

# MgcRacGAP interacts with cingulin and paracingulin to regulate Rac1 activation and development of the tight junction barrier during epithelial junction assembly

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**ABSTRACT** The regulation of Rho-family GTPases is crucial to direct the formation of cell–cell junctions and tissue barriers. Cingulin (CGN) and paracingulin (CGNL1) control RhoA activation in epithelial cells by interacting with RhoA guanidine exchange factors. CGNL1 depletion also inhibits Rac1 activation during junction assembly. Here we show that, unexpectedly, Madin–Darby canine kidney epithelial cells depleted of both CGN and CGNL1 (double-KD cells) display normal Rac1 activation and tight junction (TJ) formation, despite decreased junctional recruitment of the Rac1 activator Tiam1. The expression of the Rac1 inhibitor MgcRacGAP is decreased in double-KD cells, and the barrier development and Rac1 activation phenotypes are rescued by exogenous expression of MgcRacGAP. MgcRacGAP colocalizes with CGN and CGNL1 at TJs and forms a complex and interacts directly *in vitro* with CGN and CGNL1. Depletion of either CGN or CGNL1 in epithelial cells results in decreased junctional localization of MgcRacGAP but not of ECT2, a centralspindlin-interacting Rho GEF. These results provide new insight into coordination of Rho-family GTPase activities at junctions, since apical accumulation of CGN and CGNL1 at TJs during junction maturation provides a mechanism to spatially restrict down-regulation of Rac1 activation through the recruitment of MgcRacGAP.

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## INTRODUCTION

The precise spatiotemporal control of the activity of Rho-family GTPases is essential in many cellular processes, including the estab-

lishment and maintenance of cell–cell junctions and the formation of epithelial barriers (Nusrat *et al.*, 1995; Braga *et al.*, 1997; Takaishi *et al.*, 1997; Jou *et al.*, 1998; Yamada and Nelson, 2007). Rho-family GTPases exist in active (GTP-bound) and inactive (GDP-bound) states, and the transition between these states depends on a finely tuned antagonism between activating guanine-nucleotide exchange factors (GEFs) and inhibitory GTPase-activating proteins (GAPs; Schmidt and Hall, 2002; Rossman *et al.*, 2005; Tcherkezian and Lamarche-Vane, 2007).

Tight junctions (TJs) form, together with the zonula adhaerens (ZA), a belt-like apical junctional complex (AJC) in epithelial cells, and are uniquely responsible for the barrier function of epithelia through the formation of claudin-based paracellular channels and pores (Furuse and Tsukita, 2006; Anderson and Van Itallie, 2009). The transmembrane proteins of TJ and ZA are clustered at the sites of cell–cell contact by distinct complexes of cytoplasmic adaptor proteins, some of which provide anchoring to the actin and microtubule cytoskeletons (Shin *et al.*, 2006; Meng and Takeichi, 2009). RhoA and Rac1 are the major GTPases of the Rho family, which are

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Abbreviations used: AJC, apical junctional complex; Asef, APC-stimulated guanine nucleotide exchange factor; CGN, cingulin; CGNL1, paracingulin; ECT2, epithelial cell transforming sequence 2 oncogene; GAP, GTPase-activating protein; GEF, guanidine exchange factor; HA, hemagglutinin; KD, knockdown; KO, knockout; MDCK, Madin–Darby canine kidney; MgcRacGAP, male germ cell Rac GTPase-activating protein; MKLP1, mitotic kinesin-like protein; RICH1, RhoGAP interacting with CIP4 homologues protein 1; TER, transepithelial electrical resistance; Tiam1, T-cell lymphoma invasion and metastasis 1; TJ, tight junction; WT, wild type; ZA, zonula adhaerens.

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implicated in the regulation of TJs and the ZA through the dynamic reorganization and contractility of the actin cytoskeleton (Hall, 2012). However, the molecular mechanisms of their regulation are not completely understood. For example, although GEFs for RhoA and Rac1 have been found to interact with junctional proteins (reviewed in Citi *et al.*, 2011; McCormack *et al.*, 2013), little is known about the function of specific RhoA and Rac1 GAPs in modulating the establishment of the TJ barrier. Furthermore, it is not known whether any Rac-GAP protein interacts with specific components of the cytoplasmic plaque of the TJ. In contrast, GAPs for Cdc42 are required for TJ integrity and interact with junctional proteins (Wells *et al.*, 2006; Elbediwy *et al.*, 2012).

Two key players in the fine-tuning of RhoA activity in epithelial cells are cingulin (CGN), which is specifically localized in the cytoplasmic plaque region of TJs (Citi *et al.*, 1988), and paracingulin (CGNL1; also known as JACOP), which is localized to both the TJ and ZA (Ohnishi *et al.*, 2004; Guillemot and Citi, 2006b). CGN and CGNL1 are structurally related and recruit the Rho GEF GEF-H1 to junctions of Madin–Darby canine kidney (MDCK) cells and p114-RhoGEF to junctions of corneal cells (Citi *et al.*, 2000, 2009, 2012; Aijaz *et al.*, 2005; Guillemot and Citi, 2006a; Guillemot *et al.*, 2008; Terry *et al.*, 2011). CGNL1, unlike CGN, is also required for efficient junctional recruitment of the Rac1 GEF Tiam1 in MDCK cells, and depletion of CGNL1, but not of CGN, results in loss of the waves of increased Rac1 activation observed during junction assembly (Guillemot *et al.*, 2008). Here, by studying the junction assembly phenotype of MDCK cells depleted of both CGN and CGNL1 (double-KD cells), we find that MgcRacGAP (also known as RacGAP1, RacGAP50C, or Cyk-4; Toure *et al.*, 1998; Jantsch-Plunger *et al.*, 2000; Somers and Saint, 2003) plays a role in Rac1-dependent regulation of the dynamic establishment of the TJ barrier and interacts with both CGN and CGNL1.

## RESULTS

### In double-KD cells, Rac1 activation, junction assembly, and establishment of the TJ barrier are similar to those in wild-type cells, despite decreased junctional recruitment of Tiam1

Stable lines of MDCK epithelial cells depleted of both CGN and CGNL1 were reported previously (Guillemot *et al.*, 2013) and show increased RhoA activation at confluence, consistent with decreased junctional recruitment of GEF-H1, similar to cells depleted of either CGN alone (CGN-KD) or CGNL1 alone (CGNL1-KD; Guillemot and Citi, 2006a; Guillemot *et al.*, 2008). In addition, they show normal expression and localization of proteins of the zonula adherens and decreased expression of the transcription factor GATA-4 (Guillemot *et al.*, 2013). Here we investigate the dynamics of junction assembly of double-KD cells, starting from our previous observations that in CGNL1-KD cells Rac1 activation is decreased during junction assembly, junction assembly is delayed, and the peak in transepithelial electrical resistance (TER) observed during the calcium switch is abolished (Guillemot *et al.*, 2008).

In confluent monolayers, Rac1 activation in double-KD cells, as measured by a glutathione *S*-transferase (GST) pull-down assay, was the same as in wild-type (WT) cells (Figure 1A) and CGNL1-KD cells (Guillemot *et al.*, 2008). Rac1 activity as determined by GST-pull down on cell lysates is a reliable readout of junctional Rac1 activation during junction assembly because although Rac1 is necessary for maintenance of cell–cell contacts and protein traffic at steady state, during junction formation the zones of maximal Rac1 and lamellipodia activity are at the periphery of contacting membranes (Yamada and Nelson, 2007). So we examined Rac1 activation during

junction formation in the calcium switch, an established experimental protocol to study the dynamic formation of the TJ barrier (Gonzalez-Mariscal *et al.*, 1985). Surprisingly, Rac1 activation in double-KD cells was indistinguishable from that in WT cells; for example, the peaks of Rac1 activity observed at the early (10–30 min) and late (3 h) time points were not abolished (Figure 1B and Supplemental Figure S1A), in contrast to CGNL1-depleted cells (Guillemot *et al.*, 2008) and similar to WT and CGN(–) cells (Supplemental Figure S1A). Instead, RhoA activity of double-KD cells during the calcium switch was high and remained high at 8 h after the beginning of the switch and in confluent monolayers (Figure 1B and Supplemental Figure S1A; Guillemot *et al.*, 2008), as previously shown for single-KD cells (Guillemot and Citi, 2006a; Supplemental Figure S1A).

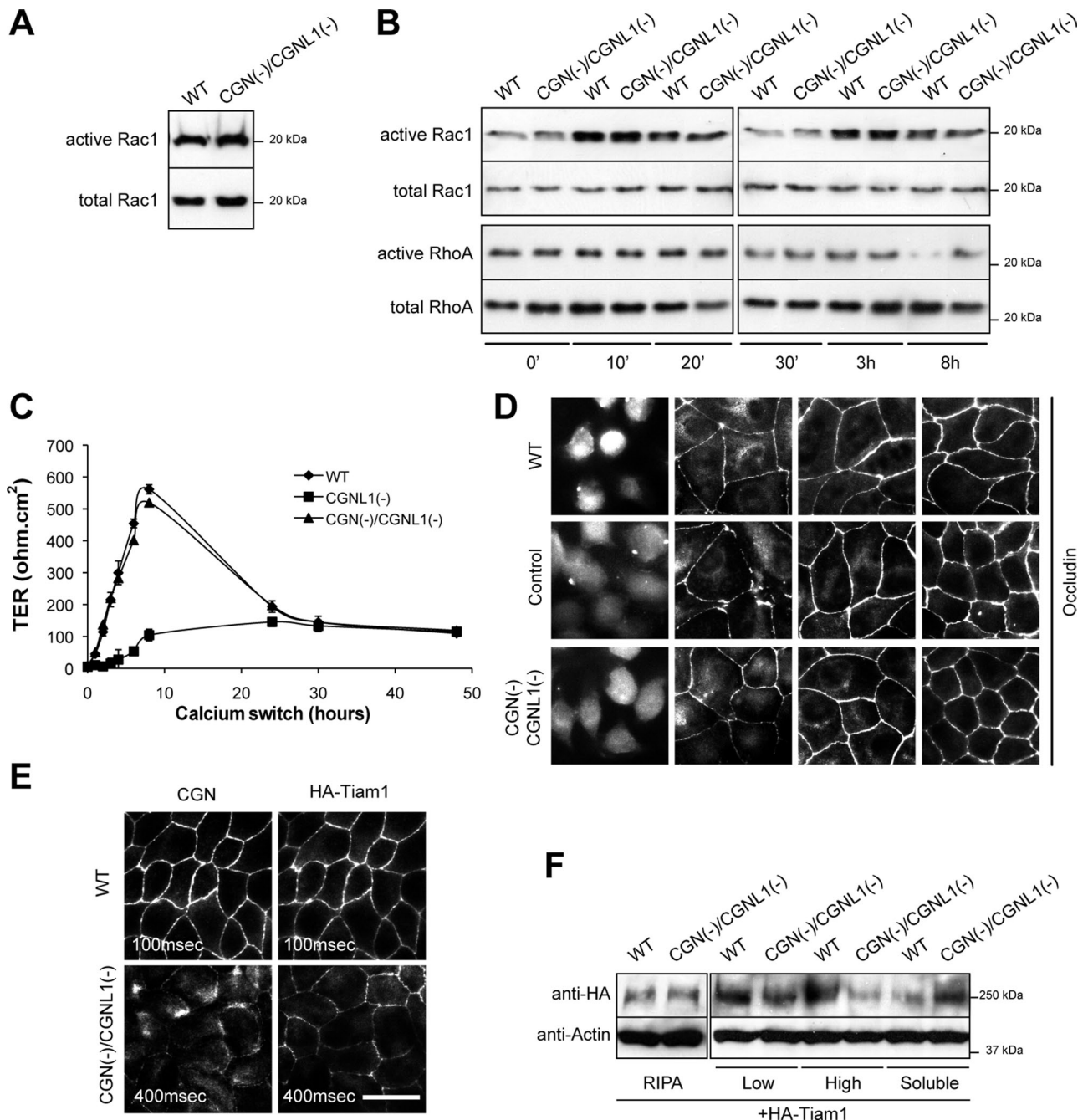
As an alternative functional assay, we assessed TJ barrier function by examining the pattern of development of the ionic permeability barrier (TER) in double-KD cells. Whereas in CGNL1(–) cells the peak in TER is abolished (Guillemot *et al.*, 2008; Figure 1C), in double-KD cells it is similar to that observed in WT cells (Figure 1C). Distinct stable clones of double-KD cells behaved similarly (Supplemental Figure S1B).

As a third functional assay, we examined the kinetics of accumulation of the transmembrane TJ protein occludin at the junctions of double-KD cells. The behavior of double-KD cells was similar to that of WT cells and cells expressing control short hairpin RNA (shRNA; Figure 1D) and not delayed, as observed in CGNL1(–) cells (Guillemot *et al.*, 2008).

Because in CGNL1(–) cells decreased Rac1 activation and delayed junction assembly correlate with decreased junctional localization of Tiam1 (Guillemot *et al.*, 2008), we asked whether the unexpected phenotype of double-KD cells was due to a rescue in the junctional localization of Tiam1, which could account for the increased Rac1 activation, and normal TJ barrier development. Immunofluorescence analysis showed that in double-KD cells there was decreased junctional localization of Tiam1 compared with WT cells, similar to what observed in single-KD CGNL1(–) cells (Figure 1E; Guillemot *et al.*, 2008). In agreement, immunoblot analysis of soluble and insoluble fractions showed a decrease in the amount of insoluble, junction-associated Tiam1 (Figure 1F). Because this assay indicated that normal Rac1 activation and TJ barrier development in double-KD cells was not due to a rescue in Tiam1 localization, we conclude that Rac1 activation during junction assembly not only depends on Tiam1, but is influenced by additional activators and/or inhibitors of Rac1.

### Expression of MgcRacGAP is decreased in double-KD cells, and rescue of normal levels of MgcRacGAP expression results in decreased Rac1 activation and delayed TJ barrier development

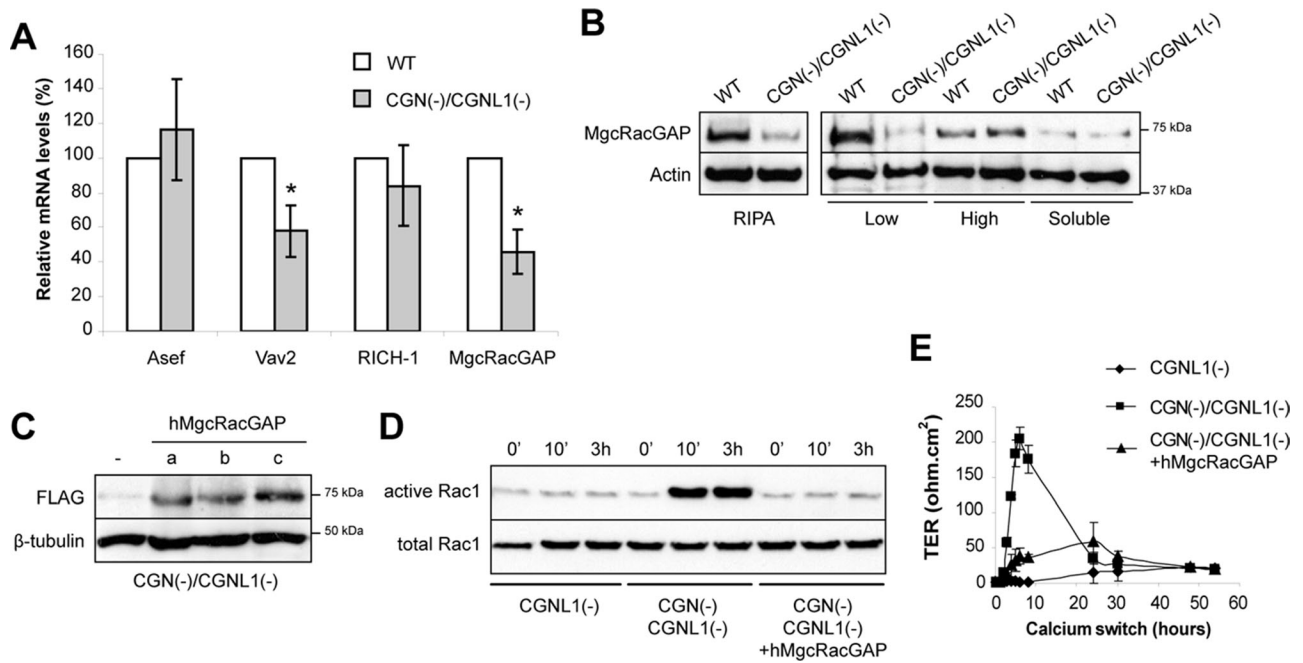
We postulated that rescue of Rac1 activation in double-KD cells could be due to either increased expression of a Rac1 GEF or decreased expression of a Rac1 GAP. So we examined the expression of Rac1 GEFs and GAPs, which are associated with junctional proteins such as Asef (Muroya *et al.*, 2007), RICH1 (Wells *et al.*, 2006), MgcRacGAP (Ratheesh *et al.*, 2012), and Vav2 (Noren *et al.*, 2000). By quantitative real-time (RT) PCR, the expression of Asef and RICH1 was not significantly altered in double-KD cells when compared with WT cells (Figure 2A and Supplemental Figure S1C). In contrast, expression of Vav2 and MgcRacGAP was significantly decreased in double-KD cells compared with WT cells (Figure 2A and Supplemental Figure S1C). Because decreased expression of Vav2 (an activator of Rac1) could not explain the increased Rac1



**FIGURE 1:** CGN(-)/CGNL1(-) (double-KD) cells show normal Rac1 activation and TJ assembly, despite reduced junctional Tiam1. (A, B) Rac1 activation at steady-state (A) and Rac1 and RhoA activation during the calcium switch (B), as determined by GST pull-down analysis. (C) TER (ohm.cm<sup>2</sup>) of WT, single-KD (CGNL1(-)), and double-KD (CGN(-)/CGNL1(-)) cells during the calcium switch. (D) Occludin immunofluorescence, showing a similar pattern of occludin accumulation at junctions in WT, control, and double-KD cells during the calcium switch (for single-KD CGNL1(-), see Guillemot *et al.*, 2008). (E) Immunofluorescence analysis of exogenous hemagglutinin (HA)-tagged Tiam1 in WT and double-KD cells, showing reduced junctional recruitment of Tiam1 in double-KD cells (exposure time in double-KD cells increased fourfold; see key in each panel). Bar, 10  $\mu$ m. (F) Immunoblotting with anti-HA (exogenous Tiam1) and anti-actin antibodies of fractionated lysates of WT and double-KD cells (Guillemot *et al.*, 2008). See Supplemental Figure S1 for additional data on single-KD cells.

activation in double-KD versus CGNL1(-) cells, we focused on MgcRacGAP, a GAP that is ~30-fold more active against Rac1 and Cdc42 than RhoA (Toure *et al.*, 1998). Immunoblotting analysis demonstrated that MgcRacGAP expression was decreased at both the mRNA and protein levels in double-KD cells compared with WT cells (Figure 2, B and C) and to either single-KD, CGN(-), or

CGNL1(-) cells (Supplemental Figure S1D). Furthermore, analysis of fractionated lysates showed that the decrease in MgcRacGAP levels was observed only in the low-speed insoluble fraction (Figure 2B and Supplemental Figure S1D), suggesting decreased association with the cytoskeleton. The reduced mRNA and protein levels for MgcRacGAP in stable double-KD cells may be due to altered



**FIGURE 2:** MgcRacGAP levels are reduced in double-KD MDCK cells, and rescue of MgcRacGAP expression inhibits Rac1 activation and the peak in TER during development of the TJ barrier. (A) Histogram showing the relative mRNA levels for Asef, Vav2, Rich-1, and MgcRacGAP in double-KD cells vs. WT cells, taking WT levels as 100, as determined by quantitative RT-PCR. (B) Immunoblotting analysis of either total lysates (RIPA) or fractionated lysates of WT and double-KD cells. Low, pellet after centrifugation at low speed ( $13,000 \times g$ ). High, pellet after high-speed ( $100,000 \times g$ ) centrifugation of the supernatant obtained after low-speed centrifugation. Soluble, Triton-soluble supernatant after centrifugation at  $100,000 \times g$  of the low-speed supernatant. (C) Immunoblotting of total (RIPA) lysates from three independent double-KD rescue clones (a–c) stably expressing or not (–) an exogenous human (h) FLAG-tagged MgcRacGAP. (D) Rac1 activation in either single-KD (CGNL1(–)) or double-KD cells expressing (clone a) or not the exogenous MgcRacGAP protein during the calcium switch. Clones b and c are shown in Supplemental Figure S1E. (E) TER profile in the calcium switch for the stable clones described in D. Clones b and c are shown in Supplemental Figure S1F.

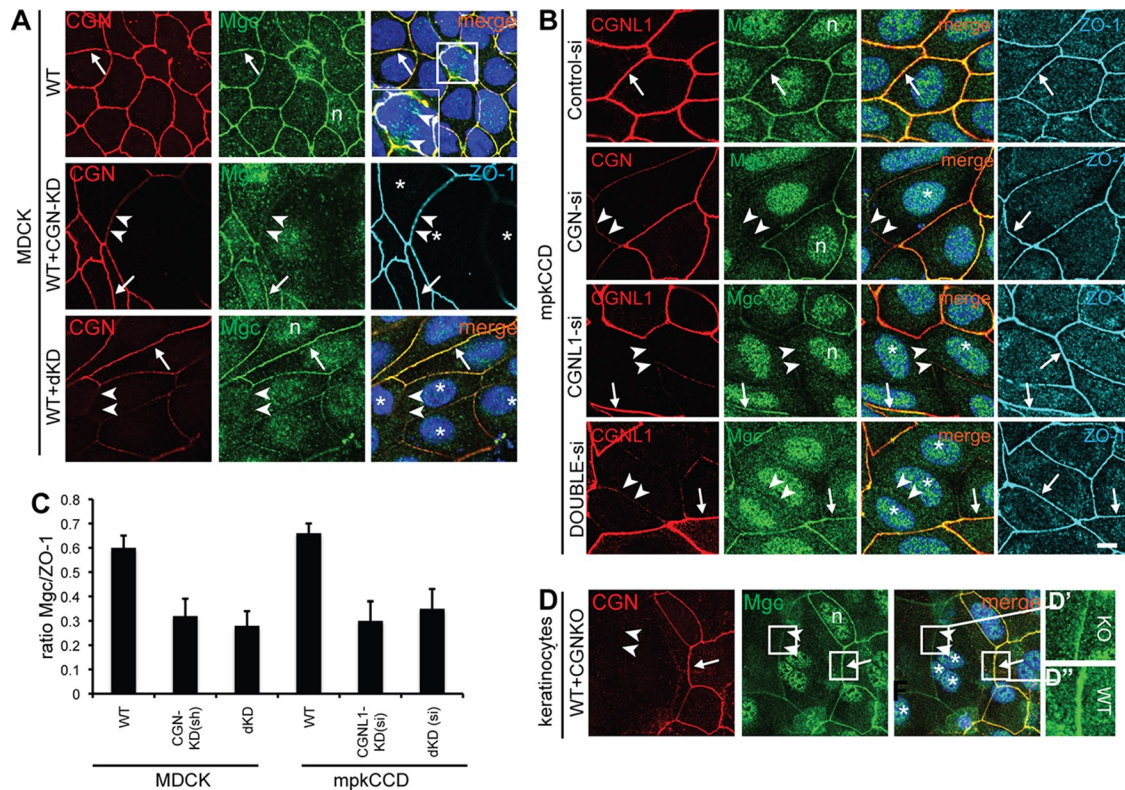
transcriptional regulation (due to reduced GATA-4 levels; Guillemot *et al.*, 2013; our unpublished results) and additional mechanisms that regulate MgcRacGAP mRNA and/or protein stability, which remain to be investigated.

Next we tested the hypothesis that the decreased expression of MgcRacGAP plays a mechanistic role in the increased Rac1 activation and normal development of the epithelial paracellular permeability barrier of double-KD cells. We established stable lines expressing exogenous FLAG-tagged MgcRacGAP in the background of double-KD cells (Figure 2C) and asked whether the exogenous MgcRacGAP expression could revert the phenotype of double-KD cells to that of CGNL1(–), single-KD cells. GST pull-down analysis of activated Rac1 showed that when exogenous MgcRacGAP was expressed, the increased Rac1 activation detected at different time points during the calcium switch in double-KD cells was suppressed, thus reverting the phenotype to that of single-KD, CGNL1(–) cells (Figure 2D and Supplemental Figure S1E). Furthermore, Rac1 inactivation induced by exogenous MgcRacGAP expression correlated with a strong reduction in the peak of TER detected at 8 h after the calcium switch in double-KD cells, resulting in a phenotype that was similar to that of single-KD, CGNL1(–) cells (Figure 2E and Supplemental Figure S1F). We also attempted to generate stable lines depleted of MgcRacGAP through shRNA expression in order to test directly the role of MgcRacGAP in junction assembly in WT cells. However, such lines could not be isolated, probably due to the essential role of MgcRacGAP in cytokinesis (Glotzer, 2009).

### Cingulin and paracingulin are required for efficient junctional recruitment of MgcRacGAP in different types of epithelial cells

Having established that modulating MgcRacGAP expression levels affects Rac1 activation and the dynamics of establishment of the TJ barrier to ions, we asked whether MgcRacGAP is localized at TJs through interaction with CGN, CGNL1, or both. To do this, we examined the localization of MgcRacGAP in epithelial cells in which CGN, CGNL1, or both were depleted through either shRNA or small interfering RNA (siRNA). In addition, we studied the localization of MgcRacGAP in mixed cultures of primary keratinocytes isolated from WT and CGN-KO mice (Figure 3).

In confluent WT MDCK cells, MgcRacGAP staining at junctions was continuous and largely colocalized with CGN (arrows in Figure 3, A, B, and D, and Supplemental Figure S2). However, in dividing cells, only MgcRacGAP and not CGN labeling was detected in the mitotic spindle (inset in Figure 3A, WT). In addition, unlike CGN and CGNL1, MgcRacGAP was also detected in the nucleus (marked “n” in Figure 3 and Supplemental Figure S2). In mixed cultures of WT MDCK cells, together with either CGN-KD or double-KD stable clonal cells (WT+dKD), MgcRacGAP labeling at junctions was reduced, albeit not abolished, in junctions between KD cells compared with junctions between WT cells (double arrowheads in Figure 3A). A similar reduction of junctional MgcRacGAP labeling was obtained by depleting CGN in MDCK cells, using siRNA instead of shRNA (Supplemental Figure S2A).



**FIGURE 3:** CGN and CGNL1 are required for the efficient recruitment of MgcRacGAP to epithelial junctions. (A, B) Double immunofluorescence of CGN and MgcRacGAP (Mgc) in WT MDCK cells in cocultures of WT and CGN-KD MDCK cells, WT and double-KD MDCK cells (A), or mouse kidney (mpkCCD<sub>C14</sub>) cells, after siRNA control, si-CGN, si-CGNL1, and si-double (CGN and CGNL1) treatment. Cells were labeled also with rat anti-ZO-1 to identify junctions. Arrows, junctions labeled by both MgcRacGAP and CGN antibodies. Double arrowheads, junctions with decreased labeling for both CGN and MgcRacGAP and normal labeling for ZO-1. The square area in A and magnified inset shows labeling for MgcRacGAP (arrowheads) in the mitotic spindle. Asterisks, positions of nuclei of KD cells. Single arrowheads, junctions with reduced CGNL1 staining and normal MgcRacGAP staining. n, nuclear labeling for MgcRacGAP. (C) Semiquantitative analysis of junctional labeling intensity for MgcRacGAP (expressed as a ratio of MgcRacGAP to ZO-1 pixel intensity in the same junctional areas) in WT, CGN-KD, dKD junctions of MDCK clonal lines or WT, CGNL1-KD, dKD si-treated mouse kidney cells. (D) Double immunofluorescence of CGN and MgcRacGAP in cocultures of primary keratinocytes derived from either WT or CGN KO mice. Magnified insets in D' and D'' show intensity-adjusted images of junctions, to show the lower levels (but not absence) of MgcRacGAP in junctional areas between KO cells (D') vs. junctions between WT cells (D''). Bar, 5  $\mu$ m.

To examine the effect of CGNL1 depletion on the junctional localization of MgcRacGAP, we used mouse kidney cells (mpkCCD<sub>C14</sub>) because the mouse anti-CGNL1 antibody required for double immunofluorescence with rabbit anti-MgcRacGAP does not recognize canine CGNL1. In mouse kidney cells, depletion of CGN, CGNL1, or both by siRNA resulted in reduced but not absent junctional staining for MgcRacGAP (Figure 3B). Semiquantitative analysis of MgcRacGAP staining at junctions, expressed as a ratio to ZO-1 staining in the same junctional area, showed a similar decrease, by ~50%, in both single- and double-KD canine and mouse kidney cells (Figure 3C).

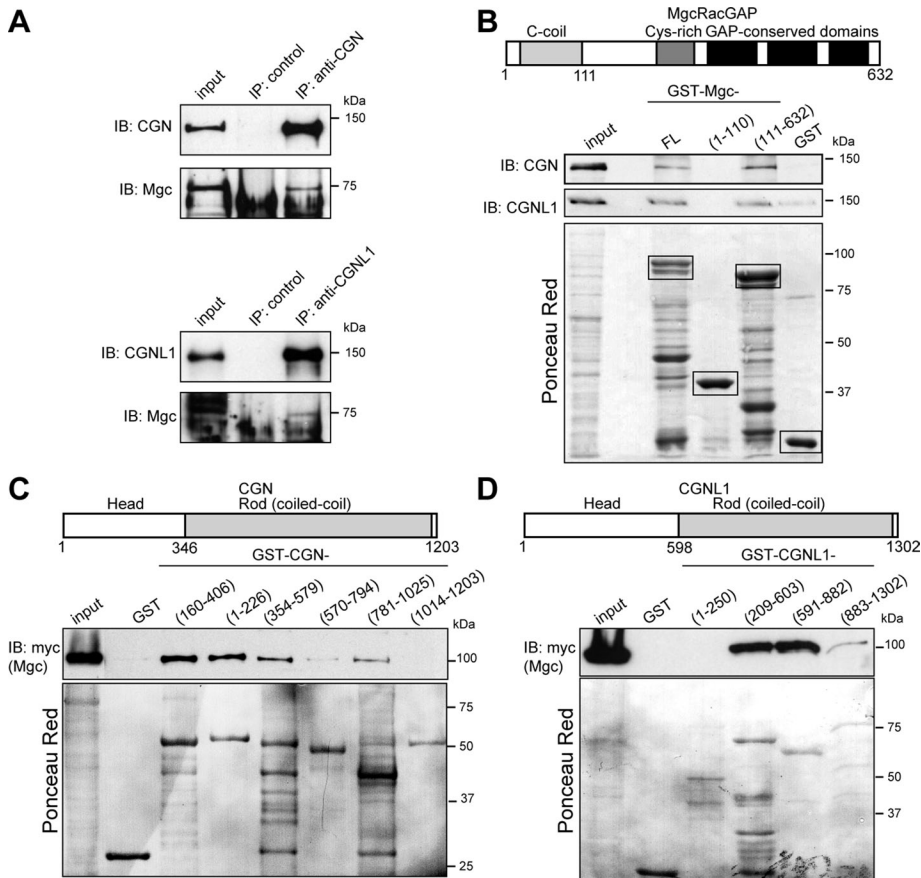
As a third cell model system, we examined differentiated keratinocytes isolated from WT and CGN-KO mice and grown in mixed cultures (Figure 3D). Both CGN and MgcRacGAP showed a continuous, linear (zonular), and mostly overlapping labeling of junctions in WT keratinocytes (arrow in Figure 3D). In contrast, in junctions between CGN-KO keratinocytes, MgcRacGAP labeling was decreased but not absent (arrowheads in Figure 3D and magnified insets in Figure 3, D' and D''), indicating that even in the absence of any junctional CGN, a fraction of MgcRacGAP remains localized at junctions.

To extend our analysis to additional epithelial cell types, we also examined the effect of either CGN or CGNL1 depletion in human intestinal colon carcinoma cells (SKCO-15) and mouse mammary epithelial cells (Eph4). In both of these cell types (and also in MCF-7 cells; unpublished data) depletion of CGN, either alone or in combination with CGNL1 resulted in decreased junctional staining for MgcRacGAP (Supplemental Figure S2, B and C). In contrast, depletion of CGNL1 alone resulted in decreased junctional labeling for MgcRacGAP only in Eph4 (Supplemental Figure S2C) but not in SKCO-15 cells (single arrowhead in Supplemental Figure S2B, CGNL1-si) or human breast cancer (MCF-7) cells (unpublished data).

Taken together, these observations demonstrate that CGN, in all the epithelial cell types we tested, and CGNL1, in all the cell types we tested with the exception of SKCO-15 and MCF-7 cells, are required for the recruitment of MgcRacGAP to the AJC.

### Cingulin and paracingulin interact with MgcRacGAP

To explore whether the requirement of CGN and CGNL1 for the junctional recruitment of MgcRacGAP depends on an interaction of MgcRacGAP with these proteins, we carried out immunoprecipitation and GST pull-down experiments. Immunoblot analysis of either



**FIGURE 4:** CGN and CGNL1 form a complex and interact directly with MgcRacGAP. (A) Immunoblotting of CGN (top, from MDCK cell lysates) or CGNL1 (bottom, from Eph4 cell lysates) immunoprecipitates, using antibodies against CGN/MgcRacGAP or CGNL1/MgcRacGAP, respectively. Input: 1/40 of volume used for immunoprecipitation. MgcRacGAP shows different mobility and forms in MDCK vs. Eph4 cells. (B) Domain organization of MgcRacGAP and immunoblotting analysis of CGN in GST pull-downs of full-length CGN interacting with either full-length (FL) or truncated constructs (1–110, 111–632) of MgcRacGAP fused to GST. (C, D) Domain organization of CGN (C) and CGNL1 (D) and immunoblotting analysis of myc-tagged MgcRacGAP in GST pull-downs of the indicated fragments of either CGN or CGNL1 fused to GST. Images of Ponceau red-labeled membranes below immunoblots show protein loadings for GST fusion proteins.

CGN or CGNL1 immunoprecipitates showed that endogenous MgcRacGAP is present in a complex with CGN and CGNL1 in epithelial cells (Figure 4A). Furthermore, specific coimmunoprecipitation was also detected between tagged, exogenously expressed MgcRacGAP, CGN, and CGNL1 in MDCK cells (Supplemental Figure S3).

Next we asked whether MgcRacGAP can interact directly with CGN and CGNL1, by incubating recombinant, bacterially expressed MgcRacGAP constructs with full-length CGN or CGNL1, the latter expressed in baculovirus-infected insect cells. Both CGN and CGNL1 were detected by immunoblotting when either full-length MgcRacGAP or an N-terminally truncated construct of MgcRacGAP (residues 111–632) were used as bait, but not when a fragment containing the coiled-coil domain of MgcRacGAP (1–111) was used (Figure 4B). Thus the region of MgcRacGAP that interacts with CGN and/or CGNL1 is distinct from the region (N-terminal 120 residues) that interacts with ECT2 and MKLP1 (Mishima *et al.*, 2002; Somers and Saint, 2003) and partially overlaps with the sites of interaction with anillin (D'Avino *et al.*, 2008; Gregory *et al.*, 2008) and PRC1 (Ban *et al.*, 2004).

To map the regions of CGN and CGNL1 that interact with MgcRacGAP, we incubated bacterially expressed constructs of either CGN or CGNL1 with myc-tagged MgcRacGAP. In the case of CGN, the strongest binding was observed with fragments of the CGN globular head domain (residues 160–406, 1–226), although fragments of the coiled-coil rod (residues 354–579, 781–1025) also interacted with MgcRacGAP (Figure 4C). In the case of CGNL1, the strongest interaction was observed with the C-terminal two-thirds of the globular head domain (residues 209–603) and the N-terminal one-third of the coiled-coil rod domain (residues 591–882; Figure 4D).

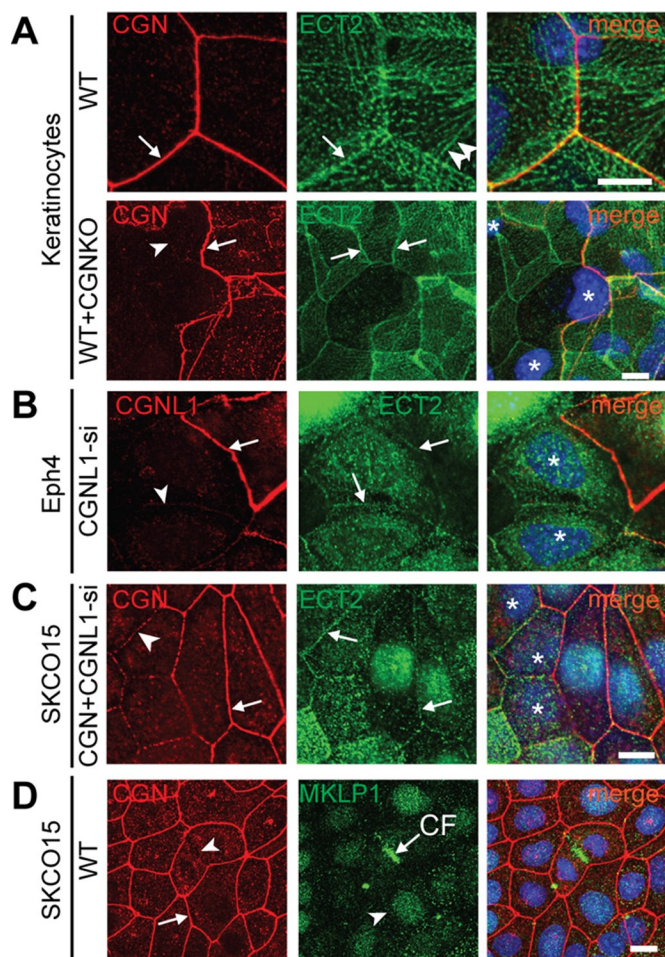
In summary, CGN and CGNL1 form a complex and interact directly with MgcRacGAP.

### Cingulin and paracingulin are not required for junctional recruitment of the Rho GEF ECT2

MgcRacGAP interacts with and is required for the junctional recruitment of ECT2, a centralspindlin-associated Rho GEF, at cadherin-based junctions of human breast cancer cells (MCF-7; Ratheesh *et al.*, 2012). Because depletion of either CGN or CGNL1 reduced the junctional localization of MgcRacGAP, we asked whether ECT2 is also affected under these conditions.

The localization of ECT2 was investigated in different types of epithelial cells: canine and mouse kidney cells, mouse primary keratinocytes, human intestinal cells, and mouse mammary epithelial cells (Figure 5 and Supplemental Figure S4). In canine and mouse kidney cells, no junctional labeling for ECT2 could be detected (unpublished data). In keratinocytes, ECT2 labeling was detected at junctions, but it was not

zonular like that of MgcRacGAP, and it only partially colocalized with CGN (arrows in Figure 5A, WT, and Supplemental Figure S4B), suggesting that it is associated with the ZA rather than the TJ. In addition, prominent ECT2 labeling was detected along lateral regions of cell–cell contact, clustered along aligned cable-like structures, some of which reached the zonular junctional region, where they apparently connected with continuing cables on the adjoining cells (double arrowheads in Figure 5A, WT, and Supplemental Figure S4A). These observations indicate that ECT2 is distributed along lateral spot-like adherens junctions associated with actomyosin bundles, which terminate at the zonula adhaerens and may be part of a transcellular network across neighboring cells that provides continuity across contractile units (Ebrahim and Kachar, 2013). Of importance, no decrease in ECT2 labeling was observed in CGN-KO keratinocytes (arrowhead in Figure 5A, WT+CGNKO) compared with junctions between WT keratinocytes (arrow in Figure 5A, WT+CGNKO). In SKCO-15 cells (Supplemental Figure S4) and Eph4 cells (unpublished data), labeling for ECT2 was highly heterogeneous in intensity and distribution in different cells, mostly detectable in the cytoplasm and nucleus in a pattern that suggested cell cycle-dependent



**FIGURE 5:** Depletion of CGN and CGNL1 does not affect the junctional recruitment of the Rho GEF ECT2. (A) Double immunofluorescence analysis (CGN, red; ECT2, green; 4',6-diamidino-2-phenylindole [DAPI], blue; in merge images) of WT keratinocytes (top) or mixed cultures of WT and CGN-KO keratinocytes (bottom). Additional images are shown in Supplemental Figure S4A. Note the presence of ECT2 (arrow) in cells lacking CGN (arrowhead). (B, C) Double immunofluorescence analysis (CGN, red; ECT2, green; DAPI, blue; in merge images) of Eph4 cells treated with siRNA against CGNL1 or SKCO-15 cells treated with siRNA against both CGN and CGNL1 (C), showing both WT cells, containing junctional CGNL1/CGN (arrow) and CGNL1/CGN-depleted cells (arrowhead). Note the presence of ECT2 (arrow) in cells lacking CGNL1/CGN. (D) Double immunofluorescence analysis (CGN, red; MKLP1, green) of confluent SKCO-15 cells, showing that MKLP1 is not detected at junctions (arrow), but only at the cleavage furrow (CF) of dividing cells. Asterisks, positions of nuclei of siRNA-CGN-depleted/KO cells. Single arrowheads, sites with reduced CGN/CGNL1 labeling (KD or KO) but detectable ECT2 labeling. Arrows, junctions between WT cells showing both CGN/CGNL1 and ECT2 labeling. Bar, 5  $\mu$ m (B–D), 2  $\mu$ m (A).

expression and localization (Liot *et al.*, 2011; Matthews *et al.*, 2012) and was more rarely detectable at junctions (Supplemental Figure S4). We found no correlation between intensity and distribution of ECT2 labeling and depletion of either CGN or CGNL1 in Eph4 or SKCO-15 cells (Figure 5, B and C, and Supplemental Figure S4B). In human mammary carcinoma cells (MCF-7), labeling for both ECT2 and MgcRacGAP was junctional, and CGN depletion decreased junctional MgcRacGAP, whereas ECT2 was not affected by depletion of either CGN or CGNL1 (unpublished data). These observa-

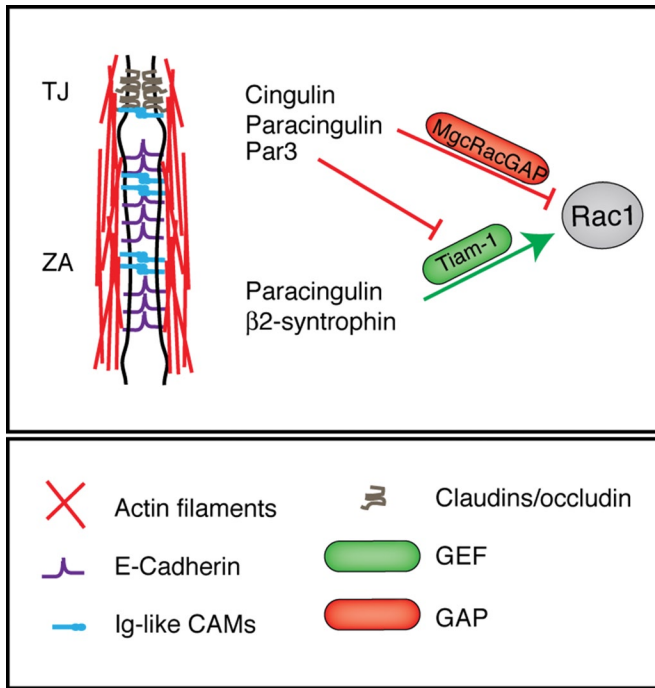
tions indicate that ECT2 distribution is highly variable depending on cell type, the decreased levels of junctional MgcRacGAP in CGN/CGNL1-depleted cells do not correlate with reduced junctional localization of ECT2, and neither CGN nor CGNL1 is required for junctional recruitment of ECT2. This is consistent with the observation that depletion of CGN, CGNL1, or both has no detectable effect on the organization of cadherin-based junctions (Guillemot *et al.*, 2008, 2013; Pulimeno *et al.*, 2011).

Finally, we examined the localization of the kinesin MKLP1 (KIF23), the second subunit of the centralspindlin complex, in SKCO-15 cells, and detected strong labeling only at the cleavage furrow of dividing cells but no junctional labeling in interphase cells (Figure 4D), suggesting that the centralspindlin complex dissociates upon completion of mitosis, and MKLP1 does not associate with junctions.

## DISCUSSION

We provide evidence for a new molecular mechanism regulating the activity of Rac1 at epithelial junctions, based on observations on the phenotype of stable MDCK lines depleted of both CGN and CGNL1. Our data indicate that a component of the centralspindlin complex, MgcRacGAP, is involved in regulation of Rac1 activation and dynamics of TJ barrier formation during epithelial junction assembly, and is recruited to apical zonular junctions by CGN and CGNL1. We also provide evidence that the localization of MgcRacGAP in interphase epithelial cells is distinct from that of the second component of the centralspindlin complex, MKLP1, and of ECT2.

MgcRacGAP is a Rac-specific GAP (Toure *et al.*, 1998) fundamental for mammalian viability, since its deficiency in mouse embryos leads to preimplantation lethality (Van de Putte *et al.*, 2001). As a component of the centralspindlin complex, MgcRacGAP plays a critical role during cytokinesis, by regulating assembly of the central spindle, in part by affecting RhoA through the Rho GEF ECT2 (Mishima *et al.*, 2002; Yuce *et al.*, 2005; White and Glotzer, 2012) but mainly by inhibiting Rac1 activity (D'Avino *et al.*, 2004; D'Avino and Glover, 2009; Canman *et al.*, 2008; Bastos *et al.*, 2012). What role, if any, MgcRacGAP plays in Rac1 regulation at junctions of interphase cells has not been determined. A study reported that the centralspindlin complex is localized at the ZA of breast cancer (MCF-7) cells by anchoring with the N-terminus of  $\alpha$ -catenin to support ECT2-controlled Rho signaling at junctions (Ratheesh *et al.*, 2012). Here we show that in interphase epithelial cells, MgcRacGAP colocalizes with CGN, a specific marker of TJ, whereas ECT2 is distributed heterogeneously at the ZA, along lateral contacts, and in the cytoplasm/nucleus in different cell types. In addition, whereas depletion of either CGN or CGNL1 results in decreased MgcRacGAP accumulation at junctions, we did not observe any decrease in the junctional localization of ECT2, in apparent disagreement with the idea that MgcRacGAP recruits ECT2 to junctions (Ratheesh *et al.*, 2012), and confirming the lack of effect of CGN/CGNL1 depletion on ZA organization (Guillemot *et al.*, 2004, 2008, 2012, 2013; Guillemot and Citi, 2006a; Pulimeno *et al.*, 2011). However, because even in double-KD kidney epithelial cells we observed residual junctional staining for MgcRacGAP, this discrepancy can be explained by the presence of two pools of MgcRacGAP, one at the TJ, which is recruited by CGN and CGNL1, and one at the ZA, recruited by the  $\alpha$ -catenin/E-cadherin complex (Ratheesh *et al.*, 2012; Priya *et al.*, 2013). The proportion of MgcRacGAP that associates with TJ versus ZA, and hence its regulation, may be different in cancer cell lines versus spontaneously immortalized cell lines and may depend on the composition and degree of "maturation" of these junctions. Further studies should characterize the interactions of MgcRacGAP with components of the ZA, and its regulation at the apical junctional



**FIGURE 6:** The control of Rac1 signaling at the apical junctional complex. Simplified scheme showing the AJC and the putative molecular interactions of GEFs and GAPs with CGN, CGNL1, and other junctional proteins. Green lines indicate activation; red lines indicate inhibition. A key for the graphical objects is shown at the bottom.

complex. On the other hand, our observations on different types of CGN/CGNL1-depleted cells, and the finding that MgcRacGAP forms a complex and interacts directly with CGN and CGNL1 strongly support the idea that CGN and CGNL1 play a major role in recruiting MgcRacGAP to the TJ.

Concerning the function of MgcRacGAP in interphase cells, we show that in double-KD MDCK cells, where MgcRacGAP levels are decreased, RhoA activation during the calcium switch is indistinguishable from WT and single-KD (CGNL1(-)) MDCK cells (Guillemot *et al.*, 2008; present study). In contrast, rescuing MgcRacGAP levels dramatically affects Rac1 signaling during junction assembly, supporting the notion that MgcRacGAP acts primarily on Rac1 and not RhoA signaling (D'Avino and Glover, 2009). The correlation between Rac1 activation and the pattern of TER development in the double-KD cell lines strengthens the idea that Rac1 activation regulates the development of the paracellular barrier to ions. The barrier to ions depends on claudins, which polymerize on a scaffold of cytoplasmic TJ proteins, which are linked directly or indirectly to the actin cytoskeleton (Umeda *et al.*, 2006; Fanning and Anderson, 2009; Citi *et al.*, 2012). Although the precise molecular mechanism that generates the peak in TER observed during junction assembly in MDCK cells is not known, it can be speculated that it is due to a transient, cytoskeleton-dependent "tightening" of the claudin-based barrier, the differential kinetics of assembly of "tight" (barrier) versus "leaky" (pore) claudin isoforms (Kolosov *et al.*, 2014), or a combination of these mechanisms. Because TER development is also modulated by Tiam1, a Rac1 GEF that interacts with Par3 and CGNL1 (Chen and Macara, 2005; Mertens *et al.*, 2005; Guillemot *et al.*, 2008); by RICH1, a Cdc42/Rac1 GAP that interacts with angiomin (Wells *et al.*, 2006); and by  $\beta$ 2-syntrophin, which modulates the localization of Tiam1 and Rac1 along the apicobasal axis (Mack *et al.*, 2012), we conclude that Rac1 is a key regulator of

TER development, and we hypothesize that this regulation occurs primarily by controlling cytoskeletal organization and cytoskeletal anchoring of claudins. Of importance, the ability of MgcRacGAP to modulate TJ barrier development must depend on its spatially restricted activity at the cytoplasmic, cytoskeleton-linked "plaque" of the TJ, and our data indicate that this is through its interaction with CGN and CGNL1. Therefore, accumulation of CGN and CGNL1 at TJs during junction maturation provides a mechanism to spatially restrict down-regulation of Rac1 activation at TJs, through recruitment of MgcRacGAP.

Taken together, the results presented here and in previous studies (Aijaz *et al.*, 2005; Guillemot and Citi, 2006a; Guillemot *et al.*, 2008) suggest a working model in which CGN and CGNL1 have multiple functions in fine-tuning RhoA and Rac1 activation in MDCK cells (Figure 6). In confluent cells, they both inhibit RhoA activation by sequestering GEF-H1, whereas during junction assembly, CGNL1 promotes Rac1 activation by supporting junctional recruitment of Tiam1, whereas both CGN and CGNL1 inhibit Rac1 activation at TJ through recruitment of MgcRacGAP. So we propose that the dynamics of junction assembly and TER development result at least in part from a balance between Tiam1-mediated Rac1 activation and MgcRacGAP-mediated Rac1 inhibition. Changes in the levels of expression of either Tiam1 or MgcRacGAP or their interacting partners at junctions, and additional regulatory mechanisms, may affect such equilibrium, resulting in altered spatiotemporal dynamics of junction assembly. To further test this model, it will be necessary to explore in more detail the phenotype of CGN and CGNL1 KO model systems, and the regulation of the molecular interactions between different GEFs, GAPs, and cytoplasmic junctional proteins.

In summary, our data reveal a new mechanism of fine-tuning of junction assembly and development of the TJ barrier, by which MgcRacGAP controls Rac1 activation at apical junctions, where it is recruited through its interaction with CGN and CGNL1. Our results also support the idea that the spatiotemporal control of Rho GTPases is coordinated by the cell context-dependent makeup of GTPase activators and inhibitors and interacting junctional proteins.

## MATERIALS AND METHODS

### Antibodies and plasmids

Commercial antibodies were purchased against hemagglutinin (Covance, Meyrin, Switzerland), FLAG (M2; Sigma-Aldrich, Buchs, Switzerland), MgcRacGAP (rabbit sc-98617; Santa Cruz), ECT2 (07-1364; Millipore), MKLP1 (sc-867; Santa Cruz), and CGNL1 (mouse sc-162681; Santa Cruz, Muttentz, Switzerland). The mouse anti-CGNL1 antibody did not cross-react with canine CGNL1 and therefore could not be used to immunolocalize CGNL1 in double labeling of MDCK cells with rabbit anti-MgcRacGAP or for immunoprecipitations of CGNL1 from MDCK cells. The rat anti-ZO-1 monoclonal antibody (R40-76) was a kind gift of D. Goodenough (Harvard University, Cambridge, MA). Antibodies against MgcRacGAP (immunoblotting) and a plasmid (pMXs-neo) encoding FLAG-tagged human MgcRacGAP were kind gifts from T. Kitamura (Institute of Medical Science, University of Tokyo, Tokyo, Japan; Hirose *et al.*, 2001) and A. Touré (Institut Cochin, Paris, France; Toure *et al.*, 1998). Secondary antibodies for immunofluorescence (Jackson ImmunoResearch Europe, Newmarket, UK) were Alexa 488 anti-rabbit, Cy3 anti-mouse, and Cy5 anti-rat. Constructs for bacterial expression of MgcRacGAP GST fusion proteins (full-length and truncated) were produced by PCR amplification and subcloning into the pGEX4T1 vector. MgcRacGAP was subcloned into pcDNA3.1myc-His (EcoRI-NotI) for establishing stable MDCK cell lines. All new constructs were verified by sequencing. Polyclonal antiserum against CGN



(Cardellini *et al.*, 1996), other constructs, and antibodies were as previously described (Guillemot *et al.*, 2004, 2008; Guillemot and Citi, 2006a; Paschoud and Citi, 2008).

### Cell culture, transfection, and other techniques

MDCK, SKCO-15 (human intestinal colon carcinoma; a kind gift from A. Nusrat, Emory University, Atlanta, GA; Ivanov *et al.*, 2010), and Eph4 cells (mouse mammary epithelial cells; a kind gift of E. Reichmann, University of Zurich, Zurich, Switzerland; Maschler *et al.*, 2005) were cultured in DMEM containing 10% fetal bovine serum (FBS), nonessential amino acids, and appropriate antibiotics for selection (Guillemot *et al.*, 2013). Mouse kidney collecting duct cells (mpkCCDC<sub>14</sub>; a kind gift of E. Feraille, University of Geneva, Geneva, Switzerland; Gonin *et al.*, 2001) were cultured as described previously (Pulimeno *et al.*, 2010). Stable clones of MDCK cells expressing shRNAs to deplete CGN, CGNL1, or both CGN and CGNL1 (double-KD) or rescue MgcRacGAP constructs were obtained as described (Guillemot and Citi, 2006a; Guillemot *et al.*, 2008, 2013). For siRNA-mediated depletion, the following target sequences were used (sense): 1) CGN: GTGACCAGGAGGTGGAACA (human and mouse), AGCTCAGAAGGCTTCCAGA (canine); 2) CGNL1: AGTGCTGAACCTCAGAGA (human), GACTTAAAGAGCCGGATTA (mouse). Transfections were carried out using Lipofectamine2000 (DNA) and RNAiMax (siRNA), and cells were analyzed by immunofluorescence 48–72 h after transfection. Calcium switch, measurement of TER, Rac1 and RhoA activation assays, immunoblotting, and immunofluorescence were as described (Citi *et al.*, 2001; Guillemot and Citi, 2006a; Guillemot *et al.*, 2008; Paschoud and Citi, 2008).

### GST pull downs, immunoprecipitation, and cell fractionation

For GST pull downs, full-length CGN and CGNL1 were from baculovirus-infected insect cells and MgcRacGAP from HEK293 cells transfected with exogenous, myc-tagged MgcRacGAP (Citi *et al.*, 2001; Guillemot *et al.*, 2008). Coimmunoprecipitation experiments were carried out as described (Guillemot *et al.*, 2008), except that lysates were sonicated, and antibodies (2 µg) were incubated with Dynabeads Protein G (Ref. 1004D; Life Technologies Europe, Zug, Switzerland; 20 µl, prewashed three times with phosphate-buffered saline containing 5% skimmed milk) for 1 h at 4°C before incubation with cell lysates (overnight at 4°C) and washing with coimmunoprecipitation buffer. MDCK lysates were used for CGN-MgcRacGAP immunoprecipitations and Eph4 cell lysates for CGNL1-MgcRacGAP immunoprecipitation because the mouse anti-CGNL1 antibody does not cross-react with canine CGNL1. The anti-MgcRacGAP antibody did not work for immunoprecipitation. For fractionation, cells were lysed in cytoskeleton stabilizing buffer (CSK; 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 6.8, 250 mM sucrose, 150 mM KCl, 1 mM ethylene glycol tetraacetic acid, 3 mM MgCl<sub>2</sub>, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail). The CSK lysate was centrifuged for 15 min at 13,000 × *g*, and the pellet was resuspended, washed with CSK buffer, centrifuged again, and taken as the “low-speed” cytoskeleton fraction. The soluble fraction was centrifuged for 120 min at 100,000 × *g*, and the pellet was washed and taken as the “high-speed” Triton X-100 insoluble cytoskeleton fraction. The supernatant from high-speed centrifugation was taken as the “soluble” fraction. Equivalent protein loadings from each fraction were analyzed by SDS-PAGE and immunoblotting.

### Primary keratinocyte cultures

Keratinocytes were isolated from WT and CGN KO (Guillemot *et al.*, 2012) newborn mice, following a protocol approved by the

Ethics Committee of the University of Geneva and the Cantonal Veterinary Office (Authorization 1027/3853/53). Skins were dissected and incubated with dispase II (04942078001; Roche), 2.5 U/ml (3.1 mg/ml) at 37°C for 2 h, in low-calcium medium (DMEM/F12 3:1, pH 7.2, supplemented with 15% calcium-free FBS, 100 U/ml penicillin/streptomycin, 0.1 nM cholera toxin, 6 × 10<sup>-4</sup> mg/ml hydrocortisone, 5 × 10<sup>-3</sup> mg/ml insulin, 5 × 10<sup>-3</sup> mg/ml transferrin, and 0.02 nM 3',5-triiodo-L-thyronine). The epidermis was then separated from the dermis, minced into pieces, and incubated with trypsin 0.25%-1× EDTA (253000062; Invitrogen) for 15 min at 37°C. The cell suspension was filtered through a 70-µm cell strainer and centrifuged at 1500 rpm for 5 min. Cells were seeded at a density of 0.25 × 10<sup>6</sup> cells/cm<sup>2</sup> and incubated at 37°C in the presence of 5% CO<sub>2</sub>. Immunofluorescence analysis of keratinocytes was performed after calcium-induced induction of differentiation, which promotes junction formation: CaCl<sub>2</sub> (0.25 M stock) was added to a final concentration of 1.2 mM, and keratinocytes were fixed (cold methanol) for immunofluorescence labeling at different times after calcium addition.

### Quantitative RT-PCR

mRNA levels for GEFs and GAPs were analyzed by SYBR green-based RT-PCR (Guillemot and Citi, 2006a; Guillemot *et al.*, 2008, 2013), using the following primers: Asef (ARHGEF4) forward, 5'-GCGGACCAACGTCATCAAC-3'; reverse, 5'-CCCAGGCTGCTTGATGTA-3'; Vav2 forward, 5'-AAGAACTCAATGACCCCTGA-3'; reverse, 5'-AGGTTGATGAAAATGGCTGCC-3'; RICH1 forward, 5'-TGGCAGCGGCTACCTCTG-3'; reverse, 5'-TCGGCGTGCTGAA-TGATG-3'; MgcRacGAP forward, 5'-TGAATTTGCGGAATCT-GTTTGA-3'; reverse, 5'-TGGAGTTCATTTCTTCACTGAGA-3'.

### Confocal microscopy

Cells were observed using Zeiss conventional (Axiovert TV100) and confocal (LSM700) microscopes. For quantification of MgcRacGAP labeling in CGN-KD and double-KD cells, cells were triple labeled with mouse anti-CGN/CGNL1, rabbit anti-MgcRacGAP, and rat anti-ZO-1. ZO-1 was used as an internal reference for junction/plane of focus to exclude the possibility that reduced junctional labeling for MgcRacGAP could be due to shift of plane of focus. For quantifications using ImageJ (National Institutes of Health, Bethesda, MD), for each marker the cytoplasmic area (devoid of junctions) was taken as background and subtracted from the junctional labeling. The mean pixel intensity of MgcRacGAP junctional labeling was measured in junctional segments or representative images in WT, single-KD, and double-KD cells (*n* = 5) and ratioed to ZO-1 labeling in the corresponding junctions (Pulimeno *et al.*, 2011).

### Statistical analysis

All experiments were carried out at least in triplicate, and histogram values show means ± SD. Values were considered statistically significant (\*) when *p* < 0.05 between experiments (Student's *t* tests). For immunoblots and immunofluorescence data, one representative example is shown.

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