Cooperation of distinct Rac-dependent pathways to stabilise E-cadherin adhesion

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A B S T R A C T

The precise mechanisms *via* which Rac1 is activated by cadherin junctions are not fully known. In keratinocytes Rac1 activation by cadherin junctions requires EGFR signalling, but how EGFR does so is unclear. To address which activator could mediate E-cadherin signalling to Rac1, we investigated EGFR and two Rac1 GEFs, SOS1 and DOCK180. EGFR RNAi prevented junction-induced Rac1 activation and led to fragmented localization of E-cadherin at cadherin contacts. In contrast, depletion of another EGFR family member, ErbB3, did not interfere with either process. DOCK180 RNAi, but not SOS1, prevented E-cadherin-induced Rac1 activation. However, in a strong divergence from EGFR RNAi phenotype, DOCK180 depletion did not perturb actin recruitment or cadherin localisation at junctions. Rather, reduced DOCK180 levels impaired the resistance to mechanical stress of pre-formed cell aggregates. Thus, within the same cell type, EGFR and DOCK180 regulate Rac1 activation by newly-formed contacts, but control separate cellular events that cooperate to stabilise junctions.

1. Introduction

E-cadherin is the classical prototype cell–cell adhesion receptor. The E-cadherin knockout is embryonic lethal, and thus its function is critical for life. In homeostasis, the dynamic regulation of E-cadherin signalling is fundamental for the differentiation, maintenance and functional integrity of epithelia. Among the different pathways activated by E-cadherin contacts is the Rac1 small GTPase (hereafter referred to as Rac). Rac plays an essential role in the differentiation of epithelial organs by regulating polarization [1], lumen formation or epidermal barrier integrity [2]. In addition to other functions in epithelia, Rac stabilizes cell–cell contacts *via* regulation of E-cadherin trafficking and actin recruitment to junctions [1,3].

A major drive has been in place to identify how the engagement of E-cadherin at contacts is able to activate Rac. Specific Rac activators or guanine nucleotide exchange factors (GEF) [4] such as Tiam1 or Asef have been reported to regulate adherens junctions in epithelial cells [3]. However, their regulation of Rac activation upon junction assembly has not been determined. More recently, ELMO2, an adaptor for the unconventional GEFs of the DOCK family [5], has been identified as a junction regulator in a genome-wide screen in *Drosophila* [6]. Depletion of ELMO2 significantly reduces Rac levels at newly formed contacts and

prevents the transient recruitment of DOCK180 to MDCK junctions [7]. However, the direct effect of DOCK180 RNAi on Rac activation could not be evaluated in MDCK cells [7].

The variety of regulators identified to date imply two important points. First, it is clear from current studies that distinct epithelial cell types employ distinct signalling mechanisms downstream of E-cadherin contacts [3]. A prediction is that alternative GEFs may be involved in multiple cell types to modulate cadherin signalling to Rac. Second, in the same cell type, different GEFs could activate Rac locally at cell–cell contacts at different time points and regulate distinct cellular events. Indeed, the number of Rac-dependent cellular processes known to stabilise junctions strongly suggests that, within the same cell type, various mechanisms modulate GTPase signalling. Thus, there is a large scope to identify Rac regulators and cellular mechanisms triggered by cadherin engagement.

These considerations led us to investigate in a single cell type the potential ways in which new cadherin contacts activate Rac and the specific functional outcome during junction stabilisation. In normal keratinocytes, cell–cell adhesion-dependent activation of Rac requires Epidermal Growth Factor Receptor (EGFR) [8]. EGFR signalling plays an essential role in epithelia. $EGFR^{-/-}$ mice die soon after birth with growth retardation, epithelial immaturity and dysfunction of multiple organs [9,10]. E-cadherin contacts can utilise EGFR signalling to regulate the assembly of adhesive structures [11,12] and activate specific pathways required for epithelial function, proliferation and survival [8,13,14]. This crosstalk represents a fundamental principle *via* which different cadherin receptors trigger signalling pathways in a cell type- and growth factor receptor-specific manner [15–17].

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Rac signalling is known to be deployed following growth factor stimulation to regulate cell motility and other processes [18]. In the context of cell–cell adhesion, it is likely that Rac may participate at least in some of the cellular processes downstream of the EGFR–E-cadherin cross-talk. We hypothesise that different Rac GEFs may participate in junction stabilisation downstream and/or independently of EGFR. Here we set out to dissect the mechanisms of Rac activation by new cadherin contacts in keratinocytes. We sought to identify: (i) which EGFR family members (EGFR, ErbB2, ErbB3 or ErbB4) can mediate E-cadherin signalling to Rac and (ii) the potential involvement of the Rac GEFs, SOS1 and DOCK180. SOS1 is a Ras and Rac dual exchange factor [19] and is activated following EGF treatment [20]. DOCK180 activates Rac upon growth factor stimulation leading to migration, integrin adhesion and cell protrusion [21,22]. DOCK180 also modulates a variety of Rac-dependent events such as cell fusion during muscle differentiation, migration of gonad cells in *Drosophila* and phagocytosis of apoptotic cells and IgG opsonised particles [5]. We investigated whether DOCK180 or SOS1 could facilitate cadherin-dependent activation of Rac, whether they operate downstream of EGFR and which cellular event is regulated.

2. Materials and methods

2.1. Cell culture and treatment

Normal human keratinocytes from neonatal foreskin (strain Sf, passages 3 to 7) were cultured on a mitomycin C (Sigma)-treated monolayer of 3T3 fibroblasts and cultures, in the absence of calcium-dependent cell–cell contacts, were grown as described [23]. Cell–cell contacts in confluent cultures were induced by addition of calcium ions (1.8 mM), rather than switching medium. For siRNA experiments, cells were grown to 90% confluence in low calcium medium and then treated as described below. Confluent cells were transfected with siRNA oligos and RNAifect (Qiagen) or INTERFERin (Peqlab) as per manufacturer's instructions.

Aggregation assays were performed essentially as described [24]. Confluent keratinocytes were trypsinised in 500 µl trypsinisation buffer (60% versene (v/v), 0.1% trypsin, 0.1 mM $CaCl_2$) to prevent cleavage of cadherins. Six drops of cell suspension per sample (20 µl of 5×10^4 cells/ml in standard calcium medium) were pipetted onto the inverted lid of a Petri dish and aggregates allowed to form in a humid chamber. After 120 min, cells were pipetted gently (trituration) to disaggregate.

For bead assays, polystyrene beads (15 µm, Polysciences) were coated with anti-E-cadherin antibody (HECD1) or heat inactivated BSA overnight at 4 °C as described previously [8,23]. Recruitment of DOCK180 or F-actin to E-cadherin clustered around latex beads for 15 or 30 min was performed as described [23].

2.2. Biochemistry

Following RNAi transfections, cells were washed twice with cold PBS and lysed in cold buffer (50 mM Tris–HCl pH 7.5, 1% Triton-X100, 150 mM NaCl, 0.1% SDS, 5 µg/ml leupeptin, 5 µg/ml pefabloc, 5 µg/ml pepstatin, 50 mM phenylmethylsulfonyl fluoride). For standard Western blots of lysates, protein concentration was measured with the BCA method (Pierce) using BSA as a standard. Equal amount of protein was separated by SDS–PAGE and transferred onto a polyvinylidene difluoride membrane (PVDF) membrane (Millipore). Incubation with primary antibodies was carried out overnight, and after incubation with secondary antibodies blots were developed with ECL™ or ECL Plus™ chemiluminescence and exposed to Hyperfilm ECL (all from GE Healthcare).

E-cadherin tail and DOCK180 interaction was determined by inducing cell–cell contacts for up to 30 min; cells were then lysed (20 mM Hepes pH 7.5, 300 mM NaCl, 1% triton, 5 mM MgCl2, 1 mM DTT, 5 µg/ml leupeptin, 5 µg/ml pefabloc, 5 µg/ml pepstatin, 50 mM phenylmethylsulfonyl fluoride, 20 mM sodium fluoride). The lysate was added to either GST–E-cadherin beads (Glutathione sepharose, GE Healthcare) or GST alone and incubated for 1 h at 4 °C, washed in lysis buffer, resuspended in sample buffer and the whole sample separated on SDS–PAGE.

2.3. Antibodies, immunofluorescence and microscopy

The following primary antibodies were used against: EGFR (1005; Santa Cruz); ErbB2 (CB11; Serotec); ErbB3 (C17; Santa Cruz); α3-integrin (VM2; gift from F. Watt); E-cadherin (HECD1); α-catenin (VB1); β-catenin (VB2); β1-integrin (gift from F Giancotti); DOCK180 (C19 and H70; Santa Cruz); SOS1 (E1; Santa Cruz); actin (C4; MP Biomedicals); β-tubulin (Tub2.1; Sigma); Rac1 (23A8; Millipore, Upstate); RhoA (26C4; Santa Cruz); and Cdc42 (44; Transduction Laboratories). Secondary antibodies (fluorescent and HRP conjugates) were bought from DAKO.

Cells were fixed in 3% PFA for 10 min, washed in PBS and permeabilised with 0.1% Triton X-100 for 10 min at room temperature [23]. For E-cadherin and staining, keratinocytes on coverslips were imaged using a Leica DCS NT confocal microscope with Leica LCS Lite software. Z-slices of 0.2 µm were taken. Z-slices were summed and images were processed using open source ImageJ software.

For aggregation assays, phase contrast images were taken before and after mechanical trituration of aggregates using an Olympus CKX41 microscope and a Colour View IIIu camera linked to Sort Imaging System software. For bead assays, images were collected using a Zeiss Axiovert 200 microscope with Velocity software.

2.4. Immunoprecipitations

Cells were induced to form cell–cell contacts for 30 min and then lysed in lysis buffer (1% Triton X-100, 15% Glycerol, 50 mM Tris–HCl pH 8, 150 mM NaCl, and 5 mM EDTA, 5 µg/ml leupeptin, 5 µg/ml pefabloc, 5 µg/ml, pepstatin, 50 mM phenylmethylsulfonyl fluoride, 20 mM sodium fluoride, 1 mM sodium pyruvate, 1 mM sodium orthovanadate and 20 mM β-glycero-phosphate). After centrifugation for 5 min, pre-cleared supernatants were immunoprecipitated for 1 h with 5 µl of the indicated antibody bound to 40 µl slur of protein A or protein G-Sepharose-4B beads (Sigma). Beads were washed three times in lysis buffer and SDS–PAGE sample buffer added to beads.

2.5. Reverse transcription polymerase chain reaction (RT-PCR)

Extraction of mRNA from cells grown in standard or low calcium media was carried out using the GTC method. A SuperScript t™Π Reverse Transcriptase (Invitrogen) reaction was performed with 1 µg total mRNA, according to the manufacturer's protocol and using specific PCR primers to amplify each family member: EGFR, (683 bp product); ErbB2 (193 bp); ErbB3 (603 bp) and ErbB4 (718 bp). PCR products were resolved on a 1% (w/v) agarose gel.

2.6. Quantification

The area of each aggregate remaining after trituration was measured (excluding clusters smaller than 3 cells) and the average size of clusters was calculated per sample. Values were expressed relative to control cells. The intensity of relevant bands obtained in Western blots was measured in the linear range, and values were corrected for control.

For bead assays, the percentage of attached beads with an enrichment of DOCK180 or F-actin immunofluorescence was quantified per image; recruitment of DOCK180 to BSA coated-beads was done as control.

For analysis of DOCK180 distribution at junctions, the E-cadherin image was thresholded to leave only junctional E-cadherin and the

area remaining was measured in pixels. The thresholded E-cadherin image was made into a binary mask and subtracted from the DOCK180 image to obtain DOCK180 that localizes at junctions. Junctional DOCK180 was further thresholded and pixel area was measured. For all channels, the percent area remaining after thresholding was calculated by dividing the thresholded pixel area by the raw image pixel area. Two to four images were analysed per time-point, per sample and per experiment. Three experiments were analysed. Mean, standard deviation and statistical analysis (Unpaired Student t-test assuming unequal variances) were calculated using pooled data from all

experiments (immunofluorescence) or average of the means of independent replicates (all other experiments).

3. Results

3.1. EGFR, but not other family members, co-precipitates with E-cadherin and participates in junction-dependent Rac activation

We and others have previously shown that EGFR co-precipitates with E-cadherin receptors [13] and that the EGFR kinase activity is
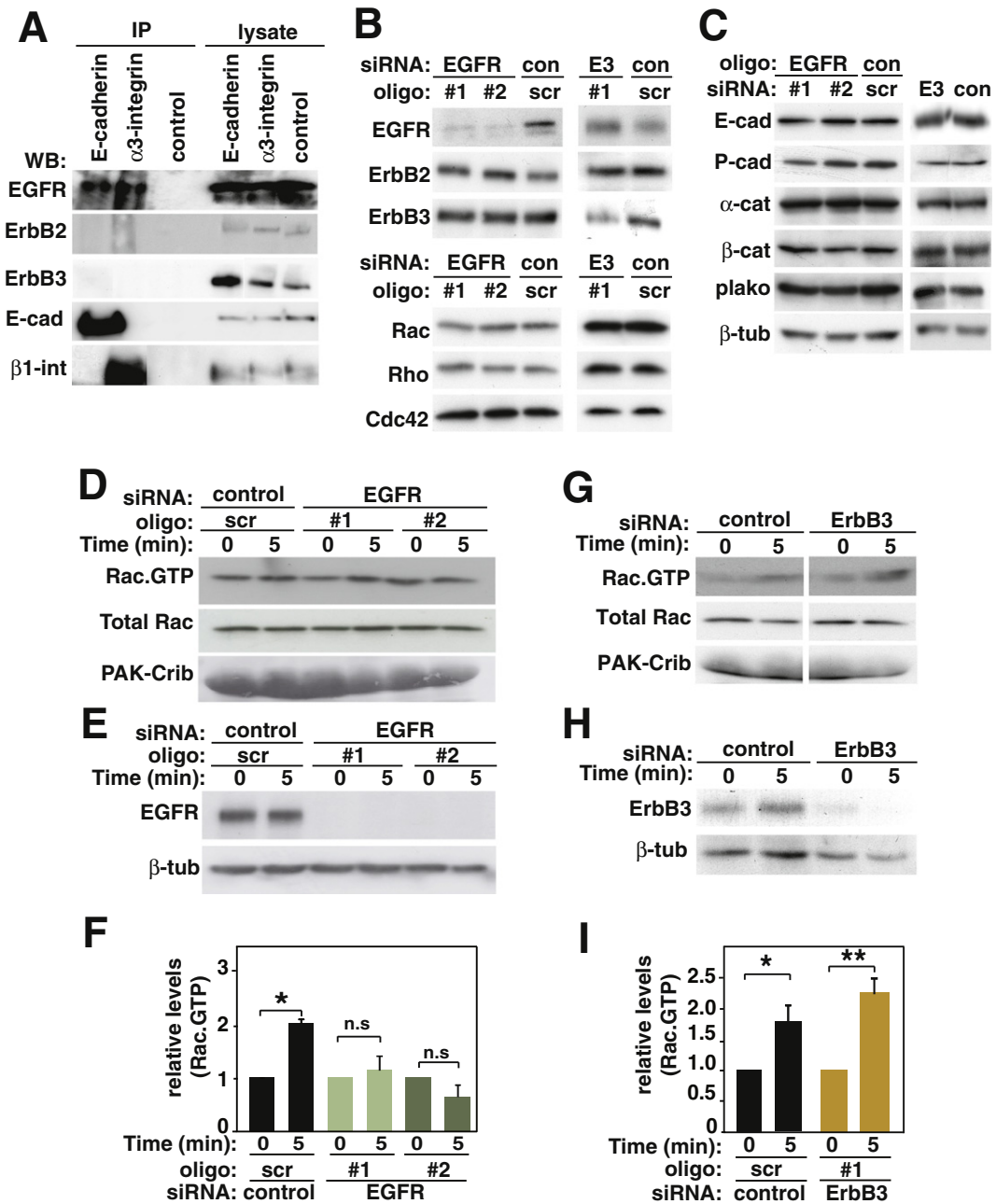

Fig. 1. EGFR, but not ErbB3, is required for cadherin-dependent Rac activation. A, Following induction of cell–cell contacts, keratinocyte lysates were immunoprecipitated with anti-E-cadherin antibodies, anti-α3-integrin (positive control) or no antibody (control) and probed with antibodies against proteins shown on the left of panels. B–C, Keratinocytes were treated with EGFR (oligo #1, oligo #2), ErbB3 (E3) or control (scr) siRNA oligos for 48 h. Equal amount of protein was separated on SDS–PAGE and probed with antibodies against proteins shown on the left of each panel. D–I, Keratinocytes were transfected with different siRNA oligos and junctions were initiated for 5 min by the addition of calcium ions and active Rac levels measured. D and G, Proteins were precipitated with GST–PAK-Crib beads (Rac·GTP) and lysates (Total Rac) were probed with anti-Rac antibodies. The amount of GST fusion protein in each sample was evaluated by Amido Black staining (PAK-Crib). E and H, Depletion of EGFR or ErbB3 are shown. Beta-tubulin is shown as a loading control. F and I, Cell–cell-adhesion-dependent Rac activation was quantified and normalised to Rac·GTP levels at time 0 (no cell–cell contacts) for each siRNA group. Data is representative of 3 independent experiments (thereafter N = 3). *, p < 0.05; **, p < 0.005; n.s., non-significant.

required for Rac activation following E-cadherin contact formation [8]. However, EGFR family members can form homo- and heterodimers, and it is not clear whether all family members are able to interact with E-cadherin. Out of the four different EGFR family members, EGFR, ErbB2 and ErbB3 are expressed in normal keratinocytes [25] as confirmed in this study (Figs. 1A, S1). From these, only EGFR was able to co-precipitate with E-cadherin when cell–cell junctions are initiated for 30 min (Fig. 1A). As a positive control, α3β1 integrin also precipitated with EGFR, but not ErbB2 or ErbB3. These results indicate that, when cell–cell junctions are formed, EGFR monomers or homo-dimers, but not other EGFR family members, may co-cluster with E-cadherin.

Pharmacological inhibition of EGFR kinase activity prevents Rac activation upon junction assembly [8]; however, EGFR depletion has not been previously examined to support the specificity of Rac activation *via* this family member. To examine these points, we performed siRNA to deplete EGFR and as a control ErbB3, which does not co-precipitate with cadherin receptors. Depletion of EGFR or ErbB3 did not change the levels of different EGFR family members, Rho GTPases or proteins found in cadherin complexes (Fig. 1B–C). Upon junction formation, Rac activation was significantly increased in controls (scrambled or ErbB3 depleted; Fig. 1D, G). In the absence of EGFR, this significant change in Rac·GTP levels following cell–cell contact assembly was abolished (Fig. 1F). In contrast, when ErbB3 was depleted, the increased levels of Rac·GTP induced by junctions were similar to controls (Fig. 1G–I). Both EGFR and ErbB3 are able to activate Rac upon ligand stimulation. Yet, our data strongly indicates that, in the context of ligand-independent junction assembly, there is a specific requirement for EGFR for Rac activation and ErbB3 cannot regulate cadherin-dependent Rac activation.

Strikingly, despite defective Rac activation upon EGFR depletion, junction assembly was not completely abolished (Fig. 2A–B). However, junctions were not uniformly stable: E-cadherin staining was fragmented, not covering the whole extension of each cell–cell contact (Fig. 2A, C, D). In contrast, treatment with ErbB3 or scramble oligos did not impair cadherin localization at junctions (Fig. 2A, D). Thus, Rac signalling downstream of EGFR is necessary for junction maintenance, rather than assembly of new cell–cell contacts.

3.2. Identification of putative GEFs necessary for Rac activation

It is feasible that E-cadherin–EGFR complexes could recruit and/or activate a Rac-specific GEF directly or indirectly. We tested whether two Rac exchange factors, SOS1 and DOCK180, could activate Rac downstream of EGFR signalling following junction induction. SOS1 and DOCK180 can activate Rac *in vitro* and have been shown to regulate Rac *in vivo* downstream of distinct cellular functions [18,19].

DOCK180 RNAi in keratinocytes did not reduce SOS1 protein levels or *vice versa* (Fig. 3A–B) nor interfered with the levels of proteins in the cadherin complex or Rho small GTPases (Fig. 3C–D). When keratinocyte cell–cell contacts were initiated for 30 min, Rac·GTP levels increased significantly following treatment with control or SOS1 siRNA oligos (Fig. 3G–H). However, cells with reduced DOCK180 protein had no significant increase in active Rac levels (Fig. 3E–F). Thus, DOCK180, but not SOS1, is likely to be one of the GEFs responsible for Rac activation by new junctions in keratinocytes. Our results suggest a selectivity among distinct GEFs *via* which Rac is activated upon engagement of cadherin receptors at cell–cell contacts. Our data is also supported by a previous report on depletion of ELMO2 in MDCK and indicates that DOCK180 may be important for junction signalling in cell types other than keratinocytes. DOCK180 was not formally tested in MDCK cells because of toxicity caused by its depletion. However, in contrast to MDCK cells [7], DOCK180 RNAi did not reduce keratinocyte cell viability (data not shown) and could be tested herein.

3.3. Mechanisms downstream of DOCK180 and EGFR to stabilise cadherins at junctions are distinct

We tested whether DOCK180 is found at keratinocyte cell–cell contacts in a time course from early to more mature contacts. E-cadherin receptors are recruited to newly formed keratinocyte junctions as early as 5 min and by 30 min, junction assembly, cytoskeletal reorganization and cell polarization are mostly complete [26,27]. In the absence of cell adhesion, endogenous DOCK180 was found dispersed in the cytoplasm (Fig. 4A). Upon addition of calcium ions, a pool of DOCK180 was clearly recruited to the junctional region (Fig. 4A–B). This re-localization
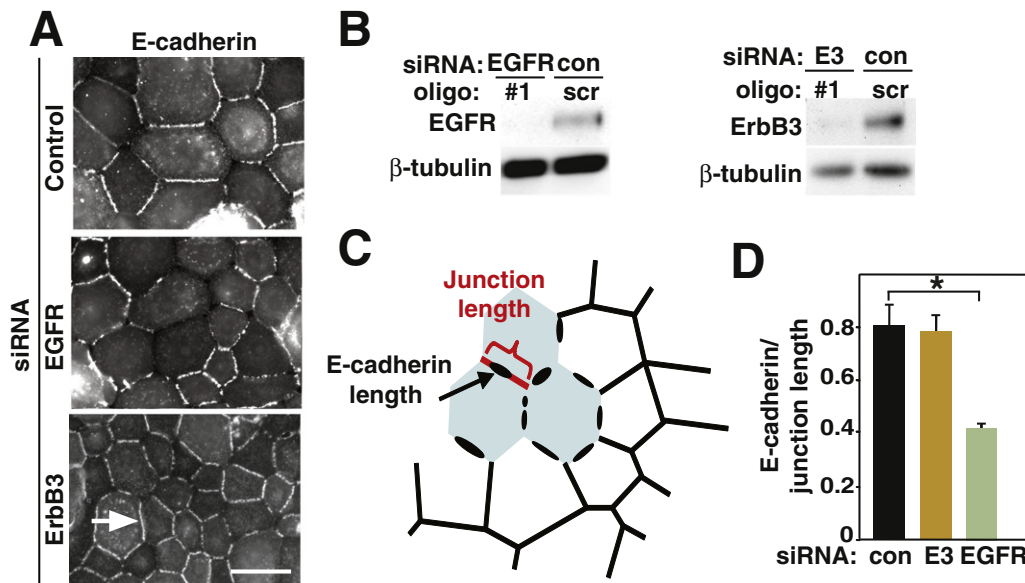

Fig. 2. EGFR depletion perturbs cell–cell contacts. A, Keratinocytes treated with different siRNA oligos were induced to form cell–cell contacts for 30 min, fixed and stained for E-cadherin. Arrows show E-cadherin at junctions; arrowhead points to perturbed junction. B, Western blot shows knockdown of EGFR or ErbB3 (E3). C, Method for quantification of junction disruption. The length of cell–cell contacts (corner to corner) and the length of E-cadherin staining were obtained for each junction and expressed as a ratio (control junctions = 1). D, Disruption of E-cadherin localization by depletion of EGFR. ErbB3 (E3) and non-targeting oligos (con) were used as controls. N = 3 or N = 2 (ErbB3); about 150 junctions quantified in each replicate. Scale bar = 40 μm. *, p < 0.05.
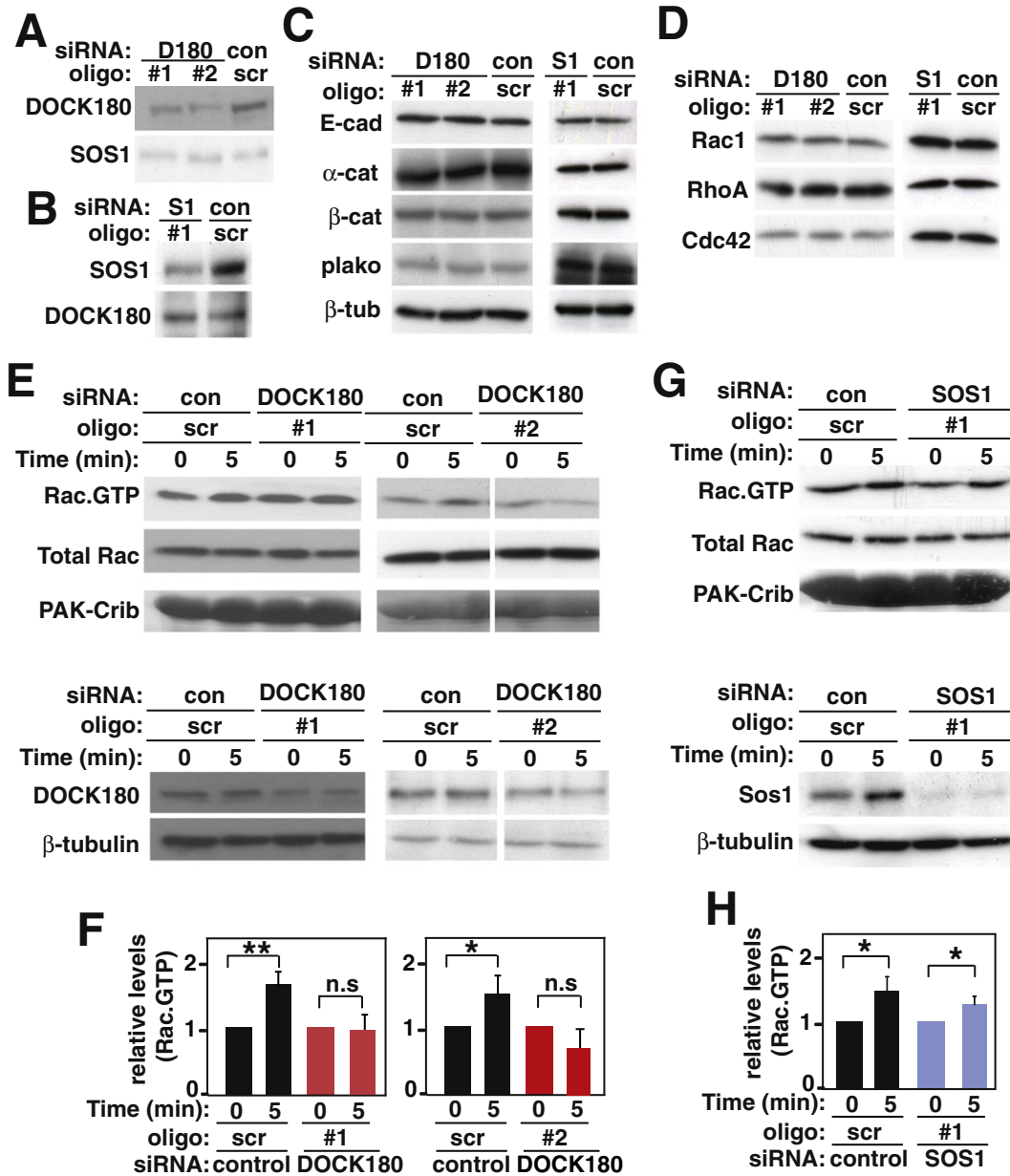
Fig. 3. DOCK180 is required for E-cadherin-mediated Rac activation. Cells were treated with DOCK180 (oligo #1, oligo #2), SOS1 (oligo #1) or control (scr) siRNA oligos. A–D, Lysates were prepared and used to determine efficiency of knockdown (A, B) and its specificity for DOCK180 or SOS1 depletion (C, D). Equal amounts of protein were separated on SDS–PAGE and probed with antibodies against proteins as shown on the left of each panel. E–H, Activation levels of Rac after 5 min of cell–cell contact formation. Pull down using GST–PAK-Crib was performed to precipitate active Rac (Rac·GTP). E and G, Samples were probed with antibodies listed on the left of panels. Fusion proteins in each sample were evaluated by Amido black staining (PAK-Crib). Depletion of DOCK180 and SOS1 is shown and β-tubulin used as a loading control. F and H, Cell–cell adhesion-dependent Rac activation. Rac·GTP was quantified at different time points and normalised to Rac·GTP levels at time 0 (no cell–cell contacts) for each siRNA group (scr, DOCK180 or SOS1). N = 3; *, p < 0.03; **, p < 0.003; n.s., non-significant.

was significant and increased with time. This result is in contrast to the transient DOCK180 recruitment to MDCK new junctions (2.5 h), but absent at more established contacts (5 h) [7].

In addition, clustering of cadherin receptors is sufficient to recruit DOCK180 to cadherins without a need for other adhesion receptors found at junctions (Fig. 4C). Counterintuitively we found that DOCK180 localisation on cadherin clusters was not perturbed in the absence of EGFR (Fig. 4D). Thus, even though Rac activation by junctions requires both EGFR and DOCK180 (Figs. 1 and 3), the ability of DOCK180 to be enriched at cell–cell contacts is EGFR-independent.

This potential association between DOCK180-E-cadherin receptors was confirmed by biochemical experiments. In the absence of junctions, DOCK180 was already found in complexes with GST–E-cadherin tail,

and remained associated for up to 30 min of junction assembly (Fig. 4E). Depletion of EGFR did not alter the interaction of DOCK180-cadherin complexes (Fig. 4F). We concluded that DOCK180 is associated with cadherin receptors at steady-state and this could explain the reason why further DOCK180 recruitment to cell–cell contacts is not modulated by EGFR.

3.4. DOCK180 and EGFR regulate distinct cellular processes

DOCK180 and EGFR are necessary for Rac activation downstream of junction assembly (Figs. 1 and 3). Yet, although DOCK180 is recruited to E-cadherin clusters in the absence of EGFR, it is feasible that EGFR signalling could activate DOCK180 locally. If the latter process occurs,
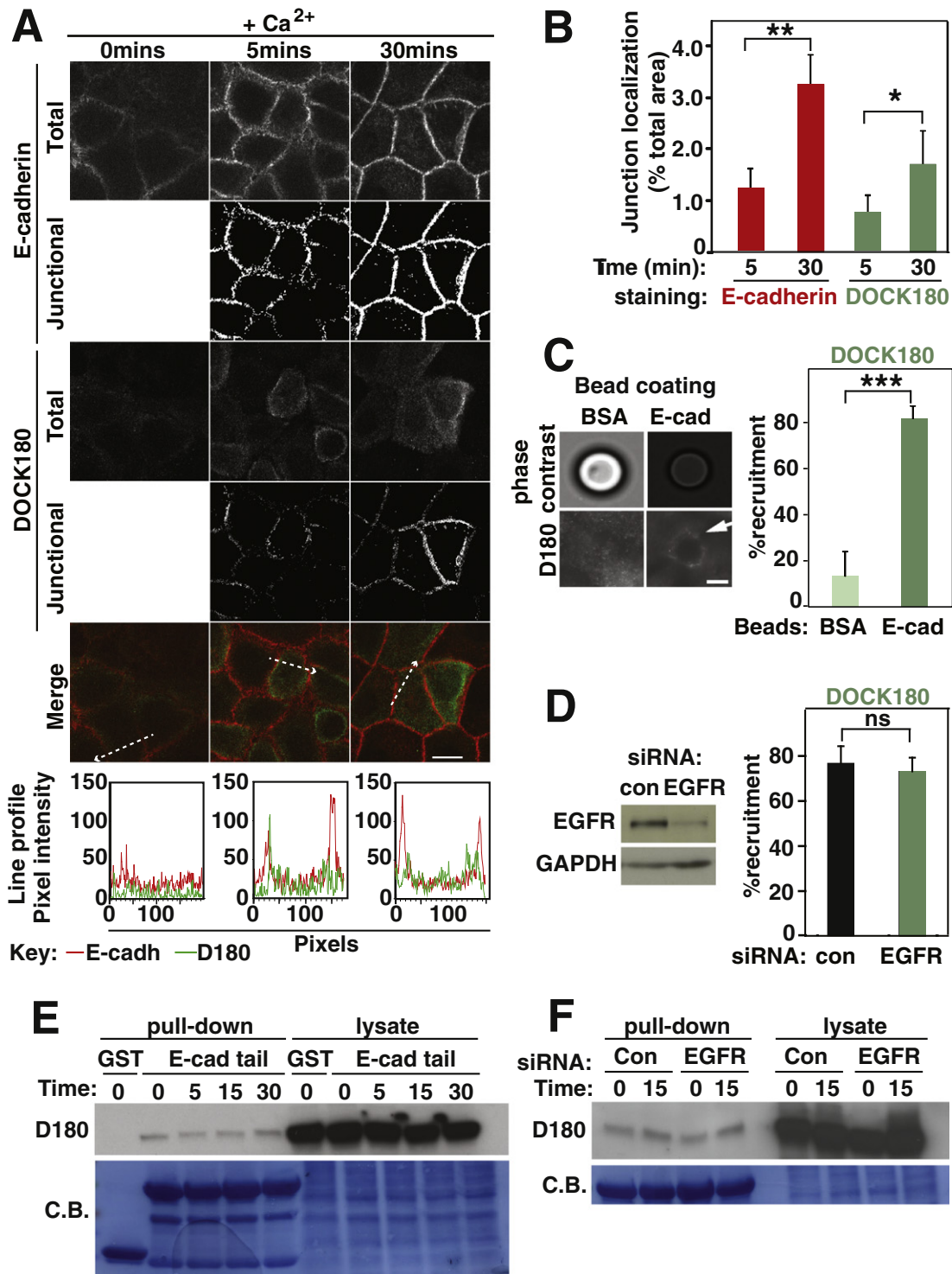
Fig. 4. DOCK180 is recruited to E-cadherin complexes. A, Cell–cell contacts were initiated for 5 or 30 min and cells stained for endogenous E-cadherin or DOCK180. Bottom graphs show intensity profile of E-cadherin or DOCK180 by line scan at sites indicated by dashed arrows. B, E-cadherin and DOCK180 staining levels in the junctional area were quantified as a percentage of the total area in the image. C–D, DOCK180 recruitment was assessed to beads coated with anti-E-cadherin or BSA in normal keratinocytes (C) or cells treated with control (con) or EGFR RNAi (D). Beads were added onto cells for 30 min, washed and stained for endogenous DOCK180. E, GST–E-cadherin tail beads were incubated with keratinocyte lysates prepared after a time course of junction induction. Precipitates were probed with anti-DOCK180 antibody (D180); fusion proteins are shown by Comassie Blue (C.B.). F, Following depletion of EGFR, cells were induced to assemble contacts for 15 min and processed as described in E.

one prediction is that DOCK180 and EGFR should contribute to the regulation of similar cellular events during junction stabilisation.

We investigated the above point further and tested whether DOCK180 and EGFR are both necessary for actin recruitment to cadherin clusters (Fig. 5), a process that is inhibited by dominant-negative Rac in

keratinocytes [23]. Similarly to Rac inhibition, EGFR RNAi caused a partial, but significant reduction of cadherin-dependent actin recruitment (Fig. 5A–B). Strikingly, actin recruitment to clustered cadherin receptors was not perturbed by DOCK180 depletion (Fig. 5A–B). These results suggest that, following junction assembly, DOCK180 may regulate
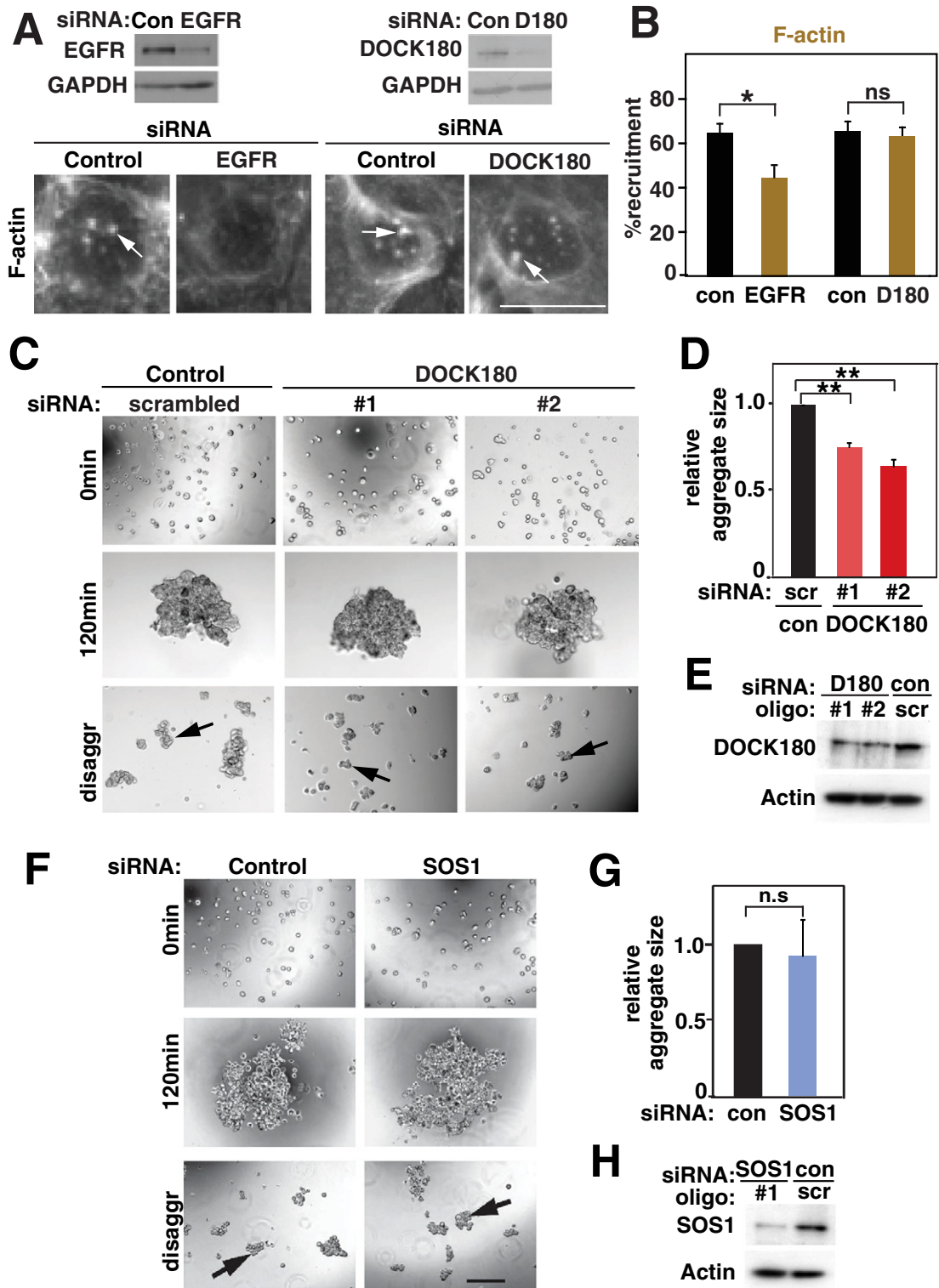
Fig. 5. DOCK180 stabilizes pre-formed junctions. Keratinocytes were treated with siRNA oligos as labelled in the different panels and processed to determine actin recruitment to clustered E-cadherin (A–B) or aggregation assays (C–H). A–B, Following depletion of EGFR or DOCK180, cells were incubated with antibody-coated latex beads for 15 min and stained for F-actin. F-actin clusters are shown by arrows (A) and the proportion of attached beads containing F-actin clusters was quantified and expressed relative to controls (B). C–H, Keratinocytes depleted of DOCK180 (C–E) or SOS1 (F–H) were trypsinised and allowed to aggregate in suspension in the presence of calcium ions for 120 min, followed by disaggregation. C and F, Phase contrast images of initial samples, following aggregation for 120 min and after mechanical stress (disaggr). Black arrows point to aggregates. D and G, Relative sizes of all remaining aggregates were measured and shown in comparison to controls (arbitrarily set as 1). E and H, Confirmation of depletion efficiency following DOCK180 or SOS1 RNAi. Equal amounts of protein were loaded, actin used as a loading control. N = 3. Scale bar = 15 μm (A) or 200 μm (C, F). *, p < 0.03; **, p < 0.0003; ***, p < 0.001; n.s., non-significant.

alternative Rac functions other than actin remodelling. In contrast, EGFR seems to be upstream of Rac in driving actin recruitment to junctions.

The question raised by the above experiments is, if actin recruitment occurs independently of DOCK180 function, does DOCK180 indeed stabilise E-cadherin contacts? Depletion of DOCK180 did not alter the

levels of E-cadherin at newly-formed junctions after 30 min (Fig. S2). This phenotype is clearly distinct from fragmented junctions observed following EGFR RNAi (Fig. 2) or the pattern of junction disruption previously reported in MDCK cells [7]. While the localization of E-cadherin at junctions is not perturbed, we tested whether junctions had similar functionality in the absence of DOCK180. The specific aspect we investigated was resistance of cell aggregates to mechanical stress. Consistent with our above results, upon DOCK180 depletion, formation of aggregates in hanging drops was not perturbed (Fig. 5C, 120 min). However, upon trituration of pre-formed aggregates, the remaining aggregates in DOCK180-depleted keratinocytes were 30–40% smaller than controls (Fig. 5C–E). In contrast, SOS1 depletion had no effect on aggregate formation or stability (Fig. 5F–H). Our results suggest that DOCK180 is not necessary for assembly of cell–cell contacts, but previously formed contacts are more fragile during mechanical stress.

4. Discussion

We present strong evidence of two pathways *via* which junctions can activate Rac in keratinocytes: EGFR and DOCK180. Perturbing the function of either protein by RNAi prevented junction-dependent Rac activation. In spite of this similar phenotype and the role of DOCK180 downstream of EGFR signalling during migration, our data indicate that DOCK180 and EGFR deploy distinct mechanisms to modulate keratinocyte cell–cell adhesion. We surmise that, in the same cell type, different GEFs are necessary for the repertoire of Rac functions that collectively stabilise E-cadherin at junctions.

We speculated that co-clustering of EGFR and E-cadherin at junctions [13] could trigger specific signalling to activate Rac locally. In the absence of EGFR, Rac cannot be activated by junction assembly, a phenotype that is not observed following ErbB3 RNAi. This result is consistent with our findings that EGFR is the only family member able to co-precipitate with E-cadherin. Although both EGFR and ErbB3 are known to activate Rac upon ligand binding, our data demonstrate that, downstream of E-cadherin engagement, the activation of Rac is specifically EGFR-dependent.

To identify potential Rac regulators that mediate the above responses, DOCK180 and SOS1 were investigated because of their *in vitro* ability to activate Rac and their participation in growth factor receptor signalling pathways. Similar to EGFR, depletion of DOCK180, but not SOS1, also prevents Rac activation by newly formed junctions. The involvement of DOCK180 in the cadherin–Rac interplay is consistent with our current thinking [3] that a specific stimulus will selectively engage a particular GEF at junctions to drive Rac activation locally and regulate a specific cellular event. The GEF selectivity guided by different stimuli would thus enable tight spatial and temporal control of Rac activation by specific molecular players.

DOCK180 appears to be associated with E-cadherin cytoplasmic tail at steady state and thus further enrichment of DOCK180 at keratinocyte cell–cell contacts occurs independently of EGFR. There is a formal possibility that, once DOCK180 is at cell–cell contacts, it could be activated indirectly by EGFR, and thus regulate some, but not all EGFR-dependent phenotypes. For example, growth factor receptor-dependent phosphorylation has been shown for DOCK180 phosphorylation by EGFRvIII in glioblastoma cells [28] or DOCK7 by ErbB2–ErbB3 heterodimers in Schwann cells [29].

However, while both EGFR and DOCK180 are required for cadherin-dependent Rac activation, we think it is unlikely that DOCK180 operates downstream of EGFR in the context of E-cadherin stabilisation. First, the defects on cell–cell contacts caused by depletion of either protein differ considerably. EGFR RNAi leads to junction fragmentation, while reduced levels of DOCK180 do not interfere with E-cadherin distribution to new adhesion sites. Yet, DOCK180 is important to provide resistance of keratinocyte aggregates to mechanical stress.

Second, mechanistically, EGFR partially regulates actin recruitment to clustered E-cadherin complexes. Surprisingly, DOCK180 does not

seem to participate in this process, even though it is well established that actin remodelling at junctions requires Rac function [2,3,30]. It is feasible that the protection to mechanical stress at junctions provided by DOCK180 may involve alternative functions of Rac, such as regulation of contraction or reinforcement of contacts by trafficking.

Third, GEF recruitment to and residence at junctions may differ depending on the cell type. In MDCK cells, DOCK180 transiently accumulates at early contacts [7], while in keratinocytes, DOCK180 remains associated with cadherin complexes for up to 30 min of junction induction. At this time point in keratinocytes, the cadherin recruitment to junctions, cytoskeletal remodelling and polarization are mostly accomplished [26,31]. The distinct pattern of DOCK180 recruitment between MDCK and keratinocytes suggests a differential regulation according to the cellular context. The latter is also reflected on the distinct outcome of DOCK180 depletion: inhibition of cadherin localization at early junctions is observed in MDCK [7], but not in keratinocytes (this work). We surmise that, upon contact initiation, distinct signalling, kinetics and membrane dynamics may account for the regulatory differences among cell types.

Clearly, further work is necessary to define mechanistically how junction assembly recruits and regulates DOCK180 in keratinocytes and the potential impact on the modulation of resistance of junctions to mechanical stress. Our results are in agreement with the ability of a distinct DOCK180 family member, DOCK3, to regulate cell–cell adhesion (also known as Modifier of Cell Adhesion or MOCA). Expression of DOCK3 increases aggregation of neuronal cell lines specifically *via* N-cadherin, but not E-cadherin [32]. DOCK3 up-regulation of N-cadherin levels may regulate the cadherin switch programme during tumour progression [33] and correlates with increased tumour invasiveness in head and neck tumour patients [34].

5. Conclusions

We uncover here two distinct pathways ways *via* which Rac can be activated by junctions in the same cell type, and their different cellular outcomes to enable stable adhesion. EGFR is the only family member able to initiate Rac signalling downstream of E-cadherin. Although DOCK180 shares this ability with EGFR, these molecules regulate distinct Rac-dependent processes: resistance to mechanical stress and actin recruitment to cadherin clusters, respectively. Thus, DOCK180 is unlikely to operate downstream of EGFR signalling triggered by junctions. We demonstrate a direct role of DOCK180 in Rac activation and the mechanical support of cadherin mature contacts. Finally, our data strengthen the notion that DOCK family members emerge as master regulators of cell–cell adhesion events in different cellular contexts.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cellsig.2015.04.014.

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