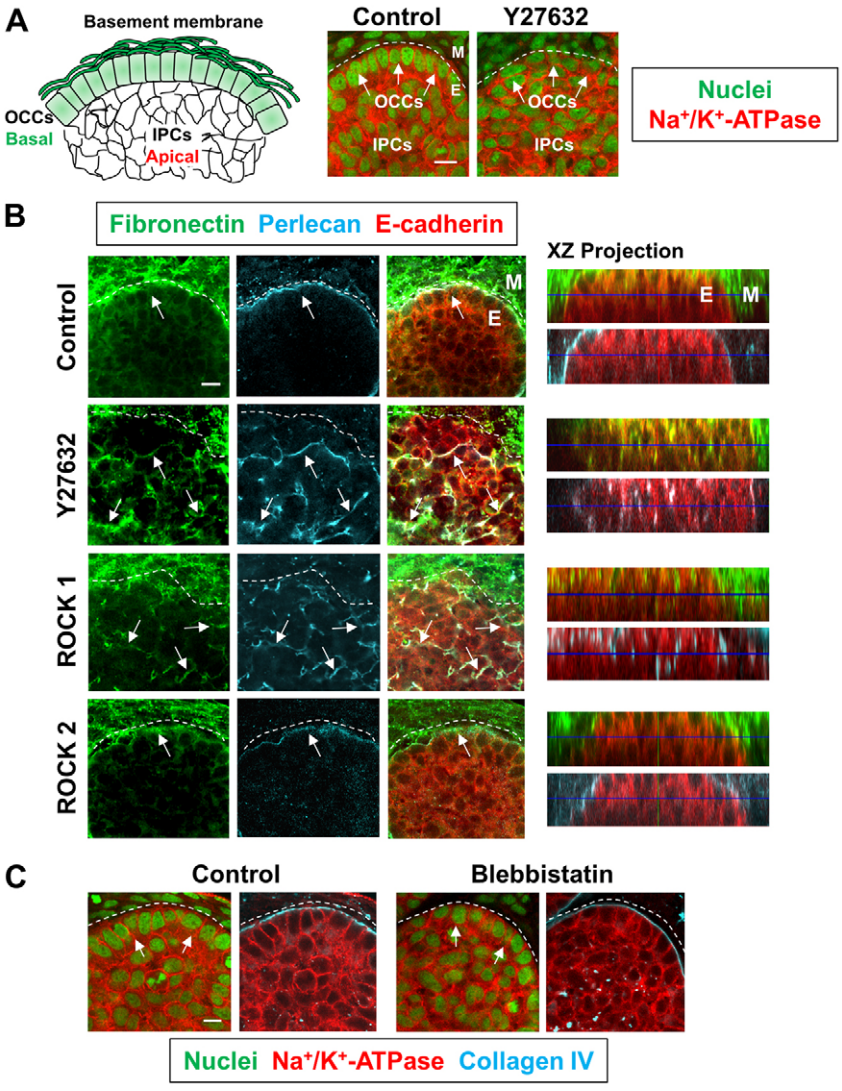

Fig. 1. ROCK1 restricts basement membrane position in developing submandibular salivary glands. (**A**) Mouse E13 salivary glands consist of outer columnar cells (OCCs) surrounding inner polymorphic cells (IPCs), as shown schematically and with the nuclear dye SYBR Green (green) and by membrane staining of Na^+/K^+-ATPase (red). With the ROCK inhibitor Y27632, OCC morphology is lost (arrows). Dashed line indicates the basal periphery of the bud. (**B**) E13 glands cultured with Y27632 or ROCK1 siRNA exhibit fibronectin (green) and perlecan (cyan) within the epithelium (E-cadherin, red), whereas vehicle control and ROCK2 siRNA-transfected glands localize these proteins solely in the basement membrane (arrows). A single central confocal section and *xz* projection are shown. (**C**) Inhibition of myosin II in E13 glands with blebbistatin does not alter OCC morphology (arrows), nor does it induce interior basement membrane (collagen IV, cyan). E, epithelium; M, mesenchyme. Scale bars: 10 μm.

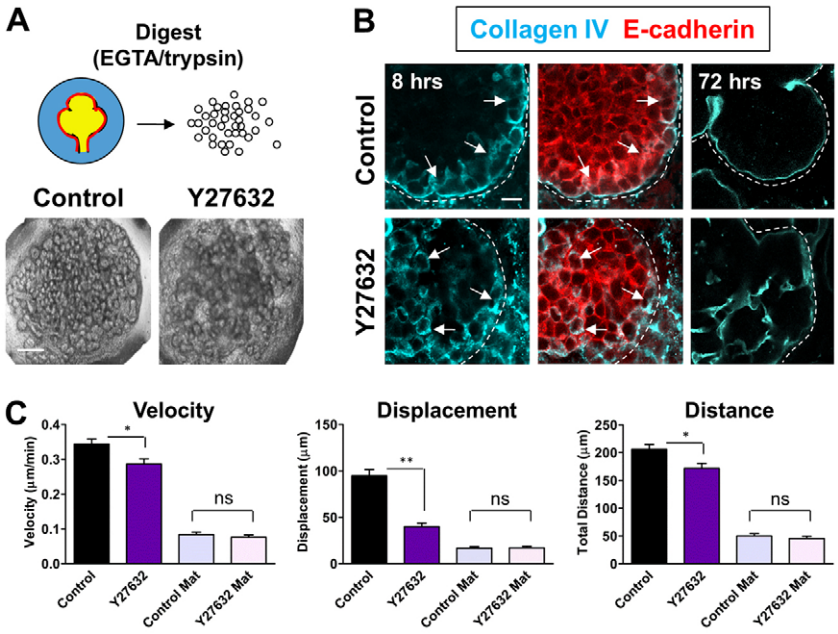

Fig. 2. Salivary gland interior cells secrete and assemble basement membrane in the absence of ROCK signal. (**A**) Schematic of cell aggregation assay. Mouse E13 salivary glands that have been dissociated into single cells undergo reaggregation. Aggregate formation occurs in the presence of Y27632, but aggregates branch less and are more disorganized than in vehicle controls. (**B**) After 8 hours, basement membrane (collagen IV, cyan) is synthesized and assembled by cells only at the periphery in vehicle controls, but by cells throughout the epithelium (E-cadherin, red) in Y27632-treated cultures (arrows). By 72 hours, collagen IV has assembled into basement membrane in vehicle controls but is present as accumulations within epithelial buds of ROCK-inhibited aggregates. (**C**) SIMS cell velocity, displacement and total distance traveled are decreased when cultured on glass, but not on Matrigel (Mat), in the presence of Y27632. Error bars indicate s.e.m. ANOVA, **P*<0.05, ***P*<0.01; ns, not significant. Scale bars: 250 μm in A; 10 μm in B.

aggregates 8 hours post-dissociation, cellular collagen IV was detected throughout the aggregate and, by 72 hours, assembled collagen IV was present throughout the epithelial bud (Fig. 2B). Fibronectin and perlecan also demonstrated inappropriate deposition within the interior of ROCK-inhibited aggregates (data not shown). These results indicate that in the absence of ROCK activity, basement membrane is synthesized, secreted and assembled by cells throughout the epithelial compartment, rather than solely by cells at the basal periphery of the tissue as in control glands.

Since salivary epithelial cells move dynamically during embryonic development (Larsen et al., 2006b), and epithelial cells within the interior of tissue aggregates synthesize and secrete basement membrane upon ROCK inhibition, we questioned whether outer cells that normally synthesize basement membrane instead migrate into the bud interior when ROCK signaling is perturbed. We analyzed the migration of SIMS salivary gland epithelial cells on glass or on the basement membrane substitute Matrigel. ROCK-inhibited SIMS cells migrated more slowly than vehicle control-treated cells and traveled a shorter distance on glass (Fig. 2C, supplementary material Fig. S2). SIMS cells grown on Matrigel to simulate salivary OCCs migrated significantly more slowly than cells on glass and traveled a shorter distance; however, in this context, ROCK inhibition had no effect on cell migration (Fig. 2C, supplementary material Fig. S2). These results indicate that basement membrane modulates the effects of ROCK inhibition on cell motility and suggest that, in the context of Y27632-treated intact tissue, enhanced migration of OCCs into the bud interior is unlikely to be the primary cellular response leading to interior basement membrane accumulations. Instead, ROCK likely functions to restrict basement membrane production to the epithelial periphery.

ROCK1 coordinates epithelial tissue organization through regulation of basement membrane placement

Our results indicate that the IPCs, which normally do not produce basement membrane, can produce it when ROCK is inhibited, and we hypothesized that these interior cells might undergo apicobasal polarization. Glandular epithelial tissues have specialized apical membrane domains, which in the adult tissue contact the lumen and are surrounded by intracellular tight junctions. In E13 salivary glands, β-actin and the tight junction protein ZO-1 (TJP1 – Mouse Genome Informatics) accumulate in the center of cellularized ducts and buds, where the lumen will later form. In timecourse assays of control glands, ZO-1 and actin progressively localized to the center of epithelial buds, so that by 96 hours each bud contained a well-defined apically localized lumen (Fig. 3A). In Y27632-treated glands, we instead observed cell clusters surrounding centrally localized accumulations of actin and ZO-1 throughout the bud, with ectopic basement membrane located opposite to the apical accumulations, as confirmed by line profiling (Fig. 3A,B). This contrasts with the single aligned OCC layer at the basal periphery in control buds resting on a continuous basement membrane and suggests that, in the absence of ROCK signaling, interior cells undergo aberrant polarization, albeit in an uncoordinated manner relative to the whole tissue.

Given the previously identified role of basement membrane in orienting epithelial apical polarity (O'Brien et al., 2001; Yu et al., 2005), we hypothesized that ectopic basement membrane might directly induce polarization of interior cells. To test whether basement membrane can induce apical polarity in salivary gland epithelial cells we compared ZO-1 localization in SIMS cells cultured on glass versus Matrigel. Whereas ZO-1 was present across the entire lateral membrane of cells on glass, it was more apically localized in cells on Matrigel (Fig. 3C). Since integrin β1 is a primary transducer of basement membrane-mediated polarity signals (Yu et al., 2005), we tested a function for integrin β1-mediated polarity signaling. When SIMS cells on Matrigel were treated with Ha2/5, a function-blocking antibody that specifically perturbs integrin β1 function, but not with isotype-matched control IgG, ZO-1 localization at cell-cell boundaries resembled that of SIMS cells on glass (Fig. 3C). A web of cortical actin was also apically localized in cells on Matrigel and was disrupted by Ha2/5 (data not shown). Thus, a basement membrane-mediated outside-in signal that is mediated through integrin β1 orients apical polarity in salivary epithelial cells and may drive the uncoordinated alignment of epithelial cells in the absence of ROCK.

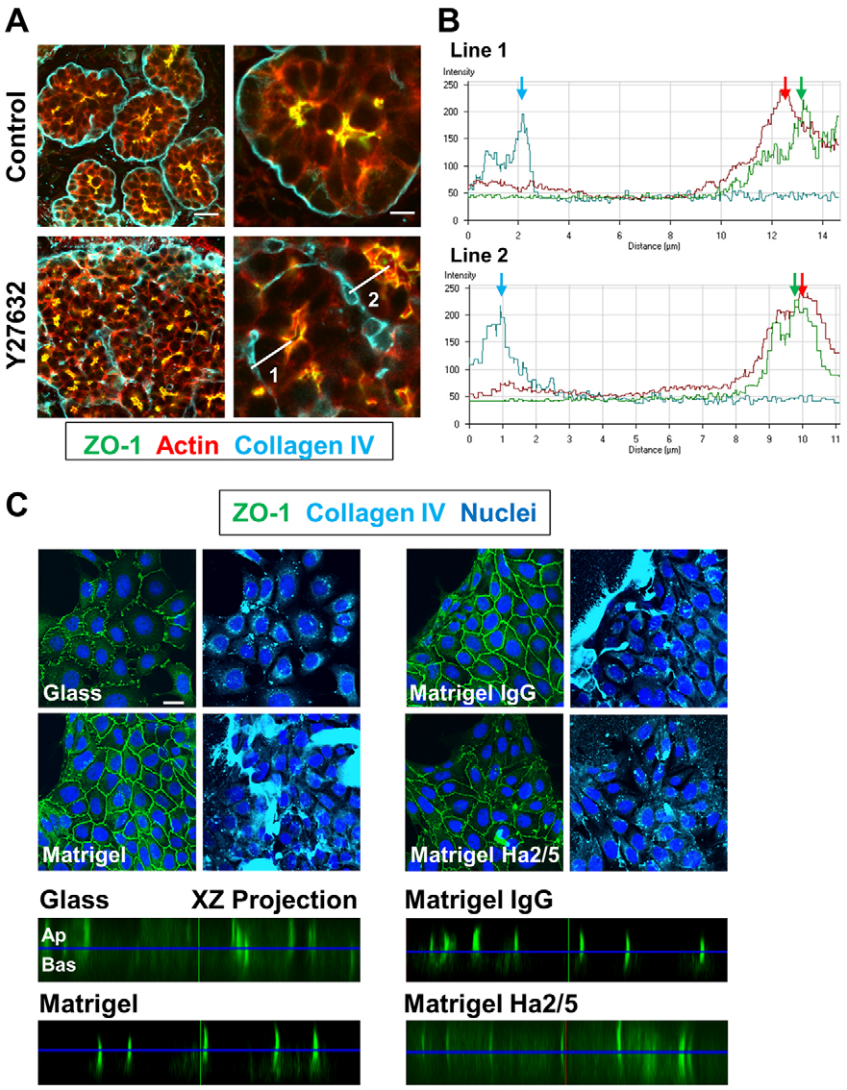

Fig. 3. ROCK coordinates individual cell polarity at the global tissue level. (**A**) After 96 hours of culture, control mouse salivary glands exhibit centralized accumulations of β-actin (red) and ZO-1 (green) within epithelial buds. By contrast, in glands treated with Y27632 these proteins are present at multiple locations throughout the distended epithelial buds. (**B**) Fluorescence intensity line profiles (at the locations indicated in A) of ROCK-inhibited epithelial buds reveal that actin (red arrows) and ZO-1 (green arrows) occur on the opposite side of cells to the basement membrane, as demarked by collagen IV (cyan arrows). (**C**) SIMS cells cultured on glass localize ZO-1 (green) along cell boundaries (*xy* projections), but apically in cells cultured on Matrigel (collagen IV, cyan) (*xz* projections) for 48 hours. SIMS cells cultured on Matrigel with the integrin β1 function-blocking antibody Ha2/5, but not with control IgG, exhibit diffuse ZO-1 in the *z* dimension. Ap, apical; Bas, basal. Scale bars: 5 μm in A right; 20 μm in A left and C.

ROCK1 regulates PAR-1b

The polarity protein PAR-1b is localized to the basolateral domains of polarized epithelial cells, where it functions in part to promote laminin accumulation within the basement membrane by restricting laminin receptor localization (Masuda-Hirata et al., 2009; Yamashita et al., 2010). Thus, ROCK might regulate PAR-1b to influence basement membrane positioning in developing salivary glands. ROCK inhibition increased total PAR-1b protein levels (Fig. 4A). Additionally, in control glands, PAR-1b was preferentially localized to the basolateral cortex of the OCCs and was diffusely localized in the IPCs (Fig. 4B), whereas in ROCK-inhibited glands the coordinated basolateral localization of PAR-1b was lost in the OCCs and an increase in cortical PAR-1b was detected in the IPCs (Fig. 4B). Significantly, cortical PAR-1b in ROCK-inhibited IPCs was closely associated with basement membrane accumulations throughout the epithelium (Fig. 4C). Consistent with the independence of OCC morphology from non-muscle myosin type II function (Fig. 1C), PAR-1b localization in blebbistatin-treated salivary glands was indistinguishable from that in controls (Fig. 4B). These data indicate that ROCK, independently of myosin II, promotes PAR-1b protein localization to the basolateral cortices of epithelial cells adjacent to the basement membrane.

PAR-1b is necessary and sufficient to drive ectopic basement membrane deposition

To determine whether PAR-1b regulates basement membrane deposition, E13 salivary glands were treated with PAR-1b-specific siRNAs and assayed for effects on basement membrane. These experiments demonstrated reduced collagen IV production (Fig. 5A) and localization (Fig. 5B) in PAR-1b siRNA-treated glands relative to controls. To determine whether the ROCK inhibitor-induced ectopic accumulations of basement membrane within the epithelial interior require PAR-1b, we treated glands with Y27632 and PAR-1b siRNA. Collagen IV accumulations inside epithelial buds were greatly reduced with PAR-1b but not control siRNAs (Fig. 5B). Furthermore, several other basement membrane proteins were decreased at the epithelial periphery in PAR-1b siRNA-treated glands and throughout the epithelial compartment with both PAR-1b siRNA and Y27632 treatment (supplementary material Fig. S3), indicating that PAR-1b is required for global basement membrane deposition.

To determine whether PAR-1b kinase activity is required for basement membrane deposition, adenovirus expressing dominant-negative PAR-1b lacking its kinase domain (Myc-PAR1b-KD) (Bohm et al., 1997; Cohen et al., 2004) was used to infect glands, and its effects on the basement membrane were

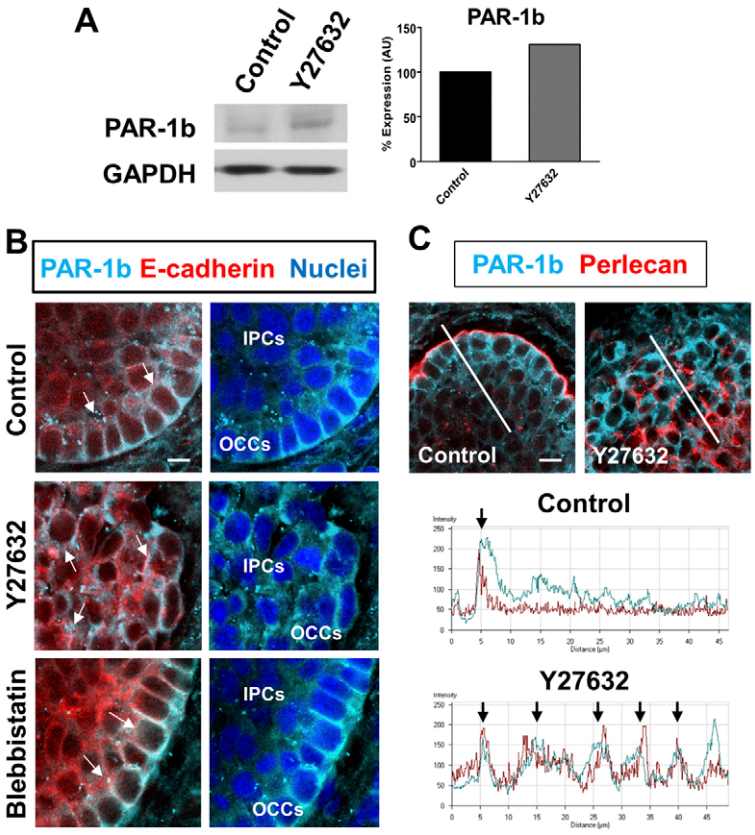

Fig. 4. ROCK, independently of myosin, regulates PAR-1b localization in developing salivary glands. (**A**) Immunoblotting reveals that PAR-1b protein levels are increased in mouse E13 salivary glands cultured for 24 hours with Y27632. Quantification is shown relative to GAPDH. (**B**) Immunocytochemistry reveals that PAR-1b (cyan) is preferentially localized to the basolateral domain of E13 salivary gland OCCs and is diffuse in IPCs. Whereas PAR-1b localization is indistinguishable from the vehicle control in blebbistatin-treated E13 glands, ROCK inhibition with Y27632 results in the coordinate loss of PAR-1b restriction to the basolateral domain of OCCs and leads to its accumulation at the cortices of IPCs (arrows). Epithelial cells are labeled with E-cadherin (red) and nuclei with SYBR Green (blue). (**C**) Line profiling of ROCK-inhibited epithelial buds reveals that mislocalized PAR-1b (cyan) at IPC cortices is closely associated with the ROCK inhibitor-induced accumulations of basement membrane (perlecan, red) (arrows). Scale bars: 5 μm in B; 10 μm in C.

compared with those of PAR-1b siRNA. Since adenoviral infection of the salivary gland epithelium requires removal of the mesenchyme (Larsen et al., 2006b), we infected epithelial rudiments with Myc-PAR1b-KD or a control GFP adenovirus and recombined epithelium and mesenchyme to recapitulate glandular structure (supplementary material Fig. S4). Significantly, collagen IV localization at the basal periphery of epithelial buds was reduced with either PAR-1b siRNA or Myc-PAR1b-KD (Fig. 5C), demonstrating that basement membrane deposition requires PAR-1b kinase activity.

Since under conditions of ROCK inhibition PAR-1b is mislocalized at IPC cortices, where it is required for the ROCK inhibitor-induced accumulations of basement membrane, we questioned whether the ectopic overexpression of wild-type PAR-1b throughout the epithelial buds would be sufficient to recapitulate this effect. As demonstrated in intact E13 organ explants (Fig. 4B), PAR-1b was also mislocalized at IPC cortices in recombination cultures treated with ROCK inhibitor (Fig. 5D). Similarly, when we infected recombination cultures with a wild-type PAR-1b adenovirus (Myc-PAR1b-WT), we observed high levels of PAR-1b expression correlating with the myc tag throughout the epithelium, even in the IPCs, where it is normally diffusely localized (Fig. 5D, supplementary material Fig. S4D). Significantly, recombination cultures infected with Myc-PAR1b-WT showed increased collagen IV expression throughout the epithelium after 48 hours that was closely associated with myc- and PAR-1b-expressing IPCs (Fig. 5D, supplementary material Fig. S4D), and by 96 hours the collagen IV appeared to be extracellular (Fig. 5E). Taken together, these data demonstrate that ectopic PAR-1b mislocalization is both necessary and sufficient for basement membrane deposition in the epithelial compartment.

PAR-1b function is required for the coordinate polarization of salivary gland epithelium

Basement membrane remodeling regulates tissue shape during salivary gland branching morphogenesis (Bernfield and Banerjee, 1982; Kadoya et al., 2003; Rebustini et al., 2009; Rebustini et al., 2007; Sakai et al., 2003). Consistent with a requirement for PAR-1b in basement membrane deposition, branching morphogenesis was decreased in intact E13 salivary gland or recombination cultures treated with PAR-1b siRNA or Myc-PAR1b-KD, respectively. Interestingly, Myc-PAR1b-WT overexpression also inhibited branching morphogenesis in recombination cultures, indicating that appropriate levels of PAR-1b are required for salivary gland development (supplementary material Fig. S5A,B).

To investigate a role for PAR-1b in the acquisition of coordinated epithelial architecture, we examined OCC morphology in E13 glands lacking PAR-1b function. As shown in Fig. 6A, when PAR-1b protein levels were reduced with PAR-1b-specific siRNAs, OCC morphology was greatly disrupted relative to negative controls (Fig. 6A). OCC organization was also disrupted in Myc-PAR1b-KD-infected recombination cultures (Fig. 5C), indicating that PAR-1b kinase activity is required to maintain OCC columnar morphology. To determine whether this loss of OCC organization was caused by decreased basement membrane deposition when PAR-1b function was inhibited, we treated E13 gland epithelial rudiments with PAR-1b siRNA or Myc-PAR1b-KD and cultured them on Matrigel. Although basement membrane deposition at the basal periphery of epithelial rudiments was reduced in the presence of PAR-1b siRNA or Myc-PAR1b-KD, the outer cells appeared to be more organized and columnar in the presence of exogenous basement

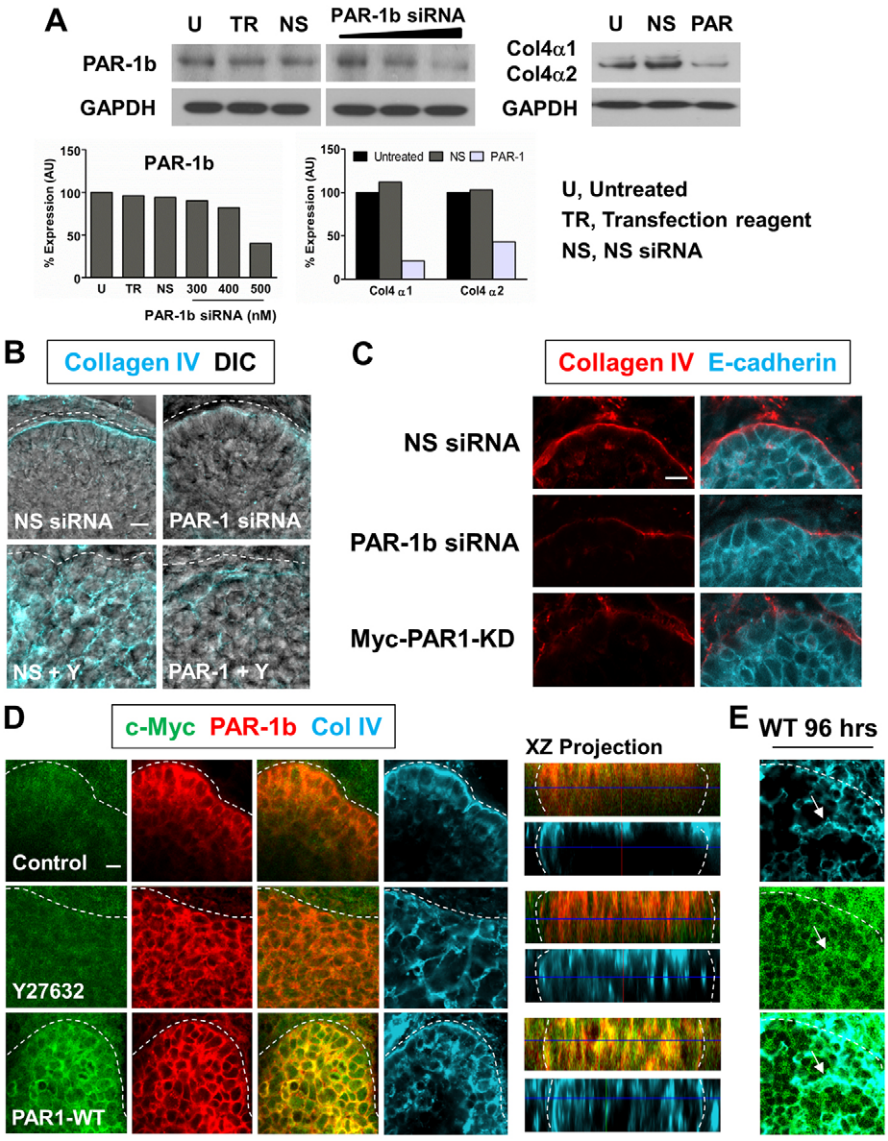

Fig. 5. PAR-1b is necessary and sufficient for basement membrane protein deposition in salivary gland epithelial buds. (**A**) Immunoblotting reveals a decrease in both PAR-1b and collagen IV α1 and α2 chain protein levels with PAR-1b, but not with non-targeting negative control (NS) siRNA, relative to GAPDH after 48 hours. (**B**) Immunocytochemistry for collagen IV (cyan) demonstrates that basal basement membrane localization in intact mouse E13 salivary glands is reduced with decreased PAR-1b. The ROCK inhibitor-induced interior basement membrane is reduced when treated with both Y27632 (Y) and PAR-1b siRNA. Images are overlaid on DIC images with the dashed white line indicating the basal periphery of the bud. (**C**) Recombination cultures transfected with PAR-1b siRNA or transduced with Myc-PAR1b-KD exhibit decreased collagen IV deposition (red) at the basal periphery of epithelial buds, with E-cadherin (cyan) demarking epithelium. (**D**,**E**) Recombination cultures infected with Myc-PAR1b-WT show ectopic expression of collagen IV (cyan), coincident with PAR-1b (red) and myc (green), inside epithelial buds after 48 (D) and 96 (E) hours. Arrows, collagen IV accumulations coincident with myc. Scale bars: 10 μm.

membrane than in intact or recombination cultures in which PAR-1b function was perturbed (Fig. 6B), despite the defect in endogenous basement membrane deposition. To directly test whether exogenous basement membrane can rescue a loss of cytoarchitecture in cells lacking PAR-1b, SIMS cells cultured on glass were treated with PAR-1b siRNA, and increased cell spreading and loss of epithelial organization were observed. By contrast, culture on a thin layer of Matrigel rescued these changes in cell morphology (supplementary material Fig. S6A,B). Since PAR-1b-mediated regulation of the basement membrane affects salivary gland tissue organization it might also regulate apical membrane accumulations in the IPCs with ROCK inhibition. As shown in Fig. 6C, treatment of E13 salivary glands with both Y27632 and PAR-1b siRNA decreased both the accumulations of basement membrane and the opposing apical protein accumulations relative to glands treated with Y27632 and control siRNA (Fig. 6C). These results demonstrate that PAR-1b kinase activity is a crucial determinant of salivary gland epithelial cell cytoarchitecture and suggest that PAR-1b mediates this function via regulation of basement membrane deposition.

A basement membrane-mediated outside-in signal downstream of ROCK1 and PAR-1b maintains epithelial cell-cell adhesions

Our data suggest that ROCK1 controls basement membrane positioning via regulation of PAR-1b localization in developing salivary glands. However, ROCK might also be required for the maintenance of cell-cell adhesions in the epithelium, and basement membrane deposition might be indirectly controlled through cell-cell interactions. In fact, previous studies demonstrated that PAR-1b is a positive regulator of cell-cell adhesions (Bohm et al., 1997; Cohen et al., 2011), and our finding of disrupted OCC morphology with ROCK or PAR-1b inhibition suggests that cell-cell adhesions might be negatively affected under these conditions. When SIMS cell monolayers cultured on glass were treated for 18 hours with Y27632, cell-cell adhesions were dramatically disrupted leading to cell scattering within the monolayer. E-cadherin (Fig. 7A) and ZO-1 (supplementary material Fig. S8B) were reduced at cell boundaries, demonstrating that, for SIMS cells cultured on glass, ROCK inhibition disrupts cell-cell adhesions, including both adherens and tight junctions. Similarly, E-cadherin was reduced in

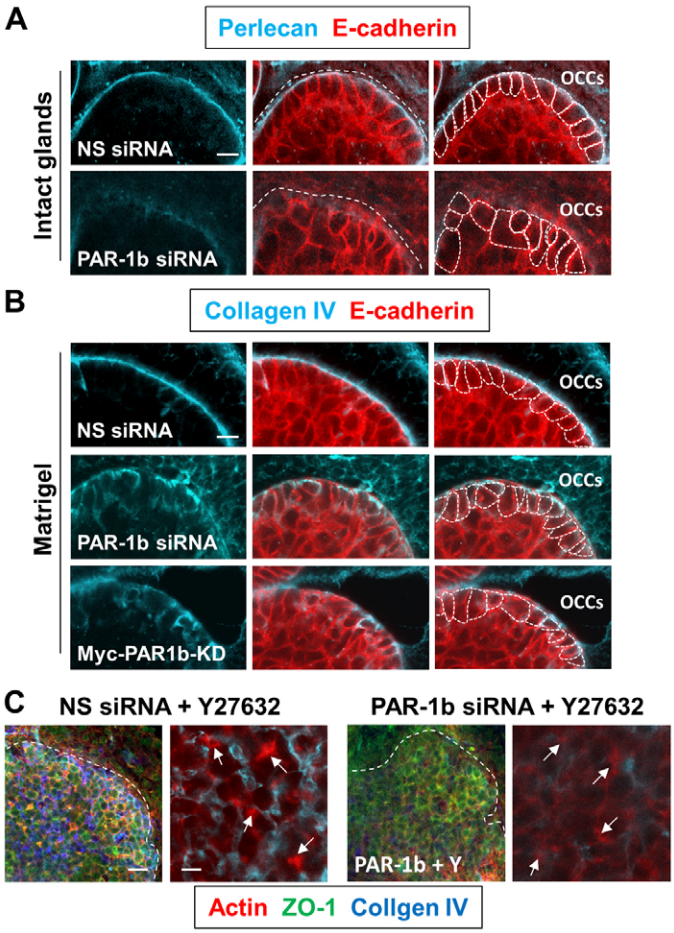

Fig. 6. PAR-1b-mediated regulation of basement membrane positioning is required for the coordinate polarization of salivary gland epithelium at the tissue level. (**A**) Mouse E13 salivary gland OCC columnar morphology (white outline) is disrupted with PAR-1b but not NS siRNAs. Peripheral basement membrane (perlecan, cyan) is also reduced with PAR-1b siRNA; E-cadherin marks epithelial membrane. (**B**) OCC organization is rescued by transfection with PAR-1b siRNA or infection with Myc-PAR1b-KD when cultured in Matrigel. Immunocytochemistry for collagen IV (cyan) shows basement membrane disruption when PAR-1b function is perturbed, but OCC morphology (E-cadherin, red; SYBR Green, blue) remains columnar with Matrigel. (**C**) Both apical actin (red) and ZO-1 (green), and basement membrane throughout the epithelial compartment (collagen IV, cyan), are reduced when E13 glands are cultured with Y27632 and PAR-1b siRNA but not with Y27632 and NS siRNA (arrows). Scale bars: 10 μm in A,B; 20 μm in C left; 5 μm in C right.

SIMS monolayers on glass treated with PAR-1b siRNA for 48 hours (Fig. 7A). Consistent with a loss of epithelial monolayer integrity, the overall height of cells in the *z* dimension decreased in the presence of either ROCK or PAR-1b inhibition (Fig. 7A). These data support a requirement for ROCK and PAR-1b in the maintenance of cell-cell adhesions in salivary epithelial cells cultured on glass.

To test the contribution of ROCK and PAR-1b signaling to the maintenance of cell-cell adhesions in 3D, we cultured cells in two 3D environments. We used hanging drop cultures (supplementary material Fig. S7A) to grow cells in 3D without basement membrane. Although basement membrane is produced by the cells over time, very little is detected after 18 hours (supplementary material Fig. S7B). SIMS cells cultured overnight in hanging drops exhibited decreased E-cadherin localization at cell-cell boundaries and appeared to adhere more loosely to one another when treated with Y27632, ROCK1 siRNA or PAR-1b siRNA (Fig. 7B, supplementary material Fig. S7C), similar to cells cultured on glass. We compared these with cells cultured on a thin layer of Matrigel. Control SIMS monolayers on Matrigel exhibited cell-cell adhesions that appeared to be more organized than those of cells on glass, as revealed by E-cadherin (Fig. 7A) and ZO-1 (Fig. 3C) staining. When SIMS cells cultured on Matrigel were treated with Y27632 or PAR-1b siRNA, the majority of cells retained E-cadherin, ZO-1 localized apically, and cells did not dissociate as they did on glass or in hanging drops. Furthermore, the decrease in SIMS height (*z* dimension) observed on glass with ROCK and PAR-1b inhibition was partially rescued by culture on Matrigel (Fig. 7A). These data demonstrate that, although ROCK and PAR-1b are required for the maintenance of salivary gland epithelial cell-cell adhesions in cells cultured on glass, the basement membrane has a dominant effect on cell-cell adhesions on exogenous basement membrane, such that loss of ROCK or PAR-1b has little effect on cell adhesions in this context. Similarly, in ROCK-inhibited salivary gland cultures we did not detect any changes in adherens junction protein expression or distribution (data not shown). Thus, in the context of the whole tissue 3D environment, basement membrane mislocalization in ROCK-inhibited salivary glands is unlikely to be due to a loss of epithelial tissue integrity caused by disrupted cell-cell adhesions. Rather, a basement membrane-mediated outside-in signal modulates the effect of ROCK inhibition to maintain cell-cell adhesions in the salivary gland epithelium.

Basement membrane-mediated maintenance of cell adhesions is integrin β1 dependent

Since culture of SIMS cell monolayers on Matrigel modulates the effects of ROCK and PAR-1b on the maintenance of adherens and tight junctions, we questioned whether integrin signaling is required for this effect. Because integrin β1 is implicated in basement membrane-mediated polarity signaling (Weir et al., 2006; Yu et al., 2005), we treated SIMS cells on Matrigel with the Ha2/5 function-blocking antibody. Importantly, whereas culture on Matrigel prevented cell scattering and junctional protein loss at cell boundaries in ROCK-inhibited SIMS monolayers, treatment with Ha2/5 prevented basement membrane-induced rescue (Fig. 7C, supplementary material Fig. S8B). We conclude that a basement membrane-mediated outside-in signal requiring integrin β1 promotes cell-cell adhesion in the absence of ROCK signaling. Interestingly, we also observed the apparent remodeling of basement membrane proteins at the basal surface of SIMS monolayers on Matrigel (supplementary material Fig. S8C). Inhibition of integrin β1 function with Ha2/5 prevented such remodeling of exogenous basement membrane in both control- and Y27632-treated monolayers (Fig. 3C, Fig. 7C). Thus, integrin β1-mediated inside-out signaling also appears to be required for basement membrane remodeling, which then may feed-forward to modulate cell-cell adhesions and cell polarity signaling.

DISCUSSION

Here, we report a mechanism by which ROCK1 activity coordinates global tissue polarity through basal restriction of basement membrane positioning. We demonstrate that inhibition of ROCK1 in embryonic mouse salivary gland cultures prevents

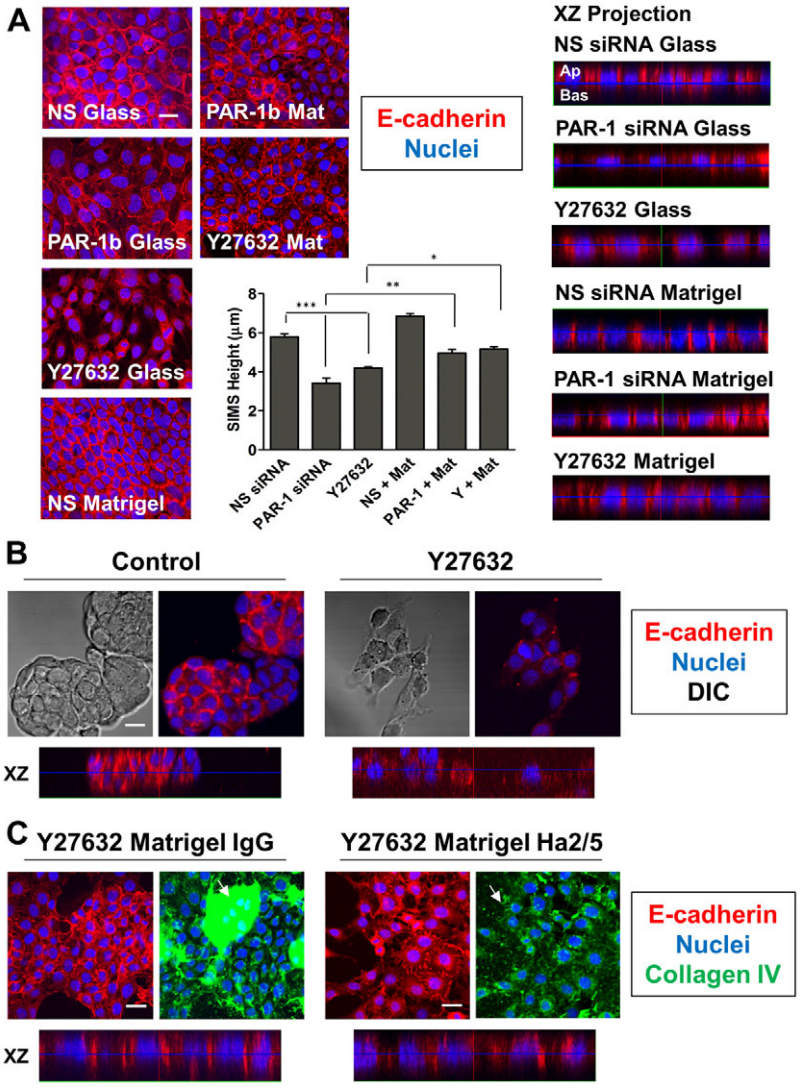

Fig. 7. A basement membrane-mediated outside-in signal promotes the maintenance of cell-cell adhesions by salivary gland epithelial cells in the absence of ROCK and PAR-1b.
(**A**) Immunocytochemistry for E-cadherin (red), shown in both the *xy* and *xz* dimensions, reveals that for SIMS cells cultured on glass in the presence of Y27632 (Y) or transfected with PAR-1b siRNA, E-cadherin localization is lost, but it is rescued by culture on Matrigel (Mat). Cell height was measured as the distance between the top and bottom of the monolayer from confocal *z*-stacks. (**B**) SIMS cells cultured as hanging drops with Y27632 fail to localize E-cadherin (red) at cell-cell contacts (*xy* and *xz* projections). (**C**) Treatment of ROCK-inhibited SIMS cells on Matrigel with Ha2/5 results in loss of E-cadherin (red) localization. Ha2/5 also interferes with the ability of SIMS cells to remodel Matrigel (collagen IV, green) at their basal surfaces (arrows). Error bars indicate s.e.m. ANOVA, $*P<0.05$, $**P<0.01$, $***P<0.001$. Scale bars: 20 μm in A,C; 10 μm in B.

basement membrane restriction to the basal periphery of epithelial buds and instead triggers inappropriate basement membrane deposition throughout the epithelial compartment. ROCK-mediated control of PAR-1b activity is the molecular mechanism responsible for basal basement membrane positioning. Our results demonstrate that ROCK maintains the localization of PAR-1b in the outer epithelial cells that normally produce basement membrane and prevents PAR-1b function in the inner cells that do not (Fig. 8). We not only demonstrate that PAR-1b is required for basement membrane deposition and that it mediates this function through its kinase activity, but also that overexpression of PAR-1b is sufficient to drive ectopic basement membrane production. Our data demonstrate that PAR-1b is a master regulator of basement membrane deposition in developing salivary glands, and that ROCK control of PAR-1b function is essential for normal epithelial integrity and organ development.

Using both organ and 3D cell culture methods we demonstrate that the basement membrane acts as a polarity cue, guiding the coordinate alignment of cells within the developing gland, and that uncoordinated basement membrane production upon loss of ROCK activity drives the uncoordinated alignment of cells within the tissue, leading to destruction of tissue architecture (Fig. 8). Since coordinated cell alignment can be rescued by exogenous basement membrane, we propose that the loss of organization in the outer cell layer when ROCK and PAR-1b functions are perturbed is due to the loss of basement membrane localization at the basal periphery of the tissue. Based on these results, as well as our finding that exogenous basement membrane directs apical polarity in a salivary gland epithelial cell line, we propose that the inappropriate positioning of basement membrane within epithelial buds drives the uncoordinated polarization of the IPCs under conditions of ROCK inhibition. Indeed, these findings are consistent with studies in MDCK cells which reported that basement membrane assembly directs apical pole orientation to the opposite side of the cell (Chambard et al., 1981; O'Brien et al., 2001; Yu et al., 2005). To the best of our knowledge, however, ours is the first study to demonstrate this principle in the context of an intact mammalian epithelial tissue, where we demonstrate that the molecular mechanism involves ROCK-mediated regulation of PAR-1b activity to coordinate the orientation of apicobasal polarity via basal basement membrane restriction. Our findings suggest that the establishment of polarity in individual cells is not in itself sufficient to give rise to an organized multicellular tissue and highlight the apparent independent nature of the establishment of polarity within

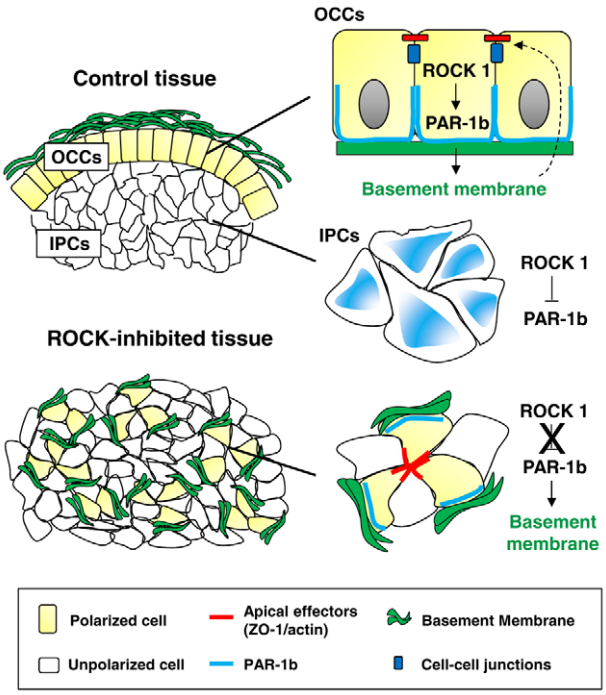

Fig. 8. Model for ROCK1 coordination of tissue polarity via basal basement membrane positioning. At the tissue level, ROCK1 restricts basement membrane positioning to maintain a polarized epithelium with coordinately aligned cell apical surfaces directed towards the center of the tissue. With ROCK inhibition, basement membrane is inappropriately positioned within the epithelial interior, leading to uncoordinated cell polarization and loss of overall tissue polarity. At the cellular level, ROCK1 promotes and inhibits PAR-1b activity in salivary gland OCCs and IPCs, respectively, with PAR-1b being necessary and sufficient for basement membrane deposition. A basement membrane-mediated signal promotes the maintenance of cell-cell adhesions in salivary epithelial cells.

individual cells and the orientation of the apicobasal axis in a coordinated manner to give rise to a higher-order tissue structure via basal basement membrane positioning.

Our findings indicate that ROCK-mediated restriction of PAR-1b activity to the basolateral domains of salivary gland OCCs is the critical downstream mediator of basement membrane positioning and subsequent coordination of tissue polarity. Other studies provide insight into the possible mechanisms by which PAR-1b regulates basement membrane deposition. PAR-1b regulates microtubule dynamics (Drewes et al., 1997) via its phosphorylation of microtubule-associated proteins (MAPs) and the acquisition of a polarized microtubule array (Cohen et al., 2004; Cox et al., 2001; Doerflinger et al., 2003). In chick epithelial cells, a polarized microtubule array is required to maintain basement membrane stability, possibly through the stabilization of basally localized basement membrane receptors (Nakaya et al., 2011; Nakaya et al., 2008). Alternatively, a polarized microtubule array may be required for the polarized secretion of basement membrane components (Gilbert et al., 1991; Grindstaff et al., 1998), which could also explain the generalized defect in basement membrane deposition observed upon PAR-1b siRNA knockdown in the salivary gland organ cultures. Our data indicate that PAR-1b also regulates basement membrane protein expression. Since multiple basement membrane proteins in the developing salivary glands can stimulate their own expression upon cell surface binding (Rebustini et al., 2009; Rebustini et al., 2007), PAR-1b regulation of basement membrane protein expression might be indirect. Taken together with our observation that the expression of PAR-1b itself increases when ROCK is inhibited, it is intriguing to speculate that a basement membrane-mediated feed-forward signal might function to promote PAR-1b activity and the continued maintenance of basement membrane protein expression and positioning.

An important outstanding question is how ROCK regulates PAR-1b localization in developing salivary glands? Although we cannot rule out the possibility that ROCK directly regulates PAR-1b localization, we could not detect a direct interaction between ROCK1 and PAR-1b by co-immunoprecipitation from cultured salivary epithelial cells or organ cultures (W.P.D., D. Trufanoff and M.L., unpublished). Alternatively, ROCK might indirectly regulate PAR-1b through interactions with other polarity proteins. In mammalian cells, ROCK phosphorylation of PAR-3 (PARD3) prevents its association with PAR-6 (PARD6) and aPKC (Nakayama et al., 2008), and in *Drosophila* ROCK phosphorylation of PAR-3 (Bazooka – FlyBase) prevents its cortical association (Simoes Sde et al., 2010). aPKC can reciprocally regulate ROCK localization (Ishiuchi and Takeichi, 2011). Thus, given the established mechanism of mutual exclusion between the apical PAR complex and PAR-1b (Goldstein and Macara, 2007; Suzuki and Ohno, 2006), ROCK regulation of PAR-1b activity might be mediated through the apical PAR complex. Polarity protein asymmetries also depend on cytoskeletal rearrangements (Harris and Peifer, 2005; Munro et al., 2004). Although myosin II is not required for the restriction of PAR-1b to salivary gland OCCs, ROCK can regulate actin polymerization and microtubule stability via LIM kinase (Narumiya et al., 2009), which may impact localization of PAR polarity proteins (Chen and Macara, 2006).

Another interesting question that arises from this study concerns the mechanism of apical polarity establishment and lumen formation in developing salivary glands. Our results demonstrate that PAR-1b can regulate the orientation of an apical membrane, consistent with results in MDCK cells (Cohen et al., 2004; Cohen et al., 2011; Cohen and Müsch, 2003), and that it does so indirectly through regulation of basement membrane deposition. Although the mechanism by which an apical membrane forms is not completely understood in the salivary gland, it occurs first in the developing ducts (Patel et al., 2011; Walker et al., 2008) and might be independent of basement membrane as apical proteins appear first in cells that do not contact basement membrane, consistent with data showing that the establishment of an axis of polarity can be a cell-autonomous process (Baas et al., 2004). Nevertheless, our results indicate that basement membrane can promote the establishment of an apical membrane in salivary gland epithelial cells.

Our data also indicate that a basement membrane-mediated outside-in signal downstream of ROCK and PAR-1b promotes the maintenance and/or reinforcement of epithelial cell-cell adhesions. Although our data indicate that integrin β1 is required for this basement membrane-mediated effect, other basement membrane receptors might also be involved. We also cannot exclude a role for the apical PAR complex, but our results are consistent with studies indicating that basolateral polarity is a prerequisite for apical polarity, and that basement membrane-mediated signals are required for the maturation of cell-cell adhesions (Li et al., 2003; Miner and Yurchenco, 2004; Plachot et al., 2009; Weir et al., 2006). Furthermore, our data help to explain why certain cell junction

molecules are more cortically localized in epithelial cells that directly contact the basement membrane than in interior cells that do not (Menko et al., 2002; Walker et al., 2008).

ROCK controls tissue architecture through myosin-independent effects on tissue polarity, as we report here, and through myosin-dependent effects on cell contractility and basement membrane remodeling, which we reported previously (Daley et al., 2009), both of which are required for proper branching morphogenesis. Thus, characterizing the molecular mechanisms that govern the subcellular localization and activation of ROCK will provide crucial insights into the molecular mechanisms controlling tissue integrity. The impact of these signaling pathways and the basement membrane on cell differentiation is also of interest. Additionally, as inhibition of ROCK activity is implicated in the regulation of embryonic stem cell survival and differentiation, and as heightened ROCK expression and activity correlate with cancer (Ohgushi et al., 2010; Olson, 2008), it will be important for the development of improved therapeutics and regenerative medicine strategies to determine how the spatiotemporal regulation and/or activation of ROCK functions to maintain tissue organization through downstream effectors.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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References

Baas, A. F., Kuipers, J., Van der Wel, N. N., Batlle, E., Koerten, H. K., Peters, P. J. and Clevers, H. C. (2004). Complete polarization of single intestinal epithelial cells upon activation of LKB1 by STRAD. *Cell* **116**, 457-466.
Benton, R. and St Johnston, D. (2003). Drosophila PAR-1 and 14-3-3 inhibit Bazooka/PAR-3 to establish complementary cortical domains in polarized cells. *Cell* **115**, 691-704.
Bernfield, M. and Banerjee, S. D. (1982). The turnover of basal lamina glycosaminoglycan correlates with epithelial morphogenesis. *Dev. Biol.* **90**, 291-305.
Bernfield, M., Banerjee, S. D., Koda, J. E. and Rapraeger, A. C. (1984). Remodelling of the basement membrane: morphogenesis and maturation. *Ciba Found. Symp.* **108**, 179-196.
Bissell, M. J., Rizki, A. and Mian, I. S. (2003). Tissue architecture: the ultimate regulator of breast epithelial function. *Curr. Opin. Cell Biol.* **15**, 753-762.
Bohm, H., Brinkmann, V., Drab, M., Henske, A. and Kurzchalia, T. V. (1997). Mammalian homologues of C. elegans PAR-1 are asymmetrically localized in epithelial cells and may influence their polarity. *Curr. Biol.* **7**, 603-606.
Bryant, D. M. and Mostov, K. E. (2008). From cells to organs: building polarized tissue. *Nat. Rev. Mol. Cell Biol.* **9**, 887-901.
Chambard, M., Gabrion, J. and Mauchamp, J. (1981). Influence of collagen gel on the orientation of epithelial cell polarity: follicle formation from isolated thyroid cells and from preformed monolayers. *J. Cell Biol.* **91**, 157-166.
Chapin, H. C. and Caplan, M. J. (2010). The cell biology of polycystic kidney disease. *J. Cell Biol.* **191**, 701-710.
Chen, X. and Macara, I. G. (2006). Par-3 mediates the inhibition of LIM kinase 2 to regulate cofilin phosphorylation and tight junction assembly. *J. Cell Biol.* **172**, 671-678.
Cohen, D. and Müsch, A. (2003). Apical surface formation in MDCK cells: regulation by the serine/threonine kinase EMK1. *Methods* **30**, 269-276.
Cohen, D., Brennwald, P. J., Rodriguez-Boulan, E. and Müsch, A. (2004). Mammalian PAR-1 determines epithelial lumen polarity by organizing the microtubule cytoskeleton. *J. Cell Biol.* **164**, 717-727.
Cohen, D., Fernandez, D., Lazaro-Dieguez, F. and Müsch, A. (2011). The serine/threonine kinase Par1b regulates epithelial lumen polarity via IRSp53-mediated cell-ECM signaling. *J. Cell Biol.* **192**, 525-540.
Collins, J. and Bonnemann, C. G. (2010). Congenital muscular dystrophies: toward molecular therapeutic interventions. *Curr. Neurol. Neurosci. Rep.* **10**, 83-91.
Cox, D. N., Lu, B., Sun, T. Q., Williams, L. T. and Jan, Y. N. (2001). Drosophila par-1 is required for oocyte differentiation and microtubule organization. *Curr. Biol.* **11**, 75-87.
Cukierman, E., Pankov, R., Stevens, D. R. and Yamada, K. M. (2001). Taking cell-matrix adhesions to the third dimension. *Science* **294**, 1708-1712.
Daley, W. P., Gulfo, K. M., Sequeira, S. J. and Larsen, M. (2009). Identification of a mechanochemical checkpoint and negative feedback loop regulating branching morphogenesis. *Dev. Biol.* **336**, 169-182.
Doerflinger, H., Benton, R., Shulman, J. M. and St Johnston, D. (2003). The role of PAR-1 in regulating the polarised microtubule cytoskeleton in the Drosophila follicular epithelium. *Development* **130**, 3965-3975.
Drewes, G., Ebneth, A., Preuss, U., Mandelkow, E. M. and Mandelkow, E. (1997). MARK, a novel family of protein kinases that phosphorylate microtubule-associated proteins and trigger microtubule disruption. *Cell* **89**, 297-308.
Etemad-Moghadam, B., Guo, S. and Kemphues, K. J. (1995). Asymmetrically distributed PAR-3 protein contributes to cell polarity and spindle alignment in early C. elegans embryos. *Cell* **83**, 743-752.
Foty, R. (2011). A simple hanging drop cell culture protocol for generation of 3D spheroids. *J. Vis. Exp.* **51**, 2720.
Foty, R. A. and Steinberg, M. S. (2005). The differential adhesion hypothesis: a direct evaluation. *Dev. Biol.* **278**, 255-263.
Gilbert, T., Le Bivic, A., Quaroni, A. and Rodriguez-Boulan, E. (1991). Microtubular organization and its involvement in the biogenetic pathways of plasma membrane proteins in Caco-2 intestinal epithelial cells. *J. Cell Biol.* **113**, 275-288.
Goldstein, B. and Macara, I. G. (2007). The PAR proteins: fundamental players in animal cell polarization. *Dev. Cell* **13**, 609-622.
Grindstaff, K. K., Bacallao, R. L. and Nelson, W. J. (1998). Apiconuclear organization of microtubules does not specify protein delivery from the trans-Golgi network to different membrane domains in polarized epithelial cells. *Mol. Biol. Cell* **9**, 685-699.
Grobstein, C. (1953). Inductive epitheliomesenchymal interaction in cultured organ rudiments of the mouse. *Science* **118**, 52-55.
Guo, S. and Kemphues, K. J. (1995). par-1, a gene required for establishing polarity in C. elegans embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. *Cell* **81**, 611-620.
Harris, T. J. and Peifer, M. (2005). The positioning and segregation of apical cues during epithelial polarity establishment in Drosophila. *J. Cell Biol.* **170**, 813-823.
Hirose, T., Izumi, Y., Nagashima, Y., Tamai-Nagai, Y., Kurihara, H., Sakai, T., Suzuki, Y., Yamanaka, T., Suzuki, A., Mizuno, K. et al. (2002). Involvement of ASIP/PAR-3 in the promotion of epithelial tight junction formation. *J. Cell Sci.* **115**, 2485-2495.
Hung, T. J. and Kemphues, K. J. (1999). PAR-6 is a conserved PDZ domain-containing protein that colocalizes with PAR-3 in Caenorhabditis elegans embryos. *Development* **126**, 127-135.
Ishiuchi, T. and Takeichi, M. (2011). Willin and Par3 cooperatively regulate epithelial apical constriction through aPKC-mediated ROCK phosphorylation. *Nat. Cell Biol.* **13**, 860-866.
Kadoya, Y. and Yamashina, S. (2010). Cellular dynamics of epithelial clefting during branching morphogenesis of the mouse submandibular gland. *Dev. Dyn.* **239**, 1739-1747.
Kadoya, Y., Mochizuki, M., Nomizu, M., Sorokin, L. and Yamashina, S. (2003). Role for laminin-alpha5 chain LG4 module in epithelial branching morphogenesis. *Dev. Biol.* **263**, 153-164.
Kalluri, R. (2003). Basement membranes: structure, assembly and role in tumour angiogenesis. *Nat. Rev. Cancer* **3**, 422-433.
Kass, L., Erler, J. T., Dembo, M. and Weaver, V. M. (2007). Mammary epithelial cell: influence of extracellular matrix composition and organization during development and tumorigenesis. *Int. J. Biochem. Cell Biol.* **39**, 1987-1994.
Laoide, B. M., Courty, Y., Gastinne, I., Thibaut, C., Kellermann, O. and Rougeon, F. (1996). Immortalised mouse submandibular epithelial cell lines retain polarised structural and functional properties. *J. Cell Sci.* **109**, 2789-2800.
Laoide, B. M., Gastinne, I. and Rougeon, F. (1999). Tubular morphogenesis and mesenchymal interactions affect renin expression and secretion in SIMS mouse submandibular cells. *Exp. Cell Res.* **248**, 172-185.
Larsen, M., Hoffman, M. P., Sakai, T., Neibaur, J. C., Mitchell, J. M. and Yamada, K. M. (2003). Role of PI 3-kinase and PIP3 in submandibular gland branching morphogenesis. *Dev. Biol.* **255**, 178-191.
Larsen, M., Artym, V. V., Green, J. A. and Yamada, K. M. (2006a). The matrix reorganized: extracellular matrix remodeling and integrin signaling. *Curr. Opin. Cell Biol.* **18**, 463-471.
Larsen, M., Wei, C. and Yamada, K. M. (2006b). Cell and fibronectin dynamics during branching morphogenesis. *J. Cell Sci.* **119**, 3376-3384.

Li, S., Edgar, D., Fassler, R., Wadsworth, W. and Yurchenco, P. D. (2003). The role of laminin in embryonic cell polarization and tissue organization. *Dev. Cell* **4**, 613-624.
Masuda-Hirata, M., Suzuki, A., Amano, Y., Yamashita, K., Ide, M., Yamanaka, T., Sakai, M., Imamura, M. and Ohno, S. (2009). Intracellular polarity protein PAR-1 regulates extracellular laminin assembly by regulating the dystroglycan complex. *Genes Cells* **14**, 835-850.
Menko, A. S., Zhang, L., Schiano, F., Kreidberg, J. A. and Kukuruzinska, M. A. (2002). Regulation of cadherin junctions during mouse submandibular gland development. *Dev. Dyn.* **224**, 321-333.
Meyer, T. N., Schwesinger, C., Sampogna, R. V., Vaughn, D. A., Stuart, R. O., Steer, D. L., Bush, K. T. and Nigam, S. K. (2006). Rho kinase acts at separate steps in ureteric bud and metanephric mesenchyme morphogenesis during kidney development. *Differentiation* **74**, 638-647.
Michael, L., Sweeney, D. E. and Davies, J. A. (2005). A role for microfilament-based contraction in branching morphogenesis of the ureteric bud. *Kidney Int.* **68**, 2010-2018.
Miner, J. H. and Yurchenco, P. D. (2004). Laminin functions in tissue morphogenesis. *Annu. Rev. Cell Dev. Biol.* **20**, 255-284.
Moore, K. A., Polte, T., Huang, S., Shi, B., Alsberg, E., Sunday, M. E. and Ingber, D. E. (2005). Control of basement membrane remodeling and epithelial branching morphogenesis in embryonic lung by Rho and cytoskeletal tension. *Dev. Dyn.* **232**, 268-281.
Munro, E., Nance, J. and Priess, J. R. (2004). Cortical flows powered by asymmetrical contraction transport PAR proteins to establish and maintain anterior-posterior polarity in the early C. elegans embryo. *Dev. Cell* **7**, 413-424.
Nakanishi, Y. and Ishii, T. (1989). Epithelial shape change in mouse embryonic submandibular gland: modulation by extracellular matrix components. *BioEssays* **11**, 163-167.
Nakaya, Y., Sukowati, E. W., Wu, Y. and Sheng, G. (2008). RhoA and microtubule dynamics control cell-basement membrane interaction in EMT during gastrulation. *Nat. Cell Biol.* **10**, 765-775.
Nakaya, Y., Sukowati, E. W., Alev, C., Nakazawa, F. and Sheng, G. (2011). Involvement of dystroglycan in epithelial-mesenchymal transition during chick gastrulation. *Cells Tissues Organs* **193**, 64-73.
Nakayama, M., Goto, T. M., Sugimoto, M., Nishimura, T., Shinagawa, T., Ohno, S., Amano, M. and Kaibuchi, K. (2008). Rho-kinase phosphorylates PAR-3 and disrupts PAR complex formation. *Dev. Cell* **14**, 205-215.
Narumiya, S., Tanji, M. and Ishizaki, T. (2009). Rho signaling, ROCK and mDia1, in transformation, metastasis and invasion. *Cancer Metastasis Rev.* **28**, 65-76.
Nelson, W. J. (2003). Adaptation of core mechanisms to generate cell polarity. *Nature* **422**, 766-774.
O'Brien, L. E., Jou, T. S., Pollack, A. L., Zhang, Q., Hansen, S. H., Yurchenco, P. and Mostov, K. E. (2001). Rac1 orientates epithelial apical polarity through effects on basolateral laminin assembly. *Nat. Cell Biol.* **3**, 831-838.
Ohgushi, M., Matsumura, M., Eiraku, M., Murakami, K., Aramaki, T., Nishiyama, A., Muguruma, K., Nakano, T., Suga, H., Ueno, M. et al. (2010). Molecular pathway and cell state responsible for dissociation-induced apoptosis in human pluripotent stem cells. *Cell Stem Cell* **7**, 225-239.
Olson, M. F. (2008). Applications for ROCK kinase inhibition. *Curr. Opin. Cell Biol.* **20**, 242-248.
Patel, N., Sharpe, P. T. and Miletich, I. (2011). Coordination of epithelial branching and salivary gland lumen formation by Wnt and FGF signals. *Dev. Biol.* **358**, 156-167.
Patel, V. N., Rebustini, I. T. and Hoffman, M. P. (2006). Salivary gland branching morphogenesis. *Differentiation* **74**, 349-364.
Plachot, C., Chaboub, L. S., Adissu, H. A., Wang, L., Urazaev, A., Sturgis, J., Asem, E. K. and Lelievre, S. A. (2009). Factors necessary to produce basoapical polarity in human glandular epithelium formed in conventional and high-throughput three-dimensional culture: example of the breast epithelium. *BMC Biol.* **7**, 77.
Rebustini, I. T. and Hoffman, M. P. (2009). ECM and FGF-dependent assay of embryonic SMG epithelial morphogenesis: investigating growth factor/matrix regulation of gene expression during submandibular gland development. *Methods Mol. Biol.* **522**, 319-330.
Rebustini, I. T., Patel, V. N., Stewart, J. S., Layvey, A., Georges-Labouesse, E., Miner, J. H. and Hoffman, M. P. (2007). Laminin alpha5 is necessary for submandibular gland epithelial morphogenesis and influences FGFR expression through beta1 integrin signaling. *Dev. Biol.* **308**, 15-29.
Rebustini, I. T., Myers, C., Lassiter, K. S., Surmak, A., Szabova, L., Holmbeck, K., Pedchenko, V., Hudson, B. G. and Hoffman, M. P. (2009). MT2-MMP-dependent release of collagen IV NC1 domains regulates submandibular gland branching morphogenesis. *Dev. Cell* **17**, 482-493.
Sakai, T. and Onodera, T. (2008). Embryonic organ culture. *Curr. Protoc. Cell Biol.* **41**, 19.8.1-19.8.8.
Sakai, T., Larsen, M. and Yamada, K. M. (2003). Fibronectin requirement in branching morphogenesis. *Nature* **423**, 876-881.
Sequeira, S. J., Larsen, M. and DeVine, T. (2010). Extracellular matrix and growth factors in salivary gland development. *Front. Oral Biol.* **14**, 48-77.
Simoes Sde, M., Blankenship, J. T., Weitz, O., Farrell, D. L., Tamada, M., Fernandez-Gonzalez, R. and Zallen, J. A. (2010). Rho-kinase directs Bazooka/Par-3 planar polarity during Drosophila axis elongation. *Dev. Cell* **19**, 377-388.
Suzuki, A. and Ohno, S. (2006). The PAR-aPKC system: lessons in polarity. *J. Cell Sci.* **119**, 979-987.
Suzuki, A., Yamanaka, T., Hirose, T., Manabe, N., Mizuno, K., Shimizu, M., Akimoto, K., Izumi, Y., Ohnishi, T. and Ohno, S. (2001). Atypical protein kinase C is involved in the evolutionarily conserved par protein complex and plays a critical role in establishing epithelia-specific junctional structures. *J. Cell Biol.* **152**, 1183-1196.
Suzuki, A., Hirata, M., Kamimura, K., Maniwa, R., Yamanaka, T., Mizuno, K., Kishikawa, M., Hirose, H., Amano, Y., Izumi, N. et al. (2004). aPKC acts upstream of PAR-1b in both the establishment and maintenance of mammalian epithelial polarity. *Curr. Biol.* **14**, 1425-1435.
Tabuse, Y., Izumi, Y., Piano, F., Kemphues, K. J., Miwa, J. and Ohno, S. (1998). Atypical protein kinase C cooperates with PAR-3 to establish embryonic polarity in Caenorhabditis elegans. *Development* **125**, 3607-3614.
Totsukawa, G., Yamakita, Y., Yamashiro, S., Hartshorne, D. J., Sasaki, Y. and Matsumura, F. (2000). Distinct roles of ROCK (Rho-kinase) and MLCK in spatial regulation of MLC phosphorylation for assembly of stress fibers and focal adhesions in 3T3 fibroblasts. *J. Cell Biol.* **150**, 797-806.
Vega-Salas, D. E., Salas, P. J., Gundersen, D. and Rodriguez-Boulan, E. (1987a). Formation of the apical pole of epithelial (Madin-Darby canine kidney) cells: polarity of an apical protein is independent of tight junctions while segregation of a basolateral marker requires cell-cell interactions. *J. Cell Biol.* **104**, 905-916.
Vega-Salas, D. E., Salas, P. J. and Rodriguez-Boulan, E. (1987b). Modulation of the expression of an apical plasma membrane protein of Madin-Darby canine kidney epithelial cells: cell-cell interactions control the appearance of a novel intracellular storage compartment. *J. Cell Biol.* **104**, 1249-1259.
Walker, J. L., Menko, A. S., Khalil, S., Rebustini, I., Hoffman, M. P., Kreidberg, J. A. and Kukuruzinska, M. A. (2008). Diverse roles of E-cadherin in the morphogenesis of the submandibular gland: insights into the formation of acinar and ductal structures. *Dev. Dyn.* **237**, 3128-3141.
Wei, C., Larsen, M., Hoffman, M. P. and Yamada, K. M. (2007). Self-organization and branching morphogenesis of primary salivary epithelial cells. *Tissue Eng.* **13**, 721-735.
Weir, M. L., Oppizzi, M. L., Henry, M. D., Onishi, A., Campbell, K. P., Bissell, M. J. and Muschler, J. L. (2006). Dystroglycan loss disrupts polarity and beta-casein induction in mammary epithelial cells by perturbing laminin anchoring. *J. Cell Sci.* **119**, 4047-4058.
Wheelock, M. J. and Johnson, K. R. (2003a). Cadherin-mediated cellular signaling. *Curr. Opin. Cell Biol.* **15**, 509-514.
Wheelock, M. J. and Johnson, K. R. (2003b). Cadherins as modulators of cellular phenotype. *Annu. Rev. Cell Dev. Biol.* **19**, 207-235.
Xu, R., Boudreau, A. and Bissell, M. J. (2009). Tissue architecture and function: dynamic reciprocity via extra- and intra-cellular matrices. *Cancer Metastasis Rev.* **28**, 167-176.
Yamashita, K., Suzuki, A., Satoh, Y., Ide, M., Amano, Y., Masuda-Hirata, M., Hayashi, Y. K., Hamada, K., Ogata, K. and Ohno, S. (2010). The 8th and 9th tandem spectrin-like repeats of utrophin cooperatively form a functional unit to interact with polarity-regulating kinase PAR-1b. *Biochem. Biophys. Res. Commun.* **391**, 812-817.
Yu, W., Datta, A., Leroy, P., O'Brien, L. E., Mak, G., Jou, T. S., Matlin, K. S., Mostov, K. E. and Zegers, M. M. (2005). Beta1-integrin orients epithelial polarity via Rac1 and laminin. *Mol. Biol. Cell* **16**, 433-445.
Yurchenco, P. D. and Wadsworth, W. G. (2004). Assembly and tissue functions of early embryonic laminins and netrins. *Curr. Opin. Cell Biol.* **16**, 572-579.
Yurchenco, P. D., Amenta, P. S. and Patton, B. L. (2004). Basement membrane assembly, stability and activities observed through a developmental lens. *Matrix Biol.* **22**, 521-538.