β2-syntrophin and Par-3 promote an apicobasal Rac activity gradient at cell–cell junctions by differentially regulating Tiam1 activity

Natalie A. Mack1, Andrew P. Porter1, Helen J. Whalley1, Juliane P. Schwarz2, Richard C. Jones3, Azharuddin Sajid Syed Khaja4, Anders Bjartell4, Kurt I. Anderson2 and Angeliki Malliri1,5

Although Rac and its activator Tiam1 are known to stimulate cell–cell adhesion, the mechanisms regulating their activity in cell–cell junction formation are poorly understood. Here, we identify β2-syntrophin as a Tiam1 interactor required for optimal cell–cell adhesion. We show that during tight-junction (TJ) assembly β2-syntrophin promotes Tiam1–Rac activity, in contrast to the function of the apical determinant Par-3 whose inhibition of Tiam1–Rac activity is necessary for TJ assembly. We further demonstrate that β2-syntrophin localizes more basally than Par-3 at cell–cell junctions, thus generating an apicobasal Rac activity gradient at developing cell–cell junctions. Targeting active Rac to TJs shows that this gradient is required for optimal TJ assembly and apical lumen formation. Consistently, β2-syntrophin depletion perturbs Tiam1 and Rac localization at cell–cell junctions and causes defects in apical lumen formation. We conclude that β2-syntrophin and Par-3 fine-tune Rac activity along cell–cell junctions controlling TJ assembly and the establishment of apicobasal polarity.

Cell–cell adhesion and apicobasal polarity are critical for epithelial function. In vertebrates, TJs define the apical–basolateral membrane border1,2, acting as a gate by regulating paracellular traffic, and a fence by limiting apicobasal diffusion, thereby maintaining apicobasal polarity. Adherens junctions (AJs), located below TJs, provide strong intercellular connections, helping to maintain tissue architecture. Cytoplasmic signalling and scaffolding protein complexes associated with AJs and TJs, such as the Par complex (Par-3–Par-6–atypical protein kinase C), regulate junction assembly and polarity3. Disrupted cell–cell adhesion and polarity contributes to tumour development and malignant progression4–6. Deciphering the molecular mechanisms regulating cell–cell adhesion and polarity will enhance our understanding of tumorigenesis and potentially improve therapies.

The small GTPase Rac and its activator Tiam1 (T-cell lymphoma invasion and metastasis 1) regulate TJs and AJs, and are implicated in tumorigenesis7. However, their exact roles at cell–cell adhesions remain controversial. One study found Tiam1–Rac inhibition to be required for TJ assembly8, whereas other studies have shown that Tiam1–Rac activity promotes TJ assembly9,10, consistent with it promoting AJs (refs 11–13). Moreover, precisely how Tiam1 contributes to tumorigenesis remains unknown, although its regulation of cell–cell adhesions, cell-cycle progression14–16 and survival17–21 are all believed to be important.

To better understand how Tiam1–Rac signalling contributes to tumorigenesis we further investigated its function at cell–cell adhesions. We identified β2-syntrophin as a Tiam1 interactor and found that in contrast to Par-3 (ref. 8), β2-syntrophin promotes Tiam1–Rac activity during TJ assembly. These differential effects result in an apicobasal Rac activity gradient at developing cell–cell junctions that controls TJ assembly and apicobasal polarity. Finally, we showed that reduced membrane-associated β2-syntrophin correlates with prostate cancer progression.

RESULTS

A PDZ-mediated interaction between Tiam1 and the β2-syntrophin–utrophin–dystrobrevin-β complex

By tandem affinity purification of tagged Tiam1 followed by mass spectrometry, we identified β2-syntrophin, utrophin and dystrobrevin-β as Tiam1 interactors among the known interactors 14–3–3, ERK1, Camk2 and Cask (refs 11,22–24 and Supplementary Table S1). β2-syntrophin, utrophin and dystrobrevin-β form a complex localizing to the basolateral membrane in MDCKII cells25; however, its role at

1Cell Signalling Group, Cancer Research UK Paterson Institute for Cancer Research, The University of Manchester, Manchester M20 4BX, UK.
2Tumour Cell Migration Group, Cancer Research UK Beatson Institute for Cancer Research, Glasgow G61 1BD, UK.
3MS Bioworks, 3950 Varsity Drive, Ann Arbor, Michigan 48108, USA.
4Department of Clinical Sciences, Division of Urological Cancers, Lund University, Skåne University Hospital, SE-205 02, Malmö, Sweden.
5Correspondence should be addressed to A.M. (e-mail: amalliri@picr.man.ac.uk)

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Figure 1  Tiam1 interacts with the β2-syntrophin PDZ domain using an internal PBM. (a) Exogenous Tiam1–myc expressed in HEK293T cells was immunoprecipitated with anti-myc antibody. Co-precipitated endogenous syntrophin and endogenous utrophin were detected by immunoblotting. (b) Endogenous Tiam1 from MDCKII cells was immunoprecipitated with anti-Tiam1 antibody and co-precipitated endogenous syntrophin was detected by immunoblotting. (c) MDCKII cells cultured in HCM were fixed and stained with immunofluorescent markers for Tiam1 and syntrophin. Scale bar, 10 μm. (d) Schematic representation of the domain structure of full-length HA-tagged Tiam1 (FL-Tiam1–HA). Horizontal arrows indicate the various N-terminally truncated Tiam1–HA constructs used. The vertical arrow indicates the approximate location of the internal KETDI PBM. Mr, myristoylation site; PH1a, PH1b, split pleckstrin homology domain; PH2, second pleckstrin homology domain; PHn-CC-Ex, N-terminal pleckstrin homology, coiled-coil, extended structure; RBD, Ras-binding domain; PDZ, PSD-95/Dlg1/ZO-1; DH, Dbl homology; PHc, C-terminal pleckstrin homology; HA, haemagglutinin tag. (e) Schematic representation of the domain structure of GFP–β2-syntrophin. GFP, green fluorescent protein tag; SU, syntrophin unique domain; PH1a and PH1b, split pleckstrin homology domain; PH2, second pleckstrin homology domain. (f) Tiam1-C196–HA–WT and Tiam1-C196–HA–ΔKETDI were transfected into HEK293T cells with the GFP-tagged PDZ domain of β2-syntrophin (GFP–Syn–PDZ). The exogenous Tiam1 fragments were immunoprecipitated with anti-HA antibody and co-precipitated GFP–Syn–PDZ was detected by immunoblotting. (g) Tiam1–HA–WT or Tiam1–HA–ΔKETDI was expressed in HEK293T cells and immunoprecipitated with anti-HA antibody. Co-precipitated endogenous syntrophin and utrophin were detected by immunoblotting. (h) The indicated HA-tagged Tiam1 fragment constructs were expressed in HEK293T cells and immunoprecipitated with anti-HA antibody. Co-precipitated endogenous syntrophin was detected by immunoblotting with anti-syntrophin antibody. (i) Tiam1–HA was immunoprecipitated from MDCKII cells that were also engineered to express shRNA targeting β2-syntrophin (β2-syntrophin RNAi#1) following the addition of doxycycline (+Dox). Co-precipitated endogenous syntrophin and utrophin were detected by immunoblotting. All data shown are representative of at least three independent experiments. IP, immunoprecipitation. Uncropped images of blots are shown in Supplementary Fig. S9.

cell–cell adhesions was unknown. We reasoned that this complex could be important for the function of Tiam1 at cell–cell adhesions. We performed co-immunoprecipitations to validate the mass spectrometry results. We found that exogenous Tiam1 co-precipitates endogenous syntrophin and utrophin from HEK293T cells (Fig. 1a). Moreover, endogenous Tiam1 co-precipitated endogenous syntrophin (Fig. 1b), and Tiam1 and β2-syntrophin co-localized at cell–cell adhesions in MDCKII cells (Fig. 1c). We next defined their interaction domains using amino-terminally truncated Tiam1–HA constructs and GFP-tagged β2-syntrophin domain constructs25 (Fig. 1d,e). We found that the carboxy-terminal 196 amino acids of Tiam1 (C196–Tiam1) and the PDZ domain of β2-syntrophin were sufficient for the interaction (Fig. 1f and Supplementary Fig. S1a,b). C196-Tiam1 contains the internal sequence KETDI matching the consensus syntrophin PDZ-binding motif (PBM), K/R-E-(S/T)-X-(V/L/I/M) (ref. 26; Fig. 1d and Supplementary Fig. S1c). Deletion of KETDI from C196-Tiam1 (Fig. 1f), and from full-length Tiam1 (Fig. 1g), abolished the interaction with β2-syntrophin, despite there being other sequences in Tiam1 matching the consensus syntrophin PBM (Supplementary Fig. S1c). Two short Tiam1 fragments containing KETDI (KETDI-19 and KETDI-27) did not bind or bound only weakly to β2-syntrophin, whereas a longer fragment (KETDI-44) did interact (Fig. 1h and Supplementary Fig. S1d), suggesting that the interaction requires residues flanking KETDI. Furthermore, KETDI was required for the
Tiam1–utrophin interaction (Fig. 1g) and β2-syntrophin knockdown abolished the Tiam1–utrophin interaction (Fig. 1i), suggesting that Tiam1 interacts with utrophin indirectly through β2-syntrophin. The KETDI sequence is highly conserved in Tiam1 orthologues (Supplementary Fig. S1e), indicating the importance of the interaction for Tiam1 function. Notably, this sequence is found in Tiam2 (Stef; Supplementary Fig. S1e).

β2-syntrophin regulates TJ assembly

We next investigated whether β2-syntrophin regulates cell–cell adhesion similarly to Tiam1 (refs 8–13). For this we engineered MDCKII cells with doxycycline-inducible expression of short hairpin RNA (shRNA) sequences targeting β2-syntrophin and a non-targeting control (Fig. 2a,b). Calcium withdrawal disassembles cell–cell adhesions, which is reversed by calcium readdition27 (calcium switch, CS). The remodelling of junctional complexes following CS mimics events accompanying epithelial cell movements during wound closure and tumour development. TJ integrity can be assessed by measuring the transepithelial electrical resistance (TER) of cell monolayers grown on Transwell filters and together with immunostaining for TJ markers has been widely used to identify TJ assembly regulators8,10,28,29. We found that β2-syntrophin knockdown in MDCKII cells markedly retards CS-induced TER development, indicating defective TJ assembly (Fig. 2c). We confirmed that the TER defect was β2-syntrophin-dependent by rescuing with expression of shRNA-resistant β2-syntrophin (Fig. 2d,e). Immunofluorescence microscopy for the TJ marker occludin after CS confirmed a delay in TJ assembly (Fig. 2f). We also observed delayed TJ assembly after plating (Fig. 2g,h), confirming that the defects are not CS-specific. Immunostaining for β-catenin revealed impaired AJ assembly in β2-syntrophin knockdown cells (Supplementary Fig. S2a). It is possible that the TJ assembly defects result from defective AJ formation. By performing hanging-drop assays to assess the aggregation ability of single cells, we also found that β2-syntrophin knockdown impedes cell–cell aggregation (Supplementary Fig. S2b,c). Moreover, a cell-dissociation assay demonstrated that β2-syntrophin knockdown compromises cell–cell adhesion strength (Supplementary Fig. S2d). Together, these results demonstrate that β2-syntrophin regulates epithelial cell–cell adhesions.

Tiam1–Rac activity impedes TJ assembly

To relate β2-syntrophin function at cell–cell adhesions to its Tiam1 interaction, we further interrogated Tiam1 function in TJ assembly, because previous studies are conflicting4–10. We found that doxycycline-inducible Tiam1 knockdown in MDCKII cells for either a short or long period did not inhibit CS-induced TER development, but slightly enhanced it (Fig. 3a–d). Tiam1 knockdown with an alternative shRNA did not alter TER, probably owing to less-efficient knockdown (Supplementary Fig. S3a–d). We next found that Tiam1–HA–WT overexpression retarded CS-induced TER development (Fig. 3e). Immunostaining for occludin (Fig. 3f) and quantifying the number of intact junctions (Fig. 3g) confirmed delayed TJ assembly. To investigate whether this was through Rac activation, we analysed the effect of overexpressing dominant-negative/GEF-dead Tiam1 (Tiam1–HA–DH+) at levels comparable to Tiam1–HA–WT (Supplementary Fig. S3e). Tiam1–HA–DH+ expression enhanced CS-induced TER development (Fig. 3h) consistent with our Tiam1 knockdown data. Immunostaining for occludin at 15 min following CS (Fig. 3i) and quantifying the number of intact junctions (Fig. 3j) confirmed accelerated TJ assembly. Collectively, these results show that Tiam1-induced Rac activity impedes TJ assembly in MDCKII cells, consistent with the findings of ref. 8.

β2-syntrophin promotes Tiam1–Rac activity during TJ assembly

We next investigated whether the ability of overexpressed Tiam1 to inhibit TJ assembly requires its interaction with β2-syntrophin. Expression of Tiam1–HA–ΔKETDI at levels comparable to Tiam1–HA–WT (Supplementary Fig. S4a) had little effect on CS-induced TER development (Fig. 4a). Likewise, no significant change in TJ assembly was observed following immunostaining for occludin (Fig. 4b) and quantification of intact junctions (Fig. 4c). As the inhibition of TJ assembly by overexpressed Tiam1 requires its Rac-GEF activity (Fig. 3j–i), we reasoned that β2-syntrophin promotes Tiam1-mediated Rac activation during TJ assembly. We therefore compared Rac activity levels in cells expressing Tiam1–HA–WT, ΔKETDI or -DH+ either maintained in high-calcium medium (HCM) or at 60 min following CS. In parental and Tiam1–HA–WT-expressing MDCKII cells Rac activity at 60 min following CS is induced to levels similar to HCM (Supplementary Fig. S4b and Fig. 4d). In contrast, Tiam1–HA–ΔKETDI-expressing cells showed reduced Rac activity at 60 min following CS (Fig. 4d), which was significantly lower than in Tiam1–HA–WT-expressing cells (Fig. 4e). Cells expressing Tiam1–HA–DH+ had low Rac activity under both HCM and CS conditions (Fig. 4d).

We next investigated whether β2-syntrophin knockdown affects Rac activity. Control and β2-syntrophin knockdown cells had comparable levels of Rac activity in HCM (Fig. 4f,g). However, β2-syntrophin knockdown cells had reduced CS-induced Rac activation (Fig. 4h,i), consistent with reduced Tiam1 activity because Tiam1 is also required for CS-induced Rac activation (Fig. 4j,k). Importantly, β2-syntrophin knockdown did not increase the endogenous Tiam1–Par-3 interaction at 60 min following CS (Supplementary Fig. S4c,d), excluding the possibility that the Rac activation defects are due to an increase in the inhibitory Par-3–Tiam1 interaction8.

We next analysed the effect of inhibiting the Tiam1–β2-syntrophin interaction on CS-induced Rac activation. Expression of the Tiam1 fragment KETDI-44 reduced the endogenous interaction (Fig. 4l). Inducible KETDI-44 expression inhibited CS-induced Rac activation (Fig. 4m,n), providing further evidence that β2-syntrophin promotes CS-induced Rac activation through binding Tiam1. Furthermore, we observed β2-syntrophin, along with E-cadherin and Par-3, localizing to cell–cell junctions at 60 min after CS (Supplementary Fig. S4e). Together, these results suggest that β2-syntrophin promotes Tiam1–Rac activity at developing cell–cell junctions in MDCKII cells.

β2-syntrophin and Par-3 localize differently along the apicobasal axis

Initially it seemed counterintuitive that β2-syntrophin, an apparent positive regulator of TJ assembly, would promote Tiam1–Rac activity, an apparent negative regulator of TJ assembly. However, the contribution of Tiam1 to CS-induced Rac activation (Fig. 4j,k) indicates also a positive role during TJ assembly. To rationalize our data, and considering the inhibitory effect of Par-3 on Tiam1 during TJ assembly8, we postulated that both inhibitors and activators of
Tiam1–Rac control TJ assembly. We reasoned that β2-syntrophin and Par-3 localize differently at cell–cell junctions, thereby spatially regulating Tiam1–Rac activity, which might in turn drive optimal TJ assembly. To investigate this, we analysed Par-3, β2-syntrophin and Tiam1 localizations along the apicobasal axis of MDCKII cells. We found that β2-syntrophin localizes more basally than Par-3, although with some overlap (Fig. 5a,b). Quantifying staining intensities at individual junctions confirmed the differential localizations of Par-3

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**Figure 2** β2-syntrophin regulates TJ assembly in MDCKII cells. (a) MDCKII cells with doxycycline-inducible expression of one of two separate shRNAs targeting β2-syntrophin (β2-syntrophin RNAi#1, #2) or a non-targeting control shRNA (Non-targeting RNAi) were treated with (+) or without (–) doxycycline (Dox) where indicated. Levels of syntrophin and tubulin were detected by immunoblotting. A representative immunoblot is shown. (b) Graph quantifying β2-syntrophin knockdown, n = 3. (c) CS TER readings from β2-syntrophin RNAi#1, #2 or non-targeting RNAi MDCKII cells treated with (Plus dox) or without (Control) doxycycline, n = 3. (d) CS TER readings from β2-syntrophin RNAi#1 MDCKII cells expressing shRNA-resistant β2-syntrophin (β2-syntrophin-rescue) treated with (Plus dox) or without (Control) doxycycline, n = 3. (e) Immunoblot showing syntrophin levels in β2-syntrophin RNAi#1 cells expressing β2-syntrophin-rescue treated with (+) or without (+) doxycycline. Actin is used as a loading control. (f) β2-syntrophin RNAi#1 MDCKII cells treated with (Plus dox) or without (Control) doxycycline were fixed from HCM, or at the indicated CS time points, and stained with immunofluorescent markers for occludin. Panels show representative images from one of three independent experiments. (g) β2-syntrophin RNAi#1 MDCKII cells treated with (Plus dox) or without (Control) doxycycline were fixed at the indicated time points after plating and stained with immunofluorescent markers for occludin. Panels show representative images from one of three independent experiments. (h) TER readings from β2-syntrophin RNAi#1 MDCKII cells treated with (Plus dox) or without (Control) doxycycline at the indicated time points after plating, n = 3.

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and β2-syntrophin (Fig. 5c,d), and revealed that although Tiam1 and Par-3 overlap, the peak of Tiam1 expression was below that of Par-3 (Fig. 5c). In β2-syntrophin knockdown cells, although both Tiam1 and Par-3 still localized to cell–cell junctions, we observed a significant shift in Tiam1 localization in the apical direction relative to Par-3 (Fig. 5g–e). It is possible that β2-syntrophin promotes TJ assembly through maintaining correct Tiam1 localization along the apicobasal axis, in addition to regulating Tiam1 activity.

Staining for the AJ marker E-cadherin revealed that both β2-syntrophin and Tiam1 are present at AJs with low levels of Par-3 (Fig. 5h,i and Supplementary Fig. S5a,b). Staining for occludin revealed that Par-3, as anticipated, is mainly present at TJs (Supplementary Fig. S5c,d). These staining results imply the presence of a Par-3–Tiam1 complex at TJs and a separate β2-syntrophin–Tiam1 complex at AJs. Consistent with this, β2-syntrophin overexpression reduced the Tiam1–Par-3 interaction (Fig. 5j,k), indicative of competition between the two complexes and supporting our conclusion that Tiam1 is regulated by different complexes at distinct cell–cell junction locations. From our immunostaining results, we cannot exclude the possibility of a ternary Tiam1–Par-3–β2-syntrophin complex, although the lack of a detectable Par-3–β2-syntrophin interaction (Supplementary Information S5e) suggests any such complex is transitory or contains only a small proportion of the available proteins.

A Rac activity gradient exists along the apicobasal axis

We next investigated whether there is a gradient of Rac localization and/or activity along the apicobasal axis of developing cell–cell junctions. Immunostaining for endogenous Rac and Par-3 after CS revealed that Rac is localized more basally than Par-3 at cell–cell junctions (Fig. 6a,b). We verified the Rac antibody for immunostaining using Rac1 knockdown and GFP–Rac1 overexpression (Supplementary Fig. S6a–c). Quantification of staining intensities at individual junctions confirmed the differential localizations of Par-3 and Rac (Fig. 6c,d). These results suggested that Rac activity is spatially regulated.
Figure 4 β2-syntrophin promotes Tiam1–Rac activity during TJ assembly. (a–c) MDCKII cells inducibly overexpressing Tiam1–HA–ΔKETDI (TetOn-Tiam1–HA–ΔKETDI) were treated with (Plus dox) or without (Control) doxycycline. (a) CS TER readings, n = 3. The dotted line represents the TER peak from doxycycline-treated TetOn-Tiam1–HA-WT cells normalized to that of control TetOn-Tiam1–HA–ΔKETDI cells. (b) Representative images of cells fixed 60 min after CS and stained with immunofluorescent markers for occludin and HA. Scale bar, 30 μm. (c) Graph showing the average number of intact junctions as a ratio of total cell number, n = 3. t-test: P = 0.223 (NS). (d) Doxycycline-treated confluent TetOn-Tiam1–HA–WT, TetOn-Tiam1–HA–ΔKETDI and TetOn-Tiam1–HA–ΔDH* cells were maintained in HCM or subjected to 60 min CS and subsequently lysed and assayed for Rac activity. (e) Quantification of d, n = 5, t-test: HCM P = 0.938 (NS), 60 min switch **P < 0.005. (f–i) Confluent β2-syntrophin RNAi#1 cells were treated with (+ or plus dox) or without (− or control) doxycycline. (f) Immunoblot from cells maintained in HCM and subsequently lysed and assayed for Rac activity. (g) Quantification of f, n = 3. Paired t-test: P = 0.381 (NS). (h) Immunoblot from cells subjected to 0, 30 or 60 min CS and subsequently lysed and assayed for Rac activity. (i) Quantification of h, n = 5. Paired t-test: 30 min **P < 0.05, 60 min ***P < 0.005. (j) Immunoblot from confluent Tiam1 RNAi cells treated with (Plus dox) or without (Control) doxycycline, subjected to 0 or 60 min CS and subsequently lysed and assayed for Rac activity. (k) Quantification of j, n = 3. t-test: *P < 0.05. (l–n) TetOn–KETDI-44 cells were treated with (+) or without (−) doxycycline. (l) Endogenous Tiam1 was immunoprecipitated with anti-Tiam1 antibody and co-precipitated endogenous syntrophin was detected by immunoblotting with anti-syntrophin antibody. (m) Immunoblot from cells subjected to 0 or 60 min CS and subsequently lysed and assayed for Rac activity. (n) Quantification of m, n = 3, t-test: 60 min **∗P < 0.005, n represents the number of independent experiments. NS, not significant. Results in a,c,e,g,i,k,n represent mean values ± s.e.m for the indicated number of independent experiments. Uncropped images of blots are shown in Supplementary Fig. S9.
along the apicobasal axis of cell–cell junctions. To address this directly, we performed FLIM–FRET (fluorescence lifetime imaging microscopy–fluorescence resonance energy transfer) analysis of a Rac biosensor in live MDCKII cells as done previously for Rho (ref. 30). We generated MDCKII cells expressing a membrane-targeted RFP–GFP variant of the Raichu–Rac FRET reporter 13 and confirmed reporter function by performing FLIM–FRET analysis before and after treatment with hepatocyte growth factor (HGF), a known stimulator of Rac activity (Supplementary Fig. S6d). Next, we performed FLIM–FRET analysis after CS in cells expressing either Raichu–Rac or membrane-targeted GFP control. We measured average fluorescence lifetimes across individual cell–cell junctions at 0.5 μm steps along the z axis and compared average lifetimes at apical junctional positions (TJs) with those at basal junctional positions. We observed no significant lifetime changes in the x–y planes at different z steps with 1.2 μm separation. (g) Quantification of Tiam1 staining intensities at apical junction positions (where Par-3 staining intensity was closest to 0.8) of individual junctions in control and β2-syntrophin knockdown cells, n = 3, t-test: *P < 0.05. (h) MDCKII cells fixed and stained with anti-Par-3, anti-E-cadherin and anti-syntrophin antibodies 6 h after CS. Representative images are shown in (i) Images of the x–z plane at a cross-section along the junction highlighted in (h) by a white arrow. (j) Endogenous Tiam1 was immunoprecipitated from GFP-alone- or GFP-β2-syntrophin-overexpressing MDCKII cells 6 h after CS with anti-Tiam1 antibody and co-precipitated endogenous Par-3 was detected by immunoblotting with anti-Par-3 antibody. (k) Quantification of j, n = 3, t-test: *P < 0.005, n represents number of independent experiments. Results in g,k represent mean values ± s.e.m for the indicated number of independent experiments. Scale bars, 10 μm. Syn, syntrophin. Uncropped images of blots are shown in Supplementary Fig. S9.
Figure 6 An apicobasal Rac activity gradient at cell–cell junctions. (a–g) β2-syntrophin RNAi#1 MDCKII cells treated with β2-syntrophin knockdown) or without (Control) doxycycline subjected to 3 h CS were fixed and stained with anti-Par-3 and anti-Rac antibodies. (a) Representative images of control cells in the x–y planes at three different z steps with 1.0 μm separation. (b) Images of Par-3 and Rac stainings in the x–z plane at a cross-section along the junction highlighted in a by a white arrow. (c) Quantification of Par-3 and Rac staining intensities (normalized to their own maximal intensity) at the junction shown in b. Distance between data points on x axis is 200 nm. (d) A representative experiment quantifying average staining intensities for Par-3 and Rac (normalized to their own maximal intensity) from individual junctions of control cells at three junctional positions (1 μm apart on the z axis). (e) Quantification of Par-3 and Rac staining intensities (normalized to their own maximal intensity) at a representative junction from a β2-syntrophin knockdown cell. Distance between data points on x axis is 200 nm. (f) Images of Par-3 and Rac stainings in the x–z plane at a cross-section along the junction quantified in e. (g) Quantification of Rac staining intensities at apical junction positions (where Par-3 staining intensity was closest to 0.8) of individual junctions in control and β2-syntrophin knockdown cells, n = 3. Paired t-test: *P < 0.05.

Our data demonstrate the existence of an apicobasal Rac activity gradient at cell–cell junctions and we propose that β2-syntrophin and Par-3 are important determinants through their differential regulation of Tiam1 activity. To directly show that β2-syntrophin contributes to the gradient, we analysed Rac localization after CS in β2-syntrophin knockdown cells. Rac was less separated from Par-3 in β2-syntrophin knockdown cells (Fig. 6e,f) compared with controls (Fig. 6a–d). Quantification revealed a significant shift of Rac in the apical direction relative to Par-3 (Fig. 6g), possibly as a consequence of shifted Tiam1 (Fig. 5g). We next performed FLIM–FRET analysis after CS in non-targeting RNA-mediated interference (RNAi) and β2-syntrophin knockdown Raichu-Rac-expressing MDCKII cells (Supplementary Fig. S6i). We observed significantly reduced fluorescence lifetimes...
at subapical junctions when compared with apical junctions in non-targeting RNAi cells (Fig. 6h,i), consistent with the results above (Supplementary Fig. S6g,h), whereas we found no significant lifetime changes in β2-syntrophin knockdown cells (Fig. 6h,i). These results together with our immunostaining data show that the Rac gradient is diminished in β2-syntrophin knockdown cells.

We next investigated the effect of Tiam1 knockdown on the Rac activity gradient using FLIM–FRET analysis. We found significantly reduced fluorescence lifetimes at subapical compared with apical junctions in Tiam1 knockdown cells, similar to what was found for the controls (Supplementary Fig. S6i–k). This indicates that a Rac activity gradient can still be formed in Tiam1 knockdown cells, consistent with their ability to form TJs (Fig. 3a,c). However, we cannot rule out changes to the steepness of the Rac gradient in Tiam1 knockdown cells, which may account for their slightly enhanced CS-induced TER development (Fig. 3a,c). In these cells, compensatory mechanisms may exist to differentially regulate apicobasal Rac activity. Alternatively, residual Tiam1 may be sufficient to generate a gradient of Rac activity.

Spatial regulation of Rac activity is important for TJ assembly and polarity
To assess the importance of the Rac activity gradient for TJ assembly, we analysed the effect of targeting constitutively active Rac1 (Rac1-V12) to TJs on CS-induced TER development. We used the GBP (GFP-binding protein) targeting system to force an interaction between occludin and GFP–Rac1-V12 or GBP–occludin+-GFP–Rac1-V12-expressing MDCKII cells grown in collagen I matrix and stained for actin (red, left panel), Gp135 (green, middle panel) as an apical marker, and with Hoechst (blue, right panel) to show nuclei. (e) Quantification of abnormal cysts from d, n = 3. The total number of cysts analysed for each cell line is shown. Paired t-test: GFP control versus Rac1-WT P = 0.572 (NS, not significant), GFP control versus Rac1-V12 *P < 0.05, n represents the number of independent experiments. Results in c and e represent mean values ± s.e.m for the indicated number of independent experiments. Scale bars, 30 μm (b) and 10 μm (d).

Figure 7 The cell-cell junction Rac activity gradient promotes TJ assembly and apicobasal polarity. (a) Schematic representation of the GBP-GFP system used to target GFP-tagged proteins to TJs. (b–e) MDCKII cell lines expressing GBP–Pk–occludin, GFP–Rac1-V12, GBP–Pk–occludin+GFP, GBP–Pk–occludin+GFP–Rac1-V12 or GBP–Pk–occludin+GFP–Rac1-WT were generated. (b) Representative images of cell lines indicated, fixed and stained with anti-GFP and anti-Pk antibodies. The images shown are from a single z section. (c) CS TER readings: GBP–occludin (n = 4), GFP–Rac1-V12 (n = 6), GBP–occludin+GFP (n = 9), GBP–occludin+GFP–Rac1-V12 (n = 7) and GBP–occludin+GFP–Rac1-WT (n = 3). (d) Images showing single cross-sections of representative cysts of GBP–occludin+GFP, GBP–occludin+GFP–Rac1-V12- or GBP–occludin+GFP–Rac1-WT-expressing MDCKII cells grown in collagen I matrix and stained for actin (red, left panel), Gp135 (green, middle panel) as an apical marker, and with Hoechst (blue, right panel) to show nuclei. (e) Quantification of abnormal cysts from d, n = 3. The total number of cysts analysed for each cell line is shown. Paired t-test: GFP control versus Rac1-WT P = 0.572 (NS, not significant), GFP control versus Rac1-V12 *P < 0.05, n represents the number of independent experiments. Results in c and e represent mean values ± s.e.m for the indicated number of independent experiments. Scale bars, 30 μm (b) and 10 μm (d).
Figure 8 β2-syntrophin promotes apicobasal polarity. (a) Images show single cross-sections of representative cysts of β2-syntrophin RNAi#1, #2 or non-targeting RNAi MDCKII cells treated with doxycycline and stained for actin (red, left panel) and Gp135 (green, middle panel) and with Hoechst (blue, right panel). Scale bar, 10 μm. (b) Quantification of apical and subapical junctions (Fig. 7b). MDCKII cells expressing β2-syntrophin alone, GFP–Rac1-V12 alone or GFP–occludin with GFP, GFP–Rac1-V12 or GFP–Rac1-WT were generated. GFP–Rac1-V12 and GFP–Rac1-WT were expressed at levels lower than endogenous Rac (Supplementary Fig. S7a) to minimize adverse effects of overexpression. Whereas targeting Rac1-V12 to TJs was anticipated to disrupt the Rac activity gradient, Rac1-WT was expected to be less disruptive owing to the low abundance of Rac activators apically. Cells expressing GBP–occludin with GFP–Rac1-V12 had reduced CS-induced TER development indicating TJ assembly defects compared with all controls, including cells expressing GBP–occludin with GFP–Rac1-WT (Fig. 7c).

As TJ assembly and apical lumen formation are tightly connected36–38, we reasoned that disrupting the Rac activity gradient may perturb apical lumen formation. Cells expressing GBP–occludin with GFP–Rac1-V12 produced more multi-lumen cysts compared with controls when grown in collagen I matrix (Fig. 7d,e), indicative of impaired apicobasal polarity43. Expression of Rac1-V12 alone did not affect cyst development (Supplementary Fig. S7d,e). These results suggest that the apicobasal Rac activity gradient at cell–cell junctions regulates TJ assembly and the establishment of apicobasal polarity.

β2-syntrophin regulates polarity and is a potential tumour suppressor in the human prostate

The above results suggest that deregulation of the Rac activity gradient can disrupt TJ integrity and apicobasal polarity, potentially promoting cell proliferation and tumour formation4,7,40–42. Therefore, we predicted that β2-syntrophin loss from cell–cell adhesions would disrupt apicobasal polarity and promote tumorigenesis. To address this, we first investigated whether β2-syntrophin regulates apical lumen formation. β2-syntrophin knockdown cells produced mostly multi-lumen cysts (Fig. 8a,b and Supplementary Fig. S8a), consistent with our results for Rac1-V12 targeted to TJs (Fig. 7d,e).

Membrane-localized β2-syntrophin has been documented in normal prostate tissue (http://www.proteinatlas.org/ENSG00000168807/normal). Moreover, Tiam1 overexpression correlates with poor prognosis of human prostate cancer49. Furthermore, Rac expression has been observed in healthy prostate tissue and increased Rac expression correlates with prostate cancer progression44,45. We therefore reasoned that the β2-syntrophin–Tiam1–Rac signalling pathway may be deregulated in human prostate cancer. We stained a prostate cancer tissue microarray (TMA) with an anti-β2-syntrophin antibody and scored its expression levels in both malignant and pre-malignant areas (Supplementary Table S2). β2-syntrophin expression was frequently reduced in malignant when compared with pre-malignant areas in the
same tumour section (Supplementary Fig. S8b,c and Tables S2 and S3). However, no significant correlation was found between malignant expression and primary Gleason scores (Supplementary Fig. S8d), indicating that β2-syntrophin downregulation is an early event in prostate cancer pathogenesis.

Subsequently, we scored subcellular localization of β2-syntrophin in malignant areas. Membrane β2-syntrophin intensity scores were obtained by combining the expression and localization scores (Supplementary Table S2). We found a significant negative correlation between membrane β2-syntrophin intensity and primary Gleason score (Supplementary Fig. S8e), revealing that β2-syntrophin is frequently lost from cell–cell adhesions during prostate cancer progression (Supplementary Fig. S8f). Performing classification regression tree analyses to divide the membrane intensity scores into weak and strong subgroups revealed a significant association between membrane β2-syntrophin intensity and BCR (time to biochemical recurrence) free time (Supplementary Fig. S8g), indicating that patients showing reduced β2-syntrophin levels at cell–cell adhesions are more likely to relapse.

**DISCUSSION**

We have shown that β2-syntrophin, and associated utrophin–dystrobrevin-β, interact with an internal PBM on Tiam1—a rare mode of PDZ recognition.10–12 We infer that the differential effects of β2-syntrophin and Par-3 on Tiam1–Rac activity, in conjunction with their differential but also overlapping localizations, enable them to promote an apicobasal Rac activity gradient at cell–cell junctions (see models; Fig. 8c,d), which is required for optimal establishment of TJs and apicolateral polarity.

Our findings support those of ref. 53, in which the data implied that differential apicolateral Rac activity exists in *Drosophila* columnar epithelia. Here, we have directly visualized an apicolateral Rac activity gradient in mammalian epithelial cells and relate it to TJ formation and apical lumen formation. Our results also have parallels with those of ref. 54, which demonstrated low apical Rac activity in MDCKII cysts. The β2-syntrophin–Tiam1–Rac pathway might also regulate apicolateral polarity by alternative means: Tiam1, Rac and the dystroglycan–Par-1β complex of which syntrophin is a part all regulate laminin deposition, which is important for apicolateral polarity.13–39

Our data enhance our understanding of tumorigenesis. We propose that deregulation of β2-syntrophin, Par-3 or Tiam1 would disrupt the Rac activity gradient, and in turn disrupt TJs and apicolateral polarity, promoting tumorigenesis.40,41,42 In agreement is our finding that increased Tiam1–Rac activity impedes TJ assembly and the findings of ref. 54 showing that Tiam1 overexpression at the entire plasma membrane disrupts apicolateral polarity. Moreover, Tiam1 overexpression occurs in many human tumours,43,45,46,63 and correlates with prostate cancer progression.64 Our TMA data suggest that membrane-associated β2-syntrophin is another mechanism by which Tiam1–Rac signalling at cell–cell junctions is deregulated and highlights the potential of β2-syntrophin as a biomarker for prostate cancer progression and prognosis. Loss-of-function mutations reported for utrophin could be another mechanism.65

The coordinated action of multiple protein complexes is a well-established mechanism of regulation of cell–cell adhesion and polarity.65 Our data show that cooperation between the Tiam1–β2-syntrophin–utrophin–dystrobrevin-β and Tiam1–Par-3 complexes generates a finely tuned spatial distribution of Rac activity at cell–cell junctions, controlling TJ formation and apicolateral polarity. This may explain previously reported cell–cell adhesion defects following either global Rac activation or inhibition (see ref. 7 for a review).

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Note:** Supplementary Information is available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

N.A.M. performed most of the experimental work, data analysis and manuscript preparation. A.P.P. contributed extensively to experiments and preparation of the manuscript. H.I.W. contributed to experiments and analysis. R.C.I. performed the mass spectrometry analysis. I.P.S. and K.I.A. contributed to and supervised the FLIM–FRET analysis. A.S.S.K. and A.B. performed the TMA staining and scoring together with N.A.M. A.M. was the grant holder and principal investigator who supervised the study and manuscript preparation and made intellectual contributions throughout.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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METHODS

Antibodies. The primary antibodies used in this study are shown in Supplementary Table S4. Secondary antibodies: IgG–peroxidase-conjugated (GE Healthcare); Alexa Fluor 488, 568 and 647 (Molecular Probes).

Constructs. HA–CTAP was generated from pcDNA4/TO-CTAP (gift from H. Clevers, Hubrecht Laboratory, The Netherlands). The following have been described elsewhere: Tiam1–HA–CTAP (ref. 22), Tiam1–myc (ref. 66), Tiam1–HA–WT (refs 11.22), C199–HA (ref. 22) and CS80–HA (ref. 22). PH–CC–EX–HA was a gift from J. Collard (The Netherlands Cancer Institute, The Netherlands). C196–HA–WT in pcDNA3.1 was generated by PCR from Tiam1–HA–WT. Tiam1–ΔKETDI constructs were generated by deleting a KET-1 residue using QuikChange mutagenesis (Stratagene). KETD1–19, KETDI–27 and KETDI–44 in pcDNA3.1 were generated by PCR from C196–HA–WT. pRETRO–X–Tight–Pur–Tiam1–HA–WT, ΔKETDI and ΔDH* were generated by ligating corresponding fragments into pRETRO–Super–Puro (gift from N. Dívecha, The Paterson Institute for Cancer Research, UK, as was pRETRO–Super–Racl1/SHRNA. The following [b2–syntrophin plasmids]: GFP–Syn–FL, GFP–Syn–PDZ, GFP–Syn–PH1b, GFP–Syn–PH2 and GFP–Syn–SU, were gifts from S. Froehner (University of Washington, USA). [b2–syntrophin–rescue (resistant to [b2–syntrophin RNAi)] was generated by mutating GFP–Syn–FL, and subsequently ligating into pcDNA4/TO. The RFP–GF–RacI–RFP–RET–reporter plasmid was generated by replacing PKN–BD and RhoA in the RFP–GF–RhoA–FRET–reporter plasmid with PKA–BD and Rac1 from pRaihuRac1 (ref. 31). LZ5S–GBP–PK–oculindin–Zeo was generated from TOPO–GBP–PK (gift from H. Leonardt, Ludwig Maximilians University, Germany) and GFP–oculindin (gift from A. Reijskerk, VU University Medical Center, The Netherlands). pEGFP–Racl1–V12 and pEGFP–Racl1–WT were gifts from F. Sanchez–Madrid (Hospital de la Princesa, Spain). pEGFP–F and pEGFP–C1 plasmids were from Clontech. Glo–myc was from A. Hurlstone (University of Manchester, UK).

Cell culture. Mouse embryonic fibroblast (MEF), MDC02K and HEK293T cells were maintained in DMEM (Invitrogen) with 10% FBS (GIBCO) or 10% charcoal–dextran treated FBS (GIBCO) for 24 h, and then maintained in DMEM (Invitrogen) with 10% FBS (GIBCO) or 10% charcoal–dextran treated FBS (GIBCO). Fibroblasts were a kind gift from M. Leonhardt, Ludwig Maximilians University, Germany) and GFP–oculindin (gift from A. Reijskerk, VU University Medical Center, The Netherlands). FRET plasmids were generated by replacing PAK1–BD and Rac1 from pRaichuRac1 (ref. 31). pHKDI and –DH* were generated by mutating the corresponding residues in the FRET-reporter plasmid. Cell treatment. Except for cysts, cells were grown on 6-cm dishes (plating 105 cells per dish) for two days. Transwell filters were used for some GFP–b2–syntrophin immunoprecipitations. Immunoblot band intensities were quantified using Genetools software (SynGene).

Cell culture. Mouse embryonic fibroblast (MEF), MDC02K and HEK293T cells were maintained in DMEM (Invitrogen) with 10% FBS (GIBCO) or 10% charcoal–dextran treated FBS (GIBCO). Fibroblasts were a kind gift from M. Leonhardt, Ludwig Maximilians University, Germany) and GFP–oculindin (gift from A. Reijskerk, VU University Medical Center, The Netherlands). FRET plasmids were generated by replacing PAK1–BD and Rac1 from pRaichuRac1 (ref. 31). pHKDI and –DH* were generated by mutating the corresponding residues in the FRET-reporter plasmid.

Cell treatment. For inducible expression, cells were treated with doxycycline (1 mg ml–1 for confluent cells, 0.2 mg ml–1 for subconfluent cells) for at least three days before analysis. Cells were treated with HGF (10 ng ml–1) for subconfluent cells, 0 ng ml–1 for confluent cells, 0.1% FBS for FLIM analysis, 2 mM EDTA, 25 mM NaF and 2 mM NaH2PO4 for Tandem affinity purification (TAP) and mass spectrometry. Protein analysis. Cells were lysed in immunoprecipitation lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% (v/v) glycerol, 2 mM EDTA, 25 mM NaF and 2 mM NaH2PO4) containing protease and phosphatase inhibitor cocktails (Sigma). Immunoprecipitation and immunoblotting were performed as described previously11, except GFP–Trap beads (Chromotech) were used for some GFP–b2–syntrophin immunoprecipitations. Immunoblot band intensities were quantified using Genetools software (SynGene).

Cysts. Basal layers (230 μl) of collagen I solution (40% (v/v) collagen IPC-50, Koken; 0.375% (v/v) sodium bicarbonate; 12 mM HEPES; 10% (v/v) 10X MEM; 21430, GIBCO; 4 mM t-glutamine; 5% (v/v) tetracycline-free FBS; 5% (v/v) PBS; 31.8% DMEM) were set in 24-well plates (20 min, 37°C), before the addition of 300 μl collagen top-layers containing 3.6 × 104 cells. Gels were cultured in 700 μl medium for 12 days before immunostaining: cyst-containing gels were washed twice in PBS (30 min, 0.5% Triton/BSA/PBS, 30 min; room temperature; washed twice in PBS; incubated with blocking solution (10% FBS/PBS) for 1 h, room temperature; incubated with primary antibody in 2% FBS/PBS overnight, 4°C; washed in PBS 6 × 30 min, 4°C; incubated with secondary antibody, phallodin, Hoechst or SYBR Gold, in 2% FBS/PBS overnight, 4°C; washed in PBS 6 × 30 min, 4°C; and then mounted on slides in mounting medium for imaging on the low-light or macro-confocal microscopes.

Microscopy. The spinning-disc confocal microscope was based around an Olympus IX81, using the Sedat filter set (Chroma, 89000), an array of imaging lenses (406, 488, 548, 645 nm) and an apo ×100 1.45 NA oil objective. The low-light microscope was based around a Zeiss Axiovert 200M, with an Andor iXon DU-897+ EMCCD camera, a 300W xenon light source, using the Sedat filter set, and a Zeiss alpha plan ×100 1.45 NA oil objective. The time-lapse microscope was based around a Zeiss Axiovert 200M, with a Roper Coollens HQ camera and a Zeiss alpha plan Fluor ×10 1.45 NA objective, using white light LED for illumination. The macro-confocal microscope was based around a Leica TCS LSI using a Leica plan apo ×5 0.50 NA objective, an array of solid-state imaging lasers (488, 532 and 635 nm) and an ultrahigh dynamic photomultiplier. Images were captured using Metamorph software (Molecular Devices) or Leica proprietary imaging software and were deconvoluted where necessary using Huygens software (SVI). Numbers of intact junctions were quantified from images acquired on the low-light microscope. The number of intact occludin–stained junctions as a ratio of total nuclei was calculated. Rac, Par–3, syntrophin, and Tiam1 staining intensities were quantified from images acquired on the spinning-disc confocal microscope, by manually drawing around junctions at each z step and recording average pixel intensities using ImageJ software (NIH). Background readings from equivalent regions adjacent to junctions were subtracted. Apical junctional positions were defined as the z steps where the apical Par–3 staining intensity was closest to 0.8.

FLIM–FRET analysis. FRET was measured using frequency-domain FLIM on a Nikon Eclipse TE 2000-U microscope with a Lambda Instruments LIFFA attachment. We used a Yokogawa CSU 22 confocal scanner unit and a ×100 1.4 NA oil objective mounted on a piezo z drive (Nano-Drive, Mad City Labs Inc) to image at different z positions. A 488 nm laser modulated at 40 MHz (60 mW, Deepster, Omicron) was used to enable measurement of fluorescence lifetimes in the frequency domain. Lifetime images were acquired at 300 nm z steps. Standard halogen illumination together with a GFP-filterblock (470/40X, T495LP, 525/50M) enabled visualization of probe expression. Erythrosin B (1 mg ml–1) was used as a reference standard (0.086 ns). Donor lifetime at cell–cell junctions was analysed by manually drawing around junctions at each z step using FLIM software (Version 1.2.1, Lambert Instruments). Apical junctions were identified as the first z step at which junction GFP was focused and subapical junctions were assumed to be 1 μm below.

Protein analysis. Cells were lysed in immunoprecipitation lysis buffer (50 mM Tris–HCl, at pH 7.5, 150 mM NaCl, 1% (v/v) Triton–X-100, 10% (v/v) glycerol, 2 mM EDTA, 25 mM NaF and 2 mM NaH2PO4) containing protease and phosphatase inhibitor cocktails (Sigma). Immunoprecipitation and immunoblotting were performed as described previously11, except GFP–Trap beads (Chromotech) were used for some GFP–b2–syntrophin immunoprecipitations. Immunoblot band intensities were quantified using Genetools software (SynGene).

Tandem affinity purification (TAP) and mass spectrometry. Tiam1 knockout MEFs expressing Tiam1–HA–CTAP or HA–CTAP were cultured in 20–40 500 μl dishes (plating 105 cells per dish) for two days. TAP purifications were performed as previously described22. Samples were run on one-dimensional gels (Nupage 4–12%), and then lanes were manually cut into 40 bands. Bands were processed robotically using a ProGest instrument. Digested gel slices (30 μl) were analysed using nano-liquid chromatography–tandem mass spectrometry on a LTQ-Orbitrap-XL or
LTQ tandem mass spectrometer (ThermoFisher). Product ion data were searched against the combined forward and reverse IPI mouse v3.29 protein database using the Mascot search engine v2.0.04 (Matrix Science) through Mascot Daemon v2.0.0. Mascot search result flat files (DAT) were parsed to the Scaffold program (Proteome Software). Criteria for accepting protein identifications were determined by calculating the false discovery rates from the concatenated forward/reverse database, resulting in the following cutoff values: 90% protein, 50% peptide level probability, and minimum two unique peptides per protein. These criteria resulted in false discovery rates of <0.5% at the protein level for each data set.

**Rac activity assay.** Rac activity was assayed as previously described.

**Hanging-drop assay.** Drops (30 μl) of cells (10⁶ cells ml⁻¹) were hung from 24-well plate lids above H₂O and allowed to aggregate for 0–3 h at 37 °C. Drops were passed through 20 μl pipette tips 10 times before imaging. Images were captured of 10–12 fields per drop using the time-lapse microscope at ×10 magnification. At least 2 drops were analysed per experiment. Aggregate areas were measured using ImageJ.

**Cell-dissociation assay.** Cell-dissociation assays were performed on MDCKII colonies as previously described.

**Immunohistochemical analysis.** The prostate cancer TMA included tumour specimens from 122 patients and has been described elsewhere. After removing patients who received neoadjuvant radiation and hormonal therapy, missing cores and cores with no cancer, 74 patients remained for analyses. Scorings were verified by two independent observers. Immunohistochemistry for β2-syntrophin and classification tree analyses were performed as described previously. BCR was defined as a blood prostate-specific antigen concentration of ≥0.2 ng ml⁻¹ and a subsequent confirmatory value.

**Statistical analysis.** The specific statistical tests used are indicated in the figure legends alongside the P values.

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Figure S1 Tiam1 interacts with the β2-syntrophin PDZ domain using an internal PDZ-binding motif. (a) The indicated HA-tagged Tiam1 constructs were expressed in HEK293T cells and immunoprecipitated with anti-HA antibody. Co-precipitated endogenous syntrophin was detected by immunoblotting with anti-syntrophin antibody. (The same exposure is shown for both pieces of the anti-HA immunoblot). (b) GFP-Syn-FL and the indicated GFP-tagged β2-syntrophin domains were immunoprecipitated with anti-GFP antibody from HEK293T cells. Co-precipitated exogenous Tiam1-HA was detected by immunoblotting with anti-HA antibody. (c) Shown is the human Tiam1 protein sequence with the three consensus syntrophin PBM highlighted. The functional β2-syntrophin PBM, KETDI, is highlighted in blue, and the non-functional syntrophin PBM's in red. (d) Amino acid sequences of KETDI-containing fragments of Tiam1-Dog with KETDI highlighted in blue. (e) The syntrophin PBM KETDI (shown in bold) is highly conserved in Tiam1 orthologs. The syntrophin PBM is also present in the Tiam1 paralog, Tiam2 (STF), as KESDI. IP indicates immunoprecipitation.
**Figure S2** β2-syntrophin regulates cell-cell adhesion in MDCKII cells. (a) β2-syntrophin RNAi#1 MDCKII cells treated with (Plus dox) or without (Control) dox in high calcium medium (HCM), or at the indicated calcium switch time-points were fixed and stained by immunofluorescence for β-catenin. Panel shows representative images from one of three independent experiments. Scale bar represents 30 µm. (b, c) β2-syntrophin RNAi#1 and Non-targeting RNAi MDCKII cells treated with (Plus dox) or without (Control) dox were subjected to a hanging-drop assay for 0 and 3 hours. Shown in (b) are representative images for Control and Plus dox cells at each time-point. Scale bar represents 200 µm. (c) The mean aggregate area for 3 hour hanging-drops is shown, n=3. t-test: β2-syntrophin RNAi#1 ** P<0.005, Non-targeting RNAi P=0.312 (ns). (d) β2-syntrophin RNAi#1, #2, or Non-targeting RNAi MDCKII cells treated with (Plus dox) or without (Control) dox were subjected to a cell-dissociation assay. The total number of particles (Np) and total number of cells (Nc) were counted. Shown is the average Np/Nc as a ratio of the control, n=4. Paired t-test: β2-syntrophin RNAi#1 and #2 * P<0.05, Non-targeting RNAi P=0.861 (ns). n represents number of independent experiments. ns indicates no significant difference. Results in c and d represent mean values±s.e.m for the indicated number of independent experiments.
Figure S3 Effect of a second Tiam1-targetting oligo on junction formation. (a) Calcium switch (CS) TER readings from Tiam1 RNAi#2 MDCKII cells treated with (Plus dox) or without (Control) dox for 7 days (short dox), n=3. (b) Representative immunoblots showing the extent of Tiam1 knockdown for cells in (a). Actin is shown as a loading control. (c) Calcium switch (CS) TER readings from Tiam1 RNAi#2 MDCKII cells treated with (Plus dox) or without (Control) dox for three weeks (long dox), n=3. (d) Representative immunoblots showing the extent of Tiam1 knockdown for cells in (c). Tubulin is shown as a loading control. (e) Representative immunoblot showing Tiam1 expression levels in TetOn-Tiam1-HA-WT and TetOn-Tiam1-HA-DH* MDCKII cells treated with (+) or without (-) dox. Actin is shown as a loading control. n represents number of independent experiments. Results in a and c represent mean values±s.e.m for the indicated number of independent experiments.
Figure S4: Syntrophin regulates Tiam1 activity after CS. (a) Representative immunoblot of Tiam1 expression levels in TetOn-Tiam1-HA-WT and TetOn-Tiam1-HA-ΔKETDI MDCKII cells treated with (+) or without (-) dox. Actin is shown as a loading control. (b) Confluent MDCKII cells were maintained in high calcium medium (HCM) or subjected to 0, 30 or 60 min CS and subsequently lysed and assayed for Rac activity. A representative immunoblot is shown. (c, d) The endogenous Tiam1-Par-3 interaction is not increased in β2-syntrophin knockdown MDCKII cells after CS. (c) Endogenous Tiam1 was immunoprecipitated with anti-Tiam1 antibody from β2-syntrophin RNAi#1 MDCKII cells treated with (+) or without (-) dox 60 min following CS. Co-precipitated endogenous Par-3 was detected by immunoblotting with anti-Par-3 antibody. IP, indicates immunoprecipitation. (d) Quantification of (c), n=3. Paired t-test: P=0.193 (ns). (e) Representative images of MDCKII cells fixed and stained with anti-Par-3, anti-E-cadherin and anti-syntrophin antibodies 0 and 60 min after CS. n represents number of independent experiments. ns indicates no significant difference. Results in d represent mean values±s.e.m for the indicated number of independent experiments. Scale bar represents 10 μm.
Figure S5 Par-3 and Tiam1 localise differently along the apicobasal junctional axis. (a) MDCKII cells fixed and stained with anti-Par-3, anti-Tiam1 and anti-E-cadherin antibodies 6 hours after CS. Representative images are shown in the x/y planes at three different z-steps with 1.2 μm separation. (b) Images of the x/z plane at a cross-section along the junction highlighted in (a) by a white arrow. (c) MDCKII cells fixed and stained with anti-Par-3, anti-occludin and anti-E-cadherin antibodies 6 hours after CS. Representative images are shown in the x/y planes at three different z-steps with 1.2 μm separation. (d) Images of the x/z plane at a cross-section along the junction highlighted in (c) by a white arrow. (e) GFP or GFP-β2-syntrophin were stably expressed in MDCKII cells and precipitated using GFP-trap 6 hours after CS. Co-precipitated endogenous Par-3 and Tiam1 were detected by immunoblotting with anti-Par-3 and anti-Tiam1 antibodies. Scale bars represent 10 μm. Syn, Syntrophin; E-cad, E-cadherin; Occ, Occludin.
**Figure S6** Rac staining and FLIM-FRET analysis reveal a gradient of Rac activity at cell-cell junctions. (a) Representative images of control or Rac1 RNAi MDCKII cells fixed and stained with anti-Rac and anti-E-cadherin antibodies 3 hours after CS. (b) Representative immunoblot showing the extent of Rac1 knockdown for cells in (a). Tubulin was used as a loading control. (c) Representative images of MDCKII cells expressing control GFP or GFP-Rac1 fixed and stained with anti-Rac antibody 3 hours after CS. GFP localisation was revealed by epifluorescence (epi). (d) Average fluorescence lifetimes from 20 junctions in Raichu-Rac expressing MDCKII cells treated with (+ HGF) or without (- HGF) hepatocyte growth factor (HGF) for 5-15 min. (e-h) FLIM-FRET analysis of MDCKII cells expressing pEGFP-F (GFP) (e and f) or Raichu-Rac (Rac) (g and h) 6 hours after CS. (e, g) Representative lifetime images in the x/y plane at apical and subapical junctional positions. White boxes highlight the junctions shown at higher magnification. Fluorescence lifetimes of GFP are shown in false colours. Red indicates high lifetime (inactive), yellow/green indicates low lifetime (active). (f) Average fluorescence lifetimes found at subapical and apical junctional positions of 40 individual junctions in MDCKII cells expressing pEGFP-F. (h) Average fluorescence lifetimes found at subapical and apical junctional positions in Raichu-Rac expressing MDCKII cells, n=3, Paired t-test; * P<0.05. (i) Representative immunobLOTS showing the extent of β2-syntrophin knockdown for cells in Figs 6h and 6i and for Tiam1 knockdown for cells in Figs S6j and S6k. Actin was used as a loading control. (j, k) FLIM-FRET analysis of Tiam1 RNAi Raichu-Rac expressing MDCKII cells 6 hours after CS. (j) Lifetime images in the x/y plane at apical and subapical positions of a single junction. (k) Quantification of average fluorescence lifetimes found at subapical and apical junctional positions, n=3. Paired t-test; * P<0.05. n represents number of independent experiments. ns indicates no significant difference. Results in h and k represent mean values±s.e.m for the indicated number of independent experiments. Scale bars represent 10 μm.
Figure S7 GBP-Occludin allows targeting of GFP-tagged proteins. (a) GBP-Pk-occludin was immunoprecipitated with anti-Pk antibody and co-precipitated GFP and GFP-Rac1-V12 were detected by immunoblotting with anti-GFP antibody. * indicates GFP-Rac1-V12 band; o indicates GFP band; IP, indicates immunoprecipitation. (b) Immunoblots showing occludin, Rac and GFP expression levels in the cell lines used in Figure 7. (c) Immunoblots showing occludin, Rac and GFP expression levels in GBP-occludin+GFP-Rac1-V12 and GBP-occludin+GFP-Rac1-WT cell lines. (d) Images show single cross-sections of representative cysts of GFP or GFP-Rac1-V12 expressing MDCKII cells grown in collagen I matrix and stained for Actin (red, left panel), Gp135 (green, middle panel) as an apical marker, and with Sybr-Gold (blue, right panel) to show nuclei. (e) Quantification of average percentage of normal cysts from (d), n=3. The total number of cysts analysed is shown. Paired t-test: P=0.754 (ns). n represents number of independent experiments. ns indicates no significant difference. Results in e represent mean values±s.e.m for the indicated number of independent experiments. Scale bar represents 10 µm.
**Figure S8** β2-syntrophin has potential tumour suppressive functions in the human prostate. (a) Images showing single cross-sections of representative cysts of β2-syntrophin RNAi#1, #2, or Non-targeting RNAi MDCKII cells without dox treatment (Control) and stained for Actin (red, left panel), Gp135 as an apical marker (green, middle panel), and with Hoechst to show nuclei (blue, right panel). Scale bar represents 10 μm. (b) Prostate tumour section with both pre-malignant (green arrow) and malignant (red arrow) areas stained for β2-syntrophin. Scale bar represents 50 μm. (c) Graph shows average β2-syntrophin expression scores for pre-malignant and malignant areas of 57 prostate tumours. Results represent mean values±s.e.m. Paired t-test: *** P<0.0005. (d) Bubble plot depicts no correlation between β2-syntrophin expression and Primary Gleason score. Spearman’s Rho correlation coefficient (ρ) is displayed, P=0.096 (ns). (e) Bubble plot depicts the significant negative correlation between membrane β2-syntrophin intensity and Primary Gleason score. Spearman’s Rho correlation coefficient (ρ) is displayed, ** P<0.01. (f) Representative malignant prostate tissue images showing the negative correlation between membrane-localised β2-syntrophin and Primary Gleason score. Scale bar represents 25 μm. (g) Kaplan-Meier survival curve with strong and weak membrane β2-syntrophin subgroups showing the significant positive correlation between membrane β2-syntrophin intensity and relapse-free survival. Each step in the curve represents a relapsed patient. Significance was determined with a Mantel-Cox test. * P<0.05.
Figure S9 Uncropped image blots.
Table 1 Mass spectrometry spectral count data for identified proteins from control (Con) and Tiam1 TAP purifications.

Table 2 Scoring data from β2-syntrophin immunostained cores of prostate tumours mounted in duplicates on a TMA. The table shows scorings of expression of β2-syntrophin in malignant areas of the tumours (malignant β2-syntrophin expression), localisation of β2-syntrophin in malignant areas of the tumours (malignant β2-syntrophin localisation) and expression of β2-syntrophin in pre-malignant areas of the tumours (pre-malignant β2-syntrophin expression), alongside the corresponding Primary Gleason scores (grade of the predominant malignant population) and BCR free time (time to biochemical recurrence) data. β2-syntrophin expression was scored using arbitrary units from 0-3, with 0 representing no staining, 1 as weak staining, 2 as moderate staining and 3 as strong staining. β2-syntrophin localisation was scored from 1-3, with 1 representing predominantly cytoplasmic, 2 disturbed membrane localisation and/or increased cytoplasmic, and 3 predominantly membrane localisation. To gain a more accurate score of the intensity of β2-syntrophin expression in the membrane in malignant areas of the tumours we multiplied the malignant β2-syntrophin expression and localisation scores obtaining scores in the range 0-9 (malignant membrane β2-syntrophin intensity).

Table 3 The number of prostate tumours with malignant β2-syntrophin expression scores at ratios 0.75-1 or <0.75 of their corresponding pre-malignant expression scores.

Table 4 Primary antibody information. IB, immunoblotting; IF, immunofluorescence; IHC, immunohistochemical analysis; IP, immunoprecipitation.

Table 5 shRNA constructs sequence information.