Rac1 meets ubiquitin: New insights in Rac1 signalling
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Chapter 6

A Rac1-Nedd4-1- Dishevelled-1 axis regulates maturation of cell-cell contact

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Summary

The formation of homotypic cell-cell contacts regulates the integrity of endothelial and epithelial monolayers and is essential for tissue morphogenesis. Conversely, loss of cell-cell contacts is a hallmark of cellular transformation and tumour cell metastasis. The Rho-GTPase Rac1 promotes actin polymerization and membrane protrusion that mediate the initiation and subsequent maturation of intercellular contacts. Here we report that activated Rac1 associates to the E3 ubiquitin ligase Nedd4-1 and that Rac1 stimulates the formation of cell-cell contact through a pathway in which Nedd4-1 acts as a central regulator. Depletion of Nedd4-1 reduced transepithelial electrical resistance (TER) and disrupted the localisation of adherens as well as tight junction markers. In addition, Nedd4-1-expressing cells show decreased levels of stable, acetylated microtubules (MTs), which alters the directionality of MT-linked traffic and may interfere with maturation of cell-cell contacts. The adapter protein Dishevelled-1 (DVL-1) is a regulator of MT stability and harbours a PPXY motif, a feature of Nedd4-1 substrates. Nedd4-1 promoted ubiquitylation and degradation of DVL-1 which reduced MT-acetylation. Conversely, induction of MT-acetylation impaired junctional maturation. Thus, we propose that Nedd4-1, in conjunction with activated Rac1, regulates DVL-1 expression and MT-stability to promote the maturation of cell-cell contacts.

Results

A mass spectrometry-based screen for proteins, interacting with a peptide resembling the Rac1 C-terminus, revealed that the HECT (Homologous to the E6-AP Carboxyl Terminus) E3 ubiquitin ligase Nedd4-1 is a Rac1-binding protein (Supplementary Information Fig. S1a). Additional pull-down experiments showed that endogenous Nedd4-1 binds most efficiently to the Rac1 C-terminus whereas binding to the C-termini of other RhoGTPases is either weak (e.g. Rac3) or undetectable (Fig. 1a). A GST-Rac1ΔC fusion protein, which lacks the Rac1 C-terminus, showed a reduced binding to Nedd4-1 as compared to full-length GST-Rac1 indicating that the Rac1 C-terminus is both necessary and sufficient for
Nedd4-1 binding (Fig. 1a,b). Use of peptides encoding mutations within the Rac1 C-terminal domain showed that the polybasic region, rather than the proline-stretch, mediates Rac1 binding to Nedd4-1 (Supplementary Information, Fig. S1b).

**Figure 1** Nedd4-1 binds to Rac1 and regulates cell-cell junctions. (a) Biotinylated peptides encoding the C-terminal hypervariable domains of different RhoGTPases were assayed for binding to endogenous Nedd4-1 in streptavidin-based peptide pull-down assays. (b) Bacterially expressed and purified GST-tagged Rac1 and Rac1ΔC, which lacked the C-terminal region, were examined for binding to HA-tagged Nedd4-1. (c) Immuno-precipitated myc-tagged inactive (N17T) and active (Q61L) Rac1 were analysed by SDS-PAGE and western blotting for binding to co-transfected HA-Nedd4-1 in HeLa cells. (d) HeLa cells, transfected with myc-Rac1Q61L were fixed and stained for endogenous Nedd4-1 and myc. Subsequent z-stacks along the indicated dashed line were analysed for co-localisation of Nedd4-1 with active Rac1. Arrows indicate co-localisation of Nedd4-1 with active Rac1 at the cell-cell junctions and membrane ruffles, with arrows at the corresponding positions in the XZ image. (Scale bar, 10 μm) (e) HeLa cells were fixed and immuno-stained for endogenous Nedd4-1 and β-catenin. Arrows indicate the absence of Nedd4-1 at jagged, immature junctions in contrast to its presence at mature junctions (Scale bar, 20 μm) (f,g) H292 lung epithelial cells were fixed 48hrs following transfection with indicated lentivirus control and Nedd4-1 shRNAs and immuno-stained for (f) endogenous β-catenin and γ-catenin as markers for adherens junctions and for endogenous ZO-1 (g) as a marker for tight junctions. X/Z images were derived from Z-stack sections along the dashed lines. (Scale bar, 15 μm) (g) Arrows in g indicate ZO-1 in tight junctions in control H292 cells which is lost following depletion of Nedd4-1.
Finally, immunoprecipitation of inactive (Rac1N17T) and active (Rac1Q61L) mutants of Rac1, expressed in HeLa cells, showed that Nedd4-1 binds most efficiently to activated Rac1 (Fig. 1c). Together, these results identify Nedd4-1 as a novel Rac1-effector that interacts with Rac1 through binding of the Rac1 hypervariable C-terminal domain. Subsequent analysis by confocal microscopy revealed that endogenous Nedd4-1 co-localises with Rac1Q61L at cell-cell contacts and peripheral membrane ruffles in HeLa cells (Fig. 1d). Activated Rac1 also localises to Focal Adhesions (FAs)\(^1\), but Nedd4-1 could not be detected at these sites (Fig. 1d). Interestingly, Nedd4-1 was absent from immature junctions characterised by a jagged \(\beta\)-catenin staining, but co-localized with \(\beta\)-catenin at mature, well organized cell-cell contacts (Fig. 1e). To define the function of Nedd4-1 at cellular junctions, we used lentiviral shRNAs to deplete endogenous Nedd4-1 in HeLa cells and in H292 lung epithelial cells (Supplementary Information, Fig. S2a). Reduction of Nedd4-1 expression notably affected the cell-cell junctions in both epithelial cell types, as shown by the disturbed integrity of adherens and tight junctions, identified by \(\beta\)- and \(\gamma\)-catenin and by ZO-I, respectively (Fig. 1f,g, Supplementary Information, Fig. S2b). Re-expression of shRNA-resistant, murine Nedd4-1 rescued junctional integrity as revealed by restored distribution of \(\beta\)-catenin (Supplementary Information, Fig. S3a).

To further establish the relevance of Nedd4-1 for epithelial integrity, Nedd4-1-depleted lung epithelial cells were seeded on gold electrodes, and TER was recorded by Electrical Cell-substrate Impedance Sensing (ECIS) in real time\(^1\). Nedd4-1 shRNA-expressing cells showed reduced epithelial monolayer integrity as compared to the controls (Fig. 2a). To analyse this putative defect in junctional maturation further, we used a calcium switch assay\(^2\), that allows analysis of loss and formation of cell-cell contact upon EGTA-mediated depletion and re-addition of extracellular calcium. Calcium depletion by EGTA resulted in a loss of Nedd4-1 accumulation at intercellular junctions of H292 lung epithelial cells (Fig. 2b). One hour after EGTA washout and re-addition of calcium, accumulation of Nedd4-1 at cell-cell contacts was not detectable. However, at 5 hours after calcium re-addition,
Nedd4-1 concentrated at intercellular junctions, confirming the notion that Nedd4-1 localizes at mature cell-cell contacts (Fig. 2b).

Nedd4-1-mediated promotion of junction maturation was next examined by analysing TER of HeLa cells expressing GFP as a control or full-length human GFP-Nedd4-1.

Following the initial spreading phase (2-4 hours), expression of GFP-Nedd4-1 significantly increased TER as compared to the GFP-expressing cells,
indicative for strong cell-cell contact and junctional maturation (Fig. 2c). Together, these findings show that Nedd4-1, in conjunction with activated Rac1\textsuperscript{13}, promotes the formation of epithelial cell-cell contacts. Because cell-cell adhesion is regulated by cytoskeletal dynamics, we examined effects of Nedd4-1 expression on the actin and microtubule cytoskeleton. We found no major changes in the distribution and organisation of F-actin in the absence or presence of Nedd4-1 (not shown). We therefore examined the organisation of microtubules (MTs) in more detail because MTs contribute to the maturation of cell-cell contacts by facilitating transport of junctional proteins such as N-cadherin and connexin-43\textsuperscript{5,7}. As deduced from confocal imaging, MTs co-localize with β-catenin at cell-cell contacts (Supplementary Information, Fig. S3b). Interestingly, we found that GFP-Nedd4-1-expressing H292 cells showed a marked decrease in the levels of acetylated MTs (Fig 2d). Posttranslational modification of MTs by acetylation is a highly conserved process comprising conjugation of an acetyl moiety at lysine residue K40 on the luminal side of α-tubulin\textsuperscript{14}. Increased levels of acetylated (Ac)-MTs mark an increase in MT stability, as deduced from resistance to MT-destabilising compounds such as nocodazole (NOC)\textsuperscript{14}. Ac-MTs, as compared to non-Ac-MTs, show different binding affinities for MT-linked motor proteins and altered directionality of motor protein-mediated vesicular traffic\textsuperscript{8,15}. As a result, increased MT stability could well interfere with the maturation of cell-cell junctions. We therefore hypothesised that Nedd4-1-mediated downregulation of MT stability promotes cell-cell junction maturation. To test this hypothesis, candidate proteins that are known to affect MT stability were screened for a PPXY or RXXQE motif, a feature of Nedd4-1 substrates\textsuperscript{9}. This search identified dishevelled-1 (DVL-1) as a potential substrate for Nedd4-1-mediated ubiquitylation. DVL-1 contains an evolutionary conserved PPXY motif downstream of its DEP (dishevelled, Egl-10 and pleckstrin homology)-domain (Fig. 3a). In addition, DVL-1 was previously found to enhance MT stability\textsuperscript{10}, suggesting it could serve as a target for Nedd4-1-regulated junctional maturation.

To test whether Nedd4-1 regulates DVL-1 stability, the protein levels of endogenous DVL-1 were examined in GFP and GFP-Nedd4-1-transfected HeLa cells treated for 6 hrs with cycloheximide to block new DVL-1 protein synthesis.
Expression of Nedd4-1 reduced the expression of endogenous DVL-1, but not DVL-2 or DVL-3 (Fig. 3a).

Figure 3 Nedd4-1 regulates ubiquitylation and proteasomal degradation of DVL-1. (a) Sequence alignment of DVL family members identified a conserved PPXY motif in DVL-1, identifying DVL-1 as a potential Nedd4-1 substrate. Protein expression levels of endogenous DVL-1, -2 and -3 were assessed in control and Nedd4-1 transfected HeLa cells, treated for 6hrs with CHX (10μg/ml), and assessed by quantification of western blots (n=3+/±s.e.m). (b) Protein stability of DVL family members were assessed in H292 cells 48 hrs after transfection with control or Nedd4-1 lentiviral shRNAs followed by 6hr incubation with CHX (10μg/ml). (c) Ubiquitylation of cotransfected DVL-1-HA was assessed in empty vector, Nedd4-1 or Nedd4-2 transfected HeLa cells, that were treated for 6hrs with MG132 (25μM) or chloroquine (100μM) prior to cell lysis and analysis of ubiquitylation. (d) Overview of the different DVL-1 truncation mutants used to map the Nedd4-1-binding region and subsequent ubiquitylation by Nedd4-1. (e) Nedd4-1-driven ubiquitylation of DVL-1 was analysed using different DVL-1-truncation mutants. (f) Bacterially expressed and purified, GST-Nedd4-1 was tested for binding to full-length and the indicated truncated versions of DVL-1. (g) GST-tagged DVL-1 was tested for binding to the C2 domain or the WW-region of Nedd4-1. (h) The requirement of the DVL-1 C-terminal region, downstream of the DEP domain, for Nedd4-1-driven ubiquitylation was further examined using DVL-1 truncation constructs. (i) Lysine residues K218,K220, K225 in DVL-1 were mutated to arginine to test their role in Nedd4-1-mediated ubiquitylation.
Conversely, cycloheximide-treated cells, which were depleted of Nedd4-1 by shRNA expression, showed an increase in the levels of endogenous DVL-1, but not DVL-2 or DVL-3, in both H292 and HeLa cells (Fig 3b, Supplementary Information, Fig. S4a). Of note, the increased protein levels of endogenous DVL-1 in Nedd4-1-depleted cells did not induce canonical Wnt signalling since the protein levels of β- and γ-catenin remained unaffected and did not show any nuclear accumulation (Fig. 1f, 3b). Next, ubiquitylation of DVL-1 by Nedd4-1 was analysed and compared with that by Nedd4-2, a closely related homologue9. Expression of Nedd4-1, but not of Nedd4-2, stimulated the ubiquitylation of full-length HA-DVL-1 (Fig. 3c). Moreover, inhibition of the proteasome with MG132 significantly increased Nedd4-1-mediated ubiquitylation of DVL-1 (Fig. 3c). In contrast, ubiquitylation of DVL-1 remained unaffected upon inhibition of lysosomal degradation (Fig. 3c), which was recently implicated to control the levels of DVL-216,17.

DVL proteins contain three characteristic domains; i.e. an N-terminal DIX (Dishevelled-Axin)-domain, involved in (self)-oligomerisation, a central PDZ (PSD95, DlgA, ZO-1)-domain and a C-terminal DEP-domain, both involved in mediating protein-protein interactions, whereas the DEP domain has also been reported to bind lipids18. To map the Nedd4-1-Dvl-1 interaction and to define the regions, required for DVL-1 ubiquitylation, we used a series of deletion and truncation mutants of DVL-1, schematically depicted in Fig. 3d. Deletion of DVL-1 DIX (ΔDIX) or PDZ (ΔPDZ) domains did not impair Nedd4-1-mediated ubiquitylation of DVL-1 (Fig. 3e). In contrast, truncation of the DEP-C-terminal region (ΔDEP-tail) completely blocked Nedd4-1-mediated ubiquitylation of DVL-1 (Fig. 3e). This could be explained by the lack of binding between Nedd4-1 and the DVL-1 protein lacking the DEP-domain and C-terminal region (ΔDEP-tail), as shown by GST-fusion protein pull-down experiments (Fig. 3f). Expression of a partial DVL-1 protein comprising the DEP-C-terminal region (DEP-tail, Fig. 3d) confirmed that this region is sufficient for binding to Nedd4-1 (Fig. 3f). Conversely, using Nedd4-1 truncation constructs we found that the WW-region of Nedd4-1,
previously reported to be required for substrate recognition\(^9\), was sufficient for binding to GST-tagged full-length DVL-1 (Fig. 3g).

To determine which regions are required for the ubiquitylation by Nedd4-1, we used deletion and point mutants within DVL-1. Deletion of the DEP domain and the adjacent C-terminal region completely abrogated Nedd4-1-dependent ubiquitylation (Fig. 3e), despite the fact that this region is sufficient to bind Nedd4-1 (Fig. 3f). A construct lacking the C-terminus, but including the DEP domain (DVL-1-Δtail) was efficiently ubiquitylated by Nedd4-1 in contrast to a DVL-1 protein lacking both the DEP-domain and adjacent C-terminal portion (Fig. 3h). Point mutations within the PPXY motif showed that this motif also contributes to efficient ubiquitylation by Nedd4-1 (Supplementary Information, Fig. S4b) These data show that the DEP domain, in conjunction with the PPXY motif, is required for efficient ubiquitylation of DVL-1 by Nedd4-1.

Although the DVL-1 DEP-C-terminal region can associate with Nedd4-1 (Fig. 3f), this portion of the protein was not sufficient for ubiquitylation by Nedd4-1 (Fig. 3h). Therefore, we reasoned that neither the DEP C-terminal region, nor the DIX and PDZ domains (Fig. 3e) encode the lysine residue(s) required for the ubiquitylation of DVL-1. Consequently, the three remaining conserved lysine residues (K118, K220, K225) C-terminal to the DIX domain are the most likely target sites for Nedd4-1-mediated ubiquitylation of DVL-1. Removal of the region in DVL-1 (DVL1 PDZ-tail, Fig. 3d) as well as substitution of these three lysines by arginines in the full length DVL-1 protein (DVL-1-3KR) showed that these residues allow efficient ubiquitylation of DVL-1 by Nedd4-1 (Fig. 3i). In summary, these data suggest that Nedd4-1 binds DVL-1 through the DEP domain and ubiquitylates a lysine-rich region between the DIX and PDZ-domain, resulting in proteasome-dependent degradation of DVL-1.

Ectopic expression of DVL-1-HA in HeLa cells showed that DVL-1 accumulated in puncta throughout the cytoplasm (Fig. 4a), in line with earlier observations\(^{19}\). DVL-1 co-localised with Nedd4-1 throughout the cytoplasm and at β-catenin-positive cell-cell contacts (Fig. 4a). To test whether Nedd4-1 localisation at cell-cell junctions was essential for DVL-1 ubiquitylation, we constructed a
Nedd4-1 mutant lacking the C2 domain (ΔC2-Nedd4-1), which regulates Nedd4-1 translocation to the plasma membrane\(^20\). This mutant did not localise to cell-cell contacts (Fig. 4b). Moreover, the ΔC2-Nedd4-1 protein, similar as the inactive C867S-Nedd4-1\(^9\), strongly reduced Nedd4-1-mediated DVL-1 ubiquitylation (Fig. 4c), even though ΔC2-Nedd4-1 is considered to be constitutively active because the C2 domain is auto-inhibitory\(^21\).

Figure 4  Nedd4-1 down-regulates DVL-1 at cell-cell junctions. (a) Co-localisation of GFP-Nedd4-1 with HA-DVL-1 was examined in H292 cells (Scale bar, 10 μm). Arrows in magnifications indicate Nedd4-1 co-localizing with DVL-1 at β-catenin-positive cell-cell junctions. (b) Subcellular distribution of GFP-ΔC2-Nedd4-1 in H292 cells reveals its absence at cell-cell contacts as further indicated in the XZ images, derived from a section corresponding with the dashed line. Arrows indicate corresponding cell-cell contact in XY and XZ images. (c) Ubiquitylation of DVL-1 by Nedd4-1 lacking the C2 domain was lost as compared to its ubiquitylation by full-length Nedd4-1. (d) Regulation by Rac1 of Nedd4-1-dependent ubiquitylation of DVL-1 was analysed using inactive (N17T) and active (Q61L) Rac1 constructs. (e) Reconstitution of cell-cell junctions, immunostained for β-catenin, in H292 cells overexpressing DVL-1-GFP after a calcium switch assay and analysed by confocal microscopy. Profile scan analysis was used to visualize the distribution of β-catenin in control and DVL-1-GFP expressing cells. Arrows in images indicate the cell-cell contacts and match with the position in the intensity line graphs (Scale bar, 10 μm).

These results suggest that, although the Nedd4-1 C2 domain does not mediate binding to DVL-1 (Fig. 3g), it does regulate the translocation of Nedd4-1 towards cell-cell contacts, which is required for DVL-1 ubiquitylation. This notion
is in good agreement with our finding that the ubiquitylation by Nedd4-1 requires the DVL-1 DEP domain (Fig. 3e), as this region mediates the association of DVL-1 with the plasma membrane. In line with these findings, NKD2, a negative regulator of DVL signalling, was recently found to stimulate proteasomal degradation of DVL-1 at the plasma-membrane. Together, these data suggest that the Nedd4-1-mediated ubiquitylation and degradation of DVL-1 occurs at cell-cell junctions. Because Nedd4-1 binds to active Rac1 and localises with Rac1 at cell-cell contacts, we tested whether Rac1 activity could promote Nedd4-1-dependent ubiquitylation of DVL-1. Supporting this hypothesis, expression of active Rac1 promoted DVL-1 ubiquitylation in the presence of Nedd4-1 (Fig. 4d). Conversely, inhibiting endogenous Rac1 activity by treating cells with the Rac1 inhibitor EHT1864 (50 μM) resulted in a reduction of Nedd4-1-mediated ubiquitylation of DVL-1 (Supplementary Information, Fig. S4c). Thus, Rac1 activity stimulates Nedd4-1 driven degradation of DVL-1. Moreover, the Nedd4-1-dependent degradation of DVL-1 is important for maturation of cell-junctions, because overexpression of DVL-1, similar to the depletion of Nedd4-1, impairs maturation of cell-cell junctions, as deduced from a calcium-switch experiment (Fig. 4e).

Since maturation of cellular junctions required Nedd4-1 and expression of Nedd4-1 correlated with decreased levels of Ac-MTs (Fig. 2d), we next investigated whether Nedd4-1-driven proteasomal degradation of DVL-1 modulated Ac-MTs as a means to promote junction maturation. Previously, we found that DVL-1 increases the level of AcMTs in neuroblastoma cells, a result we confirmed in HeLa and H292 cells (Fig. 5a and data not shown). Moreover, the elevated expression of endogenous DVL-1 in Nedd4-1-depleted cells also correlated positively with a strong increase in the level of Ac-MTs (Fig. 5b). We next assessed whether MTs have a role in Nedd4-1-mediated ubiquitylation and degradation of DVL-1 by treating cells with NOC (10 μM) for 6 hours, which completely disrupted the MT network as shown by the loss of Ac-MTs (Supplementary Information, Fig. S4d). NOC-induced depletion of MTs did not prevent degradation of endogenous DVL-1 (Fig. 5c, Supplementary Information Fig. S4e), indicating that Nedd4-1-mediated degradation of DVL-1 occurs upstream of MT dynamics.
Interestingly, a subset of Ac-MTs-associated DVL-1 was found to co-localise with β-catenin-positive cell-cell contacts (Fig. 5a). The interaction of β-catenin with the MT network has been proposed previously, because the MT-associated motor protein dynein directly interacts with β-catenin.25

Figure 5 Nedd4-1-mediated down-regulation of DVL-1 modulates MT acetylation and affects junction maturation. (a) H292 cells ectopically expressing DVL-1-phiYFP were examined for MT acetylation by immunostaining for acetylated α-tubulin. β-catenin immunostaining was used to identify the cell junctions. (Scale bar, 10 μm). Arrows in magnification indicate a subset of DVL-1 co-localizing with Ac-MTs at β-catenin-positive junctions. (b) Levels of acetylated MTs were assessed in H292 cells 72hrs after transfection with control or Nedd4-1 lentiviral shRNA. (c) Protein stability of endogenous DVL-1 was examined in control and Nedd4-1 transfected HeLa cells, treated for 6hrs with CHX (10μg/ml) or with CHX in combination with NOC (10μM). (d) Analysis of TSA (0.5μM)-induced acetylation of MTs 4hrs in H292 cells by Western-blot. (e) Monolayer integrity of HeLa cells, as reflected by TER, was recorded before and after stimulating the cell with TSA. (f) Still images from movies of control and TSA-stimulated HeLa cells. Arrows show the reformation of cell-cell junctions in control- and TSA-treated cells. (Scale bar,10 μm). (g) Model for the regulation of cell-cell contacts. Nedd4-1, in conjunction with activated Rac1, controls the poly-ubiquitylation and degradation of DVL-1, which reduces the levels of Ac-MTs. This promotes the maturation of cell-cell junctions.
Because MT acetylation redirects trafficking of MT-linked cargo\(^5\), DVL-1-induced stabilisation of MTs could well impair the formation of cell junctions by interfering with transport of junctional proteins. We therefore examined whether increased stability of MTs hampered maturation of cell-cell junctions. Treatment of cells with TrichostatinA (TSA), an inhibitor of class I and II histone deacetylases including HDAC6 which deacetylates MTs\(^26\), resulted in a strong increase in Ac-MTs (Fig. 5d) and a reduction in TER of H292 cells (Supplementary Information, Fig. S4f), comparable to what we observed in Nedd4-1-depleted cells (Fig. 2a). Cell-cell junctions of HeLa cells are, in contrast to those of H292 cells, very dynamic and are disrupted and reformed continuously.

Recording of TER of HeLa monolayers upon the addition of TSA showed that monolayer TER dropped markedly after the inhibition of MT acetylation, indicative of a reduction in junctional stability (Fig. 5e). In line with this result, live-cell imaging of control and TSA-stimulated HeLa cells showed that membrane extensions in TSA-stimulated cells collapsed upon cell contact and failed to form stable cell-cell contacts, in contrast to those in control cells (Fig. 5f, Supplementary movies 1,2). This response indicates that MT acetylation impairs junctional maturation. Consequently, these data imply that the accumulation of Ac-MTs in Nedd4-1-depleted cells, resulting from increased stability of DVL-1, leads to a reduction of stable cell-cell contacts.

It is well established that Rac1 activity is required for the formation of cell-cell contacts\(^1\). Our findings indicate that binding of Rac1 to Nedd4-1 contributes to a novel Rac1-dependent pathway that results in epithelial junction maturation, by stimulation of Nedd4-1-mediated degradation of DVL-1, consequently reducing MT stability (Fig. 5g). MT-dependent vesicular transport towards cell-cell contacts has been shown to promote junction maturation\(^6,7,27,28\). Conversely, DVL-1-driven stabilisation of MTs could reduce junction formation, as MT acetylation reverses MT-associated transport\(^8\). Thus, the enhanced accumulation of Ac-MTs following expression of DVL-1 can explain its inhibition of junctional maturation.

These data indicate that Rac1-stimulated degradation of DVL-1, similar to Rac1 binding to IQGAP1\(^29\), is an important MT-dependent mechanism that serves to
promote formation of strong cell-cell contacts. Our current findings identify the E3 ubiquitin ligase Nedd4-1 as a new and potent regulator of DVL-1, but not of DVL-2 or DVL-3, expression. DVL-1 mutant mice exhibit a defect in social behaviour and deficits in synapse formation\(^\text{30}\). Moreover, DVL-1 expression was recently found to be up-regulated in primary human lung tissue-derived tumour cells which correlated with metastatic capacity\(^\text{31}\). In line with this, acetylation of MTs promotes neuronal migration \textit{in vivo}\(^\text{32}\). Hence, the pathway comprising DVL-1-regulated, MT-driven modulation of epithelial junctions could be employed by cancer cells to promote cell migration. The mechanism by which DVL-1 stabilises MTs represents a key aspect for future research. Interestingly, we recently found that the tumour-suppressor CYLD de-ubiquitylates DVL-1, thereby modulating DVL-1 signalling\(^\text{33}\). CYLD also reduces HDAC-6 activity, thereby altering the levels of Ac-MTs\(^\text{34}\). As a result, these proteins are obvious targets for future experiments aimed at identifying the mechanisms that regulate MT stability and its role in the control of epithelial integrity.

**Supplementary Information**

**Figure S1** Nedd4-1 interacts with the Rac1 C-terminal polybasic region. (a) Image of a protein gel following silver staining, revealing proteins isolated after a control (Ctrl) or Rac1 C-terminal peptide pull-down. The protein migrating around Mw 115 kD was identified by mass spectrometry as Nedd4-1. (b) The interaction of Rac1 with Nedd4-1 was further examined using different peptides resembling part of the Rac1 effector domain (17-32) and the Rac1 hypervariable C-terminal region (Rac1) or mutated versions thereof in which the triple prolines (Rac1 P-A) or the polybasic region (Rac1 PBQR) were mutated to alanines. Binding of the peptides to the Rac1 GEF beta-PIX\(^\text{31}\) was included as a control.

**Figure S2** shRNA-mediated down-regulation of Nedd4-1. (a) Two different shRNA constructs were used to down-regulate endogenous Nedd4-1 expression in H292 cells as analysed by western-blotting of cell lysates. (b) Distribution of \(\beta\)-catenin as a marker for adherens junction integrity was analysed by immunostainings in control and Nedd4-1-depleted HeLa cells.
**Figure S3** Nedd4-1 regulates junctional integrity. (a) Analysis of junctional integrity by immunostainings for β-catenin in HeLa cells transfected with a shRNA to Nedd4-1 and following re-expression of shRNA-insensitive murine (m)Nedd4-1 (Scale bar, 20 μm). (b) Distribution of MTs, identified by staining for endogenous α-tubulin, were examined for their (co)localisation with β-catenin at cell-cell junctions in H292 cells. Beta-catenin distribution is also analysed by the indicated profile analysis along the dashed line.

**Figure S4** Inhibition of Rac1 activity impairs Nedd4-1-mediated ubiquitylation of DVL-1. (a) Protein stability of DVL-1 was examined in HeLa cells 48 hrs after transfection with control or Nedd4-1 lentiviral shRNAs followed by 6hr incubation with CHX (10μg/ml) prior to cell lysis (b) Nedd4-1 driven ubiquitylation of HA-tagged DVL-1 was compared with DVL-1 mutants (P551A/Y553F and P545-547A) that showed reduced levels of ubiquitylation. (c) Nedd4-1-mediated ubiquitylation of DVL-1 was examined in cells treated with carrier (ctrl) or with the Rac-1 inhibitor EHT1864 (50μM). (d) H292 cells treated for 6 hrs with NOC (10μM) and immuno-stained for acetylated α-tubulin and β-catenin were fixed and analysed by immuno-staining for tubulin and β-catenin. (e) TER of control and TSA-treated H292 cells was recorded by ECIS (data represent mean +/-s.e.m, n=3). (f) Relative expression, as determined by Western-blot, of endogenous DVL-1 protein expression levels in control and Nedd4-1-overexpressing HeLa cells treated or not with NOC (10μM) for 6 hours. Alpha-catenin was included as a control.
METHODS

Cell culture. HeLa and H292 lung epithelial cells were maintained in Iscove’s Modified Dulbecco’s Medium (IMDM; BioWhittaker) containing 10% heat inactivated FCS (Bodinco), 2mM L-glutamine and Penicillin/streptomycin (all purchased from PAA Cell Culture Company) 37°C and 5% CO2. Cells were passaged by trypsinization.

Cell transfection, DNA constructs and immunofluorescence microscopy. Cells were transiently transfected with FuGene (Roche) as described by the manufacturer. Briefly, 1 μg DNA was mixed with 6 μl of Fugene in 100μl of Optitrem (51985026) (Invitrogen) and incubated for 30 min at RT, after which the transfection mix was applied to 50% confluent cells. The following constructs were used: GST-Rac, GST-RacΔC (a kind gift from R. Ahmadian, European Molecular and Cell Biology Laboratory, Heidelberg, Germany); GFP-Nedd4-1, originally from D. Rotin (The Hospital for Sick Children, Canada); TRCN000007550 (#a) and -7551 (#b). The human Nedd4-1-specific constructs used were: TRCN000007550 (#a) and -7551 (#b). The SHC002 scrambled shRNA construct (Sigma-Aldrich) was used as a negative control. All shRNA constructs were in the pLKO.1 vector backbone, shRNA-expressing lentiviral particles were prepared by transfection of HEK293T cells with pLKO.1 shRNA plasmid, together with the pMD2.G, pMDLg/RRE, and pRSV-Rev third-generation HIV-1 packaging plasmids obtained through Addgene, Cambridge, MA), using Fugene HD (Roche) for transfection. Generated lentiviral particles were collected for three days post-transfection and cleaned up by filtration through a 0.45 µm PVDF filter unit (Millipore), anti-DVL3(AB5974; Millipore), anti-DVL-1 (AB5970; Millipore) , anti-DVL-2 (AB5972; Millipore), anti-β-catenin (610154; BDBioscience), anti-tubulin (ATN02; Cytoskeleton), anti-c- myc (13-2500; Zymed). F-actin was stained with rhodamine-labeled phalloidin (Invitrogen). Rac1 inhibitor EHT1864 and HDAC6 inhibitor TSA were obtained from Sigma.

Pull-down assays. Peptide pull-down assays were performed as described previously. In short, each assay was performed with 5 μg of indicated biotin-labelled peptide, 25μl streptavidin-coated beads (Sigma-Aldrich) in NP-40 lysis buffer (50mM Tris-HCl, 150mM NaCl, 10mM MgCl2, 10% glycerol, 1% NP-40). All peptides were fused to a protein transduction domain sequence: YARAAARQARA, which was also used as a control in the pull down experiments. GST-fusion proteins were purified from BL21
bacteria as described\textsuperscript{17} and 100 µg of the indicated GST-fusion protein was used per pull-down. Mass spectrometry analysis was performed as described\textsuperscript{18} and was used for the initial identification of Nedd4-1.

Peptide synthesis. Peptides were synthesized on a peptide synthesizer (Syro II) using Fmoc solid phase chemistry. Peptides encoded a biotinylated protein transduction domain (Biotin-YARAAARQARAG)\textsuperscript{41} followed by the 10 amino acids proceeding the CAAX domain for all used RhoGTPase peptides. The sequences of the Rac1 (P-A) and the Rac1 (PBQR) mutants are respectively: CAAAVKKRKRK and CPPPVKKAAT.

Ubiquitylation assay. To detect ubiquitylated DVL-1, β-catenin and γ-catenin, HeLa cells, cells were transfected with 6xHis-myc-tagged ubiquitin. Twenty-four hrs after transfection, cells were washed with PBS (containing Mg\textsuperscript{2+} and Ca\textsuperscript{2+}) at RT and lysed for 5 min in Urea buffer (200mM Tris–HCl (pH 7.5) 200 mM NaCl, 10 mM Imidazol, 0.1% Triton X-100 and 8M urea). Cells were scraped, collected and incubated for 5 min at 37°C and centrifuged 5 min at 14000 rpm, after which the supernatant was incubated with 25 µl of pre-washed, blocked (1hr RT with 200 µg/ml BSA) Talon beads (Clontech) at RT for 1hr while rotating. Beads were washed 5 times with urea buffer and resuspended in SDS sample buffer. Peptides were subsequently analysed by western-blot.

Electrical resistance measurements. ECIS-based cell spreading experiments were performed as previously described\textsuperscript{35}. Briefly, ECIS electrodes (8W10E; Applied Biophysics) were coated with 10 µg/ml fibronectin (Sigma) in PBS for 1 h at 37°C. 400,000 HeLa- or H292 cells were seeded per well in 400 μl of the indicated bacterial growth medium. ECIS electrodes were incubated with 25 µl of pre-washed, blocked (1hr RT with 200 µg/ml BSA) Talon beads (Clontech) at RT for 1hr while rotating. Beads were washed 5 times with urea buffer and resuspended in SDS sample buffer. Cells were subsequently analysed by western-blot.

Reference List

34. Wickstrom, S.A., Massoumi, K.C., Khochbin, S., Fassler, R. & Massoumi, R. CYLD negatively regulates cell-cycle progression by inactivating HDAC6 and increasing the levels of acetylated tubulin. *EMBO J* 29, 131-144 (2010).