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## Rictor regulates cell migration by suppressing RhoGDI2

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### Abstract

Rictor and its binding partner Sin1 are indispensable components of mTORC2 (mammalian Target of Rapamycin Complex 2). The mTORC2 signaling complex functions as the regulatory kinase of the distinct members of AGC kinase family known to regulate cell proliferation and survival. In the early chemotaxis studies in *Dictyostelium*, the rictor's ortholog has been identified as a regulator of cell migration. How rictor regulates cell migration is poorly characterized. Here we show that rictor regulates cell migration by controlling a potent inhibitor of Rho proteins known as the Rho-GDP dissociation inhibitor 2 (RhoGDI2). Based on our proteomics study we identified that the rictor-dependent deficiency in cell migration is caused by up-regulation of RhoGDI2 leading to a low activity of Rac and Cdc42. We found that a suppression of RhoGDI2 by rictor is not related to the Sin1 or raptor function that excludes a role of mTORC2 or mTORC1 in regulation of RhoGDI2. Our study reveals that rictor by suppressing RhoGDI2 promotes activity of the Rho proteins and cell migration.

### Keywords

rictor (rapamycin-insensitive companion of mTOR); cell migration; Rho proteins; RhoGDI2 (Rho-GDP dissociation inhibitor 2)

### Introduction

The functional characterization of the mammalian Target of Rapamycin (mTOR), a central component of the essential and highly conserved signaling pathway (1, 2), identified rictor (rapamycin-insensitive companion of mTOR) as the mTOR interacting protein (3, 4). Biochemical studies revealed that mTOR with its binding proteins mLST8 and DEPTOR exists at least in two distinct complexes. The binding of raptor to mTOR defines its nutrient-

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sensitive mTOR Complex 1 (mTORC1) that regulates protein synthesis by phosphorylating its substrates S6K1 and 4EBP1. The second mTOR complex identified as mTORC2 is assembled by binding to mTOR of its essential components rictor and Sin1 (3–5).

The mTORC2 kinase complex by phosphorylation of the distinct AGC kinase family members including Akt (also known as protein kinase B, PKB), PKC $\alpha$  (protein kinase C $\alpha$ ) and SGK (serum and glucocorticoid-inducible kinase) on the hydrophobic and turn motif sites regulates variety cellular processes such as proliferation, metabolism, and survival (6). Rictor together with Sin1 is believed to function as an adaptor protein controlling the mTORC2 substrate binding and specificity. The recent study identified that a single substitution of human rictor Gly-934 to any charged amino acid prevents its binding to Sin1 causing deficiency in assembly of mTORC2 (7). As an anabolic signaling complex mTORC2 is dependent on a functional activity of endoplasmic reticulum (ER), a cellular organelle critical in protein and fatty acid synthesis. It has been identified that ER is a major localization site of mTORC2 (8) and the mechanism how ER stress inhibits the mTORC2 kinase activity by phosphorylation of rictor on a specific site mediated by the stress responsive glycogen synthase kinase-3 $\beta$  has been identified (9). Importantly, activation of mTORC2 mediated by association with ribosomes has been also recently reported (10, 11).

Rictor has been initially identified as a regulator of cell migration. In the original study by Devreotes group in Dictyostelium, the rictor's ortholog has been linked to regulation of chemotaxis (12). Several studies in human cancer cells have indicated a role of rictor in cytoskeleton regulation (3, 4) and cell migration (13). The functional study of mTORC2 carried by knock down of rictor demonstrated a role of mTORC2 in regulation of the cAMP- and RhoA-dependent chemotaxis in neutrophils (14). Although the functional role of rictor has been mostly assigned to its role as the essential component of mTORC2, the recent studies provide evidence that rictor also carries the mTORC2 independent functions with a potential role in cell migration. Rictor alone apart from other components of mTORC2 has been shown to interact with the regulators of cell morphology and migration known as the integrin-linked kinase (ILK) and actin-based molecular motor myosin 1c (15, 16). It is likely that rictor regulates cell morphology and migration by playing a role as the indispensable component of mTORC2 and also by carrying its mTORC2 independent functions. How rictor regulates cell migration remains poorly characterized.

## Results

### Cell migration of mouse embryonic fibroblasts is dependent on rictor

The functional studies of rictor indicate its role in regulation of cytoskeleton and cell migration (3, 4, 13). We observed a substantial difference in cell morphology between the immortalized wild type and rictor null mouse embryonic fibroblasts (MEFs) under serum-starved cell culture conditions (Fig. 1A). The rictor deficient MEFs appear as the long and thin cells suggesting that this elongated cell morphology caused by an impaired cell spreading on culture dishes. This distinct phenotype in cell morphology is rictor dependent because the reconstitution of its expression in the rictor null cells has reversed the elongated cell shape similar to the wild type well-spread cell morphology. The elongated thin cell morphology of the rictor null cells correlates well with the impaired cell migration (Fig. 1B).

By staining cells adhered to the membrane (no swab, Fig. 1B, the upper panel) we observed that the rictor deficiency did not alter cell adherence indicating no dramatic rictor-dependent effect on cell survival. Whereas, by performing of swab to detect cells migrated to the opposite side of the membrane (with swab, Fig. 1B, the lower panel), we found that the rictor null cells show only approximately 25% efficiency in cell migration when compared to the wild type cells (Fig. 1C). The stable rictor expression in the null cells has rescued substantially the cell migration capacity that has reached the 60% level observed in the wild type cells. The partial rescue of cell migration by the stable ectopic rictor expression might be linked to its lower expression level. The myc-tagged rictor expressed in the null cells has been functionally validated by analysing the rictor-dependent mTORC2 function in cells as detected by phosphorylation of Akt on its regulatory Ser-473 site (Fig. 1D).

### The proteomics study reveals a high abundance of RhoGDI2 in rictor null cells

To define a role of rictor in regulation of cell migration we performed an unbiased proteomic analysis of the paired wild type and rictor null MEF extracts as previously described (17). The total cellular protein lysates were separated on the two dimensional (2D) gels, the proteins were detected by Silver staining and analyzed (Fig. 2A). Only protein spots indicating significant differences in their intensity were cut and further analyzed by Mass Spectrometry. Based on this approach we have identified the actin filaments assembly and stress responsive proteins such as cofilin, peroxiredoxin 6, and HSP27 as the low expressed proteins in the rictor null cells. These differences might reflect a lower cytoskeleton dynamics of the elongated rictor null cells. On the contrary, we also detected an intensively stained protein spot in the gel obtained from the rictor null cell protein lysate and the similar spot showed only a faint signal in the resolved protein lysate of the wild type MEFs (Fig. 2A, lower panel). This protein spot has been identified as the Rho GDP-dissociation inhibitor 2 (RhoGDI2) known to function as a potent negative regulator of Rho proteins (18). It has been also reported that a low RhoGDI2 expression associates with the human metastatic tumors (19).

To address whether rictor regulates the RhoGDI2 expression, we studied the primary rictor null with the paired wild type MEFs. Indeed, we found a high protein level of RhoGDI2 in the rictor null but not in the wild type cellular lysates (Fig. 2B), whereas the HSP27 abundance showed opposite relationship consistent with the 2D gel data (Figs. 2A and 2B). Our finding indicates that the RhoGDI2 protein expression is rictor dependent. Rictor and Sin1 have been defined as the essential components of mTORC2 and a role of rictor in regulation of RhoGDI2 might be related to the mTORC2 function. In this case, we expected the similar regulation of RhoGDI2 by the mTORC2 component Sin1 (5). We found that the Sin1 null cells did not show any change in the RhoGDI2 protein abundance (Fig. 2C). This finding correlated well with the observation that the Sin1 null cells were not defective in cell migration (Fig. S1). Besides Sin1, other components of the mTOR signalling including mTOR and raptor were not linked to regulation of the RhoGDI2 expression because only knock down of rictor but not mTOR or raptor caused an increase in RhoGDI2 abundance in MDA-MB-435 cells (Fig. S2A and B). Coherent with the knock down study of the essential mTORC1 component raptor, the prolonged treatment of cells with rapamycin known to target and inhibit mTORC1 also did not show any effect on expression of RhoGDI2 (Fig.

S2C). Furthermore, an effective inhibition of the mTOR kinase activity by its specific inhibitor pp242 (11) for 6 or 24 hrs as detected by phosphorylation of the mTOR substrates S6K1 and Akt did not alter expression of RhoGDI2 (Fig. S2D). Thus, we found that regulation of the RhoGDI2 expression is carried by a distinct function of rictor and is not related to the function of mTORC1 and mTORC2.

### **The rictor-dependent up-regulation of RhoGDI2 associates with inhibition of Rac1 and Cdc42**

To assure that the RhoGDI2 protein expression depends on rictor, we have reintroduced the rictor expression to the immortalized rictor null MEFs and found that the RhoGDI2 abundance has been decreased substantially to the similar level detected in the wild type cells (Fig 3A, the right panel). The knock down of rictor by the different shRNA hairpins in the wild type MEFs (Fig. 3A, the left panel) or in the human cancer cell lines HT29D4 and HeLa (Fig. 3B) showed the similar rictor-dependent RhoGDI2 expression in the mouse and human cells. Interestingly, the highly homologous isoform of RhoGDI2 known as RhoGDI1 (or RhoGDI $\alpha$ ) has not been regulated by rictor (Fig. 3 A and B) indicating that rictor specifically controls RhoGDI2. The increased abundance of RhoGDI2 in the rictor null cells has not been carried by regulation of the gene expression, because we did not detect any difference in the levels of mRNA encoding RhoGDI2 in the wild type or rictor deficient MEFs (Fig. S3).

RhoGDIs are the critical and potent inhibitors of the Rho proteins (20). They act by binding to the GDP-bound forms of Rho GTPases and maintaining them at inactive soluble state by masking their membrane-anchoring isoprenylated C-terminus domains and blocking the access to guanine nucleotide exchange factors (18). Our data indicate that a loss of rictor associates with a substantial increase in abundance of RhoGDI2. Based on this finding we hypothesized that if the abundant RhoGDI2 protein in the rictor null cells is functional, it will interfere with activation of the Rho proteins. By a specific pull down of the active GTP-bound form of Rho proteins we detected a substantial level of the active Rac1 and Cdc42 proteins in the wild type but not in the rictor null cells (Figure 3C). Inhibition of the Rho proteins in the rictor null cells was rictor dependent because its ectopic expression induced activation of Rac1 and Cdc42. The similar study of the Sin1 null cells with its matched wild type MEFs did not show any changes in activity of the studied Rho proteins (Fig. 3D) indicating that rictor carries a role in regulation of RhoGDI2 and Rac/Cdc42 proteins independent of its binding partner Sin1 and mTORC2. Our finding is consistent with the previous studies indicating that RhoGDI2 binds to Rac1 and Cdc42 but with a higher affinity toward Rac1 (21) carrying a substantial inhibitory effect on activity of Rac1 (22). Thus, the rictor-dependent increased abundance of RhoGDI2 associates with an effective inhibition of the Rac1 and Cdc42 proteins.

### **The cell migration deficiency of rictor null cell is dependent on RhoGDI2**

Our functional study identified rictor as a positive regulator of the Rho proteins Rac1 and Cdc42 that acts by suppressing RhoGDI2. A poor cell migration capacity of the rictor null cells associates with a high abundance of RhoGDI2 in these cells. We have assumed if RhoGDI2 mediates the cell migration deficiency in the rictor null cells, a loss of RhoGDI2

will rescue the rictor-dependent inhibition of cell migration. To address a functional role of RhoGDI2 in suppression of cell migration of the rictor null MEFs, we carried out the knock down of RhoGDI2 by expressing the specific shRNAs (Fig. 4C, upper panel) and performed the cell migration assay. We found that the RhoGDI2 knock down in the rictor null cells caused a substantial increase in cell migration efficiency compare to the cells expressing the non-specific luciferase shRNA as a control (Fig. 4A and B). Importantly, RhoGDI2 inhibited Rac1 in the rictor null MEFs, because the knock down of RhoGDI2 has induced activation of Rac1 as detected by the pull down of its GTP bound form (Fig. 4C, the lower panel). Our data indicate that rictor promotes cell migration by suppressing RhoGDI2 and activating the Rac1 and Cdc42 proteins, whereas the rictor deficiency caused up-regulation of RhoGDI2 and inhibition of cell migration. Most likely, the rictor deficiency causes activation of RhoGDI2 that results in induction of its protein abundance because the stable ectopic expression of RhoGDI2 in the wild type MEFs was not sufficient to interfere with cell migration (Fig. S4).

## Discussion

To address a role of rictor in cell migration we have carried out the proteomics study of the rictor null cells and identified that rictor regulates cell migration by suppressing RhoGDI2. This protein belongs to the RhoGDI family represented by three (RhoGDI1, 2, and 3) members that function as the potent negative regulators of Rho proteins (18). The small Rho GTPase family members known as Rho, Rac, and Cdc42 play a critical role in cell migration by regulating dynamic assembly of actin filaments. Rho GTPases cycle between an inactive (GDP bound) state located in cytosole and active (GTP bound) state localized on membrane (23). RhoGDIs inhibit Rho GTPases by direct interaction and maintaining Rho proteins at the inactive state in cytoplasm and restraining from the activation site on membrane (18). In our study we found that the up-regulation of RhoGDI2 in the rictor null cells caused inhibition of Rac1 and Cdc42. Preferential binding of RhoGDI2 to Rac1 or Cdc42 is related to a distinctive regulation of Rho proteins by RhoGDI2 reported in the previous studies (21, 22). It is consistent with the RhoGDI2-dependnet inhibition of Rac1 and Cdc42 detected in the rictor null cells.

Within the RhoGDI family members RhoGDI1 has been well characterized as the ubiquitously expressed member of this family, whereas RhoGDI2 and RhoGDI3 have been shown to carry the tissue specific expression (18). Although RhoGDI2 is known to be highly expressed particular in B- and T-lymphocytes, the widespread tissue distribution of its mRNA has been recently reported (24). The facts that both RhoGDI1 and 2 are highly conserved in evolution and identified in *Dictyostelium* (25) support a biological significance of both isoforms. The functional distinction between RhoGDI1 and RhoGDI2 remains not well characterized but a loss of RhoGDI2 but not RhoGDI1 has been reported to associate with metastatic human cancers (19, 20, 26) that attracted attention to RhoGDI2 as a potential invasion and metastatic suppressor.

Our study shows that rictor by controlling RhoGDI2 maintains activity of the Rho proteins and promotes cell migration. Loss of rictor caused an increase in abundance of RhoGDI2 carrying the negative impact on cell migration associated with deregulation of the Rho

proteins. How a distinctive regulation of RhoGDI2 but not RhoGDI1 takes place by rictor remains to be addressed. This regulation is not related to the gene expression mechanism and it is likely to be mediated by a rictor-dependent post-translational modification of the RhoGDI2 protein leading to its higher activity and stability. Our data suggest that the rictor-dependent functional regulation of RhoGDI2 is critical in suppressing of cell migration because the overexpression of RhoGDI2 itself is not effective in inhibition of cell migration.

Rictor is an essential component of mTORC2 and by binding to Sin1 rictor forms the mTORC2 kinase complex known to phosphorylate and regulate Akt (2). Interestingly, regulation of RhoGDI2 by rictor is independent of mTORC2 because deficiency of Sin1 did not show any regulatory effect on RhoGDI2 and activity of Rho proteins. The previous studies have reported that rictor carries its mTORC2 independent functions by binding to ILK or myosin 1c. Both ILK and myosin 1c are known to regulate cell morphology and motility (27, 28) and it is possible that rictor by binding to these proteins mediates regulation of RhoGDI2. Recently, it has been also shown that rictor carries the mTORC2-independent function by forming a complex with Cullin-1 and inducing ubiquitination of SGK1 leading to its degradation (29). Although an increased abundance of SGK1 in absence of rictor represents a potential lead in understanding of the rictor-dependent regulation of RhoGDI2, restriction of the SGK1 induction to serum stimulation makes it less likely to play a role in the serum-independent regulation of RhoGDI2 by rictor.

Our functional studies indicate that rictor promotes cell migration by suppressing RhoGDI2 and increasing a ratio of the active Rac1 and Cdc42 proteins. We believe that the functional role of rictor in regulation of RhoGDI2 might contribute to the rictor-dependent chemotaxis described in the original study in *Dictyostelium* (12) and also in the recent study in neutrophils (14).

Importantly, a loss of RhoGDI2 has been shown to associate with metastatic human cancers (19) and our finding indicating a role of rictor in suppressing RhoGDI2 carries a novel incite in regulation of cell migration and metastasis. Understanding of the mechanism how a loss of rictor activates RhoGDI2 might lead to a novel approach to interfere with cancer cell migration and metastasis.

## Materials and methods

### Cell lines and cell culture

HeLa, HT29D4 and HEK293T cells were obtained from the American Type Culture Collection. Immortalized wild and rictor null MEFs were kindly provided by Mark A. Magnuson (Shiota et al., 2006). Wild and Sin1 null MEFs were generous provided by Bin Su (5). MEFs, HeLa, HT29D4 and 293T cells were cultured in DMEM/F12 (50:50) media containing 10mM D-glucose supplemented with 10 % fetal calf serum, 1 % L-glutamine and 1 % penicillin streptomycin at 37°C in an atmosphere of 5% CO<sub>2</sub>. 293T cells were transfected with calcium phosphate method. MEFs were exclusively transfected with Dreamfect™ reagent (OZ Biosciences, Marseille, France) following the manufacturer's protocol. When indicated, cells were serum starved for 16 hr, followed by 60 min incubation with serum free DMEM media for growth factor starvation.



### Cell spreading and transwell migration assay

Immortalized wild and rictor null MEFs were plated in 6-well tissue culture plates. Twenty-four hours later, cells were serum starved, rinsed with prewarmed PBS and images were taken using an inverted light microscope. The spread cells were defined as cells with extended processes whereas the non-spread cells were thin and elongated. Transwell migration assay was performed as described previously (30). To exclude the cell attachment and adhesion effects, as a control the same set of experiment was performed without swabbing the cells detected on the inserts. Five random fields of migrated or total cells were selected and counted using an inverted microscope. Quantified results are normalized with total number of cells and presented as percentage of means  $\pm$  standard deviation.

### Preparation of cell lysates, immunoblotting and immunoprecipitation

Cells lysis and immunoblotting were performed as described previously (31). For immunoprecipitation experiments, immortalized rictor null MEFs were transiently transfected with the Flag-tagged expression plasmids of vector, RhoGDI1 and RhoGDI2. After 48 hr, cells were lysed in ice cold lysis buffer containing 10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 10mM MgCl<sub>2</sub>, 1% NP40, 4% glycerol, complete protease inhibitor cocktail and immunoprecipitated with the anti-Flag M2 Affinity agarose beads (Sigma-Aldrich Corp., St. Louis, MO, USA) for 2 hr at 4°C. Immobilized Flag-RhoGDIs complexes were washed with ice cold lysis buffer, eluted and subjected for immunoblotting. The following antibodies were used in immunoblotting: Rictor (S-20), p70-S6 Kinase (C-18), Tubulin (Tu-02), RhoGDI1 (A20) from Santa Cruz Biotechnology, Santa Cruz, CA, USA; Rac1 (23A8), Sin1 and Cdc42 from Millipore, Billerica, MA, USA; Raptor (24C12), HSPB1, mTOR (L27D4), phospho-S6K1 Thr-389 (108D2), and phospho-Akt Ser-473 (193H12) from Cell Signaling Technology, Danvers, MA, USA, FLAG from Rockland immunochemical Inc, Gilbertsville, PA, USA; RhoGDI2 from Spring Bioscience, Pleasanton, CA, USA.

### Resolution of proteins by two-dimensional gels and mass spectrometry analysis

Immortalized wild and rictor null MEFs were plated in tissue culture plates. Twenty-four hours later, cells were placed in serum free DMEM media for 16 hr, rinsed with ice-cold PBS and lysed on ice for 20 min in 0.3–0.5 ml of ice-cold lysis buffer containing 1% Triton X-100, 10 mM Tris HCl (pH 7.5), 120 mM NaCl, and complete EDTA-free protease inhibitors. After clearing of the cell extracts by centrifugation at 13,000  $\times$  g for 10 min, samples containing 200–300  $\mu$ g of clear cell extracts were precipitate with ice-cold 80% acetone for 16 hr at  $-20^{\circ}\text{C}$ . Samples were subjected to centrifugation at 13,000  $\times$  g for 10 min, dried briefly and redissolved in rehydration buffer containing 8M urea, 2% CHAPS, 50mM DTT and 0.2% bio-lyte ampholytes. 30  $\mu$ g of purified wild and rictor-deficient protein extracts were resolved by two dimensional gel electrophoresis as previously described (17). Resolution in the first dimension was achieved using 11 cm IPG dry strips (pH 5–8) (BioRad, Hercules, CA, USA), and a 10% Bis-Tris SDS polyacrylamide gel (BioRad, Hercules, CA, USA) was used to resolve in the second dimension. Proteins were visualized by silver staining, digitized on a GS-800 calibrated densitometer using QuantityOne software (BioRad, Hercules, CA, USA) and analyzed with ImageJ software.

Bands of interest were excised, reduced and alkylated and digested with trypsin. The resulting peptide mixture was separated by reversed-phase HPLC and introduced into a tandem mass spectrometer operated in a data dependant manner. The resulting MS/MS spectra were searched against a human database of known proteins using SEQUEST as described previously (32).

### **Plasmids, lentiviral infections, and small interfering RNA infection**

Human Myc-tagged rictor in pRK5 vector were described previously (Aimbetov et al., 2011). The FLAG-tagged was added to the expression plasmids of RhoGDI1 (plasmid # 4923219) and RhoGDI2 (plasmid # 3583026) obtained from Open Biosystems (Huntsville, AL, USA). Lentiviral short hairpin RNAs (shRNAs) targeting human luciferase, rictor, raptor, and mTOR genes were described previously (3, 33). Short hairpin RNA (shRNA) constructs of mouse rictor (TRCN0000123394 and TRCN0000123395), and RhoGDI2 (TRCN0000106183 and TRCN0000106184) were obtained from Sigma Mission shRNA (Sigma-Aldrich Corp., St. Louis, MO, USA). The lentiviral expression plasmid encoding RhoGDI2 (PLOHS\_100005933) has been obtained from the MD Anderson shRNA and ORFeome Core Facility distributing Precision LentiORF Open Biosystems library cDNAs (Open Biosystems, Lafayette, CO, USA). Transfection, lentiviral production and infection were described (33).

### **Affinity-precipitation of cellular GTP-Rac1/Cdc42 and GTP-Rho proteins**

Wild, rictor or mSin1 null MEFs were washed with ice-cold PBS and lysed in ice-cold lysis buffer containing 50 mM Tris, pH 7.2, 1% Triton X-100, 150 mM NaCl, 10 mM MgCl<sub>2</sub>, 4% glycerol and complete protease inhibitors. Cell lysates were clarified by centrifugation at 13,000 g at 4°C for 10 min, and incubated with 20 µl PAK1 p21-binding domain agarose beads (Millipore, Billerica, MA, USA) at 4°C for 45 min. The beads were washed four times with same ice-cold lysis buffer and eluted agarose bound proteins were subjected for immunoblotting against Rac1 and Cdc42 antibodies.

### **Primer sequences for real-time RT-PCR**

Mouse RhoGDI1, 5'-TGTGACTCGACTGACCTTGG-3' (forward) and 5'-CAATTTGACCCCTTCCTG-3' (reverse); mouse RhoGDI2, 5'-GGACTGGCATGAGAGTGGAT-3' (forward) and 5'-CAGGTGAGGTGGTCCTGTTT-3' (reverse).

### **Statistical analysis**

Statistical analysis was carried out using the Student's *t*-test, and statistical significance was assumed for *p* values less than 0.001.

### **Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.



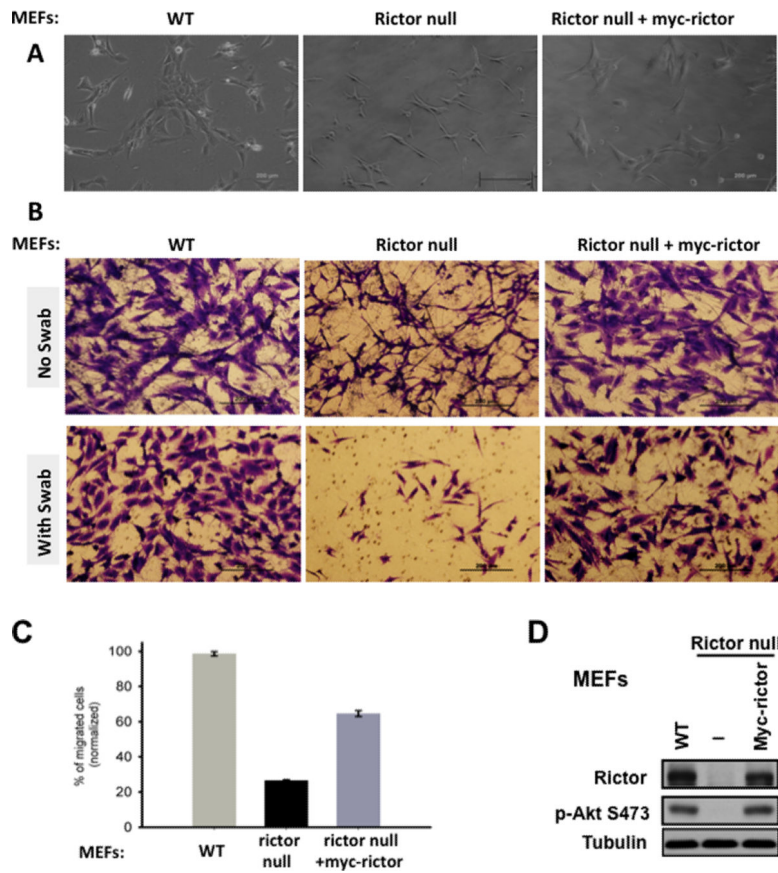
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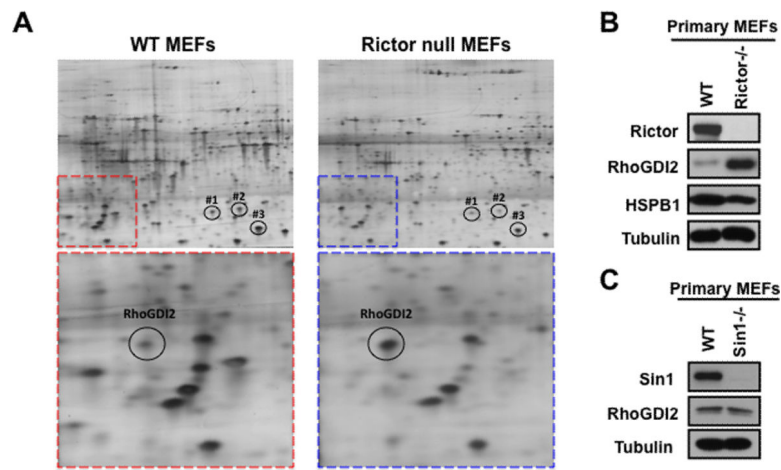
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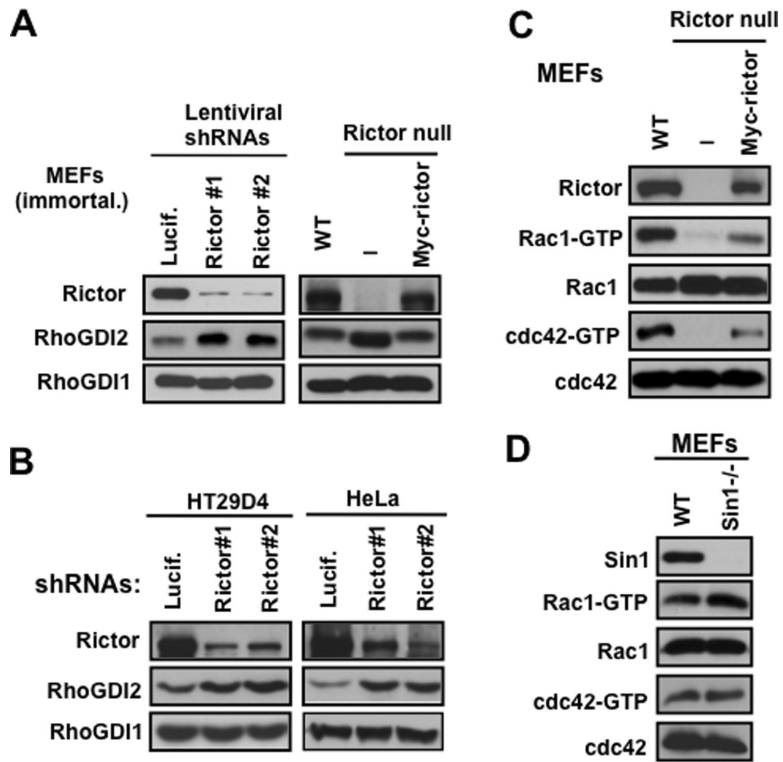


**Figure 1.** Loss of rictor causes deficiency in cell migration. Images of the wild type, rictor null and rictor null with reintroduced rictor expression taken by light microscope (a) or following transwell cell migration assay (b). The percentage of migrated cells shown in (b) is depicted in the diagram (c). (d) The immunoblotting was used to detect the level of rictor expression and Akt phosphorylation of cells lysates obtained from cells shown in (a) and (b).

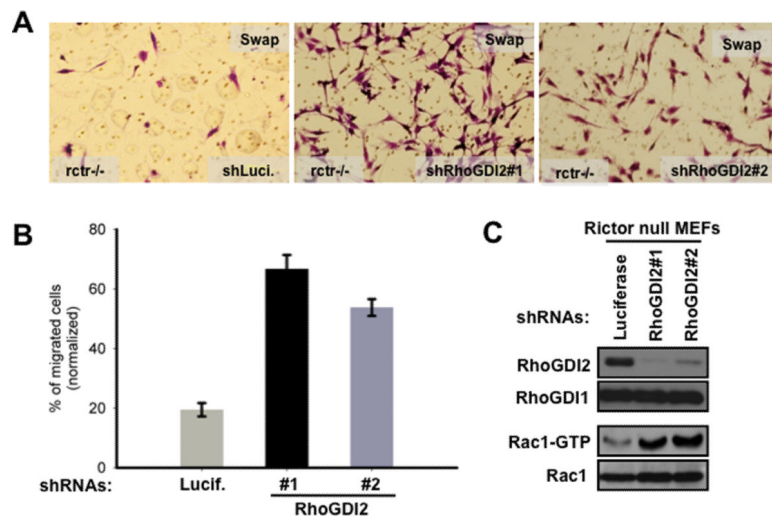


**Figure 2.**

The proteomics study: RhoGDI2 is abundant protein in the rictor null cells. **(a)** The upper panel shows the images of the total cellular lysates from the wild type and rictor null immortalized MEFs resolved by 2D gels and stained by silver staining. The lower panel shows the spot corresponding to RhoGDI2 in the zoomed areas indicated by the red and blue boxes in the upper panel. On the upper panel the spots indicated by numbers have been identified as the following: #1 - Peroxiredoxin 6; #2 - HSPB1; #3 - Cofilin1. The immunoblotting was used to detect the level of the indicated proteins obtained from the wild type and rictor null **(b)** and Sin1 null **(c)** primary MEFs.



**Figure 3.** Abundance of RhoGDI2 is rictor dependent in MEFs and human cancer cells. (a) The immunoblotting was performed to detect the levels of rictor and RhoGDIs in rictor knocked down MEFs (left panel) or the rictor null cells with the reintroduced rictor expression (right panel). (b) The similar analysis as in (a) of the human cancer HT29D4 and HeLa cells with the rictor knock down by three specific shRNAs. (c) The pull-down and detection of active GTP bound form of Rac and Cdc42 and their protein levels in the wild type and rictor null cells with or without myc-rictor expression. (d) The similar analysis as in (c) of the Sin1 null MEFs.



**Figure 4.** RhoGDI2 suppresses activity of Rac and Cdc42 GTPases and inhibits cell migration in the rictor null cells. **(a)** The cell images indicating the cell migration efficiency of the rictor null cells with or without knock down of RhoGDI2. The transwell cell migration assay was performed as in Fig. 1A. **(b)** The diagram indicating the percentage of migrated cells shown in (a). **(c)** Immunoblotting of the cellular lysates and pull down of the GTP-bound Rac1 obtained from cells shown in (a) to detect abundance of RhoGDI2 and Rac1 activity.