Receptor protein tyrosine phosphatase RPTPα controls epithelial adherens junctions, linking E-cadherin engagement to c-Src signaling to cortactin

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SUMMARY

Epithelial junctions are fundamental determinants of tissue organization, subject to regulation by tyrosine phosphorylation. E-cadherin homophilic binding activates tyrosine kinases, such as Src, that controls junctional integrity. Protein tyrosine phosphatases (PTPs) also contribute to cadherin-based adhesion and signaling, but little is known about their specific identity or functions at epithelial junctions.

We report here that the receptor-PTP RPTPα is recruited to epithelial adherens junctions at the time of cell-cell contact, where it is in molecular proximity to E-cadherin. RPTPα is required for appropriate cadherin-dependent adhesion, and for cyst architecture in 3-dimensional culture. Loss of RPTPα impairs adherens junction integrity, manifested by defective E-cadherin accumulation and perijunctional F-actin density. These effects correlate with a role for RPTPα in c-Src activation at sites of E-cadherin engagement. Mechanistically, RPTPα is required for appropriate tyrosine phosphorylation of cortactin, a major Src substrate and a cytoskeletal actin organizer. Expression of a phosphomimetic cortactin mutant in RPTPα-depleted cells partially rescues F-actin and E-cadherin accumulation at intercellular contacts.

These findings indicate that RPTPα controls cadherin-mediated signaling by linking homophilic E-cadherin engagement to cortactin tyrosine phosphorylation via c-Src.
INTRODUCTION

Cadherin-based epithelial junctions are fundamental determinants of tissue organization, dysfunction of which is associated with multiple pathologies. They mediate intercellular adhesion through homophilic binding of E-cadherin and its interaction with the actin cytoskeleton (Harris and Tepass, 2010; Mège et al., 2006). Diverse signaling pathways, such as involving protein tyrosine phosphorylation and small G proteins, contribute to junction assembly, re-modeling and turnover (Braga and Yap, 2005; Maher et al., 1985; McLachlan and Yap, 2007; Nelson, 2008). Locally accumulated tyrosine kinases of the Src family (Src family kinases; SFKs) are activated in response to homophilic binding of E-cadherin at the cell-cell interface (McLachlan et al., 2007), participate in signal transmission, and regulate junctional properties. Inhibition of c-Src activity significantly perturbs E-cadherin distribution, homophilic adhesion, and actin cytoskeleton organization (Calautti et al., 1998; Takahashi et al., 2005). At the same time, constitutively active v-Src also disrupts the integrity of cell-cell contacts, impairing contact formation and extension (Behrens et al., 1993; Volberg et al., 1991; Warren and Nelson, 1987). Clearly, SFK signaling can both positively and negatively affect E-cadherin function, depending on SFK signal strength (McLachlan et al., 2007). The complexity of SFK functions at cadherin-based junctions mandates the identification of their regulators at this cellular locale, so as to understand how they are affected by locally received signals, such as cell-cell contact or mechanical force.

Among several mechanisms, Src kinases are subject to regulation through phosphorylation on a number of discrete tyrosine residues, with positive or negative contributions to kinase activity (Roskoski, 2005). Recent data also point to a role for protein tyrosine phosphatase (PTP) activity in the maintenance of intercellular junction integrity. Inhibition of PTP activity by pervanadate treatment disrupts epithelial junctions, and interestingly impairs E-cadherin-activated Src signaling (McLachlan and Yap, 2011). While some studies have reported a role for cell contact-associated PTPs in controlling integrity and function of epithelial junctions (Anders et al., 2006; Espejo et al., 2010; Fuchs et al., 1996; Müller et al., 1999; Sheth et al., 2007), none of these identified PTPs has been shown to control junctional SFKs.

Here we report that the transmembrane receptor protein tyrosine phosphatase RPTPα, a well-established physiological regulator of c-Src (Ponniah et al., 1999; Su et al., 1999), localizes to intercellular junctions in epithelial cells, and is required for their integrity. RPTPα is already known to participate in fibroblastic adhesion-mediated processes, such as integrin-
dependent spreading, cell motility, and force transduction at integrin-cytoskeleton linkages (Jiang et al., 2006; Su et al., 1999; von Wichert et al., 2003), and to function with various neuronal cell adhesion molecules in neurons (Bodrikov et al., 2005; Ye et al., 2008). PTPs are now recognized to affect cell behaviors related to oncogenicity in complex ways (Labbé et al., 2012). Overexpression of RPTPα can induce fibroblast transformation, presumably through its Src-activating activity (Tremper-Wells et al., 2010; Zheng et al., 1992). Although in vitro and in vivo observations indicate that RPTPα can also control carcinoma tumorigenesis and/or invasiveness (Ardini et al., 2000; Huang et al., 2011; Krndija et al., 2010; Meyer et al., 2013), its cell biological functionality in epithelial context has hardly been investigated. Identifying RPTPα as a mediator of E-cadherin-mediated c-Src activation provides new insight into the relevance of receptor PTPs for the properties of normal and neoplastic epithelia.

RESULTS

RPTPα localizes to cadherin-based intercellular junctions in epithelial cells, and is in molecular proximity to E-cadherin

To start investigating the intracellular localization of RPTPα in the epithelial context, we expressed a fusion protein between human RPTPα and the fluorescent protein Venus (Nagai et al., 2002) in various epithelial cell lines. This revealed a striking pattern of accumulation of RPTPα at cell-cell junctions (Fig. 1A). In order to document the kinetics of this localization, we followed the process of cell-cell contact formation by time lapse imaging. We noticed that RPTPα was recruited to intercellular adhesion sites from the early stages of contact formation, when lamellipodial protrusions from two cells touched each other, and subsequently remained at local contact sites, accumulating along the cell-cell interface (Fig. 1B; supplementary movie M1).

To characterize the epithelial localization of RPTPα we investigated its colocalization with junctional complexes, focusing on adherens junctions marked by E-cadherin (Fig. 1C), and on tight junctions marked by ZO-1 (Fig. 1D). Extensive overlay between RPTPα and E-cadherin was observed, while a differential localization was detected with respect to ZO-1. We found RPTPα to colocalize with E-cadherin at the lateral membrane (Pearson’s correlation coefficient r=0.7); by contrast, RPTPα and ZO-1 clearly occupied distinct membrane portions (Pearson’s correlation r=0.3), as also shown by the Z-axis views. These
data reveal that RPTPα specifically accumulates at adherens junctions, characterized by the presence of E-cadherin.

We then investigated whether RPTPα and E-cadherin might be in molecular proximity to one another. Co-immunoprecipitation experiments in Caco-2 cells revealed weak (130%) but significant (p=0.027 in 9 independent experiments; data not shown) enrichment of E-cadherin within anti-RPTPα immune complexes as compared to within control IgG complexes. Subsequently we measured fluorescence resonance energy transfer (FRET) between both molecules in live Caco-2 cells, using the E-cadherin/β-catenin and E-cadherin/VAMP3 pairs as positive and negative controls, respectively (Ferrari et al., 2012). This analysis revealed FRET efficiency between RPTPα and E-cadherin at junctions similar to that between E-cadherin and β-catenin (Fig. 1E).

RPTPα depletion compromises Caco-2 morphogenesis, and results in aberrant positioning of the apical compartment in three-dimension cultures

Adherens junctions are important determinants of epithelial tissue integrity and morphogenesis (Gumbiner, 2005; Halbleib and Nelson, 2006; Nishimura and Takeichi, 2009). To assess whether RPTPα is involved in epithelial organization, we examined if it is required for the formation of cysts, typical three-dimensional structures generated by epithelial cells. When cultivated in matrigel matrix, Caco-2 cells assemble into ordered structures consisting of highly polarized cells interconnected by epithelial junctions (Jaffe et al., 2008). We compared cyst formation between pools of Caco-2 cells that had undergone stable lentiviral-induced knockdown (KD) of RPTPα, and control Caco-2 cells expressing a non-targeting control shRNA. The two pools of RPTPα KD cells retained different levels of residual RPTPα protein (Fig. 2A,B). Cyst formation was assessed after 14 days of culture, through emergence of a central lumen, as revealed by F-actin. While the majority of control cells efficiently developed single lumen cysts, RPTPα-depleted cysts more often appeared abnormal, with multiple lumina opening in various positions within the cyst. Quantitation revealed that RPTPα depletion significantly increased the population of cysts with multiple lumina from 37% to 53% (Fig. 2C,D).

To gain insight into the mechanism by which RPTPα depletion results in multiple lumina, the effect of RPTPα KD on development of cysts was also examined at early time points (24 and 72 hours after seeding). As expected, in control cells a symmetrically placed F-
actin-marked precursor apical surface appeared in the middle of the nascent cyst from the two-cell stage onward. As cysts grew, the single apical region was maintained at the center of the structure, where the lumen should progressively open (Fig. 2E, upper panels). By contrast, RPTPα-depleted cells often displayed a lateral shift of their apical surface with respect to the center of the nascent cyst, and, as cysts grew, multiple apical membrane patches were detected within the same structure (Fig. 2E, lower panels). Counting cysts with single or multiple apical surfaces after 72 hours of culture, we found that the percentage of cysts with multiple apical surfaces was significantly higher in RPTPα KD cells (55%) than in controls (22%) (Fig. 2F). We conclude that RPTPα KD leads to defective cyst morphogenesis of Caco-2 cells, and that this effect probably results from aberrant positioning of the apical compartment with respect to the growing cyst.

**RPTPα depletion impairs cadherin-based intercellular aggregation**

Given the colocalization of RPTPα with E-cadherin, we speculated that the appearance of aberrant three-dimensional structures resulting from RPTPα depletion might reflect a role for RPTPα in regulating establishment and functions of E-cadherin-based adherens junctions. As a first step to address this possibility, we determined whether knockdown of RPTPα affected the ability of isolated Caco-2 cells to aggregate in suspension, a process promoted by homophilic E-cadherin adhesion (Nagafuchi et al., 1987). Control or RPTPα-depleted cells were incubated under shaking for 4 hours (Fig. 3A). At this time, control cells underwent calcium-dependent aggregation. By contrast, RPTPα-depleted cells tended to remain isolated or formed aggregates of reduced size compared to control (Fig. 3B). We conclude that RPTPα KD compromises E-cadherin-mediated intercellular aggregation in suspension.

**RPTPα is necessary for the integrity of cell-cell contacts in epithelial cell monolayers**

We subsequently analyzed the effect of transient knockdown of RPTPα on junctional morphology in monolayer culture. Transfection of siRNA reduced overall RPTPα levels in confluent Caco-2 cells by ~90% (Fig. 4A). RPTPα depletion perturbed E-cadherin staining (Fig. 4B, left panels) while it did not affect total E-cadherin level (Fig. 4A), suggesting defects in protein distribution at the cell membrane. Contrasting with the sharp and linear
staining in control cells, reducing RPTPα levels induced a broadening of E-cadherin staining throughout the lateral cell-cell interface, with a discontinuous distribution. To quantify such alterations we measured the width and average pixel intensity of E-cadherin staining at the cell-cell contacts. As shown in Fig. 4C, RPTPα KD was associated with significantly broader E-cadherin staining and a significant reduction in average intensity at cell-cell contacts. The distribution of E-cadherin signal, measured along intercellular contacts, also revealed that the continuous staining in control cells was replaced by a more discontinuous signal in RPTPα-depleted cells. The perijunctional actin cytoskeleton, linked with E-cadherin at the adherens junctions, was also greatly perturbed by RPTPα KD (Fig. 4B, middle panels). Whereas control cells showed an intense ring of F-actin at the interface between cells, RPTPα KD cells showed reduced F-actin density at contacts (Fig. 4D). Both aberrant E-cadherin and F-actin distribution caused by RPTPα depletion could be rescued by expressing siRNA-resistant mouse RPTPα (Fig. 7C).

Contrasting with these observations, the integrity of tight junctions seemed not to be impaired by RPTPα KD, as observed by staining with ZO-1 (Fig. 4B, right panels). However, RPTPα depletion reduced junctional linearity at the apical intercellular boundaries: cell-cell junctions appeared more wavy and less straight, compared to control cells (Fig. 4B, right panels and Fig. 4E), suggesting that loss of RPTPα reduces junctional tension (McLachlan and Yap, 2011; Otani et al., 2006).

Similar junctional defects were observed when RPTPα was knocked down by siRNA in the breast carcinoma cell line MCF7 (Fig. S1). Altogether, our data indicate that RPTPα is required for the integrity and organization of E-cadherin-based intercellular junctions and the underlying actin cytoskeleton.

**RPTPα is required for tonic Src activity in epithelial cells, and controls tyrosine phosphorylation at epithelial junctions**

Tyrosine phosphorylation is crucial for both assembly and stability of cadherin-based adhesive complexes. Broad-spectrum PTP inhibition using vanadate indicates that PTP activity is required for keeping a balanced phospho-tyrosine content at cell-cell junctions, and for maintenance of these structures (McLachlan and Yap, 2011). Decreased tyrosine phosphorylation upon PTP inhibition might reflect the ability of certain PTPs to positively
modulate kinase activity and signaling (Burns et al., 1994; Su et al., 1999; McLachlan and Yap, 2011). Indeed, RPTPα is an established physiological activator of SFKs in multiple contexts, due to its ability to dephosphorylate their inhibitory C-terminal residue (Su et al., 1999; Ponniah et al., 1999; Zheng et al., 2000; Bodrikov et al., 2005; Vacaresse et al., 2008).

Since c-Src is present at cell-cell contacts, we checked whether RPTPα depletion might affect its kinase activity in epithelial cells. For this purpose we used an antibody against phosphorylated tyrosine 419 (pY419-Src), an activation loop residue in c-Src, phosphorylation of which correlates with the active state. As shown in Fig. 5A,B, depletion of RPTPα significantly reduced the fraction of pY419-Src, by an extent (50-60%) comparable to that seen in other settings (Su et al., 1999; Ponniah et al., 1999; Zheng and Shalloway, 2001; Roskoski, 2005). We then compared the distribution of total protein tyrosine phosphorylation between control and RPTPα-depleted cells. Anti-phosphotyrosine immunostaining revealed a local decrease in tyrosine phosphorylation at cell-cell contacts following RPTPα KD (Fig. 5C,D). This decrease in junctional tyrosine phosphorylation was accompanied by a local decrease in c-Src activation: transient RPTPα KD reduced the fraction of Y419-phosphorylated c-Src at intercellular contacts (Fig. 5E,F).

Our finding revealed that RPTPα contributes to Src activity in epithelial cells, particularly at intercellular contacts, where consequently it likely promotes tyrosine phosphorylation.

**RPTPα is required for E-cadherin-mediated Src activation**

Cadherin-dependent cell-cell adhesion activates c-Src and other SFKs (McLachlan et al., 2007; Yap and Kovacs, 2003); however, the molecular mechanism of this process remains as yet unknown. Since our findings demonstrated that RPTPα is essential for Src activation in epithelial cells, we proceeded to test the involvement of RPTPα in early responses to E-cadherin-dependent cell adhesion. To this end we homophilically activated E-cadherin at the surface of isolated cells using a recombinant ligand consisting of the ectodomain of human E-cadherin fused to the Fc moiety of IgG (Ecad-Fc) (Adams et al., 1996). This approach allowed us to isolate the effect of E-cadherin homophilic ligation from other juxtacrine signals that are exchanged during cell-cell contacts (Yap and Kovacs, 2003).

Control and RPTPα KD cells were allowed to adhere to substrata coated with Ecad-Fc for 90 minutes. We observed RPTPα specifically accumulating at the leading edge of control
cells spreading on Ecad-Fc, while it was absent from the leading edge of RPTPα KD cells (Fig. 6A). Wondering whether RPTPα accumulation at active leading edges might have a role in cadherin-based cellular spreading, we compared the extent of spreading of control and RPTPα KD cells on Ecad-Fc- or non-signaling adhesive ligand poly-D-lysine (PDL)-coated surfaces. As expected (Kovacs et al., 2002b), cells did not spread on PDL-coated surface, but engaged in rapid spreading upon adhesion to substrate coated with the recombinant E-cadherin, as revealed by the appearance of prominent lamellipodia (Fig. 6B). Spreading on Ecad-Fc was cadherin-dependent as it was inhibited by E-cadherin blocking antibody (Fig. S2A,B).

We then studied the involvement of RPTPα in spreading on Ecad-Fc. Strikingly, RPTPα depletion was associated with a spreading defect, since the total surface area of RPTPα KD cells was significantly reduced respective to controls for the most effective shRNA (RPTPα shRNA1), with a tendency toward reduction for the less effective shRNA2 (Fig. 6C). We previously showed that RPTPα associates with αvβ3 integrin at the leading edge of cells spreading on vitronectin (von Wichert et al., 2003b). To rule out interference by integrin-dependent effects during spreading on Ecad-Fc, we used the integrin-blocking peptide GPenGRGDSPCA (cRGD), an αvβ3 antagonist. cRGD impaired spreading of Caco-2 cells on fibronectin (Fig. S2C), reflecting additional blocking of fibronectin-binding integrins, but left unchanged the decrease (by 35%) in spreading on Ecad-Fc of RPTPα KD cells compared to controls (compare Fig. 6C and Fig. S2E).

Previous studies have shown that adhesion to E-cadherin substrate stimulates activation of SFKs (McLachlan et al., 2007). We therefore monitored the effect of RPTPα KD on E-cadherin-mediated Src activation. Ninety minutes after initial adhesion to recombinant cadherin, immunostaining for pY419-Src in control cells revealed active c-Src at the E-cadherin-driven lamellipodia. By contrast, the ability of RPTPα shRNA1 cells in activating c-Src at these sites was compromised, as revealed by decreased pY419-Src staining at cadherin-based lamellipodia compared to controls, while the less effectively interfering shRNA2 caused a tendency decrease (Fig. 6D,E).

Altogether, our observations provide evidence that RPTPα is required for Src activation downstream of E-cadherin.
RPTPα regulates cadherin-based junctions by affecting cortactin tyrosine phosphorylation

We wished to determine which among the multiple signaling events triggered by c-Src mediates RPTPα-mediated control of epithelial junctions. In previous studies we have shown that the actin-binding protein cortactin is necessary for E-cadherin-mediated contact formation and actin reorganization, and that it is a target of E-cadherin-activated Src (Helwani et al., 2004; Ren et al., 2009). Cortactin depletion affects junctional E-cadherin accumulation, and rescue of this phenotype requires tyrosine phosphorylation of cortactin (Ren et al., 2009). We observed presence of cortactin in an in vitro pull-down assay from Caco-2 cells aimed at characterizing interaction partners and substrates for RPTPα (Fig. S3). These observations led us to investigate the functional interaction between cortactin and RPTPα at epithelial junctions.

We first determined whether RPTPα depletion affects tyrosine phosphorylation of cortactin. Cortactin immunoprecipitation, followed by anti-phosphotyrosine immunoblotting, revealed reduced cortactin tyrosine phosphorylation in RPTPα KD cells as compared to controls, while total cortactin recovery was comparable (Fig. 7A).

This finding suggested that cortactin might be a functional downstream effector of RPTPα-activated c-Src at cadherin-based junctions. To test this possibility, we examined how expression of phosphorylation mutants of cortactin affected the junctional phenotype induced by RPTPα KD. Accordingly, we co-transfected Caco-2 cells with RPTPα siRNA, singly or together with a number of cortactin variants: wild type cortactin (WT), a non-phosphorylatable variant (3YF, in which three principal tyrosine residues, Y421, Y470 and Y486, were mutated to phenylalanine), or a phosphomimetic variant (3YD, in which these three tyrosine residues were replaced by aspartic acid) (Ren et al., 2009), or with a fluorescence-tagged mouse RPTPα construct (mRPTPα-cherry). We subsequently monitored the intensity of E-cadherin signal and the density of F-actin along junctions as a measure of the junctional RPTPα KD phenotype. Overexpression of either WT cortactin or the 3YF variant had no effect on the reduction of junctional E-cadherin and F-actin induced by RPTPα depletion. By contrast, expression of cortactin 3YD significantly increased junctional E-cadherin and F-actin in RPTPα KD cells up to a level comparable to what observed when mRPTPα was used to rescue the phenotype (Fig. 7B,C,D).
These results indicate that RPTPα KD reduces cortactin tyrosine phosphorylation, and suggest that this reduction is responsible for the perturbed junctional organization associated with RPTPα depletion.

DISCUSSION

RPTPα controls a c-Src/cortactin signaling pathway important for the organization of epithelial adherens junctions

Epithelial integrity and organization are collective cellular functions that accordingly require coordinated intercellular adhesion and communication, processes in which E-cadherin-mediated phosphotyrosine signaling by SFKs is crucial. The v-Src oncogene has long been known to disturb epithelial communality, and more recently c-Src signaling was reported to be activated by E-cadherin homophilic binding and to mediate junctional organization (McLachlan et al., 2007). Yet the actors controlling c-Src at adherens junctions have hardly been identified. The tyrosine phosphatase RPTPα has been implicated in SFK regulation in fibroblasts, neurons, and other cell types, by virtue of its ability to specifically remove an inhibitory phosphate on the critical regulatory C-terminal tyrosine residue in these kinases. In spite of the potential relevance of RPTPα in carcinomas (in which SFKs are commonly found to be activated), the epithelial cell biology of RPTPα remained unexplored.

We observe here that RPTPα is enriched at cadherin-based adherens junctions, where it is in close molecular proximity to E-cadherin, and that it participates in activation and regulation of epithelial c-Src at this cellular locale. This effect is accompanied by control of the ability of epithelial cells to organize in well-ordered three-dimensional structures, to form calcium-dependent aggregates, and to maintain integrity of intercellular junctions. In cellular monolayers, knockdown of RPTPα affects E-cadherin organization, the perijunctional F-actin ring, and the linearity of cell-cell junctions.

As a downstream effector of RPTPα-activated c-Src at cadherin-based junctions, we identify the c-Src substrate cortactin. The junctional effects of RPTPα KD are similar to those of cortactin depletion in MCF7 cells (Ren et al. 2009), and in Caco-2 cells (data not shown). Here we show that adequate tyrosine phosphorylation of cortactin depends on RPTPα, and expression of the 3YD mutant form of cortactin normalizes the aberrant E-cadherin
distribution associated with loss of RPTPα. This reveals a molecular link between RPTPα and epithelial junction integrity. It is possible to interpret the behavior of the 3YD mutant as mimicking (c-Src-mediated) cortactin phosphorylation. While this requires caution given the significant structural differences between aspartic acid and phosphotyrosine, this interpretation is supported by the observation that the 3YF mutant did not rescue the effect of either cortactin or RPTPα depletion.

As a target of E-cadherin-activated SFK signaling (Helwani et al., 2004; Ren et al., 2009), cortactin is a positive regulator of the Arp2/3 complex, involved in control and homeostasis of branched actin filaments. By showing RPTPα-mediated regulation of the junctional cortactin pool, our studies thus implicate RPTPα in coordination between E-cadherin and the actin cytoskeleton, a crucial process for intercellular adhesion (Lambert et al., 2002; Thomas et al., 2013).

**Functions of RPTPα at the epithelial junctions**

Our observations indicate that RPTPα depletion may reduce intercellular adhesion strengthening by the perijunctional actin cytoskeleton by deregulating cortactin function, with consequent aberrant organization of E-cadherin at adherens junctions. However, the potential for additional targets of RPTPα-mediated Src signaling at epithelial junctions merits exploration. Junctional integrity requires cortical actomyosin contractility, which is promoted by E-cadherin homophilic binding and contributes to extension and strengthening of E-cadherin-based adhesions, by generating tension along the junctions (Gomez et al., 2011). RPTPα has already been linked to cytoskeletal contractility and stress fiber formation at integrin-based focal adhesion sites; these effects depend on substrate stiffness and require RPTPα-mediated SFK activation (Krndija et al., 2010; von Wichert et al., 2003; Zeng et al., 2003). Our observation of reduced junctional linearity in epithelial monolayers as a consequence of RPTPα KD suggests imbalanced junctional tension between adjacent cells. In this sense an additional relevant target of RPTPα at epithelial junctions may be the nonmuscle myosin IIB, which is required for E-cadherin organization at intercellular contacts (Smutny et al., 2010), and is recruited to the adherens junction through SFK signaling (McLachlan and Yap, 2011).

Surface organization of E-cadherin also relies on dynamic endocytosis and/or recycling, dysfunction of which might contribute to the discontinuous and punctuated distribution of E-
cadherin observed upon RPTPα KD. Potentially, by regulating c-Src, RPTPα could control events in the endocytic pathway, given the implication of the former in epithelial E-cadherin endocytosis (Canel et al., 2010; Swaminathan and Cartwright, 2012), and in neuronal neurotransmitter receptor trafficking in neurons (Ohnishi et al., 2011).

The complex and multi-step nature of junctional organization and maturation, though ill-understood, raises the question at what stage of maturation RPTPα action is most crucial. RPTPα KD-induced defects in cell aggregation and spreading on Ecad-Fc suggest an early intervention of RPTPα, consistent with its early recruitment at cell-cell contacts. It will be informative to characterize in detail the contribution of RPTPα to formation and fusion of transient junctional intermediates (spot-like adherens junctions; Yonemura et al., 1995) during junctional maturation, to E-cadherin dynamics, e.g. basal-to-apical flow (Kametani and Takeichi, 2007), and to endocytic recycling.

Finally, our data do not rule out epithelial functions of RPTPα beyond E-cadherin-mediated adhesion: e.g. RPTPα’s relevance for 3D organization into cysts may suggest a role in the maintenance of apicobasal polarity.

**PTPs and integrity of cadherin-based junctions**

Our findings concretize and expand understanding of the involvement of PTPs in cell-cell adhesion. Global PTP inhibition had suggested that PTPs support cadherin-based junctional integrity (McLachlan and Yap, 2011). However, such effects could merely reflect indirect activation of tyrosine kinases that are negatively controlled by PTPs. By contrast, our findings directly implicate RPTPα in adherens junction integrity. Several other PTPs also act at junctions (Anders et al., 2006; Espejo et al., 2010; Fuchs et al., 1996; Müller et al., 1999; Sheth et al., 2007). To the best of our knowledge, our study identifies RPTPα as the first PTP controlling c-Src signaling at cadherin-based adhesions. It is, however, not unlikely that additional PTPs will be found to contribute to c-Src activation at cell-cell contacts.

**Mechanistic aspects of E-cadherin/RPTPα interactions**

Our results suggest that RPTPα is responsible for local activation of c-Src signaling upon E-cadherin engagement. It remains to be elucidated how signals from E-cadherin adhesion would be transduced to RPTPα. RPTPα has been reported to transduce signals from
cell adhesion molecules, such as NCAM or integrin, by physically associating with them. The occurrence of FRET between RPTPα and E-cadherin indicates close proximity and the potential for direct interaction, while the low level of co-immunoprecipitation between both suggests that their interaction may be weak and/or transient. Such interaction may be mediated by the RPTPα ectodomain, similarly to what has been claimed for RPTPα association with the neural adhesion molecule contactin (Zeng et al., 1999); in this case homophilic engagement of E-cadherin itself might be able to transmit a conformational signal to the RPTPα ectodomain, resulting in altered RPTPα function. Alternatively, the association may involve intracellular interactions, analogous to what was suggested for RPTPα-NCAM crosstalk (Bodrikov et al., 2005). The dynamics of RPTPα recruitment as an E-cadherin partner will likely be a rewarding topic for study.

A second issue requiring further study concerns the possibility and mechanism of local activation step of RPTPα at epithelial junctions. Indeed, multiple regulatory mechanisms are known to control RPTPα: dimerization, post-translational modifications such as serine and tyrosine phosphorylation and cysteine oxidation, and conformational changes (Groen et al., 2008; Jiang et al., 1999; Su et al., 1994; Zheng and Shalloway, 2001). Whether and how E-cadherin homophilic binding, and the presence of particular actors or conditions at adherens junctions, affects one or several of these processes is an interesting question for future follow-up. The answers should also be instructive concerning the other types of adhesion molecules and junctions that rely on PTPs for appropriate function.

MATERIALS AND METHODS

Cell culture

Caco-2 cells were cultured in RPMI medium containing 10% fetal bovine serum (FBS), non-essential amino acids (Gibco), penicillin (100 units/ml) and streptomycin (100 µg/ml). MCF-7, A431 and HEK 293T cells were cultured in DMEM supplemented with 10% FBS, penicillin (100 units/ml) and streptomycin (100 µg/ml).

For three-dimensional (3D) growth, Caco-2 cells were trypsinized to obtain a single-cell suspension. A suspension of 5.8 x 10⁴ cells/ml was mixed with a solution containing 40% Matrigel (BD #354230), 1 mg/ml type I collagen (Trevigen #3440-100-01) and 0.02 M
Hepes. Cells embedded in this matrigel solution were plated into 8-well chamber slides (Becton Dickinson). The matrigel matrix was allowed to solidify by incubation at 37°C for 30 minutes prior to addition of medium. Medium was renewed every 3–4 days and up to 14 days to follow cyst formation.

Plasmids and siRNA cell transfection

Y421D/Y470D/Y486D (3YD) and Y421F/Y470/Y486F (3YF) cortactin mutants were generated by two-step PCR-based mutagenesis and introduced into pEGFP-N1 vector. RPTPα RNAi oligonucleotide duplex (SASI_Hs01_00169093) and scrambled control RNA (SIC001) were from Sigma. Transient DNA and siRNA transfections were performed using Lipofectamine 2000 or RNAiMAX (Invitrogen) according to manufacturer’s instructions (3:1 Lipofectamine reagent:DNA; 50 nM siRNA final concentration).

Human β-catenin-mCherry and murine RPTPα-mCherry were obtained by cloning the corresponding cDNA into pmCherry-N1 vector (Clontech). Similarly, murine E-cadherin-GFP was obtained by cloning the murine E-cadherin cDNA into pEGFP-N1 (Clontech; Smutny et al. 2011). mCherry-VAMP3 was a generous gift from Dr. Jenny Stow, Institute for Molecular Bioscience, Australia. GFP-mCherry tandem construct was obtained by cloning the GFP coding sequence into NheI and XhoI sites of pmCherry-N1.

Generation of lentiviral particles and infection

cDNA encoding human RPTPα was fused at its C-terminus to the fluorescent protein Venus and introduced into a pLenti derivative. Infected A431 cells were selected with 10 µg/ml blasticidin for 10 days.

To knockdown RPTPα, we used the pLKO.1-puro lentiviral vector (Sigma). The sense targeting sequences for human PTPRA were 5’-GGCGAAGAGAATACAGACTAT-3’ (RPTPα shRNA1), and 5’-AGCCCTTCTGGAGCATTATCT-3’ (RPTPα shRNA2); and for the non-targeting control 5’-CAACAAGATGAAGAGCACCAA-3’ (Sigma TRC1.5 clone SHC002). A 2nd generation packaging system was used to produce virus: 5 µg transfer vector were co-transfected together with 10 µg pMD2G (VSV-G, Addgene 12259) and 5 µg psPax2 (gag-pol, tat-rev; Addgene 12260) using calcium phosphate coprecipitation into HEK 293T cells seeded 24 hours before transfection. Supernatant containing viral particles was
harvested 48 hours post-transfection, and concentrated 100-fold by ultracentrifugation at 80,000 x g for 2 hours at 4°C using the SW32 Ti rotor (Beckman). The viral pellet was resuspended in PBS and stored at -80°C. Infected cells were selected in 4 µg/ml puromycin for 10 days, then maintained in 2 µg/ml puromycin.

**Cell aggregation assay**

Nearly confluent cells were isolated by calcium sequestration in 4 mM EDTA in Hanks’ Balanced Salt Solution (HBSS) (40 mg/L KCl, 60 mg/L KH2PO4, 8 g/L NaCl, 90 mg/L Na2HPO4·7H2O, 100 mg/L D-glucose). Isolated cells were resuspended at 2 x 10⁵/ml in 10 mM calcium-supplemented medium or in presence of 4 mM EDTA, plated in culture dishes previously coated with 2% BSA, 10 mM CaCl2 in HBSS overnight at 4°C, and further shaken at 75 rpm for 4 hours at 37°C to allow aggregation. Cells were fixed in 2% paraformaldehyde (PFA), and phase contrast images taken using Upright Leica DMR inverted microscope.

**Antibodies**

Primary antibodies used were mouse monoclonal anti-E-Cadherin (BD transduction laboratories), mouse monoclonal HECD-1 against E-cadherin ectodomain (a gift from Peggy Wheelock Omaha, NE), mouse monoclonal anti-E-cadherin blocking antibody SHE78-7 (Invitrogen), mouse monoclonal anti-Cortactin 4F11 (Millipore), mouse anti-phosphotyrosine 4G10 (hybridoma culture supernatant), mouse monoclonal anti-v-Src 327 (Sigma-Aldrich), mouse monoclonal anti-c-Src antibody 2-17 (Sandilands et al., 2007), rabbit polyclonal anti-Tyr(P)-419-Src family (Cell signaling), mouse monoclonal anti-α-tubulin (Sigma-Aldrich), rabbit polyclonal anti-RPTPα (Su et al., 1999) and mouse monoclonal anti-ZO-1 (Invitrogen).

Secondary antibodies used were goat anti-rabbit or anti-mouse Alexa Fluor 488, 594 or 647 (Invitrogen), donkey anti-mouse or anti-rabbit Cy3 (Jackson ImmunoResearch).

**Extracts and Immunoblot analysis**

Protein extracts were prepared in Triton lysis buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EDTA, 10% Glycerol and 1% Triton X-100), containing 1 mM Na3VO4, 10 mM NaF, 4% Protease Inhibitor Cocktail (Roche) and 1 mM PMSF, and cleared at 12,000 rpm for 15 min. 20 µg of proteins were resuspended in Laemmli buffer, resolved on 8% polyacrylamide gels and transferred to Immobilon membrane (Millipore). Membranes
were blocked with 5% skim milk or 5% BSA in TBS for 1 hour at room temperature (RT) and incubated overnight at 4°C with primary antibody. Membranes were washed, incubated for 1 hour with secondary antibody coupled with horseradish peroxidase (Jackson ImmunoResearch) and revealed by enhanced chemiluminescence. When required, membranes were incubated in stripping buffer (62.5 mM Tris pH 6.8, 2% SDS, 100 mM β-mercaptoethanol) for 1 hour at 60°C under agitation and washed in TBS-0.1% Tween-20.

**Immunoprecipitation**

Protein G-agarose beads (50% slurry, Upstate-16266) coupled with anti-Cortactin antibody were incubated with 1.5 mg lysate overnight at 4°C on a rotating wheel. Beads were washed six times with Triton lysis buffer containing 1 mM Na₃VO₄ and 10 mM NaF, and resuspended in Laemmli buffer. Coimmunoprecipitation of RPTPα and E-cadherin was done using lysis buffer containing 20 mM Hepes pH 7.5, 150 mM NaCl, 0.5% NP-40, 50 mM KCl, 2 mM CaCl₂ plus protease and phosphatase inhibitors.

**Recombinant Protein Production and Purification**

Expression vector (pEE14 backbone) containing the extracellular region of human E-cadherin fused to the IgG Fc (Ecad-Fc) was transiently transfected into HEK 293T cells. The secreted protein was purified from conditioned media by protein A affinity chromatography. Protein samples were dialyzed at 4°C into calcium-containing storage buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM CaCl₂, pH 7.4).

**Cell spreading assay**

IbiTreat (ibidi) 8-well microslides were coated with Ecad-Fc (50 µg/ml), or Poly-D lysine (0.1 mg/ml) overnight at 4°C then blocked with 10 mg/ml BSA, 5 mM CaCl₂ in HBSS for 30 min, as described (Kovacs et al., 2002a).

Control and RPTPα KD cells were trypsinized for 5 minutes at 37°C and maintained in single-cell suspension (2 x 10⁵ cells/ml in serum containing medium) for 1 h at 37°C under constant agitation. Where applicable, during the last 30 minutes in suspension, the E-cadherin blocking antibody SHE78-7 or the integrin-blocking peptide GPenGRGDSPGA (cRGD) (Bachem H-3964) were introduced. The cell suspension was then seeded on coated plates and incubated at 37°C for 90 minutes. Cells were fixed with 1% PFA for immunofluorescence or
staining with 0.5% Cresyl Violet. Total cell surface area (in pixel) of adherent cells was determined on phase contrast images obtained by Upright Leica DMR inverted microscope and analyzed by Metamorph or imageJ.

**In vitro substrate trapping**

Human PTPRA catalytic site cysteine residues 442 and 732 were simultaneously mutated to serine, yielding the C/S mutant cDNA. The intracellular domain of wild type or mutant (C/S) PTPRA was subcloned as an XmaIII-EcoRV fragment (after blunting the XmaIII overhang with Klenow) into the SmaI site of pGEX3X. GST fusion proteins were produced in BCL bacteria, purified using glutathione-Sepharose beads (GE Healthcare) and stored in bound form at -80°C.

Nearly confluent cells were subjected to pervanadate treatment for 20 minutes by adding 1 mM Na₃VO₄ and 1 mM H₂O₂ into the culture medium. Cell extracts were prepared in Triton lysis buffer containing 5 mM iodoacetic acid, 10 mM NaF, 4% Protease Inhibitor Cocktail (Roche) and 1 mM PMSF, further incubated at 4°C for 15 minutes with 10 mM dithiothreitol (BioChemika) to inactivate unreacted iodoacetic acid and then incubated with GST fusion proteins coupled to glutathione-Sepharose beads for 90 minutes at 4°C. Beads were washed four times with lysis buffer, resuspended in Laemmli buffer and boiled before SDS-PAGE.

**Immunofluorescence and image analysis**

For antibody penetration in 3D cultures, collagen was digested in 50 units/ml collagenase III for 15 minutes at RT prior to fixation in 4% PFA for 30 minutes, and immunofluorescence performed as described (Jaffe et al., 2008). Otherwise cells were grown on glass coverslips until confluence and fixed for 5-15 minutes with 1-4% PFA. For immunofluorescence using anti-E-cadherin antibody cells were permeabilized in 0.025% saponin for 30 minutes, and blocked in 10% FBS, 0.025% saponin. For all other antibodies cells were permeabilized with 0.3% Triton X-100 in PBS for 5 minutes and blocked with 0.5% BSA, 0.2% gelatin, 150 mM NaCl, 0.1% Triton X-100. Incubation with primary antibody dilutions was performed overnight at 4°C; after washing incubation with secondary antibodies was performed for 1 hour at RT. Nuclei were stained by DAPI (100 ng/ml, Sigma). Coverslips were mounted in Mowiol (Sigma).
Epiillumination images were acquired with an Upright Leica DMR inverted microscope and a DFC Leica camera or an IX81 Olympus microscope and a Hamamatsu Orca-1 ER camera, or with Leica TCS SP5 AOBS Tandem resonant Scanner confocal microscope when indicated. Images were analyzed with Metamorph or Image J software.

To analyze E-cadherin signal spread, intensity, and continuity, or F-actin density at cell-cell contacts, the Line Scan function of Metamorph was used. In brief, spreading of the E-cadherin signal was measured from the spread of the signal intensity plots along a 50 pixels line orthogonal to individual cell-cell contacts. Fluorescence intensity at cell-cell contacts was analyzed by drawing regions of interest covering individual cell-cell contacts and measuring average total signal intensity (for E-cadherin or F-actin) and the distribution of the signal, as intensity standard deviation (for E-cadherin) within it. The junctional linearity index was calculated as the ratio of the linear distance between intercellular vertices to the real junction length, as described (Otani et al., 2006), analyzing individual randomly picked junctions from ZO-1 images. pY419-Src and total c-Src signal intensity on monolayers were analyzed by measuring mean signal intensity along the same line (10 pixel large) covering individual junctions. The analysis of the activation status of c-Src at the cadherin-driven lamellipodia in the spreading assay was done by drawing a line (15 pixel large) covering the entire perimeter of individual spread cells and measuring average pY419-Src signal intensity along the line.

Colocalization analysis was performed on individual cell-cell contact Z-axis views with the Image J plugin JacoP, using Costes’ method with 100 randomization rounds.

For FRET analysis, Caco-2 cells were cultured on glass-bottom dishes (Shengyou Biotechnology Co. Ltd, China) and transiently transfected with pEGFP-N1, pmCherry-N1 and GFP-mCherry tandem construct, or co-transfected with E-cad-GFP and β-Cat-mCherry or RPTPα-mCherry or mCherry-VAMP3. Live cells were imaged 48h post-transfection at 37°C on an LSM510 Meta Zeiss confocal microscope. During imaging, cells were incubated in HBSS supplemented with 10 mM HEPES pH7.4 and 5 mM CaCl₂. Images were acquired with a 63x oil Immersion objective (Plan Apochromat 1.4 NA, Zeiss) with a resolution of 0.14 µm/pixel. Donor and FRET channels were recorded by scanning using a 488 nm laser line and collecting the emission in the donor emission region (BP 500-530 nm) and acceptor emission region (LP 560 nm), respectively. In addition, the acceptor channel was collected by using the 546 nm laser line for excitation and collecting the emission in the acceptor emission regions. Images were acquired by sequential line acquisition.
For FRET measurements, the corrected FRET (cFRET) signal from images from the FRET channel was obtained in a pixel-by-pixel basis using the following equation (Grashoff et al., 2010; Ferrari et al., 2012)

\[ cFRET = FRET - dbt(I_D)*I_D - abt(I_A)*I_A \]

where FRET is the FRET channel image, \(dbt(I_D)\) and \(abt(I_A)\) are the donor and acceptor bleedthrough fractions expressed as functions of donor (\(I_D\)) and acceptor (\(I_A\)) intensities.

\(dbt(I_D)\) and \(abt(I_A)\) functions and cFRET calculations were performed as described previously (Grashoff et al., 2010) using a custom made MatLab routine (Ratheesh et al., 2012).

The FRET efficiency was calculated for those pixels located in the selected regions of interest (ROI) corresponding to the zonula adherens of cells identified by Ecad-GFP fluorescence signal or the cell cytoplasm when FRET efficiency was determined for the GFP-mCherry tandem construct. FRET efficiency was calculated for every image using the following equation (Ferrari et al., 2012).

\[ E = 1 - \frac{I_D}{I_D + cFRET} \]

**Statistical analyses**

Statistical significance of the data was assessed by calculating the p values by two-tailed Student’s t-test except for FRET measurements where One-Way ANOVA, Tukey’s multiple comparisons test was used. Statistical data is shown as: ns: non significant (\(p>0.05\)); *: \(p<0.05\); **: \(p<0.01\); ***: \(p<0.001\); ****: \(p<0.0001\).
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Figure 1: RPTPα localizes at the cadherin-based intercellular junctions in epithelial cells where it is in close proximity to E-cadherin.

(A) Detection of Venus-tagged RPTPα in monolayer cultures of Caco-2, A431 and MCF7 cells. Scale bar: 20 µm. (B) Time lapse analysis of RPTPα during cell-cell contact establishment. Intercellular contact formation was followed by time-lapse video-microscopy of A431 cells expressing Venus-tagged RPTPα: phase-contrast (PH) and RPTPα-Venus epifluorescence images were acquired every 10 minutes with selected time points shown. White arrowheads show the presence of RPTPα in early cell-cell contact establishment. Scale bar: 10 µm. (C) and (D) Colocalization analysis of RPTPα with E-cadherin and ZO-1. Caco-2 cells expressing RPTPα-venus (green) were immunolabeled for (C) E-cadherin or (D) ZO-1 (purple) and analyzed by confocal microscopy. Single optical sections are shown. A Z-axis view of individual contacts (following white arrow direction) illustrates the distribution of RPTPα and E-cadherin (C), or RPTPα and ZO-1 (D). Overlap between RPTPα and E-cadherin (C) or RPTPα and ZO-1 (D) at junctions (n=23, 2 independent experiments) was analyzed by calculation of Pearson’s correlation coefficient (r). Scale bar: 10 µm. (E) FRET efficiency measurements in Caco-2 cells between E-cadherin and β-catenin (positive control); E-cadherin and RPTPα; E-cadherin and Vesicle-associated membrane protein 3 (VAMP3, negative control); and within a tandem GFP-Cherry fusion protein. Data presented are representative of 3 independent experiments and correspond to mean FRET efficiency values calculated across different images (~75 cell-cell contacts) and their standard errors.

Figure 2: RPTPα depletion inhibits Caco-2 morphogenesis, and results in aberrant positioning of the apical compartment in three-dimension cultures.

Stable RPTPα KD (RPTPα shRNA1 and shRNA2) or control (ctrl shRNA) Caco-2 cells were generated by lentiviral infection. (A) Western blot analysis of RPTPα in control and RNAi-treated cells. Densitometric quantification of RPTPα levels normalized to α-tubulin and compared to control is shown as means ± standard error of the mean (s.e.m.) of 5 independent experiments. (B) Immunofluorescence of RPTPα (green) in control and RPTPα KD cells; nuclei are stained with DAPI (white). Scale bar: 10 µm. (C) Cysts formed after two weeks culture in a matrigel matrix. Representative confocal pictures of cysts with normal morphology (single, central lumen) or with multiple lumina result from immunofluorescence
for F-actin (purple); nuclei are stained with DAPI (white). Scale bar: 20 µm. (D) Quantification of cysts with normal morphology or with multiple lumina in both control and RPTPα KD cells is shown as mean ± s.e.m. of 3 independent experiments (n= 100). (E) Early stages (24h and 72h as indicated) of cyst formation in both control and RPTPα KD cells. Immunostaining for actin (purple); nuclei are stained with DAPI (white). Scale bar: 10 µm. (F) Quantification of cysts with a central single apical surface or with multiple apical surfaces after 72 hours culture in matrigel matrix. Data shown are the mean ± s.e.m. of 3 independent experiments (n= 100).

**Figure 3: RPTPα is required for cell aggregation.**

Control and RPTPα-depleted Caco-2 cells were isolated in presence of EDTA, incubated under shaking condition and analyzed for aggregate formation after 90 minutes. (A) Phase-contrast images of transduced Caco-2 cells expressing shRNA against human RPTPα (RPTPα shRNA1, shRNA2) or a non targeting shRNA (ctrl shRNA) aggregated in absence (+EDTA) or presence (+Ca++) of calcium. (B) Quantification of aggregate size. Data shown are means ± s.e.m. of 3 independent experiments, for each 5 fields were analyzed. Scale bar: 20 µm.

**Figure 4: RPTPα is necessary for the integrity of junctions in epithelial cell monolayers.**

Caco-2 cells were transiently transfected with small interfering RNA against RPTPα (RPTPα siRNA), or with scrambled control RNA (ctrl siRNA), and analyzed 72 hours after transfection. (A) Western blot analysis of RPTPα in both conditions. Densitometric quantification of RPTPα and E-cadherin expression normalized to α-tubulin, shown as mean ± s.e.m. of at least 3 independent experiments. (B) Immunofluorescence for E-cadherin (left panel), F-actin (middle panel) or ZO-1 (right panel) in control and RNAi-treated cells. Single Z confocal pictures are shown. White arrowheads point to defective distribution of E-cadherin and white arrows to overly bended junctions. Scale bar: 10 µm. (C) Quantification of intensity and heterogeneity of E-cadherin signal along junctions, and spread of E-cadherin signal across junctions. (D) Quantification of the perijunctional F-actin intensity. (E) Quantification of the junctional linearity assessed by analysis of ZO-1 staining. Data shown are the mean ±
s.e.m. relative to the control from 3 independent transfections; for each at least 30 junctions were analyzed.

Figure 5: RPTPα is required for tonic Src activity in epithelial cells, and controls tyrosine phosphorylation at the epithelial junctions

(A) Western blot analysis of activated c-Src (pY419-Src) and total c-Src (c-Src) in total cell extracts of Caco-2 cells expressing shRNAs against human RPTPα (RPTPα shRNA1 or RPTPα shRNA2) or a non-targeting shRNA (ctrl shRNA). (B) Densitometric quantification of pY419-Src levels relative to total c-Src levels; data shown are means ± s.e.m. of 3 experiments. (C) Immunofluorescence for total phosphotyrosine (pY) in control (ctrl shRNA) and stable RPTPα KD (RPTPα shRNA1) cells. Scale bar: 10 µm. (D) Quantification of phosphotyrosine signal intensity along junctions. Data are means ± s.e.m. from 3 independent experiments; for each at least 20 junctions were analyzed. (E) Immunostaining for total c-Src (green) and activated c-Src (pY419-Src, purple) in Caco-2 cells transiently transfected with siRNA against RPTPα (RPTPα siRNA) or with scrambled control RNA (ctrl siRNA). (F) Quantification of relative pY419-Src versus c-Src signal intensity along junctions. Data are shown as the ratio of intensities of the two signals; a representative of two experiments is shown; 200 junctions were analyzed.

Figure 6: RPTPα is required for E-cadherin-dependent cell spreading and Src activation.

Cell spreading assay of Caco-2 cells expressing shRNAs against human RPTPα (RPTPα shRNA1 and shRNA2) or a non-targeting shRNA (ctrl shRNA) on poly-D-lysine (PDL) or E-cadherin (Ecad-Fc)-coated surfaces. (A) Immunofluorescence for RPTPα of cells spreading on Ecad-Fc for 90 minutes. Single Z confocal pictures are shown. Higher magnifications of cadherin-driven lamellipodia are highlighted by the squared box (upper right corner). Scale bar: 10 µm. (B) Representative phase-contrast images of control and RPTPα KD spreading cells. A dashed line delimits the surface area in contact with the substrate. Scale bar: 10 µm. (C) Quantification of the cell surface area in contact with Ecad-Fc-coated substrate expressed as mean ± s.e.m. of 3 independent experiments; for each at least 60 cells were analyzed. (D) Co-immunofluorescence for activated c-Src (pY419-Src,
green) and E-cadherin (purple) of cells spreading on Ecad-Fc for 90 minutes (in presence of the RGD peptide). Higher magnifications of cadherin-driven lamellipodia are highlighted by the squared box (upper right corner). Scale bar: 10 µm. (E) Quantification of the pY419-Src signal intensity, measured at the cadherin-based lamellipodia of single cells spreading on Ecad-Fc (in presence of the RGD peptide). Data are mean ± s.e.m. from 3 independent experiments; for each at least 9 cells were analyzed.

Figure 7: RPTPα regulates cadherin-based junctions by affecting cortactin tyrosine phosphorylation.

(A) Analysis of cortactin tyrosine phosphorylation of Caco-2 cells expressing shRNAs against human RPTPα (RPTPα shRNA1 and shRNA2) or a non-targeting shRNA (ctrl shRNA). Cortactin was immunoprecipitated from cell lysates (input), and analyzed by anti-phosphotyrosine (pY), or anti-cortactin (Cort) immunoblotting. Densitometric quantification of pY-cortactin relative to total cortactin recovered after cortactin IP is shown as mean ± s.e.m. of 5 independent experiments. (B) Rescue of RPTPα KD-induced junctional defects by overexpression of non-phosphorylatable versus phosphomimetic mutants of cortactin. Caco-2 cells transfected with siRNA against RPTPα (cortactin KD), together with plasmids expressing GFP-tagged wild-type (cortWT-GFP), mutated (cort3YF-GFP and cort3YD-GFP) human cortactin, or mouse RPTPα fused to Cherry (mRPTPα-cherry) were fixed and immunostained for E-cadherin or F-actin. Representative confocal images show the distribution of the fluorescent tag (white), E-cadherin (green) or F-actin (purple) at intercellular junctions under the various conditions. Scale bar: 10 µm. (C) Quantitative analysis of junctional E-cadherin (upper graph) or F-actin intensity (lower graph). Data are means ± s.e.m. of 3 independent experiments; n=21. (D) Western blot analysis of E-cadherin (E-cadh), RPTPα and cortactin (Cort) under the various conditions. Anti-GAPDH blot is shown as loading control.

Supplementary Movie M1

Time-lapse analysis of RPTPα during cell-cell contact establishment. Intercellular contact formation was followed by time-lapse video-microscopy of A431 cells expressing Venus-tagged RPTPα: phase-contrast and RPTPα-venus epifluorescence images were acquired at 10
minutes intervals. White arrows, arrowheads and asterisks show the presence of RPTPα in nascent, early and stable cell-cell junctions respectively. Single Z optical sections are shown for each time point.

**Figure S1**

Effects of RPTPα KD on junctional E-cadherin distribution in MCF7 mammary carcinoma cells. MCF7 cells were transiently transfected with siRNA against RPTPα (RPTPα siRNA), or with scrambled control RNA (control siRNA), and analyzed 72 hours after transfection. (A) Immunofluorescence for E-cadherin in control and RNAi-treated cells. Scale bar: 10 µm. (B) Quantification of intensity and heterogeneity of E-cadherin along junctions, and width of E-cadherin signal across junctions; mean ± standard deviation (s.d.) relative to one representative experiment, n=50 junctions.

**Figure S2**

Validation of E-cadherin-dependence, and analysis of integrin blocking in the spreading assay on immobilized E-cadherin (Ecad-Fc). (A,B) Influence of E-cadherin-function-blocking antibody SHE78-7 on spreading of control shRNA-transduced Caco-2 cells. (A) Quantification of the cell surface area in contact with Ecad-Fc-coated substrate, assayed in presence of various concentrations of the SHE78-7 antibody; at least 100 cells were analyzed per condition. (B) Quantification of cell surface area in contact with Ecad-Fc-, fibronectin (FN)- or poly-D-lysine (PDL)-coated substrates assayed in presence of 0 or 10 µg/ml of SHE78-7; mean ± s.e.m. of 3 independent experiments; for each at least 60 cells were analyzed. (C-E) Effect of cyclic peptide (cRGD) on Caco-2 cells spreading. (C) Quantification of cell surface area of control shRNA-transduced cells in contact with fibronectin-coated substrate assayed in presence of various concentrations of cRGD; at least 50 cells were analyzed. (D) Representative phase-contrast images of control (control shRNA) and RPTPα KD (RPTPα shRNA1 and RPTPα shRNA2) cells spreading on Ecad-Fc-coated substrate; dashed line delimits surface area in contact with substrate. Scale bar: 10 µm. (E) Quantification of cell surface area in contact with Ecad-Fc-coated substrate, assayed in presence of 10 µg/ml cRGD, expressed as mean ± s.e.m. of 3 independent experiments; ≥60 cells analyzed for each. For panels A and C, note that the baseline for spreading (i.e. cellular surface of unspread cells on PDL) corresponds to approximately 0.2.
Figure S3

Interaction of catalytically inactive mutant (C/S) RPTPα with cortactin by substrate-trapping. Caco-2 extract was incubated with equal amounts of GST alone or GST fused with wild type (WT) or mutant (C/S) RPTPα intracellular domain. After pull-down, bound proteins together with an aliquot of lysate of pervanadate-treated or untreated cells were analyzed by SDS-PAGE and immunoblotted with anti-phosphotyrosine (pY) (A) or anti-cortactin (B) antibodies. As positive control, immunoblotting against c-Src, a known substrate of RPTPα, was performed.
**FIGURE 1**

**A**

Caco2

MCF7

A431

**B**

PH

RPTPα-venus

0 min

40 min

80 min

**C**

E-cadherin

RPTPα-venus

merge

r=0.7±0.04

Z-axis

apical

**D**

ZO-1

RPTPα-venus

merge

r=0.3±0.02

Z-axis

apical

**E**

FRET efficiency

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* ns

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FIGURE 3

A

(+Ca²⁺, EDTA) ctrl shRNA  \quad RPTPα shRNA1  \quad RPTPα shRNA2

B

ctrl shRNA  \quad RPTPα shRNA1  \quad RPTPα shRNA2

[Graph showing data with significance levels (*) and (**) indicated]
FIGURE 7

(A) Western blot analysis showing cortactin immunoprecipitation (IP) and input with control (ctrl shRNA) and RPTPα shRNA samples. The blot shows phosphorylated cortactin (pY+) and total cortactin (Cort+) levels.

(B) Immunofluorescence images of TAG, E-cadherin, and actin in control (Ctrl), siRNA, and GFP conditions. The images indicate changes in the distribution of these proteins.

(C) Bar graphs showing junctional E-cadherin and F-actin levels with statistical significance indicated by asterisks (*, **, ***), and ns for non-significant differences.

(D) Western blot analysis of E-cadherin, RPTPα, Cort, and GAPDH proteins in control and RPTPα siRNA conditions. The blots show the protein expression levels at different molecular weights (kDa) for each protein.