DEPDC1B Coordinates De-adhesion Events and Cell-Cycle Progression at Mitosis

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http://dx.doi.org/10.1016/j.devcel.2014.09.009

SUMMARY

Cells entering mitosis become rounded, lose attachment to the substrate, and increase their cortical rigidity. Pivotal to these events is the dismantling of focal adhesions (FAs). How mitotic reshaping is linked to commitment to divide is unclear. Here, we show that DEPDC1B, a protein that accumulates in G2, coordinates de-adhesion events and cell-cycle progression at mitosis. DEPDC1B functions as an inhibitor of a RhoA-based signaling complex, which assembles on the FA-associated protein tyrosine phosphatase, receptor type, F (PTPRF) and mediates the integrity of FAs. By competing with RhoA for the interaction with PTPRF, DEPDC1B promotes the dismantling of FAs, which is necessary for the morphological changes preceding mitosis. The circuitry is relevant in whole organisms, as shown by the control exerted by the DEPDC1B/RhoA/PTPRF axis on mitotic dynamics during zebrafish development. Our results uncover an adhesion-dependent signaling mechanism that coordinates adhesion events with the control of cell-cycle progression.

INTRODUCTION

The cell cycle is a sequence of coordinated events leading to genome duplication and its correct segregation into the daughter cells at mitosis. The fidelity of this process is secured by mechanisms that are activated at specific restriction points: the cellular checkpoints (Gérard and Goldbeter, 2009; Hartwell and Weinert, 1989; Tyson and Novak, 2008). The G2/M checkpoint occurs at the onset of mitosis and is in charge of preserving genomic integrity and its inheritance without damage or mutations (Branzei and Foiani, 2008; Löbrich and Jeggo, 2007). The G2/M transition is driven by several mitotic kinases, including the Aurora, Polo, and the cyclin-dependent kinases (CDKs) (Hochegger et al., 2008; Lindqvist et al., 2009; Smits and Medema, 2001). The activation of the CDK1/cyclin B complex (mitosis-promoting factor [MPF]) is key in the control of mitotic entry and depends on multiple mechanisms that modulate the expression and/or localization of cyclin B and the phosphorylation status of CDK1 (Gavet and Pines, 2010; Lindqvist et al., 2009; Nigg, 2001; Norbury et al., 1991; Santos et al., 2012). Once activated, the MPF phosphorylates a series of molecular targets that trigger downstream mitotic events, such as nuclear envelope breakdown and chromosome condensation (Nigg, 2001; Oh and Gould, 1999).

At mitotic entry, cells also become rounded, lose attachments to the substrate, and display increased cortical rigidity (Cramer and Mitchison, 1997; Kunda and Baum, 2009; Théry and Borns, 2009). This reshaping is thought to be necessary to set the axes for symmetric or asymmetric partitioning of cell determinants and to establish a correct spindle orientation (Kunda and Baum, 2009; Théry et al., 2009).

Adhesion to the extracellular matrix (ECM) is mainly mediated by structures called focal adhesions (FAs), in which establishment, maturation, and dismantling are tightly controlled (Parsons et al., 2010; Zamir and Geiger, 2001). FAs exert a mechanostuctural role by physically connecting the actin cytoskeleton to ECM via integrin receptors, and a signaling role, serving as hubs to assemble signaling complexes (Mitra and Schlaepfer, 2006; Parsons et al., 2010). As cells approach mitosis, they dismantle FAs via inactivation of FA kinase (FAK) and downmodulation of Rap1-GTPase activity (Dao et al., 2009; Kunda and Baum, 2009; Pugacheva et al., 2006; Yamakita et al., 1999). Concomitantly, cells experience mitotic rounding and cortical stiffening caused by actomyosin remodeling through RhoA (Maddox and Burridge, 2003; Matthews et al., 2012), ezrin, radixin, and moesin complex (ERM) proteins (Carreno et al., 2008), and myosin II (Maddox and Burridge, 2003).

A mechanistic picture of how the cell coordinates detachment/rounding and entry into mitosis is, however, still lacking. Here we show that DEPDC1B, a cell-cycle-regulated gene (Nicassio et al., 2005), mediates the interplay between cell-cycle progression and de-adhesion events at the mitotic entry. The DEPDC1B protein specifically accumulates at the G2 phase of the cell cycle and inhibits RhoA recruitment to and activation by the FA-associated receptor protein tyrosine phosphatase, receptor type, F (PTPRF). By this mechanism, DEPDC1B functions as an inhibitor of the RhoA/Rho-associate protein kinase (ROCK)/MLC2
pathway during the G2/M transition, thereby allowing FA 
disruption and cell detachment. Ablation of DEPDC1B 
impairs de-adhesion events and delayed mitotic entry. 
Similarly, conditions that induced persistent adhesion to the 
substrate, independently of DEPDC1B, inhibited mitotic 
entry, suggesting that adhesion per se controls cell-cycle progression. 
Thus, we have identified a feedback loop in which the nuclear 
signals to cell periphery the need to initiate mitotic 
reshaping through the synthesis of DEPDC1B. In turn, adhesion-dependent mechanisms 
delay progression into the M phase until mitotic 
reshaping is correctly executed.

RESULTS

DEPDC1B Regulates Mitotic Entry

DEPDC1B is a proliferation-associated gene expressed in a cell-
cycle-dependent fashion through an Rb/E2F-dependent transcrip-
tional mechanism (Nicassio et al., 2005). We examined 
the pattern of expression of DEPDC1B mRNA and protein in 
HeLa cells synchronized by double-thymidine block (D-THY; 
Figure S1A available online). As cells entered the G2 phase 
(4 hr after release), DEPDC1B mRNA was induced, and the protein 
accumulated until mitosis (M phase, 8 hr), closely resembling 
the behavior of cyclin B. In addition, similar to cyclin B, 
DEPDC1B protein was degraded during mitosis in a protea-
some-dependent manner (Hershko, 1999) (Figure S1B).

Knockdown (KD) of DEPDC1B with three different short interfering 
RNA (siRNA) oligos (1B-KD1, 1B-KD2, 1B-KD3; Figures 
1A, 1B, and S1C) in HeLa cells synchronized by D-THY reduced 
the number of cells that reached mitosis (Figures 1A–1C; Movie 
S1), an effect that could be rescued by the concomitant expres-
sion of a siRNA resistant GFP-tagged DEPDC1B (Figures 1B 
and 1C). Flow-cytometry analysis showed that DEPDC1B-KD 
cells progressed normally from S to G2 (G2 phase, Figure 1D), 
while the transition from G2 to mitosis (mitosis, Figure 1D) was 
inhibited. Silencing of DEPDC1B also inhibited mitotic entry in

Figure 1. DEPDC1B Silencing Delays Mitotic Entry in Human Cells 
(A and B) HeLa cells were synchronized in S phase by D-THY 
and released in fresh medium with nocodazole (100 nM) to follow cell-cycle progression 
from S phase to mitosis. During synchronization, endogenous DEPDC1B was silenced using 
different siRNA oligos (1B-KD1, 1B-KD2, or 1B-KD3). A custom non-targeting oligo was used as a 
control. Mitotic cells were monitored using mitotic-specific phosphorylation of histone H3 (Ser-10) as 
a marker (mean ± SEM of three experiments). (B) Cells were transduced with an inducible EGFP-
DEPDC1B transgene (EGFP-1B) and synchronized as in (A). Endogenous DEPDC1B was silenced 
using a 3’UTR-targeting oligo (1B-KD3). Upon release, siRNA-resistant EGFP-1B was induced 
by doxycycline (100 ng/ml), and mitotic cells were counted. Western blot shows levels of endoge-

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Figure 2. DEPDC1B Silencing Perturbs Adhesion and Actin Cytoskeleton Dynamics in G2 Phase

(A and B) FA dynamics were observed using GFP-paxillin (A) or vinculin (B) as reporters in control (Ctrl) or DEPDC1B-silenced (1B-KD1) HeLa cells synchronized in early G2 phase (D-THY plus 4 hr release). (A) The mean number, size (area, \(\mu m^2\)), and the duration of FAs, determined by total internal reflection fluorescence (TIRF) microscopy, in control (Ctrl, blue) and DEPDC1B-silenced cells (KD-1B, red) are shown. (B) The average number and area (\(\mu m^2\)) of FAs per cell, determined by confocal microscopy using vinculin staining, are shown.

(C) The actin cytoskeleton (FITC-phalloidin) and the activation of myosin light chain (phospho-MLC2-Ser19) were analyzed by immunofluorescence and confocal microscopy in HeLa cells synchronized in G2 phase. The percentage (mean ± SEM of three experiments) of cells with high/low phospho-MLC2 staining is reported.

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other cell types, including nontransformed and cancer cell lines (Figure S1D).

A DEPDC1B-like gene, DEPDC1A, encodes two isoforms (Figure S1E) whose expression is also regulated during the cell cycle (Figure S1F). Silencing of DEPDC1A caused a mitotic phenotype similar to that of DEPDC1B-KD (Figures 1C, S1G, and S1H). Importantly, the simultaneous depletion of both genes had additive and robust effects (Figures 1C, S1G, and S1H), arguing for functional redundancy and tight cooperative control over the G2/M transition.

We investigated the effects of DEPDC1B silencing on the key molecular events of the G2/M transition (Güttinger et al., 2009; Lindqvist et al., 2009). Upon DEPDC1B silencing, the nuclear membrane abnormally persisted in the majority of cells (lamin B staining, Figure 1E), while no differences were found in cyclin B expression (Figures 1F–1G). However, nuclear accumulation of cyclin B was decreased (Figure 1F), suggesting that activation of the MPF could be impaired. Thus, we investigated the phosphorylation status of CDK1 since dephosphorylation on Tyr14/15 is required for progression into mitosis (Hunter, 1995). The phosphorylation of CDK1 in DEPDC1B-KD cells was increased and sustained in time compared with control cells, confirming that MPF activation was delayed (Figure 1G). Together these results indicate that DEPDC1B is a regulator of the G2/M transition, acting upstream of the MPF activation.

DEPDC1B Modulates Adhesion and Actin Cytoskeleton Dynamics in G2

We employed the GFP-tagged version of DEPDC1B to analyze its subcellular distribution. In G2-synchronized cells, we observed a plasma membrane (PM) localization of DEPDC1B that persisted during mitosis (Figures S2A and S2B). In addition, while control cells lost attachment to the substrate and became rounded as they approached mitosis, DEPDC1B-KD cells appeared flattened, more motile, and often failed to detach from the substrate and become rounded (Movie S1). DEPDC1B might, therefore, act at the PM to regulate cellular adhesion. We investigated this possibility by following the dynamics of GFP-paxillin, a marker of FAs (Parsons et al., 2010; Zamir and Geiger, 2001). In control cells, the typical punctuate staining of GFP-paxillin at the ventral membrane, which marks FAs in interphase, quickly disappeared as cells approached mitosis (Figure 2A; Movie S2). Conversely, DEPDC1B-KD cells displayed larger FAs that persisted in G2 (Figure 2A; Movie S2). A quantitative analysis revealed that the absolute number of FAs per cell was unaffected; however, their size was significantly increased and their mitotic dismantling delayed (Figures 2A, 2B, S2C, and S2D).

We also detected significant modifications of actin dynamics in DEPDC1B-KD HeLa cells in G2 phase, with cells displaying an altered pattern of actin stress fibers and increased phosphorylation of the actin regulator myosin light chain 2 (MLC2-Ser19), a typical downstream target of the ROCK, and coflin (pCofilin-Ser3) (Figures 2C and 2D). These observations could be extended to other cell types, including fibroblasts and nontransformed epithelial cells (Figure 2E). Finally, the silencing of DEPDC1B also altered the dynamics of cells spreading, an effect that could be rescued by the ectopic expression of the siRNA-resistant GFP-DEPDC1B (Figures 2F and S2E).

These results point to a role for DEPDC1B in the control of cellular adhesion and actin dynamics during the G2 phase of the cell cycle.

DEPDC1B-KD Induces an Adhesion-Dependent Checkpoint at the G2/M Transition

We investigated the relationship between the mitotic and the adhesion phenotypes caused by DEPDC1B silencing. Initially, we took advantage of a HeLa derivative clone (HeLa-S3) adapted to growth in suspension (Puck et al., 1956) (Figure 3A). In these cells, DEPDC1B-KD did not affect the G2/M transition (Figure 3B), arguing that in the absence of cell adhesion the mitotic phenotype of DEPDC1B-KD cells could be suppressed. If so, it should be possible to abrogate the said phenotype by directly interfering with FAs. Thus, we silenced structural (vinculin, alpha-actinin) and catalytic (FAK) components of FAs in DEPDC1B-silenced cells (Figure 3C). In all cases, the downmodulation of FA components completely rescued the DEPDC1B-KD-dependent mitotic delay (Figure 3D), indicating that, in DEPDC1B-KD cells, the cell-cycle phenotype is linked to the abnormal persistence of FAs at the G2/M transition phase.

We also analyzed the involvement of actomyosin contractility, which was altered upon DEPDC1B silencing (see Figure 2). As mentioned above, this process is closely linked to cell adhesion mechanisms and is controlled by RhoA/ROCK/MLC2 signaling. Therefore, we treated G2 synchronized DEPDC1B-KD cells with a ROCK inhibitor (Y27632, 10 μM). The treatment normalized the levels of phospho-MLC2 and concomitantly rescued the mitotic phenotype in DEPDC1B-KD cells (Figures 3E and 3F). If the impairment in G2/M transition observed in DEPDC1B-KD cells were indeed due to the persistence of FAs, then induction of persistent adhesive structures should phenocopy the DEPDC1B silencing. To investigate this, we employed two tools: (1) an auto-inhibition-deficient mutant of vinculin (VincT12 mutant), which increases adhesion strength and force transmission (Humphries et al., 2007), and (2) manganese treatment (Mn2+, 1 mM), which induces αβ3 integrin activation and clustering (Czuel et al., 2005; Gailit and Ruoslahti, 1988). In G2-synchronized HeLa cells, both treatments induced cell spreading on the substrate, formation of actin stress fibers, and high levels of phospho-MLC2.
These results suggest the existence of a DEPDC1B-based mechanism that controls the coordination of adhesion and actin cytoskeleton dynamics with entry into mitosis. Of note, the G2/M arrest induced by DNA damage-inducing agents (i.e., doxorubicin) appeared stronger than the cell-cycle arrest induced by persistent adhesion (Figures S3E and 3F).

DEPDC1B Modulates RhoA-Dependent Cell Adhesion at G2/M Transition

Since both actin cytoskeleton and adhesion dynamics are regulated by Rho-GTPases, we silenced the expression of each of the three prototypical members of this family, RhoA, Rac1, and Cdc42, alone and in conjunction with DEPDC1B-KD. The depletion of any of the three Rho-GTPases alone had no major effect on G2/M transition (Figure 4A). However, RhoA silencing, but not Rac1 or Cdc42 silencing, completely rescued the mitotic delay induced by DEPDC1B silencing (Figures 4A and 4B). Furthermore, the significant increase in the size of FAs in DEPDC1B-KD cells (see Figures 2A and 2B) was fully rescued by silencing RhoA (Figures 4C–4E). Finally, the DEPDC1B-KD-dependent spreading defect was rescued by silencing of RhoA, but not of Rac1 or Cdc42 (Figure S4A). These results link the function of DEPDC1B to the control of RhoA activity, likely through inhibition of the latter at the G2/M transition. This notion is further supported by the observations that (1) the DEPDC1B silencing increased the activity of RhoA (RBD pull-down assay, Figure 2D), (2) the levels of downstream targets, such as phospho-MLC2 (Figures 2C–2E), (3) RhoA overexpression in G2 cells phenocopied DEPDC1B KD, causing an increase in the size of FAs and in the levels of phospho-MLC2 (Figures 4F–4G) and a decrease in the mitotic index (Figure 4H).

PTPRF Controls RhoA-Dependent Signaling at Mitotic Entry

Despite having a RhoGAP-like (GTPase-activating) domain (Figure S1E), DEPDC1B is most likely not an active GAP since it lacks the catalytic arginine typical of true RhoGAPs (Graham et al., 1999; Rittinger et al., 1997) (Figure S4B). Indeed, we failed to detect RhoGAP activity of the recombinant RhoGAP domain of DEPDC1B (Figure S4C).
To understand how DEPDC1B modulates RhoA signaling, we performed a yeast-two-hybrid (Y2H) screening to identify DEPDC1B-interacting proteins. Most of the hits were represented by PTPRF (Figure 5A; Table S1), a transmembrane receptor that has been suggested to function as a molecular hub at adhesive sites that coordinates adhesion and migration events (Chagnon et al., 2004; Serra-Pagés et al., 1995; Tsujikawa et al., 2002). We confirmed the direct biochemical interaction between the DEPDC1B and PTPRF by glutathione S transferase (GST) pull-down experiments performed with cell lysates.

Figure 4. DEPDC1B Controls RhoA-Dependent Cell Adhesion at the G2/M Transition

(A and B) Control (Ctrl) or DEPDC1B-silenced (1B-KD1) D-THY synchronized HeLa cells were treated with RhoA, Rac1, or Cdc42 siRNA oligos. Mitotic cells were measured using pH3 staining (A) or by time-lapse microscopy (B).

(C–E) HeLa cells were treated with control, DEPDC1B and RhoA siRNA oligos as indicated and synchronized in G2 phase. Staining for pH3 was used to distinguish G2 from mitotic cells. G2 cells were examined for FAs (using vinculin as marker) and actin cytoskeleton (Phalloidin). (D and E) The distribution of FA mean area per cell (D) or percentage of cells displaying enlarged FAs and actin stress fibers (E) are shown. (F–H) HeLa cells were transfected with RhoA-myc and synchronized in G2 (F and G) or M phase (H). (F) Western blot analysis shows levels of RhoA-myc and phospho-MLC2. Total MLC2 was used as loading control. In parallel, FA dynamics were analyzed by vinculin staining. (G and H) Bar graphs show the percentage of cells with normal/enlarged FAs and actin stress fibers (G) and the percentage of pH3-positive cells (H). Scale bar graphs report the mean ± SEM of three (A, D, and E) or two (F and H) experiments. Asterisks mark significant values (p < 0.05, Student’s t test). Representative images are shown. Scale bars represent 10 μm.
Figure 5. The Membrane Receptor PTPRF Interacts Biochemically and Genetically with DEPDC1B and RhoA

(A) An Y2H screen was employed to search for DEPDC1B interactors. The chart shows the distribution of positive clones.

(B and C) GST-PTPRF-c (cytoplasmic fragment, 1 μM) was incubated with total lysates (2 mg) from HeLa cells overexpressing EGFP-1B (B) or from control HeLa cells (C). Western blot analysis was performed using anti-GFP or anti-DEPDC1B antibodies. Asterisk marks a non-specific band detected by the anti-GFP. Ponceau staining is shown.

(D) GST-PTPRF-c (1 μM) was incubated with the purified GAP domain of DEPDC1B (1B-GAP, 3 μM). Proteins were resolved by SDS-PAGE and detected by Coomassie staining to reveal the amount of 1B-GAP pulled down by PTPRF-c.

(E and F) Control (Ctrl) or DEPDC1B-silenced (1B-KD1) HeLa cells were synchronized and treated or not with PTPRF siRNA. (E) Mitotic cells were measured using pH3-staining (mean ± SEM of three experiments). Asterisks mark significant values (p < 0.05, Student’s t test). (F) Western blot analysis shows levels of DEPDC1B, PTPRF, and phospho-MLC2. Vinculin and total MLC2 were used as loading controls.

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Western blot analysis shows levels of RhoA-myc, PTPRF, and vinculin (loading control). Scale bar graphs report the percentage of cells entering mitosis. HeLa cells were transfected with RhoA-myc, treated with control or PTPRF-targeting siRNA oligo, and synchronized in S phase to follow mitotic entry. Phenotype induced by DEPDC1B silencing; overexpression of GEF-H1 phenocopied the effect of RhoA activation and to RhoA (Figures S6C–S6E); the interaction between PTPRF and ROCK2, and mDia1 (RhoA effectors) to PTPRF were unaffected by DEPDC1B overexpression (Figures 6A and 6F). Conversely, silencing DEPDC1B significantly increased the interaction between GST-PTPRF-c and RhoA in the same assay (Figure 6G) and the interaction between RhoA and ROCK2 in the RBD pull-down assay (Figure 6H). Finally, the purified DEPDC1B fragment that interacts with PTPRF-c (Figure 5D) halved the interaction of the latter with RhoA in vitro pull-down experiments (Figure 6I).

These results suggest a role for the DEPDC1B-PTPRF axis in the control of RhoA signaling at mitotic entry, with DEPDC1B acting as negative regulator of the recruitment of RhoA to PTPRF-based complexes (Figure 6J).

DEPDC1B Modulates Cell Proliferation during Zebrafish Embryogenesis

To analyze the relevance of the DEPDC1B/RhoA/PTPRF axis at the organismal level, we turned to zebrafish, which express a DEPDC1B ortholog (depdc1b) that has an exon/intron organization conserved with the human gene (Figure S7A) and ~75% similarity to the human protein. By in situ hybridization and RT-qPCR of zebrafish embryos, we showed that depdc1b mRNA has no maternal contribution, first appeared at 3 hr postfertilization (hpf), and gradually accumulated until the end of segmentation period (32 hpf), with more intense expression detected in the head and dorsal regions (Figures 7A and S7B).

The function of Depdc1b during zebrafish development was investigated by taking advantage of a specific splice-blocking morpholino (MOSB) to induce the formation of a truncated protein (Figure S7C). The injection of a depdc1b-MOSB caused a severe morphological defect, already visible at the late gastrula stage (10 hpf; Figure 7B), possibly due to alterations in morphogenetic mechanisms, and finally displaying a phenotype at 24 hpf characteristic of mutations with delayed or incomplete epiboly (Kane et al., 1996; Figure 7B). We investigated the effects of Depdc1b ablation on cell proliferation by measuring the mitotic rate in the anterodorsal side of the embryo, where Depdc1b is preponderantly expressed. We used the Tg(h2afva:GFP)kca6 transgenic line (Pauls et al., 2001), in which the fusion protein histone variant H2A.F/Z:GFP is expressed from the start of zygotic transcription, to monitor mitosis, Real-time analysis in the dorsal head and dorsal regions (Figures 7A and S7B).

RhoA:PTPRF Interaction Depends on DEPDC1B Levels

The sum of our results strongly supports a model in which RhoA/ROCK signaling in G2 phase is induced by PTPRF/GEF-H1 and is inhibited by DEPDC1B. One mechanism through which this might occur is competition of the interaction of RhoA with PTPRF by DEPDC1B, with ensuing inhibition of the RhoA signaling complex and the consequent dismantling of adhesion structures at the G2/M transition. We tested this hypothesis through a series of experiments. We showed by in vitro pull-down assays performed on total cellular lysates that the interaction between the cytoplasmic domain of PTPRF (GST-PTPRF-c) and RhoA was almost completely inhibited by the simultaneous presence of excess DEPDC1B (obtained by ectopic expression) in the cell lysate (Figure 6D). This effect on PTPRF and RhoA binding was also reproduced in stable isotope labeling by amino acids in cell culture (SILAC) experiments (Figure 6E) and appears specific for RhoA itself since binding of GEF-H1 (RhoA activator), ROCK2, and mDia1 (RhoA effectors) to PTPRF were unaffected by DEPDC1B overexpression (Figures 6A and 6F). Conversely, silencing DEPDC1B significantly increased the interaction between GST-PTPRF-c and RhoA in the same assay (Figure 6G) and the interaction between RhoA and ROCK2 in the RBD pull-down assay (Figure 6H). Finally, the purified DEPDC1B fragment that interacts with PTPRF-c (Figure 5D) halved the interaction of the latter with RhoA in in vitro pull-down experiments (Figure 6I).

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Figure 6. PTPRF as a Hub for the Activation of the RhoA Signaling Complex, which Is Dependent on DEPDC1B Protein Levels

(A) GST-PTPRF-c pull-down assay with total lysates (1 mg) from HeLa control cells or cells overexpressing 1B-GFP. Western blot analysis shows levels of GEF-H1 and 1B-GFP.

(B and C) The effects of silencing or overexpression of GEF-H1 has been analyzed on HeLa cells. Bar graphs report the percentage of cells entering mitosis (mean ± SEM of two experiments) using pH3 staining as mitotic marker. Asterisks mark significant values (p < 0.05, Student’s t test). (B) A bar graph on the right shows the relative mRNA expression of another RhoA GEF, PDZ-GEF, upon silencing. (C, below) Western blot shows levels of GEF-H1 upon silencing or overexpression and vinculin (loading control). (Above) Representative images of FAs (vinculin) and actin cytoskeleton (phalloidin) of control HeLa cells or cells overexpressing GEF-H1. Scale bars represent 10 μm.

(D–G) GST-PTPRF-c pull-down assays were performed with total lysates (1 mg) from control cells, cells overexpressing both 1B-GFP and RhoA-GFP (D), cells overexpressing just 1B-GFP (E and F), cells overexpressing RhoA-myc and silenced for endogenous DEPDC1B (G). Western blot analysis was performed using the anti-GFP antibody for both 1B-GFP and RhoA-GFP, the anti-myc antibody for RhoA-myc, the anti-mDia1 or anti-ROCK2 antibodies (RhoA downstream effectors), or anti-DEPDC1B antibody. The asterisk marks a nonspecific band detected by the anti-GFP antibody (D and F). In (E), protein bound to PTPRF-c were

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region after midblastula transition (MBT, ~4 hpf; Figures 7C and 7D) scored a defect upon depdc1b-MOSB injection in mitotic events occurring between 5 and 6 hpf. We did not detect alterations in mitotic duration per se (Figure 7E), suggesting that the defect in mitotic peaks was caused by a premitotic defect, possibly in the control of the G2/M transition as observed in mammalian cells. At 10 hpf the developmental defects of depdc1b-morphant embryos were mirrored by an increase in proliferation (number of pH3+ cells) of anterior dorsal region (Figure 7F). At this stage, we also observed a series of cytoskeleton related phenotypes, such as cells with much irregular shape, thickening of cortical actin, and frequent appearance of actin protrusions on cell edges (Figures 7G and 7H). All of these defects were rescued by the coexpression of human DEPDC1B mRNA, suggesting that the phenotype was specifically caused by Depdc1b ablation and that Depdc1b function is conserved (Figures 7F–7H and S7D). To corroborate this possibility, we tested whether the DEPDC1B genetic interactions identified in mammals were conserved in zebrafish, by analyzing the effect of ablation of the RhoA and PTPRF zebrafish orthologs (rhoab and ptprf, respectively). KD of either rhoab or ptprf by MOB injection (Figure S7C) rescued all of the defects of the depdc1b morphants observed at 6, 10, and 24 hpf (Figures 7I, 7F, and S7D), suggesting that the DEPDC1B/RhoA/PTPRF axis is conserved. Of note, neither the isolated DEPDC1A nor the pseudo-RhoGAP domain of DEPDC1B was sufficient to rescue the MOSB phenotype, suggesting that both domains are required for complementing DEPDC1B functions in vivo (Figure S7E).

**DISCUSSION**

We have uncovered a feedback mechanism of communication between the nucleus and the cell periphery that is centered on DEPDC1B and that allows the coordination and control of a series of events critical to the correct execution of the mitotic program. Mechanistically, this occurs through the RhoA-dependent regulation of FA clustering and actin dynamics and the PTPRF-dependent regulation of the RhoA signaling complex in proximity to FAs. The impact of the DEPDC1B/RhoA/PTPRF circuitry is not limited to 2D cell culture settings but extends to real 3D situations, as observed in zebrafish development.

**Signaling from the Nucleus to the Cell Periphery**

The levels of DEPDC1B oscillate during the cell cycle with behavior and mechanisms indistinguishable from those of checkpoint proteins, such as cyclin B. This cyclin-like regulation is critical for mammalian cell proliferation. Indeed, a lack of DEPDC1B produces two clear phenotypes: (1) a cell-cycle effect, consisting in a significant delay in the transition to mitosis (an effect greatly augmented by the concomitant depletion of DEPDC1A), and (2) an adhesion/cytoskeleton phenotype, with cells displaying enlarged and persistent FAs and aberrant actin stress fibers formation. The two phenotypes are intimately connected, since under conditions that promote de-adhesion (or upon growth in suspension) the mitotic defect is completely bypassed. Accordingly, impairment of the de-adhesion process, produced by outside-in (integrin clustering and increased force transmission) or inside-out (RhoA ectopic expression) mechanisms, resulted in a defect in cell-cycle progression that was comparable in all experimental conditions (Figure S3E and summarized in Figure 6J).

We conclude that we have identified an “adhesion-dependent checkpoint” that participates in the regulation of the G2/M transition and affects most, but not all the cells (a few still reach mitosis). Thus, the “adhesion-dependent checkpoint” appears a little less strong than a typical G2 checkpoint, such as the one induced upon DNA damage, which usually affects all the cells.

**Signaling at the Cell Periphery**

At the PM, two major processes contribute to cell shape remodeling of cells that enter mitosis: FA dismantling (de-adhesion) and cortical stiffening (mitotic rounding). The process of de-adhesion was previously shown to be under the control of the Rap1-GTPase, whose cell-cycle-specific downmodulation, by an as-yet-unknown mechanism, is critical to inhibit the formation of new FA sites and to shut down integrin signaling (Dao et al., 2009). Conversely, mitotic rounding does not apparently involve attenuation of Rap1 signaling, as it was shown that cells expressing a Rap1 dominant-negative mutant (Rap1*) could enter mitosis and displayed some kind of rounding and contractility while remaining attached to the substrate (Dao et al., 2009; Lancaster et al., 2013). Cell rounding, instead, is thought to be due to RhoA- and ERK-dependent control of cortical rigidity (Kunda and Baum, 2009; Kunda et al., 2008; Maddox and Burridge, 2003; Matthews et al., 2012). Therefore, it is likely that two pathways, one regulating FA dismantling (Rap1 dependent) and the other regulating mitotic rounding (RhoA and ERK dependent), exist and cooperate to induce cell reshaping at mitosis. Our data argue that both these pathways are under the control of DEPDC1B.

The control on FA dynamics exerted by DEPDC1B is due to its ability to bind to PTPRF and to compete specifically with RhoA for binding to this hub, thereby inhibiting activation of the RhoA signaling (likely due to GEF-H1) and actomyosin contractility at adhesion sites. It remains to be established how the identified and quantified by SILAC in presence or not of DEPDC1B-GFP overexpression (two independent experiments, forward and reverse, with swapping of isotope-encoded amino acid among the two channels). Among PTPRF interactors, 39 proteins were found with binding consistently upmodulated or downmodulated by DEPDC1B overexpression (>1 log2 fold), such as DEPDC1B itself and RhoA.

(H) Western blot analysis shows levels of ROCK2 (RhoA effector) bound to active RhoA (RBD-pull-down) upon DEPDC1B silencing or Mn2+ treatment. As positive control, the lysate was activated by GTPγS stimulation.

(I) GST-PTPRF-c (1 μM) was incubated with purified RhoA (3 μM) and/or 1B-GAP (3 μM). Proteins were resolved by SDS-PAGE and detected by Coomassie staining to reveal the amount of protein pulled down by PTPRF-c. The densitometry analysis of the PTPRF-c/RhoA (and 1B-GAP) interaction in three experiments is shown.

(J) The scheme depicts the working model for DEPDC1B as a competitive inhibitor of the RhoA/PTPRF interaction resulting in inhibition of RhoA-dependent signaling that regulates the adhesion dynamics of G2 cells and mitotic entry. Positive (left) and negative (right) inducers of the G2/M adhesion checkpoint are also reported, according to the findings of this study.
Figure 7. DEPDC1B Controls Mitotic Events in Zebrafish Embryo

(A) Zebrafish (ZF) embryos were collected at different times from fertilization (hpf) and levels of depdc1b or depdc1a mRNA expression measured by RT-qPCR. Actin B (Actb) was used as a normalizer.

(B) ZF embryos were injected with control or depdc1b splice-blocking morpholino (MOSB) at the one-cell stage. Pictures show representative images of the resulting morphological defects observed at the late gastrula (10 hpf, left) or late segmentation (24 hpf, right) stages. Scale bars represent 125 μm (left) and 500 μm (right).

(C–I) Zebrafish embryos were treated as in (B), and mitotic figures were monitored in the embryonic dorsal region of the Tg(h2afva:GFP)kca6 transgenic line using confocal microscopy. Representative images are shown in (C). The scale bar represents 10 μm.

(D) The graphs show the number of mitotic events (mean ± SD of two experiments) occurring from 4 to 6 hpf. DEPDC1B human mRNA was microinjected together with depdc1b MOSB to rescue embryo defects.

(E) Duration of mitotic phases (mean ± SEM) was measured by confocal time-lapse microscopy, after MBT.

(legend continued on next page)
DEPDC1B/RhoA/PTPRF axis connects with and/or controls Rap1-dependent de-adhesion. The role of DEPDC1B in cortical actin dynamics, on the other hand, presents us with an apparent paradox, since DEPDC1B is a functional inhibitor of RhoA signaling at PTPRF-based hubs, while the increase in cortical rigidity needed for mitotic rounding requires active RhoA. This latter event occurs just before prophase, through the Rho-GEF ECT2 (Matthews et al., 2012). Based on our results, a simple reconciling scenario might be that RhoA, once displaced by DEPDC1B from PTPRF sites during late G2, becomes available for ECT2-dependent activation at other sites of the PM later in the cell cycle when cells are in prophase. The precise order of events should be guaranteed by the fact that lack of DEPDC1B accumulation impacts on MPF activation, which, in turn, controls ECT2 translocation from the nucleus to the PM (Matthews et al., 2012).

**Signal from the Cell Periphery to the Nucleus**

Our data show that DEPDC1B not only coordinates FA disassembly with mitotic rounding at the PM but also signals to the nucleus to time entry of cells into mitosis, acting at a point upstream of the MPF complex. This signaling appears dependent on the decrease of RhoA activity at FAs, as suggested by the comprehensive analysis of our molecular genetics and biochemical evidence. The exact nature of the molecular network linking adhesion signaling to nuclear MPF activation at the G2/M transition remains to be elucidated. Possible candidates include members of MAPK-ERK cascade, which are known to have a role in cyclin B activation during mitotic entry (Patel et al., 1999; Wang et al., 2007), as well as to be activated upon FA establishment and stabilization (reviewed in Margadant et al., 2013).

In summary, we uncovered a mechanism coordinating cell adhesion with cell-cycle progression that acts as a cell-cycle “adhesion-dependent checkpoint” and demonstrated the relevance of this mechanism in vivo. Of note, the molecular components of this checkpoint, including DEPDC1B, PTPRF and RhoA, are frequently altered in cancer. Thus, the subversion of the adhesion-dependent checkpoint might also be relevant to human pathology: a possibility that warrants further investigations.

**Experimental Procedures**

**Cell-Cycle Synchronization and Procedures**

HeLa, Phoenix, and U2OS cells were cultured in Dulbecco’s modified Eagle’s medium plus fetal bovine serum (10% v/v), l-glutamine (2 mM), and sodium pyruvate (1 mM). MCF10A cells were cultured in DMEM/F12 medium plus horse serum (final concentration: 5% v/v), insulin (10 mg/ml), epidermal growth factor (20 ng/ml), cholera toxin (100 ng/ml), and hydrocortisone (0.5 mg/ml). IMR90 fibroblasts were cultured in Eagle’s medium in addition to serum, plus folic acid (10% v/v), l-glutamine (2 mM), and nonessential amino acids (1% v/v). HeLa S3 cells were seeded on poly-HEMA-coated plates (P9392 from Sigma, final concentration: 12 g/l). Synchronization in G2/M phase was performed by D-thymidine treatment (final concentration: 2.5 mM Sigma) for 16–18 hr followed by release in complete medium with nocodazole (final concentration: 200 ng/ml Sigma). Synchronization in M phase was performed by nocodazole treatment for 16 hr, followed by mitotic shake-off and replating on poly-d-lysine-coated plates (final concentration: 10 µg/ml) and monitored by immunofluorescence (FITC-phalloidin) or by Real-Time Cell Analyzer Technology (xCELLigence Roche). Integrin activation by Mn2+ was achieved by treating cells with MnCl2 (final concentration: 1 mM). Y27632 was purchased from Merck (B88001) and used in mitotic experiments at a final concentration of 10 µM. DNA damage was induced by 1 hr doxorubicin (final concentration: 10 µM) after 2 hr of release from D-thymidine treatment. RWJ-60475 (AM1) was purchased from Abbacm (ab141729) and used at a final concentration of 10 µM. DNA constructs expressing DEPDC1B, RhoA, PTPRF, and GEF-H1 were transfected into Phoenix cells using calcium phosphate or into HeLa cells by Lipofectamine 2000. Details are reported in the Supplemental Experimental Procedures. Custom or predesigned siRNA oligos were used to silence endogenous expression of DEPDC1B, DEPDC1A, vinculin, actin, FAK, RhoA, Rac1, Cdc42, and PTPRF (see Supplemental Experimental Procedures for siRNA sequence information). A custom non-targeting oligo was used as a negative control. HiPerfect Transfection Reagent from QIAGEN was used for siRNA transfection (final oligo concentration: 50 nM), according to the manufacturer’s instructions.

**Microscopy and Data Analysis**

Mitotic and adhesion phenotypes were quantified by epifluorescence microscopy under a fluorescent (DM5500B; Leica) microscope using a 20×/0.15 objective lens at room temperature. All antibodies used for immunofluorescence were employed according to manufacturer’s instructions (a complete list is reported in the Supplemental Experimental Procedures). Fluorescent images used were Cy3 (cGFP, lamin B, vinculin, pMLC2, Flag-tag), Alexa488 (ph3), FITC-/TRITC-phalloidin, or Cy5 (vinculin [ph3], Myc-tag). All images were acquired with a camera (DCF350FX; Leica) and LAS-AF image software (Leica). Images were processed with the same settings (brightness, contrast, crop, image size) using Adobe Photoshop CS5.1, and imaged figures were constructed in Adobe Illustrator.

**Confocal analysis of DEPDC1B localization, actin cytoskeleton, and FAs** was performed on a Leica TCS SP2 AOBS microscope, using a 40×/NA 1.25 oil-immersion objective and processed in Adobe Photoshop. Images were taken with identical settings, and the number and average area of FAs per cell were determined using ImageJ software with a mask with a fixed threshold that identifies vinculin- or paxillin-GFP positive FAs. Details on live microscopy (time-lapse and TIRF) are in the Supplemental Experimental Procedures.

**Protein Purification and Pull-Down Experiments**

pGEX-SH3BP1 (GAP domain) and pGEX-RhoA constructs were a kind gift from Professor Axel Ullrich (Max Planck Institute). GST-tagged proteins were expressed in High-5 insect cells with the MultiBac expression system, purified by glutathione sepharose beads (GE Healthcare). For GST pull-down experiments, RhoA was further solubilized by 3C-Precipitation Pre-treatment. The pseudo-RhoGAP domain (aa 137–400) of DEPDC1B was amplified by PCR using specific primers and cloned into pFastBac1- HisMBPVT, a custom-made vector derived from pFastBac1 (Life Technologies), expressed in High-5 insect cells with the MultiBac expression system (Berger et al., 2004) and purified by MBP affinity followed by size exclusion chromatography. Pull-downs with total cell lysate were performed in complete JS buffer, whereas those with purified proteins were performed in HEPES...
10 mM (pH 7.5), NaCl 100 mM, glycerol 5%, Tween 0.1%. Details on labeling and quantification of protein bound by SILAC are reported in the Supplemental Experimental Procedures. GST-RBD assays were performed by incubating 1 mg of protein lysate with 50 μg of GST-RBD beads for 1 hr at 4°C in a final volume of 0.5 ml. Beads were washed three times with RBD Wash Buffer (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, Triton X-100 1% v/v, 10 mM MgCl2 freshly added) and resuspended in 30 μl of SDS-PAGE sample buffer.

**Zebrafish Morpholinos and Time-Lapse Microscopy**

A depdc1b splice-blocking morpholino was synthesized by GeneTools according to sequence GTGTAAGAGCTGCAGAAGGCTTG and used at a maximum final concentration of 0.7 mM. rhoab ATG-morpholino was previously described (MO1-rhoa; Zhu et al., 2008). To inhibit ptprf genes, a mixture 1:1 of ptpra and ptprb morpholinos was used (MO1-ptpra and MO1-ptprb; Wang et al., 2012). All morpholinos were injected in one-cell stage zebrafish embryos. Human DEPDC-1B mRNA was co-injected at a final concentration of 50 ng/μl. The specificity of morpholinos was checked by RT-PCR on total cDNA (details are in the Supplemental Experimental Procedures and Figure S7C).

Time-lapse analysis was performed using embryos from the Tg(h2afv:GFP)kca6 mice. Zebrafish embryos were collected every 10 min with a 20× water-immersion objective. Actin cytoskeleton was analyzed by FITC-phalloidin (Sigma, P5282) staining (2 hr, final concentration: 5 μg/ml, 1% sodium) on 10 hpf embryos fixed with 4% PFA in PBS (overnight at 4°C) and permeabilized with Triton X-100 2% for 2 hr. Stained embryos were mounted and analyzed as above.

**Statistical Analysis**

Microsoft Excel was used to generate bar graphs and to perform statistical analyses. Dot plots were produced and analyzed with Prism 6 software. Fitting curves of mitotic time-lapse experiments were generated using JMP 10 (SAS) software.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, seven figures, two tables, and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2014.09.009.

**ACKNOWLEDGMENTS**

We thank Sebastiano Pasqualato and Nicoletta Caridi (IEO Crystallography Unit) for the production and purification of MBP-1B-GAP protein, Paolo Soffientini (IFOM Mass Spectrometry Facility) for the identification of PTPRF protein, and Paolo Asperti, C., Astro, V., Totaro, A., Paris, S., and de Curtis, I. (2009). Liprin-alpha1 promotes cell spreading on the extracellular matrix by affecting the distribution of activated integrins. J. Cell Sci. 122, 3225–3232.

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DEPDC1B/RhoA/PTPRF Axis Controls G2/M Transition

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DEPDC1B Coordinates De-adhesion Events and Cell-Cycle Progression at Mitosis

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Figure S1
A

DEPDC-1B::GFP

Median Basal Vertical section

G2 PHASE

DEPDC-1B::GFP pSer10 H3 DAPI

G2 PHASE

PROPHASE

G2 PHASE

METAPHASE

ANATELOPHASE

G1 PHASE

B

DAPI 1B-GFP pSer10-H3 Vinculin

C

Ctrl

linear fit

n=15364

Duration (sec)

Area (μm²)

Ctrl KD

linear fit

n=10790

Duration (sec)

Area (μm²)

Ctrl KD Ctrl KD Ctrl KD Ctrl KD

* ***

D

mean

* = p<0.01

***= p<0.001

E

actin cytoskeleton

stag 1

stag 2

stag 3

Figure S2

plot a

plot b

plot c

plot d

plot e
**Figure S3**

**A**

ACTIN | VINCULIN | MERGE

Ctrl

Mn++

**B**

Actin Stress Fibres  | Enlarged FAs

| Mn++: [1 mM] | 0 | 20 | 40 | 60 | 80 | 100 |
|---------------|---|---|---|---|---|-----|
| -             | ■ | ■ | ■ | ■ | ■ |     |
| +             | ■ | ■ | ■ | ■ | ■ |     |

**C**

TRTC-phalloidin

Vinculin-T12

RFP-Paxillin

**D**

Actin Stress Fibres  | Enlarged FAs

| Ctrl | VincT12 | VincT12 |
|------|---------|---------|
|     | *       | *       |

**E**

DNA Damage  | Persistent adhesion

| pH3+ over ctrl (A.U.) |
|-----------------------|
| UNT | Doxo | CTRL | KD-1A | KD-1B | KD-1A/CTRL | Mn++ | CTRL | VincT12 | CTRL | RhoG |
| A   | 0.1   | 0.2   | 0.3   | 0.4   | 0.5   | 0.6   | 0.7   | 0.8   | 0.9   | 1.0   |

**F**

γH2AX

% of positive cells

| γH2AX |
|-------|
| UNT   | 0.1  |
| Doxo  | 0.2  |
| CTRL  | 0.3  |
| KD-1B | 0.4  |

Figure S3
**Figure S5**

**A**

| Input | IP: DEPDC1B |
|-------|-------------|
| DEPDC1B: | + + + + |
| PTPRF: | + + + + |

**B**

DEPDC1B-GFP PTPRF-FLAG MERGE

**C**

![Graph showing cell distribution over stages](image)

**D**

PTPRF pull down

GST-PTPRF

Interacting partners (31)

**E**

![Western blot analysis](image)

**ANALYSIS:** Molecular Pathways (IPA)
Figure S6

A

PTPRF/C inhibitor

mitosis (pH3+)

|          | Ctrl | KD-1B | KD-1B | KD-PTPRF |
|----------|------|-------|-------|----------|
| Unt      |      |       |       |          |
| RWJ [10μM] |      |       |       |          |

% positive cells

n.s. *

B

Phosphatase treatment

PPase: 1B-GFP:

INPUT | Pull-down | INPUT

DEPDC-1B-GFP

pan phospho-Tyr

Ponceau

C

DAPI
PTPRF-flag
RhoA-myc
MERGE

D

DAPI
PTPRF-flag
Vinculin
MERGE

E

DAPI
Phalloidin-TRITC
Paxillin-GFP
PTPRF-flag
MERGE
**Figure S1 – related to Figure 1. DEPDC1B is a cell cycle protein that regulates mitotic entry in human cells**

**A.** HeLa cells were synchronized in S-phase by double-thymidine block (D-THY) and released in fresh medium to follow cell cycle transitions from S-phase (0 - 2 h) to G2 (4 - 6 h), mitosis (8 h) and the following interphase (10-15 h). Total RNA and protein lysate were collected for western blot (upper panel) and qPCR (lower panel) analyses of DEPDC1B expression. Cyclin B mRNA and protein levels were quantified and used as a reference for a typical G2/M-regulated gene. Vinculin was used as loading control for western blot. GAPDH was used as normalizer for qPCR analysis. **B.** HeLa and U2OS mitotic cells were purified by shake-off after synchronization in pro-metaphase by nocodazole (100 nM, 16 h). Mitotic cells were replated in fresh medium and whole cell lysate was obtained at the indicated time points. Western blot analyses of DEPDC1B and Cyclin B (used as positive control) proteins were performed. To inhibit proteasomal degradation, MG132 (MG) was used at a final concentration of 10 µM. **C.** DEPDC1B was silenced in synchronized HeLa cells (D-THY) using two specific custom siRNAs (1B-KD1 and -KD2). A custom non-targeting oligo was used as a control. Relative levels of DEPDC1B mRNA and protein are shown. Vinculin was used as loading control for western blot. GAPDH was used as normalizer for qPCR analysis. **D.** DEPDC1B was silenced using a specific siRNA oligo (KD1) in U2OS and MCF10A cells synchronized in S-phase (D-THY) and released with nocodazole. Mitotic cells were monitored at 12 h from release using phospho-H3 staining as a mitotic marker (mean ± s.e.m. of two independent experiments). Asterisks mark significant values (p<0.05, Student’s t-test). Representative images are shown (bars: 20 µm). **E.** The picture shows the protein structure and genomic organization of DEPDC1B and DEPDC1A genes, according to UNIPROT and NCBI database. Both proteins are characterized by the presence of a DEP domain at the N-terminus (red), and of a pseudo Rho-GAP domain at the C-terminus (green). siRNA oligos and qPCR probes used in the study are also reported. **F.** Expression analysis by qPCR of DEPDC1A (isoforms A and B) and DEPDC1B genes in D-THY block synchronized HeLa cells (as in panel A). **G.** DEPDC1B and DEPDC1A were silenced in HeLa cells
using specific custom siRNAs. A custom non-targeting oligo was used as a negative control. Bar graph shows the relative expression of DEPDC1A and DEPDC1B mRNAs (mean ± STDV). GAPDH was used as normalizer for qPCR analysis. H. Bar graph shows the percentage of pH3-positive cells at indicated time points (mean ± s.e.m. of three independent experiments). Asterisks mark significant values (p < 0.05, Student’s t-test).

**Figure S2 – related to Figure 2. DEPDC1B subcellular localization and spreading phenotype**

A-B. HeLa cells transduced with an inducible EGFP-DEPDC1B transgene (pSLIK-DEPDC1B) were synchronized by double-thymidine block (D-THY) and released in fresh medium to follow cell cycle transitions. EGFP-DEPDC1B expression was activated by doxycycline (100 ng/ml) upon release and EGFP was used to analyze DEPDC1B localization by confocal microscopy at interphase (G1 and G2 phase) or during mitosis. In the upper panel, DEPDC1B localization was analyzed in G2 cells at different focal planes by confocal microscopy. DAPI and pH3 staining were used to distinguish different mitotic phases. B. DEPDC1B (green) ventral localization was analyzed on synchronized cells along with vinculin (magenta) as a FA marker. Staining with pH3 (red) was used to distinguish different cell cycle phases (G2= G2 phase, M= mitosis). C. Scatter plots show the duration (sec) against the size (area, µm²) of FA as determined by live TIRF microscopy in control (Ctrl, in blue) and DEPDC1B silenced cells (KD-1B, in red). Linear fitting and confidence curves are also reported. The total number of FAs analyzed is shown. The box plot reports the size or the lifetime of FAs. Asterisks mark significant values (p<0.05, Wilcoxon test). D. According to the quartile distribution of all FAs acquired by TIRF, four classes of FAs were distinguished. The box-plot shows the lifetime of FAs (duration, sec) in control (Ctrl, blue) or DEPDC1B-silenced cells (KD, red) for each class of FAs. Asterisks mark significant values. E. The picture summarizes the dynamics of cell spreading upon adhesion on a substrate. Based on the staining of the actin cytoskeleton (Phalloidin-FITC) it is possible to distinguish three stages of cell spreading (stage 1-3). Control (Ctrl) or DEPDC1B-silenced (KD1, KD2, KD3) HeLa cells synchronized in G2 phase
were challenged to cell adhesion and spreading dynamics. Bar graph shows the percentage of cells in different stages of spreading at 30 or 60 min after replating, according to cell morphology (actin cytoskeleton). Knockdown of DEPDC1B was rescued by the expression of exogenous EGFP-DEPDC1B. Asterisks mark significant values (p<0.05, Student’s t-test). Shown are results of one out of two independent experiments (A-E). Representative images are also shown. Bars: 10 µm.

Figure S3 – related to Figure 3. DEPDC1B controls the activation of a G2/M adhesion-dependent checkpoint

A. HeLa cells were synchronized by double-thymidine block (D-THY) and released in fresh medium, then treated with MnCl₂[1 mM]. Control and treated cells were examined for FAs (using vinculin staining as a marker) and actin cytoskeleton (phalloidin staining). B. Bar graph shows the percentage of cells (mean ± s.e.m. of two experiments) with actin stress fibers (in green) and enlarged FAs (in red) following Mn²⁺ treatment. C. HeLa cells were synchronized by double-thymidine block (D-THY) as in panel A, and transfected with pEGFP-Vinculin (T12 mutant) during synchronization. Cells in G2-phase were examined for FAs (using paxillin-RFP as a marker) and actin cytoskeleton (phalloidin-TRITC staining). Representative images are shown. Bars: 10 µm. D. Bar graph shows the percentage of cells (mean ± s.e.m. of two experiments) with actin stress fibers (in green) and enlarged FAs (in red). Asterisks mark significant values (p<0.05, Student’s t-test). E. Bar graph shows the percentage of mitotic cells upon different treatments, inducing DNA damage (doxorubicin, 10 µM, 1 h) or persistent adhesion (knockdown of DEPDC1B, DEPDC1A or both 1A and 1B; Mn²⁺ treatment; VincT12 or RhoA overexpression). F. The presence of DNA damage in cells in G2 phase was assessed by measuring the number of nuclei positive for the phosphorylated form of histone H2AX (γH2AX) upon treatment with doxorubicin (as in E) or upon DEPDC1B silencing.
**Figure S4 – related to Figure 4. DEPDC1B and RhoA control the adhesion-dependent checkpoint**

**A.** HeLa cells were synchronized by double-thymidine block (D-THY), released in fresh medium, and then challenged to cell adhesion dynamics. Cells were treated with different combinations of siRNA oligos during synchronization, including a non-targeting oligo (Ctrl) used as a negative control, a DEPDC1B targeting oligo (1B-KD1) and oligos targeting Rho-GTPases (RhoA, Rac1 and Cdc42). Bar graphs show the percentage of cells (mean ± s.e.m. of three experiments) in different stages of spreading at 30 (left) and 60 (right) min after replating, according to cell morphology (actin cytoskeleton). Asterisks mark significant values (p<0.05, Student’s t-test). Representative images are also shown. Bars: 10 µm. Results from one of three independent experiments are shown.

**B.** Primary sequences of DEPDC1B orthologs in vertebrates were aligned using ClustalW software. The picture shows the boundaries and conserved residues of the pseudo RhoGAP domain within the human DEPDC1B protein. K235 residue is highlighted. In the lower panel, the primary sequences of the RhoGAP domain of DEPDC1B (in green) and ARHGAP1 (in yellow) were super-imposed using Pymol software, on the basis of a previously solved structure including GTP-RhoA [shown in red, ref. (Rittinger et al., 1997) ]. The catalytic cleft is shown in the magnified screenshot: R283 in ARHGAP1 (grey) and K235 in DEPDC1B (white).

**C.** GAP-assay using GTP-loaded GST-RhoA [0.1 µM] incubated with increasing concentrations of the GAP domain of either 3BP1 (used as positive control, right) or DEPDC1B (left). Shown are results of one out of three independent experiments.

**Figure S5 – related to Figure 5. Interaction and co-localization analysis of PTPRF with DEPDC1B and RhoA**

**A.** Co-immunoprecipitation of PTPRF using DEPDC1B antibody (2.5 µg/mg) in 293 cells overexpressing the two proteins (1mg lysate). Western blot analysis of DEPDC1B and PTPRF was performed on input (50 µg) and immunoprecipitated proteins. DEPDC1B was overexpressed with
no tag, and runs close the 62 KDa marker. **B.** HeLa cells previously transduced with an inducible EGFP-DEPDC1B transgene (pSLIK-DEPDC1B) were microinjected with PTPRF-Flag construct. EGFP-DEPDC1B expression was activated by doxycycline (100 ng/ml). The localization of DEPDC1B (EGFP) and PTPRF (Flag-tag, Cy5) was analyzed on the ventral plane by confocal microscopy. Representative images are shown. Bars: 10 µm. **C.** Bar graph shows the dynamics of cell spreading at 30 min from replating of G2-synchronized HeLa cells that were treated with control or PTPRF-targeting siRNA oligos. Asterisks mark significant values (p<0.05, Student’s t-test). **D.** The picture summarizes the results of the pathway analysis performed on GST-PTPRF interacting candidates, identified by direct mass spectrometry approach and Ingenuity Pathway Analysis (IPA). **E.** GST-PTPRF-c pull-down assay [1 µM], using purified RhoA [3 µM]. SDS-PAGE and Coomassie Staining were used to reveal the amount of RhoA pulled down by PTPRF-c. Before incubation with GST-PTPRF-c, purified RhoA was loaded with GDP or GTPγS to induce its inactive or active conformation respectively. An RBD-pulldown assay (right panel) is shown to control for RhoA activation upon GTPγS/GDP stimulation. Shown are results of one out of three independent experiments (A-B-C-E).

**Figure S6 – related to Figures 5 and 6. Role of PTPRF phosphatase activity and details on PTPRF localization**

**A.** HeLa control or DEPDC1B-silenced cells were treated with the PTPRF/PTPRC inhibitor RWJ-60475 (10 µM) or not (Unt) upon release from D-THY block. Mitotic events were counted by pH3 staining. Cell morphology has been checked by transmission microscopy to confirm PTPRF/PTPRC inhibitor efficacy. Bar graphs show the percentage of cells (mean ± s.e.m. of two independent experiments). Asterisk marks significant values (p<0.05). **B.** To verify whether the binding of DEPDC1B to PTPRF was dependent on phosphorylation sites, a GST-PTPRF-c pull-down assay was performed with total lysates (1 mg) from control HeLa cells or cells overexpressing DEPDC1B-GFP (1B-GFP). Lysates were treated or not with Calf Intestinal Phosphatase (PPase)
before GST-pull down, to induce protein de-phosphorylation. After pull-down, 1B-GFP binding was detected by western blot. An asterisk marks a non-specific band detected by GFP antibody. In the right panel, western blot analysis for pan-phosphoTyrosine levels to verify the phosphatase treatment is shown. C-D-E. PTPRF-flag ventral localization was analyzed by confocal microscopy in HeLa cells together with RhoA (panel C), or FA markers, vinculin or paxillin-GFP (panels D-E). In panel E, cells were previously transduced with a lentivirus expressing GFP-paxillin. PTPRF-Flag construct was microinjected at 80 ng/µL. Representative images are shown. Bars: 10 µm.

Figure S7 – related to Figure 7. Details of DEPDC1B analyses in zebrafish

A. The picture summarizes the intronic/exonic organization of human (Hs) and zebrafish (Dr) DEPDC1B genes. The DEP and pseudo-RhoGAP protein domains are highlighted in red and green, respectively. The region of the depdc1b gene used to design the splice-blocking morpholino (MOSB) and to perform PCR analysis is also reported. The identification and characterization of the zebrafish genes was based on the Ensembl database and manually curated. B. Zebrafish embryos were fixed at different stages from fertilization and stained for depdc1b mRNA expression by in situ hybridization (ISH) (Thisse and Thisse, 2008) . Bars: 125 µm. C. A scheme summarizes the treatments and analyses performed on zebrafish embryos. In the lower panel are shown the details of depdc1b, depdc1a and ptprafalb splice-blocking morpholinos. The targeted sequences are located at the junction between exon 1 and intron 2 on depdc1b, depdc1a or ptprif mRNA. Upon morpholino treatment, an intronic fragment is inserted in the mature mRNA producing a premature stop codon. Morpholino efficiency could be monitored following the formation of an aberrant transcript by RT-PCR analysis (lower panel) using PCR primers annealing to exons. D. Zebrafish embryos were injected with control or the indicated morpholinos (MOSBs for depdc1b [d1B], rhoalb or ptprafalb) at the one cell-stage. Pictures show representative images of the resulting morphological defects observed at late segmentation (24 hpf, right). Human DEPDC1B mRNA (human-1B) was also microinjected together with depdc1b MOSB. A bar graph summarizes the
frequency of wild-type vs. mutant phenotypes observed for each treatment with a color code (orange “mild”, red “harsh” phenotype). Approx. 200 morphants were counted from at least two independent experiments. Representative images are shown. Bars: 500 µm. E. The graphs show the number of mitotic cells (pH3+) in the anterodorsal region at 10 hpf upon treatment with MOSBs for \textit{depdc1a} (d1A), \textit{depdc1b} (d1B), or both (d1A+d1B, upper panel) or co-injection with mRNA from different human DEPDC1B constructs (FL, full-length, DEP and GAP domains). Results are reported as the mean ± s.e.m. of two independent experiments. Asterisks mark significant values.
Table S1 – Results of the Y2H screen – related to Figure 5

| Symbol | Fragment (nt) | Score  | Complete Gene Name                                      | Biological Function                                                                 |
|--------|--------------|--------|--------------------------------------------------------|-------------------------------------------------------------------------------------|
| CDC23  | 3 - 1551     | B (high)| cell division cycle 23 homolog                         | APC complex subunit (meta-anaphase transition)                                      |
| CDC23  | 3 - 1407     | B (high)| cell division cycle 23 homolog                         | APC complex subunit (meta-anaphase transition)                                      |
| CDC23  | 6 - 1629     | B (high)| cell division cycle 23 homolog                         | APC complex subunit (meta-anaphase transition)                                      |
| GRIP1  | 980 - 2021   | A (very high)| glutamate receptor interacting protein 1                | scaffold (trafficking of transmembrane proteins)                                    |
| GRIP1  | 1050 - 2018  | A (very high)| glutamate receptor interacting protein 1                | scaffold (trafficking of transmembrane proteins)                                    |
| PTPRF  | 3276 - 3723  | A (very high)| protein tyrosine phosphatase receptor type F            | phosphatase receptor (signaling and adhesion dynamics)                              |
| PTPRF  | 3282 - 3721  | A (very high)| protein tyrosine phosphatase receptor type F            | phosphatase receptor (signaling and adhesion dynamics)                              |
| PTPRF  | 3285 - 3969  | A (very high)| protein tyrosine phosphatase receptor type F            | phosphatase receptor (signaling and adhesion dynamics)                              |
| PTPRF  | 3285 - 3974  | A (very high)| protein tyrosine phosphatase receptor type F            | phosphatase receptor (signaling and adhesion dynamics)                              |
| PTPRF  | 3291 - 3718  | A (very high)| protein tyrosine phosphatase receptor type F            | phosphatase receptor (signaling and adhesion dynamics)                              |
| PTPRF  | 3297 - 4289  | A (very high)| protein tyrosine phosphatase receptor type F            | phosphatase receptor (signaling and adhesion dynamics)                              |
| PTPRF  | 3300 - 4264  | A (very high)| protein tyrosine phosphatase receptor type F            | phosphatase receptor (signaling and adhesion dynamics)                              |
| PTPRF  | 3306 - 4268  | A (very high)| protein tyrosine phosphatase receptor type F            | phosphatase receptor (signaling and adhesion dynamics)                              |
| PTPRF  | 3306 - 4131  | A (very high)| protein tyrosine phosphatase receptor type F            | phosphatase receptor (signaling and adhesion dynamics)                              |
| PTPRF  | 3306 - 4228  | A (very high)| protein tyrosine phosphatase receptor type F            | phosphatase receptor (signaling and adhesion dynamics)                              |
| PTPRF  | 3342 - 4017  | A (very high)| protein tyrosine phosphatase receptor type F            | phosphatase receptor (signaling and adhesion dynamics)                              |
| RGS3   | 31 - 860     | B (high)| regulator of G-protein signaling 3                      | GTPase-activating protein for heterotrimeric G proteins                              |
| RGS3   | 58 - 860     | B (high)| regulator of G-protein signaling 3                      | GTPase-activating protein for heterotrimeric G proteins                              |
| UHMK1  | 162 - 2090   | C (moderate)| U2AF homology motif (UHM) kinase 1                      | serine/threonine kinase for G1/S progression                                        |

The table displays the complete list of positive clones identified by the Yeast-two-Hybrid (Y2H) screen performed using full-length DEPDC1B against human placental cDNAs [pB27 (N-LexA-bait-C fusion) + pB66 (N-GAL4-bait-C fusion)]. Shown are the names of the interacting proteins, the fragments cloned (nt), and the confidence score of the interaction, as well as the complete gene names and biological functions.
### Table S2 – Mass Spectrometry analysis of the GST-PTPRF-c pull-down – related to Figures 5 and S5

| Protein | peptides A,B,C | Mascot Score | RefSeq protein | Symbol | Entrez Gene Name | Location | Type(s) | Putative function |
|---------|----------------|--------------|----------------|--------|-----------------|----------|---------|------------------|
| gi|4557237 | 12 | 809 | NP_000010.1 | ACAT1 | acetyl-CoA acetyltransferase 1 | Cytoplasm | enzyme | metabolism |
| gi|4757810 | 5 | 310 | NP_001001937.1 | ATP5A1 | ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle | Cytoplasm | transporter | metabolism |
| gi|306891 | 9 | 713 | NP_001017963.2 | HSP90AA1 | heat shock protein 90kDa alpha (cytosolic), class A member 1 | Cytoplasm | enzyme | chaperone |
| gi|28336 | 6 | 484 | NP_001092.1 | ACTB | actin, beta | Cytoplasm | other | cytoskeleton |
| gi|55030192 | 22 | 1765 | NP_001093896.1 | RAP1GDS1 | RAP1, GTP-GDP dissociation stimulator 1 | unknown | other | RhoGAP |
| gi|106557 | 2 | 176 | NP_00110963.1 | SEPT9 | septin 9 | Cytoplasm | enzyme | RhoGTPase |
| gi|31092 | 7 | 432 | NP_001395.1 | EEF1A1 | eukaryotic translation elongation factor 1 alpha 1 | Cytoplasm | translation regulator | gene expression |
| gi|503481 | 5 | 234 | NP_001395.1 | EEF1G | eukaryotic translation elongation factor 1 gamma | Cytoplasm | translation regulator | gene expression |
| gi|635686 | 19 | 1386 | NP_001655.1 | RHOA | ras homolog family member A | Cytoplasm | enzyme | RhoA |
| gi|502599 | 20 | 1707 | NP_001748.1 | CBP1 | carbonyl reductase 1 | Cytoplasm | enzyme | metabolism |
| gi|31645 | 2 | 129 | NP_002037.2 | GAPDH | glyceraldehyde-3-phosphate dehydrogenase | Cytoplasm | enzyme | metabolism |
| gi|557032 | 4 | 280 | NP_002999.1 | LDHB | lactate dehydrogenase B | Cytoplasm | enzyme | metabolism |
| gi|34267 | 18 | 1054 | NP_002831.2 | PTPRF | protein tyrosine phosphatase, receptor type F | Plasma Membrane | phosphatase | bait (GST-PTPRF) |
| gi|487420 | 5 | 246 | NP_002999.1 | SEMG1 | semenogelin I | Extracellular Space | other | spermatogenesis |
| gi|506885 | 10 | 602 | NP_004125.3 | SEMG2 | semenogelin II | Extracellular Space | other | spermatogenesis |
| gi|292059 | 12 | 669 | NP_004125.3 | HSPA9 | heat shock 70kDa protein 9 (mortalin) | Cytoplasm | other | chaperone |
| gi|5011974 | 10 | 426 | NP_004714.2 | ARHGEF2 | Rho/Rac guanine nucleotide exchange factor (GEF) 2 | Cytoplasm | other | RhoGAP (GEF-H1) |
| gi|4520225 | 3 | 184 | NP_004841.2 | ROCK2 | Rho-associated, coiled-coil containing protein kinase 2 | Cytoplasm | kinase | RhoA effector |
| gi|62088544 | 41 | 2420 | NP_005210 | DIAPH1 | diaphanous homolog 1 (Drosophila) | Plasma Membrane | other | RhoA effector |
| gi|88492 | 2 | 308 | NP_005336.3 | HSPA1A/HSPA1B | heat shock 70kDa protein 1A | Cytoplasm | other | chaperone |
| gi6470150  | 3   | 297   | NP_005338.1 | HSPA5  | heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa) | Cytoplasm | enzyme    | apoptosis |
|------------|-----|-------|-------------|--------|---------------------------------------------------------------|-----------|-----------|-----------|
| gi32488    | 3   | 619   | NP_005339.3 | HSP90AA1 | heat shock 70kDa protein 1-like                             | unknown   | other     | chaperone |
| gi28317    | 7   | 402   | NP_005518.3 | HSPA1L  | lactate dehydrogenase A                                     | Cytoplasm | enzyme    | metabolism |
| gi5031857  | 3   | 238   | NP_005557.1 | LDHA    | tubulin, alpha 1b                                          | Cytoplasm | other     | cytoskeleton |
| gi37492    | 7   | 459   | NP_006073.2 | TUBA1B  | heat shock 70kDa protein 8                                  | Cytoplasm | enzyme    | chaperone |
| gi5174735  | 7   | 439   | NP_006079.1 | TUBB4B  | heat shock 70kDa protein 8                                  | Cytoplasm | enzyme    | chaperone |
| gi5729877  | 10  | 509   | NP_006588.1 | HSPA8   | Rho guanine nucleotide exchange factor (GEF) 11            | Cytoplasm | other     | RhoGEF (PDZ-GEF) |
| gi7662086  | 4   | 204   | NP_055599.1 | ARHGEF11| Rho guanine nucleotide exchange factor (GEF) 12            | Cytoplasm | other     | RhoGEF (LARG) |
| gi7662088  | 11  | 543   | NP_056128.1 | ARHGEF12| Rho guanine nucleotide exchange factor (GEF) 12            | Cytoplasm | other     | RhoGEF (LARG) |
| gi54607077  | 8   | 659   | NP_075392.2 | SOWAHC  | sosondowah ankyrin repeat domain family member C           | Nucleus   | transcription regulator | scaffold |
| gi595783   | 6   | 415   | NP_116246.2 | LACTB   | lactamase, beta                                            | Cytoplasm | other     | metabolism |

The table displays the complete lists of protein identified by the mass spectrometry analysis performed using GST-PTPRF-c as bait against whole cell lysate of HeLa cells synchronized in the G2 phase. Shown are the gene symbols and full names of each interacting protein, the accession number and ref. seq., the number of peptides used for the identification and the Mascot score, as well as the protein subcellular localization and biological function. The proteins involved in the RhoA pathway and actin cytoskeleton regulation (according to IPA) are highlighted in blue. The bait (PTPRF-c) is highlighted in red.
Movie S1 – Time-lapse microscopy of mitotic events upon DEPDC1B silencing—related to Figure 1

HeLa cells were synchronized in S-phase by double-thymidine block (D-THY) and released in fresh medium to follow cell cycle progression from S-phase to mitosis by time-lapse videomicroscopy. During synchronization, endogenous DEPDC1B was silenced (1B-KD1). A custom non-targeting oligo was used as a negative control.

Movie S2 – GFP-paxillin TIRF microscopy of cell at the G2/M phase – related to Figure 2

Control (Ctrl) or DEPDC1B-silenced (1B-KD1) HeLa cells, stably transduced with the GFP-paxillin construct, were synchronized in S-phase by double-thymidine block (D-THY). Upon release, FA dynamics were observed by Total Internal Reflection Fluorescence (TIRF) microscopy, using GFP-paxillin as reporter.
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Western Blotting

Total protein lysate was obtained by cell lysis with complete JS buffer, completed with protease and (if required) phosphatase inhibitors. Sample concentration was determined using Bradford quantification and equal amounts of protein were loaded onto an SDS-PAGE gel before transfer onto the nitrocellulose membrane by western blotting. Primary antibodies were employed according to manufacturer’s instructions (the complete list is reported below).

| CODE          | ANTIBODY     | PURCHASED BY | USED FOR |
|---------------|--------------|--------------|----------|
| In-house      | anti-DEPDC1B | -            | WB       |
| V9131         | anti-Vinculin| Sigma        | WB, IF   |
| 06-570        | anti-pH3     | Upstate      | IF, FC   |
| 610219        | anti-CyclinB | BD Transduction | WB, IF, FC |
| sc-6216       | anti-LaminB  | SantaCruz    | IF       |
| 9111          | anti-pCDK1   | Cell Signaling | WB       |
| 9112          | anti-CDK1    | Cell Signaling | WB       |
| 3675          | anti-pMLC2   | Cell Signaling | WB, IF   |
| 3672          | anti-MLC2    | Cell Signaling | WB       |
| 2117          | anti-RhoA    | Cell Signaling | WB       |
| 610650        | anti-Rac1    | BD Transduction | WB       |
| 2462          | anti-Cdc42   | Cell Signaling | WB       |
| sc-135969     | anti-PTPRF   | SantaCruz    | WB       |
| G1544         | anti-GFP     | Sigma        | WB, IP   |
| F3165         | anti-FLAG    | Sigma        | IF       |
| 06-549        | anti-MYC     | Upstate      | WB, IF   |
| WH0009475M1   | anti-ROCK2   | Sigma        | WB       |
| 5486          | anti-mDia1   | Cell Signaling | WB       |
| 4076          | anti-GEF.H1  | Cell Signaling | WB       |
| 05-321        | anti-pTyrosine | Millipore  | WB       |
| 3313          | anti-pCofilin| Cell Signaling | WB       |
| 5175          | anti-Cofilin | Cell Signaling | WB       |

WB = western blotting; IF = immunofluorescence; FC = flow cytometry; IP = Immunoprecipitation
DNA constructs

The cDNA of human full-length DEPDC-1B was amplified by PCR using specific primers, cloned into the pEGFP C2 vector and sequence verified. EGFP-DEPDC1B and the RhoA-myc fragment were cloned into pSLIK-Dest vector by LR Gateway recombination (Invitrogen), according to (Shin et al., 2006). Stable pSLIK-DEPDC1B and pSLIK-RhoA HeLa cells were produced by lentiviral transduction and neomycin selection. Stable GFP-Paxillin HeLa cells were produced by transduction with LentiBrite Lentiviral Biosensor (Millipore) and GFP sorting. pcDNA3 EGFP-RhoA, pRK5 RhoA-myc and pEGFP Vinculin [T12 mutant] constructs were obtained from Addgene. pCMV-SPORT6 PTPRF construct was purchased from GeneScript and successively engineered by FLAG-tag insertion at the PTPRF C-terminus. pCMV-SPORT6 GEF-H1 construct was purchased from ImaGenes. Human DEPDC-1B cDNA and the isolated DEP domain (aa 1-112) and pseudo-RhoGAP domain (aa 154 – 428) were amplified by PCR using specific primers and cloned into pENTR-D-TOPO vector, then transferred into pCS2-Dest vector by LR Gateway recombination (Invitrogen), according to the Lawson Lab protocol (http://lawsonlab.umassmed.edu/gateway.html).

Time-lapse and live TIRF microscopy

Live time-lapse analysis of G2/M transition was performed with a ScanR screening station (Olympus-SIS) equipped with a microscope incubation chamber (Evotec), imaging cells in bright field with a 20X/NA 0.45 objective every 10 min starting at 4 h after D-THY release and reconstructed with ImageJ software. Live TIRF microscopy was used to follow FA dynamics and mitotic detachment using a Leica AM TIRF MC system mounted on a Leica AF 6000LX workstation. Living cells at 6 h from D-THY release were placed onto a sample stage within an incubator chamber set to 37°C, in an atmosphere of 5% CO2, 20% humidity and imaged every 2 minutes with a 63X/NA 1.40 oil-immersion objective. Laser penetration depth was set at 110 nm. FA mean number, size and duration were measured using ImageJ software or MATLAB scripts.
**Real-Time PCR Analysis**

Total RNA was obtained using Trizol (Invitrogen), according to manufacturer’s instructions. Reverse-transcription was performed using the VILO kit for cDNA synthesis (Invitrogen). Real-time PCR was performed with the LightCycler480 System (Roche), using specific pre-designed assays for gene expression (see below for RT-qPCR primer list).

**Purification of GST-proteins**

Bacterial pellets were resuspended in Lysis Buffer (2xTBS, 0.5 mM EDTA, 10% glycerol, 1 mM DTT) and lysed by sonication. After clearing by ultracentrifugation, GST-tagged proteins were isolated by incubating the supernatant with Glutathione-Sepharose beads (GE-Healthcare). Beads were washed three times with Wash Buffer (50 mM Tris pH 8, 0.5 mM EDTA, 10% glycerol, 1 M NaCl) and stored at -80°C in Desalting Buffer (10 mM Hepes pH 7.5, 150 mM NaCl, 5% glycerol).

**GST-RBD protein purification.**

GST-RBD protein was expressed in pLysS BL21 bacterial strain upon IPTG induction (0.2 mM) overnight at room temperature. Bacterial pellets were resuspended in GST-RBD Lysis Buffer (20 mM HEPES, 150 mM NaCl, 5 mM MgCl₂, Triton X-100 1% v/v, 1 mM DTT, protease inhibitors) and lysed by sonication. GST-tagged proteins were isolated as previously described, then beads were washed thrice with GST-RBD Wash Buffer (HBS, 5 mM MgCl₂, Glycerol 10%, 1 mM DTT) and stored at 4°C. For the GST-RBD pull-down assay, cells were lysed in ice-cold RBD Buffer (50 mM Tris-HCl pH 7.6, 500 mM NaCl, Triton X-100 1% v/v, 10 mM 5 mM MgCl₂ freshly added, protease inhibitors) and centrifuged. GTP stimulation was achieved by treating the lysate with GTPγS (final conc.: 1 mM) for 30 minutes at 37°C and then blocking the reaction with 20 mM MgCl₂.
GAP assay

GST-RhoA protein (final conc.: 0.1 µM) was loaded at 30°C for 30 minutes with $^{32}$P-GTP (60 mCi) in GAP-Loading Buffer (25 mM Tris pH 7.4, 50 mM NaCl, 1 mg/ml BSA, 0.1 mM DTT) and stabilized by adding MgCl$_2$ (final conc.: 50 mM). The GTP hydrolysis reaction was performed in GAP Buffer (25 mM Tris pH 7.4, 100 mM NaCl, 25 mM MgCl$_2$, 1 mg/ml BSA, 1 mM GTP, 0.1 mM DTT), by incubating GST-SH3BP1 [GAP] or GST-DEPDC1B [GAP] (final conc.: 2 or 4 µM) at 30°C for 30 minutes. GTP-loaded RhoA was withdrawn at the desired time points, incubated with GAP-Wash Buffer (100 mM Tris pH7.5, 50 mM NaCl, 25 mM MgCl$_2$, 1 mM DTT) and measured using a scintillation counter.

Yeast Two-Hybrid Screen

The screen was performed by Hybrigenics (Paris) using a N-LexA-bait/N-GAL4-bait system. The cDNA of human full length DEPDC-1B was tested against a human placental library (RP5), a total of $66.2 \times 10^6$ interactions were analyzed.

Mass Spectrometry and IPA analysis

PTPRF interactors were identified by performing a GST-pulldown with Phoenix lysate, using PTPRF-c as bait. Isolated proteins were separated in discrete bands by SDS-PAGE and processed using a MALDI-TOF mass spectrometer. As a control, GST-alone was used in parallel to identify possible contaminants or non-specific binders. The list of PTPRF interactors was used as the input for Ingenuity Pathway Analysis (IPA), running a core analysis and considering the enriched functions ($p < 0.001$). The RhoA signaling network was then selected and the members (9 proteins) are listed in Table S2 of Supplemental Materials.
SILAC labeling and GST-pulldown

HeLa cells previously transduced with an inducible DEPDC1B-GFP transgene were cultured for two weeks in Heavy or Light SILAC media, to induce cellular proteome labeling. SILAC DMEM (EuroClone, B7511L), depleted of lysine and arginine, was supplemented with 10% dialyzed FBS (Invitrogen, 26400-044). Light medium contains $^{12}\text{C}_6^{14}\text{N}_2$ L-lysine (Lys 0, Sigma, L8662) and $^{12}\text{C}_6^{14}\text{N}_4$ L-arginine (Arg0, Sigma, A6969), while Heavy medium is supplemented by $^{13}\text{C}_6^{15}\text{N}_2$ L-lysine (Lys 8, Sigma, 68041) and $^{13}\text{C}_6^{15}\text{N}_4$ L-arginine (Arg10, Sigma, 608033), at a concentration of 146 mg/L and 84 mg/L respectively. After labeling, DEPDC1B-GFP expression was induced in one of the two SILAC channels (Heavy in the forward experiment, Light in the reverse, respectively). Total protein lysates from both cultures (Heavy and Light) were obtained by lysis with complete JS buffer, mixed together in equal amount (1:1) and subjected to GST-PTPRF-c pulldown, as previously described. Interactors were identified by nano Liquid Chromatography-tandem Mass Spectrometry (nanoLC-MS/MS) analysis on a high-resolution instrument (LQT-Velos Orbitrap, Thermo Fisher Scientific) and proteins were identified and accurately quantified based on their SILAC H/L ration using MaxQuant algorithm (Cox and Mann, 2008). Variations in the binding to PTPRF following DEPDC1B induction were determined as Heavy/Light log2 ratio (or Light/Heavy in the REV experiment).

Phosphatase assay

Total protein lysate was obtained by cell lysis with complete JS buffer, supplemented with protease inhibitors. Total lysate (1 mg) was treated for de-phosphorylation, by incubation with 200 units of Calf Intestinal Phosphatases (CIP, from NEB) in CIP Buffer (50 mM Potassium Acetate, 20 mM Tris-Acetate, 10 mM Magnesium Acetate) at 37°C for 1 hour. Lysate was then subjected to GST-PTPRF-c pull-down and SDS-PAGE. Lysate de-phosphorylation was checked by western blotting using a pan-phosphotyrosine antibody.
**Zebrafish ISH, cDNA expression and RT-PCR experiments.**

For *in situ* hybridization (ISH) experiments, *depc1b* cDNA from *Danio rerio* embryos was cloned into the *pCR2.1 TOPO* vector. Antisense RNA probe was prepared by transcribing linearized cDNA with SP6 polymerase and digoxigenin labeling mix (Roche). Embryos were fixed in paraformaldehyde (4% w/v) overnight and stained according to the standard protocol (Thisse and Thisse, 2008).

RT-PCR primers used to verify the action of splice-blocking morpholinos using specific primers located at the exon junctions (FW_*depc1b* exon 1 target sequence: CGGGATGTTTACACTCACACAT; REV_*depc1b* exon 2 target sequence: CTGTACAGGTGTCCGCTGTC). To analyze DEPDC-1A and DEPDC-1B expression during zebrafish development, total RNA from successive embryonic stages (from 1 to 72 hours post fertilization) was extracted with Trizol and retro-transcribed in cDNA with VILO kit (Invitrogen). Real time PCR was performed with LightCycler480 System (Roche), using specific custom assays for gene expression. For immunofluorescence analysis, embryos were fixed in paraformaldehyde (4% w/v) overnight and stained for pH3 using a monoclonal rabbit primary antibody (06-570, Upstate) and an anti-rabbit Alexa-546 conjugated secondary antibody (Upstate) following standard procedures (Costagli et al., 2002). mRNA of full-length, DEP and GAP domain of human DEPDC1B were injected in one-cell stage embryos, at a concentration of 35 ng/ml.
Complete list of siRNAs used

| TYPE       | OLIGO NAME       | SEQUENCE                       | TARGET GENE | PURCHASED FROM |
|------------|------------------|--------------------------------|-------------|----------------|
| custom     | SCRAMBLE         | AGACGAACAAAGUCACCGAC           | negative control | Invitrogen     |
| custom     | DEPDC1B oligo 1  | GUACUGGGUUUGUUACAGA            | DEPDC1B     | Invitrogen     |
| custom     | DEPDC1B oligo 2  | GAUCAUAUGGCUCUCAGGAUU         | DEPDC1B     | Invitrogen     |
| custom     | DEPDC1B oligo 3  | CCUAGCUACUGAUACUUUUUU         | DEPDC1B     | Invitrogen     |
| custom     | DEPDC1A          | GAGCUGUGUUAGGGAUAAACUAUA      | DEPDC1A     | Invitrogen     |
| custom     | RhoA             | GAUACCGAUGUUAUCUGAUGUGU       | RhoA        | Invitrogen     |
| custom     | Rac1             | CACAAACCAUGCAUUCCUGGAGAA      | Rac1        | Invitrogen     |
| custom     | Cdc42            | CCUCUACUAAUGAGAAACUUGCCAA     | Cdc42       | Invitrogen     |
| pre-designed | VINCULIN        | HSS111260                     | Vinculin    | Invitrogen     |
| pre-designed | ACTININ         | HSS100130                     | Actinin     | Invitrogen     |
| pre-designed | FAK             | HSS183869                     | FAK         | Invitrogen     |
| pre-designed | PTPRF           | SI02658796                    | PTPRF       | Qiagen         |
| pre-designed | GEF-H1          | HSS113587                     | GEF-H1      | Invitrogen     |
| pre-designed | PDZ-GEF         | HSS114830                     | PDZ-GEF     | Invitrogen     |
Complete list of qPCR assays

| TYPE                        | TARGET mRNA | CODE          | PURCHASED FROM |
|-----------------------------|-------------|---------------|----------------|
| Quantitect Primer Assay     | DEPDC1B     | QT01680833    | Qiagen         |
| Quantitect Primer Assay     | Vinculin    | QT00078302    | Qiagen         |
| Quantitect Primer Assay     | Actinin     | QT00083678    | Qiagen         |
| Quantitect Primer Assay     | FAK         | QT00057687    | Qiagen         |
| Quantitect Primer Assay     | RhoA        | QT00044723    | Qiagen         |
| Quantitect Primer Assay     | Rac1        | QT00065856    | Qiagen         |
| Quantitect Primer Assay     | Cdc42       | QT01674442    | Qiagen         |
| Quantitect Primer Assay     | GAPDH       | QT01192646    | Qiagen         |
| Quantitect Primer Assay     | PDZ-GEF     | QT00082635    | Qiagen         |
| Taqman Gene Expression Assay| DEPDC1A long| Hs00873599_g1| Applied Biosystems |
| Taqman Gene Expression Assay| DEPDC1A short| Hs01374267_g1| Applied Biosystems |
| Taqman Gene Expression Assay| DEPDC1A both| Hs01372374_g1| Applied Biosystems |
SUPPLEMENTAL REFERENCES

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