Supplemental Materials

Molecular Biology of the Cell

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Supplementary Material

Supplementary Figure Legends

Figure S1. (A) Western blotting of mTOR, Rictor and Raptor in undifferentiated HL-60 cells (uHL-60), differentiated HL-60 cells (dHL-60) and human primary neutrophils (1^{o} neutrophil). GAPDH was a loading control. (B) Outlines (top) and migration speeds (bottom) of cells responding to the fMLP stimulation in the microfluidic chamber, as shown in Figure 2C. Each set of outlines represents a single cell observed at indicated intervals (denoted by different colors) after exposure to fMLP, and two representative cells are shown for each condition. Values for migration speeds are mean ± SEM (n >40). (C) Cells containing non-target (NT) shRNA, mTOR shRNA-3, Rictor shRNA-1 or Raptor shRNA-1 were allowed to migrate toward chemoattractant-containing micropipette (fMLP, 10 μM) on a fibrinogen-coated substrate. The two images in each row show the positions of individual cells (identified with a superimposed letter) after exposure to fMLP. Bar. 10 μm. Movies for control and Rictor-depleted cells are available as Movie S4 and Movie S5. (D) Apoptotic assay of control cells or mTOR and Rictor-depleted cells. Cells were infected with lenti-viral particles containing NT shRNA, mTOR shRNA-3 or Rictor shRNA-1 and differentiated for 5 days. Cells were prepared according to the manufacturer's instructions (FITC Annexin V Apoptosis Detection Kit I, BD Pharmingen) and analyzed by flow cytometry. (E) Flow cytometry analysis of surface expression of β2 integrin in control, mTOR- and Rictor-depleted cells after fMLP stimulation (100 nM, 10 min). The black line indicates background fluorescence from the isotype control IgG. (F) Analysis of intracellular [Ca^{2+}] in cells containing NT shRNA or Rictor shRNA in response to fMLP stimulation. Fluorescence was recorded using SpectraMax M2 spectrometer (excitation: 340-380 nm; emission: 510 nm). Results are plotted as emission ratio *vs.* time. Arrow indicates the time of addition of fMLP (100 nM). A representative experiment from eight separate experiments is shown.

Figure S2. (A) Western blotting of phosphorylated AKT (p-T308) in dHL-60 cells with or without Rictor depletion. Cells were stimulated with 100 nM fMLP for 1 minute or unstimulated. Total AKT was the loading control for each condition. The Rictor blot is also shown. (B) Western blotting of mSin1 in dHL-60 with or without the treatment of mSin1 shRNAs. α-tubulin was a loading control. (C) Cells containing NT shRNA, mSin1 shRNA-1 or mSin1 shRNA-2 were allowed to migrate toward chemoattractant-containing micropipette (fMLP, 10 μM) on a fibrinogen-coated substrate. The three images in each row show the positions of individual cells after exposure to fMLP for indicated times. Bar, 10 μm. (D) Top: Western blotting of AKT (p-S473) phosphorylation in cells with or without mSin1 depletion. Cells were stimulated with fMLP (100 nM) for 1 min. Total AKT was a loading control. Bottom: Quantification of relative levels of p-S473 AKT in cells with and without mSin1 depletion. Each bar represents the mean±SEM (n=4). Values are normalized to the level of p-S473 AKT (=100%) in cells treated with NT shRNA.

Figure S3. (A) dHL-60 cells were stimulated by fMLP (100 nM) for indicated times in suspension and lysed. AKT phosphorylation at serine 473 (p-S473) was assessed by western blotting. Total AKT was a loading control. (B) Quantification of relative p-S473 AKT levels after fMLP stimulation for indicated times. Values are normalized to the

level in cells 60 sec after fMLP stimulation (=100%) and are means±SEM (n=4). (C) Western blotting of p-S473 AKT and total AKT in cells with or without Rictor depletion. dHL-60 cells were stimulated with 100 nM of fMLP for 1 or 5 min in suspension and lysed. The blot for Rictor is also shown. (D) Quantification of relative p-S473 AKT levels in dHL-60 cells with or without Rictor depletion 1 min after fMLP stimulation. Each bar represents the mean±SEM (error bars, n=4). Values are normalized to the level of p-S473 AKT in cells without fMLP stimulation (*, $p<0.001$). (E) Western blotting of p-S473 AKT and p-T308 AKT in dHL-60 cells treated with AKTi-1/2 of indicated concentrations. Cells were pretreated with AKTi-1/2 for 30 min, stimulated with 100 nM fMLP for 1 minute and lysed. Total AKT was used as a loading control. (F) The number of polarized cells with or without AKTi-1/2 treatment (1 μM) in an fMLP gradient produced by the microfluidic device. 51 control cells and 43 AKTi-1/2 treated cells were analyzed. Values are normalized to the number (100%) of cells without AKTi-1/2 treatment. (G) Migration speeds of control and AKTi-1/2 treated dHL-60 cells (1 μM) in an fMLP gradient produced by the microfluidic device. 42 control cells and 39 AKTi-1/2 treated cells were analyzed.

Figure S4. (A) Quantification of S6K1 phosphorylation (p-T389) in dHL-60 cells with or without rapamycin or Torin1 treatment. The values are normalized to the level of S6K1 phosphorylation 5 min after stimulation and are means ± SEM (n = 4). (B) Quantification of AKT phosphorylation (p-S473) in dHL-60 cells with or without rapamycin or Torin1 treatment. The values are normalized to the level of AKT phosphorylation in control cells 1 min after fMLP stimulation and are means ± SEM (n = 4).

Figure S5. (A) Quantification of F-actin levels in suspended dHL-60 cells. Cells with or without mTOR depletion were stimulated with fMLP (100 nM) for various times in suspension and fixed for staining with fluorescently-labeled phalloidin. Fluorescence in stained cells was determined by flow cytometry. Values are mean ± SEM (n = 4). (B) Western blotting of Rictor in differentiated PLB-985 (dPLB-985) cells transfected with Rictor or non–targeting (NT) shRNAs. GAPDH was a loading control. PLB-985 cells were infected with lentiviruses containing the various shRNAs and were differentiated for 5 d in the presence of DMSO. (C-D) Quantification of F-actin levels in suspended dPLB-985 cells. Cells with or without Rictor depletion were stimulated with 100 nM (C) or 1 μM fMLP (D) for various times in suspension and fixed for staining with fluorescently-labeled phalloidin. Fluorescence in stained cells was determined by flow cytometry. Values are mean ± SEM (n = 4).

Figure S6. (A) The levels of Rac-GTP in suspended dHL-60 cells with or without mTOR depletion. Cells were stimulated with 100 nM of fMLP for 30 sec and lysed for the pull-down assay. Levels of total Rac were used to show cell lysate input. (B) Quantification of relative levels of Rac-GTP level with and without mTOR depletion. Each bar represents the mean±SEM (error bars). Values are normalized to the level of Rac-GTP (=100%) in cells without mTOR depletion (*, $p<0.001$). (C) The levels of Cdc42-GTP in dHL-60 cells with or without mTOR depletion. dHL-60 cells with or without mTOR depletion were stimulated with 100 nM of fMLP for 30 sec in suspension and lysed for the pull-down assay. Levels of total Cdc42 were used to show cell lysate input. (D) Quantification

of relative levels of Cdc42-GTP with and without mTOR depletion. Each bar represents the mean±SEM (n=4). Values are normalized to the level of Cdc42-GTP (=100%) in cells without Rictor depletion (*, $p<0.001$).

Figure S7. (A) Quantification of RhoA-GTP in suspended dHL-60 cells with or with Rictor depletion. Cells were stimulated with fMLP (100 nM) for times indicated and lysed. The level of RhoA-GTP was determined by the use of an absorbance-based RhoA G-LISA kit. The y axis represents the normalized level of RhoA-GTP. Each bar represents the mean ± SEM (error bars) (n=4). Student's t test was performed. The asterisk indicates that the level of Rctor-depleted cells differ statistically from the control cells (*, $p < 0.01$). (B) Quantification of RhoA-GTP in adherent dHL-60 cells with or with Rictor depletion. Cells were plated on fibrinogen-coated plates for 30 minutes. Cells were then stimulated by 100 nM fMLP for indicated times and lysed. The level of RhoA-GTP was determined as described above. Each bar represents the mean ± SEM (error bars) (n=4). (C) Western blotting of Rictor and RhoGDI2 in dHL-60 cells with or without Rictor depletion. Two separate Rictor shRNAs were used. α-tubulin was a loading control

Text for Supplementary Movies

Supplementary Movie S1 shows the responses of control dHL-60 cells (NT shRNA) to a gradient of fMLP generated by the microfluidic device. Two time points are shown in Figure 2C.

Supplementary Movie S2 shows the responses of dHL-60 cells with mTOR depletion (mTOR shRNA-3) to a gradient of fMLP generated by the microfluidic device. Two time points are shown in Figure 2C.

Supplementary Movie S3 shows the responses of dHL-60 cells with Rictor depletion (Rictor shRNA-1) to a gradient of fMLP generated by the microfluidic device. Two time points are shown in Figure 2C.

Supplementary Movie S4 shows the responses of dHL-60 cells to a gradient of fMLP generated by the micropipette. Two time points are shown in Figure S1C.

Supplementary Movie S5 shows the responses of dHL-60 cells with Rictor depletion (Rictor shRNA-1) to a gradient of fMLP generated by the micropipette. Two time points are shown in Figure S1C.

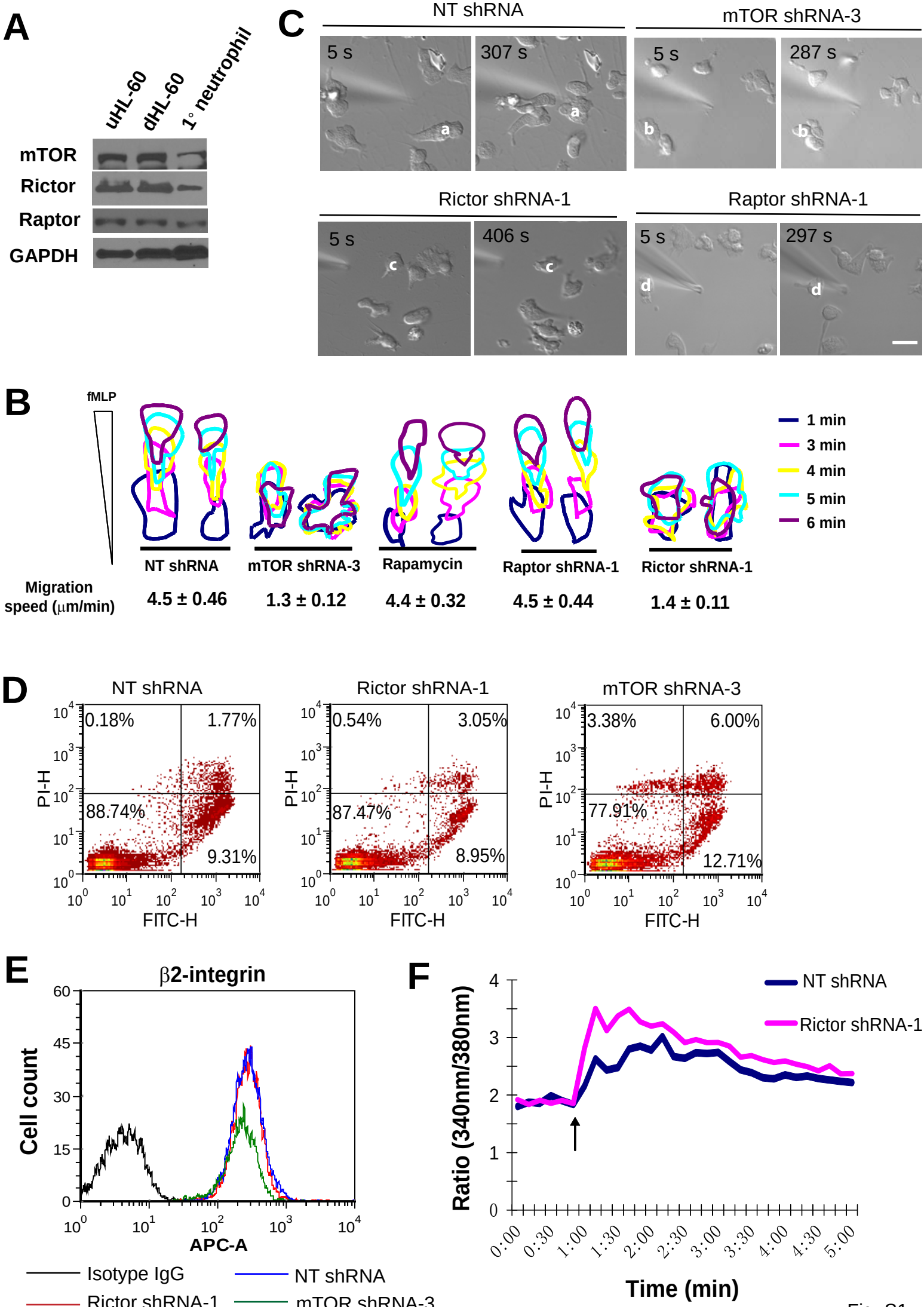
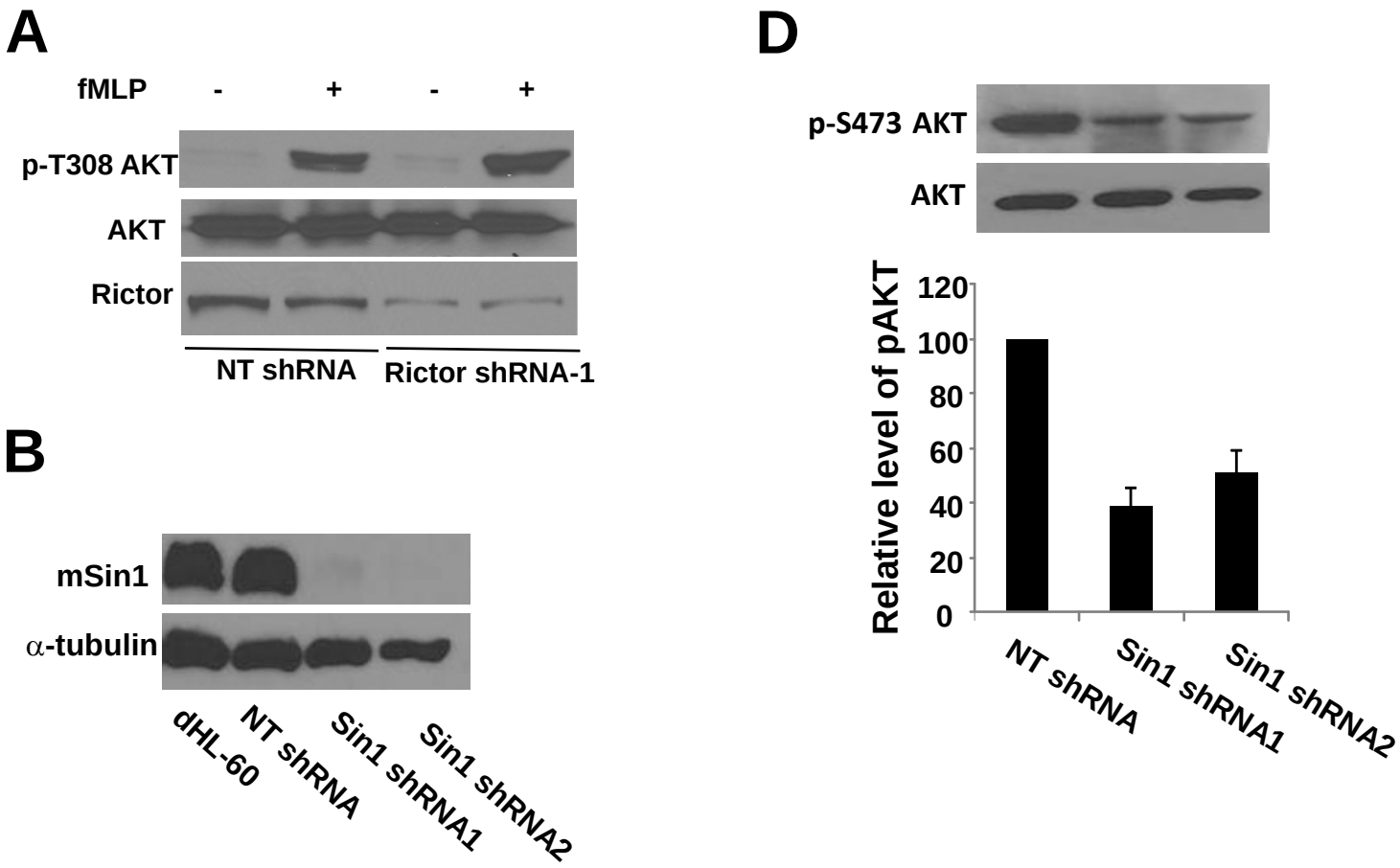

A
uHL-60
dHL-60
1° neutrophil
mTOR
Rictor
Raptor
GAPDH
C
NT shRNA
mTOR shRNA-3
5 s
307 s
5 s
287 s
Rictor shRNA-1
Raptor shRNA-1
5 s
406 s
5 s
297 s
B
fMLP
NT shRNA
mTOR shRNA-3
Rapamycin
Raptor shRNA-1
Rictor shRNA-1
1 min
3 min
4 min
5 min
6 min
Migration speed (μm/min)
4.5 ± 0.46
1.3 ± 0.12
4.4 ± 0.32
4.5 ± 0.44
1.4 ± 0.11
D
NT shRNA
Rictor shRNA-1
mTOR shRNA-3
0.18%
1.77%
88.74%
9.31%
0.54%
3.05%
87.47%
8.95%
3.38%
6.00%
77.91%
12.71%
PI-H
FITC-H
E
β2-integrin
Cell count
APC-A
Isotype IgG
NT shRNA
Rictor shRNA-1
mTOR shRNA-3
F
Ratio (340nm/380nm)
Time (min)
NT shRNA
Rictor shRNA-1

Fig. S1


A
fMLP - + - +
p-T308 AKT
AKT
Rictor
NT shRNA
Rictor shRNA-1
B
mSin1
α-tubulin
dHL-60
NT shRNA
Sin1 shRNA1
Sin1 shRNA2
D
p-S473 AKT
AKT
Relative level of pAKT
120
100
80
60
40
20
0
NT shRNA
Sin1 shRNA1
Sin1 shRNA2

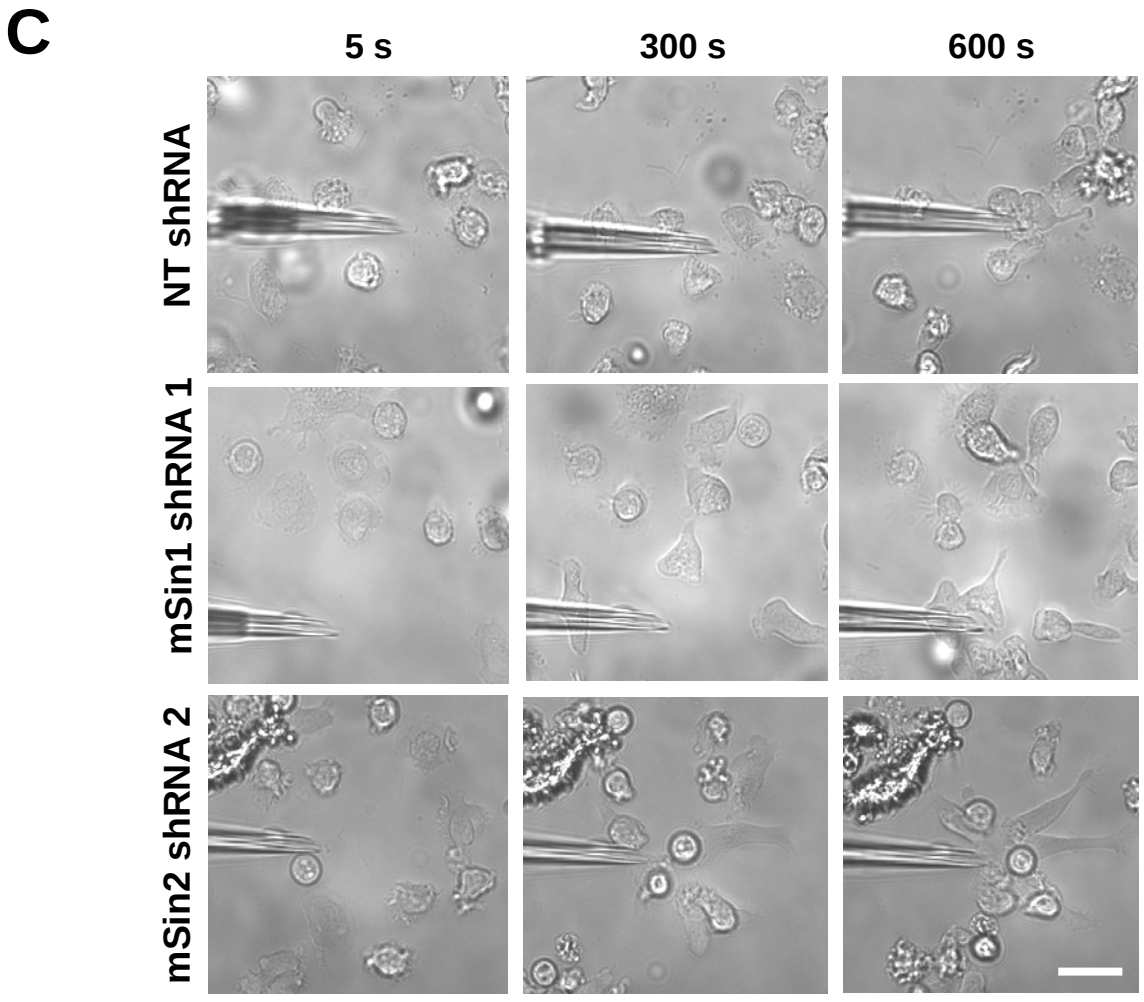

C
5 s
300 s
600 s
NT shRNA
mSin1 shRNA 1
mSin2 shRNA 2

Fig. S2

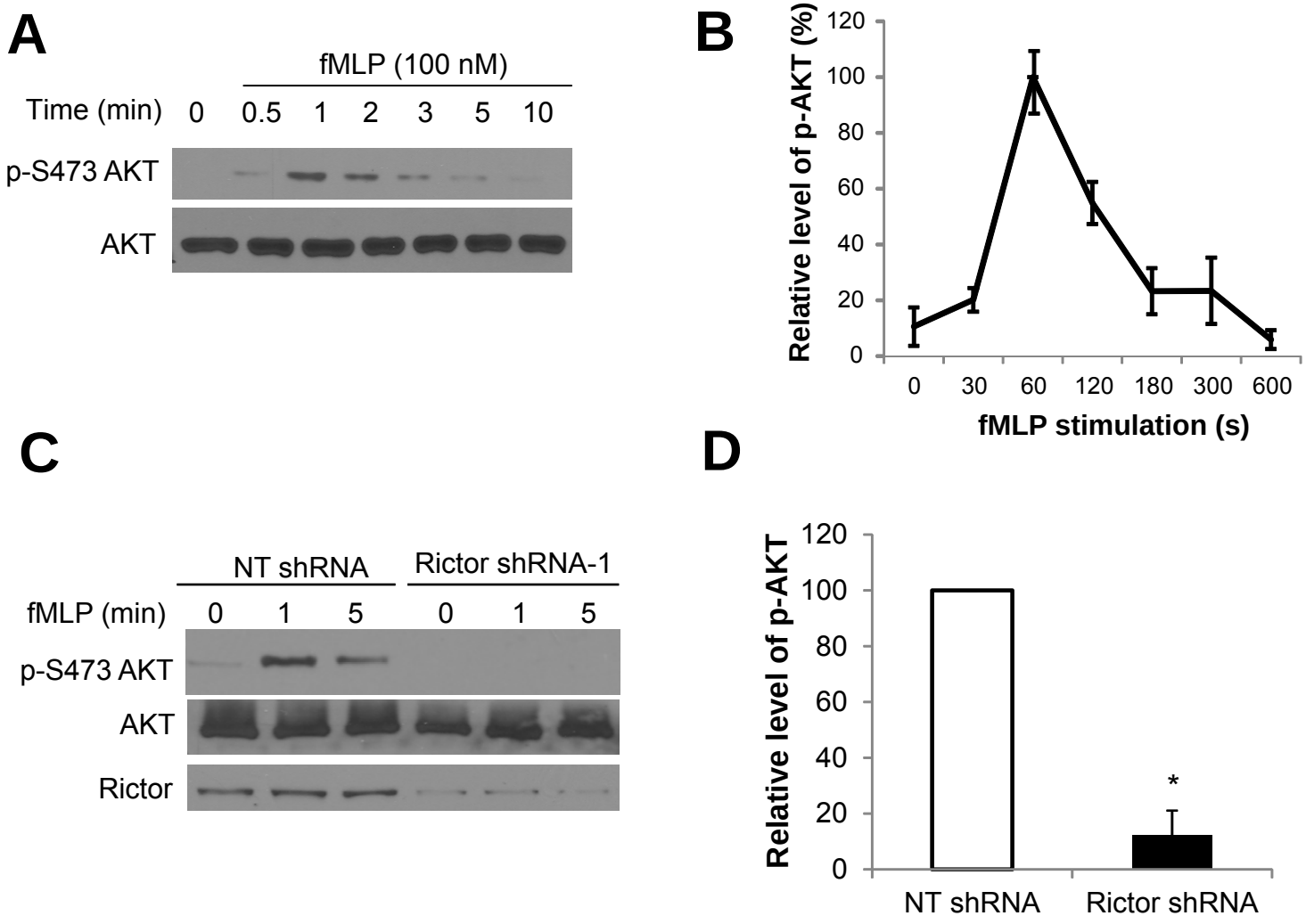

A
fMLP (100 nM)
Time (min) 0 0.5 1 2 3 5 10
p-S473 AKT
AKT
B
Relative level of p-AKT (%)
120
100
80
60
40
20
0
0 30 60 120 180 300 600
fMLP stimulation (s)
C
NT shRNA
Rictor shRNA-1
fMLP (min) 0 1 5 0 1 5
p-S473 AKT
AKT
Rictor
D
Relative level of p-AKT
120
100
80
60
40
20
0
*
NT shRNA
Rictor shRNA
E
unstim.
fMLP (100 nM)
AKTi-1/2 (μM) 0 0.1 0.5 1 2 5 10
p-S473 AKT
p-T308 AKT
AKT
F
Polarized cells (%)
120
100
80
60
40
20
0
DMSO
AKTi-1/2
G
Migration speed (μm/min)
6
5
4
3
2
1
0
DMSO
AKTi-1/2

Fig. S3

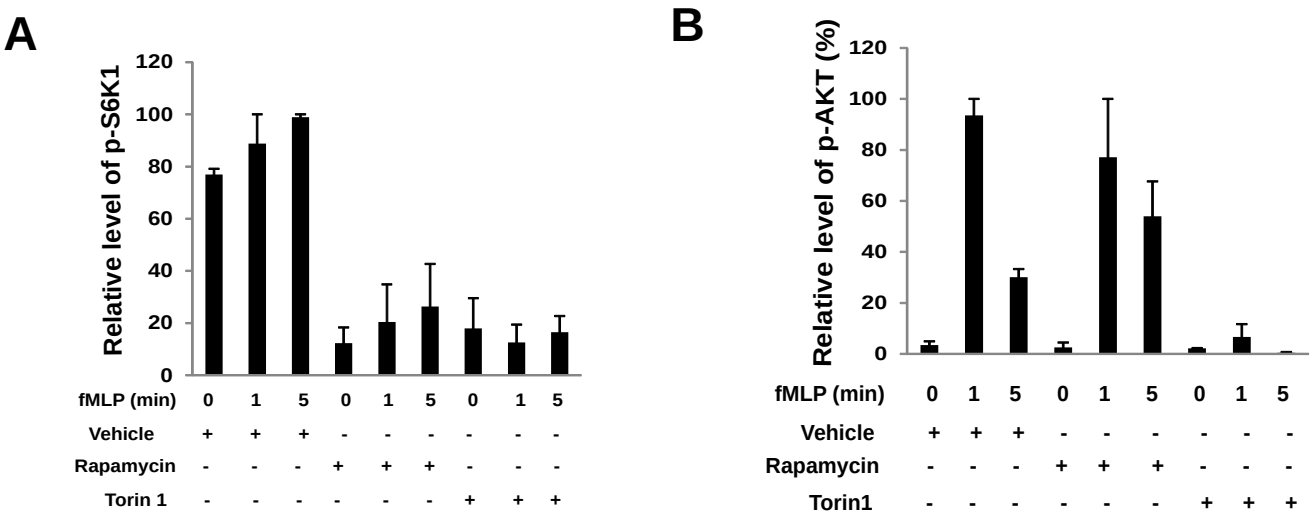

A
Relative level of p-S6K1
120
100
80
60
40
20
0
fMLP (min) 0 1 5 0 1 5 0 1 5
Vehicle + + + - - - - - -
Rapamycin - - - + + + - - -
Torin 1 - - - - - - + + +
B
Relative level of p-AKT (%)
120
100
80
60
40
20
0
fMLP (min) 0 1 5 0 1 5 0 1 5
Vehicle + + + - - - - - -
Rapamycin - - - + + + - - -
Torin1 - - - - - - + + +

Fig. S4

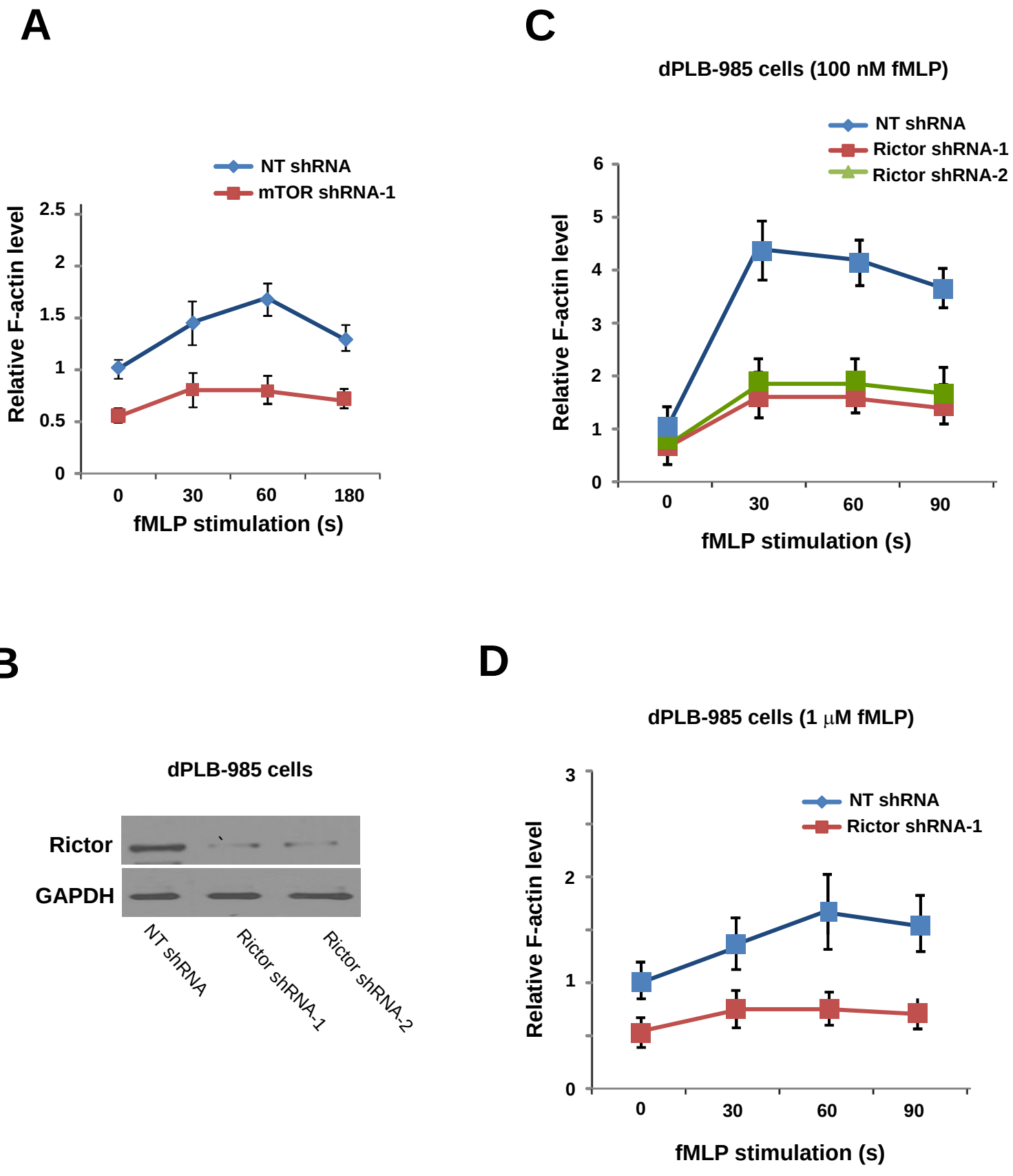

A
NT shRNA
mTOR shRNA-1
Relative F-actin level
2.5
2
1.5
1
0.5
0
0
30
60
180
fMLP stimulation (s)
C
dPLB-985 cells (100 nM fMLP)
NT shRNA
Rictor shRNA-1
Rictor shRNA-2
Relative F-actin level
6
5
4
3
2
1
0
0
30
60
90
fMLP stimulation (s)
B
dPLB-985 cells
Rictor
GAPDH
NT shRNA
Rictor shRNA-1
Rictor shRNA-2
D
dPLB-985 cells (1 μM fMLP)
NT shRNA
Rictor shRNA-1
Relative F-actin level
3
2
1
0
0
30
60
90
fMLP stimulation (s)

Fig. S5

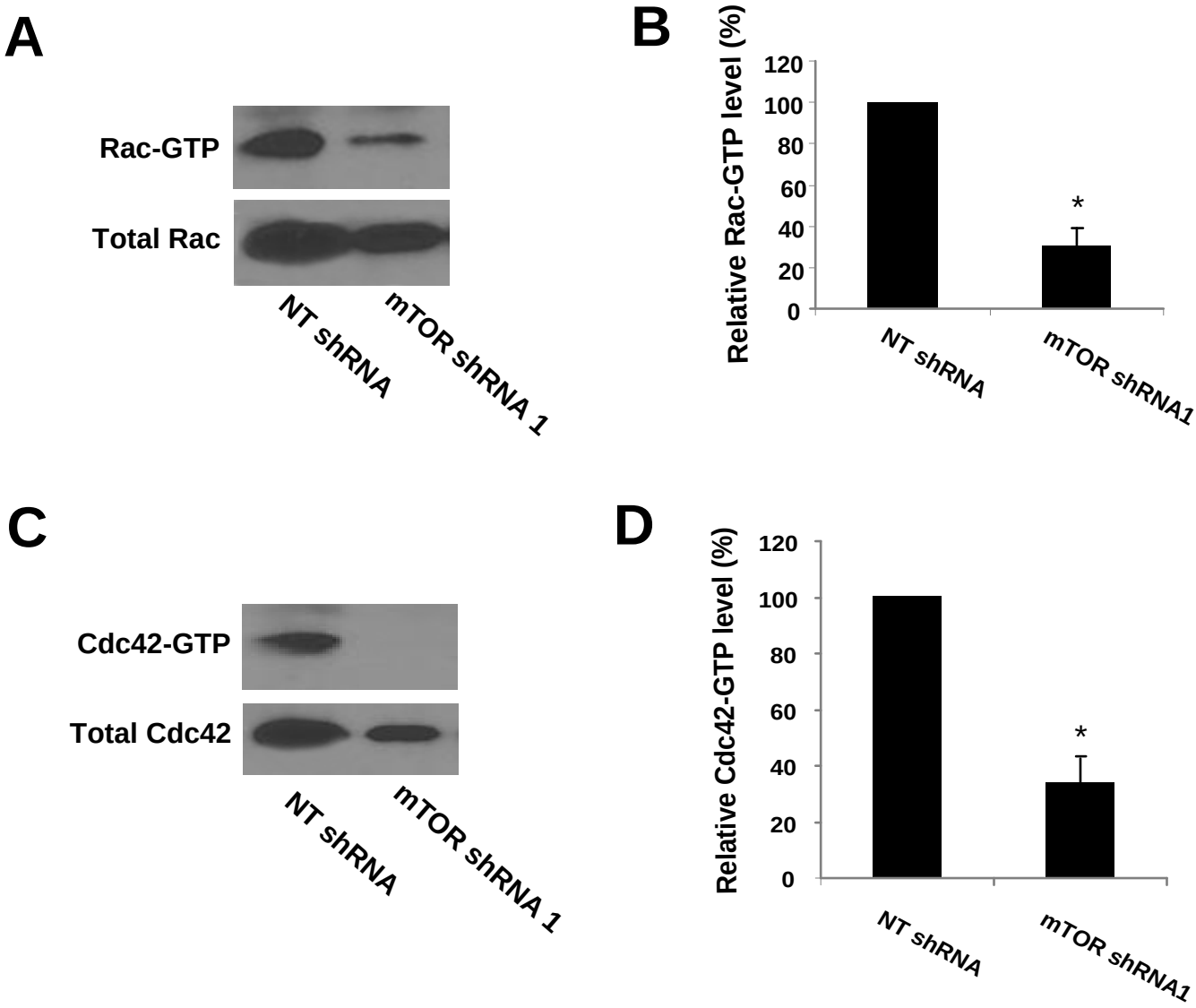

A
Rac-GTP
Total Rac
NT shRNA
mTOR shRNA 1
B
Relative Rac-GTP level (%)
120
100
80
60
40
20
0
*
NT shRNA
mTOR shRNA1
C
Cdc42-GTP
Total Cdc42
NT shRNA
mTOR shRNA 1
D
Relative Cdc42-GTP level (%)
120
100
80
60
40
20
0
*
NT shRNA
mTOR shRNA1

Fig S6

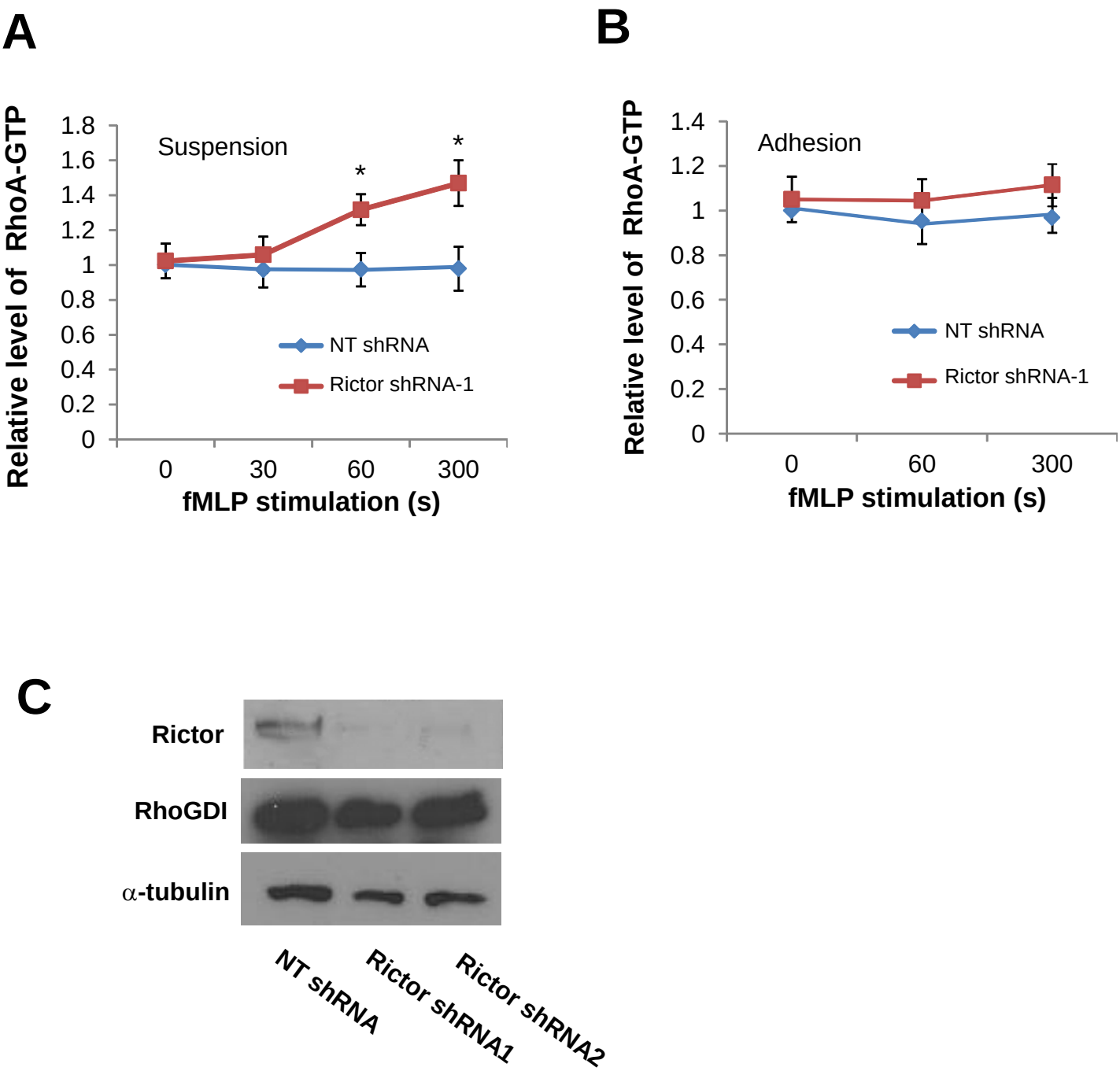

A
Relative level of RhoA-GTP
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Suspension
*
*
NT shRNA
Rictor shRNA-1
0
30
60
300
fMLP stimulation (s)
B
Relative level of RhoA-GTP
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Adhesion
NT shRNA
Rictor shRNA-1
0
60
300
fMLP stimulation (s)
C
Rictor
RhoGDI
α-tubulin
NT shRNA
Rictor shRNA1
Rictor shRNA2

Fig. S7