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mTORC1 Mediates Biphasic Mechano-Response to Orchestrate Adhesion-Dependent Cell Growth and Anoikis Resistance

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Supplementary Materials for

- **mTORC1 mediates biphasic mechano-response to orchestrate adhesion-dependent cell growth and anoikis resistance**

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This PDF file includes:

Figure S1

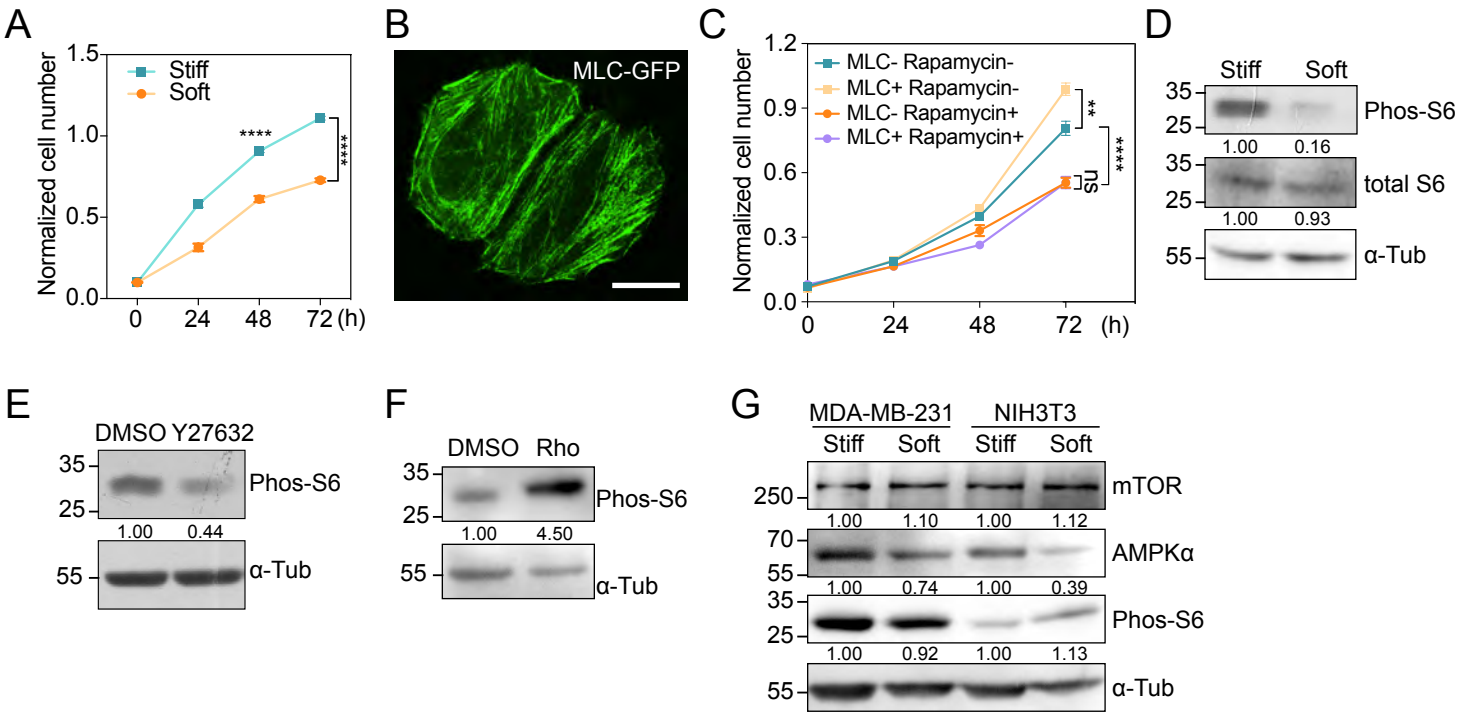
A
Normalized cell number
1.5
1.0
0.5
0.0
Stiff
Soft
****
****
0
24
48
72 (h)
B
MLC-GFP
C
Normalized cell number
1.2
0.9
0.6
0.3
0.0
MLC- Rapamycin-
MLC+ Rapamycin-
MLC- Rapamycin+
MLC+ Rapamycin+
**
ns
****
0
24
48
72
(h)
D
Stiff
Soft
35
25
Phos-S6
1.00
0.16
35
25
total S6
1.00
0.93
55
α-Tub
E
DMSO Y27632
35
25
Phos-S6
1.00
0.44
55
α-Tub
F
DMSO
Rho
35
25
Phos-S6
1.00
4.50
55
α-Tub
G
MDA-MB-231
NIH3T3
Stiff
Soft
Stiff
Soft
250
mTOR
1.00
1.10
1.00
1.12
70
55
AMPKα
1.00
0.74
1.00
0.39
35
25
Phos-S6
1.00
0.92
1.00
1.13
55
α-Tub

Fig. S1. mTORC1 signaling responds to different mechanical conditions

(A) Cell growth curve determined by CCK-8 assay at 0, 24, 48 and 72 h. Cell number of MDA-MB-231 was measured on stiff or soft substrates ($N_{stiff} = N_{soft} = 3$ independent samples, error bar: mean with SEM, ****$p < 0.0001$ by unpaired Student's *t* test).

(B) Representative images of overexpressed MLC-GFP in MDA-MB-231 cells. Scale bar, 20 μm.

(C) Cell growth curve determined by CCK-8 assay at 0, 24, 48 and 72 h under blebbistatin treatment. Cell number of MDA-MB-231 was measured in MLC- Rapamycin-, MLC+ Rapamycin-, MLC- Rapamycin+, MLC+ Rapamycin+ groups (error bar: mean with SEM, $ns_{MLC- Rapamycin+ vs MLC- Rapamycin+} > 0.9999$, ****$p < 0.0001$, **$p = 0.0036$ by one-way *ANOVA* for multiple comparison).

(D) Western blot showing Phos-S6 and total S6 levels in HRPE cells plated on stiff or soft substrates. α-tubulin is used as loading control.

(E) Western blot showing the Phos-S6 levels in stiff cultured cells treated with DMSO or ROCK inhibitor Y27632. α-tubulin is used as loading control.

(F) Western blot showing the Phos-S6 levels in soft cultured cells treated with DMSO or Rho activator II (Rho). α-tubulin is used as loading control.

(G) Western blot showing the mTOR, AMPKα and Phos-S6 levels in MDA-MB-231 and NIH3T3 cells cultured on stiff or soft substrates, with serum concentrations reducing to 1% during experiments. α-tubulin is used as loading control.

Figure S2

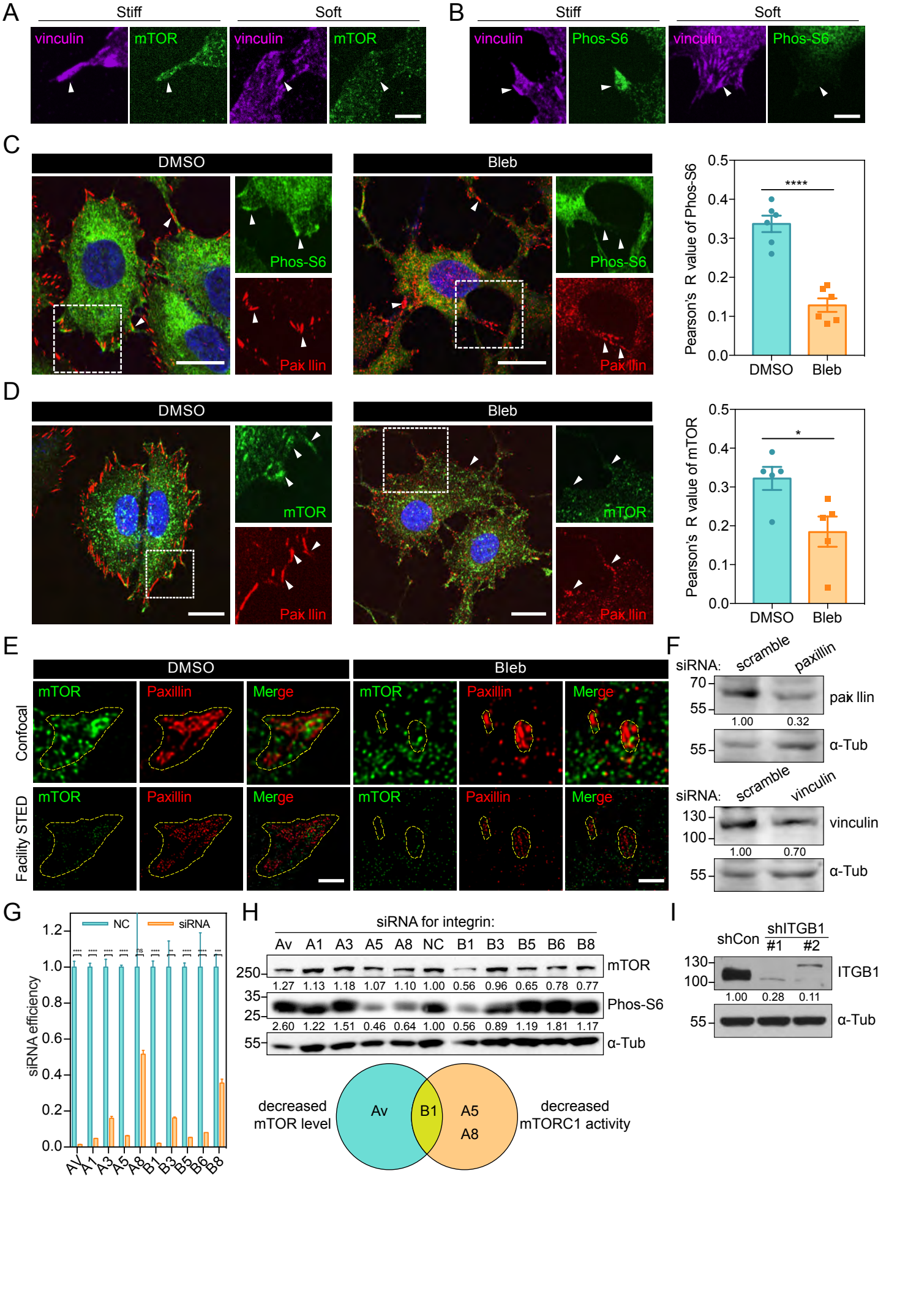
A
Stiff
Soft
vinculin
mTOR
B
Phos-S6
C
DMSO
Bleb
Pak1lin
Pearson's R value of Phos-S6
****
D
Pearson's R value of mTOR
*
E
Confocal
Facility STED
Paxillin
Merge
F
siRNA:
scramble
paxillin
pak1lin
α-Tub
vinculin
G
NC
siRNA
siRNA efficiency
H
siRNA for integrin:
Av A1 A3 A5 A8 NC B1 B3 B5 B6 B8
mTOR
Phos-S6
decreased mTOR level
decreased mTORC1 activity
I
shCon
shITGB1 #1 #2
ITGB1

Fig. S2. Cell contractility induced mTORC1 activation is regulated by integrin-FAs

(A) Representative immunofluorescence images stained with mTOR (green) and vinculin (magenta) antibodies in MDA-MB-231 cells plated on stiff or soft substrates. Scale bar, 5 μm.

(B) Representative immunofluorescence images stained with Phos-S6 (green) and vinculin (magenta) antibodies in MDA-MB-231 cells plated on stiff or soft substrates. Scale bar, 5 μm.

(C) Left: representative immunofluorescence images stained with Phos-S6 (green) and paxillin (red) antibodies in MDA-MB-231 cells treated with DMSO or Bleb. Co-localization of Phos-S6 and paxillin is shown in enlarged boxes by white arrow heads. Scale bar, 20 μm. Right: Pearson's R value represents the overlap coefficient of Phos-S6 and paxillin (N_{DMSO} = 6, N_{Bleb} = 6, error bar: mean with SEM, ****$p < 0.0001$ by unpaired Student's *t* test).

(D) Left: representative immunofluorescence images stained with mTOR (green) and paxillin (red) antibodies in MDA-MB-231 cells treated with DMSO or Bleb. Co-localization of mTOR and paxillin is shown in enlarged boxes by white arrow heads. Scale bar, 20 μm. Right: Pearson's R value represents the overlap coefficient of mTOR and paxillin (N_{DMSO} = 5, N_{Bleb} = 5, error bar: mean with SEM, *$p = 0.0247$ by unpaired Student's *t* test).

(E) Representative images of confocal (top) and STED (bottom) showing the co-localization of mTOR (green) and paxillin (red) in MDA-MB-231 cells treated with DMSO or Bleb. Scale bar, 5 μm.

(F) Western blot showing the successful silencing of paxillin (top) and vinculin (bottom) in MDA-MB-231 cells using siRNA. α-tubulin is used as loading control.

(G) The knocked down efficiency of siRNA for integrin AV/A1/A3/A5/A8/B1/B3/B5/B6/B8 quantified by qPCR assay (error bar: mean with SEM, ****$p < 0.0001$, ***$p = 0.0010$, **p = 0.0046, ns = 0.4953 by unpaired Student's *t* test).

(H) Top: western blot showing the mTOR and Phos-S6 levels in siNC and integrin AV/A1/A3/A5/A8/B1/B3/B5/B6/B8 gene-silenced MDA-MB-231 cells. Bottom: Venn diagram showing the types of integrin that result in decreased mTOR protein levels and mTORC1 activity.

(I) Western blot showing the successful silencing of ITGB1 in MDA-MB-231 cells. α-tubulin is used as loading control.

Figure S3

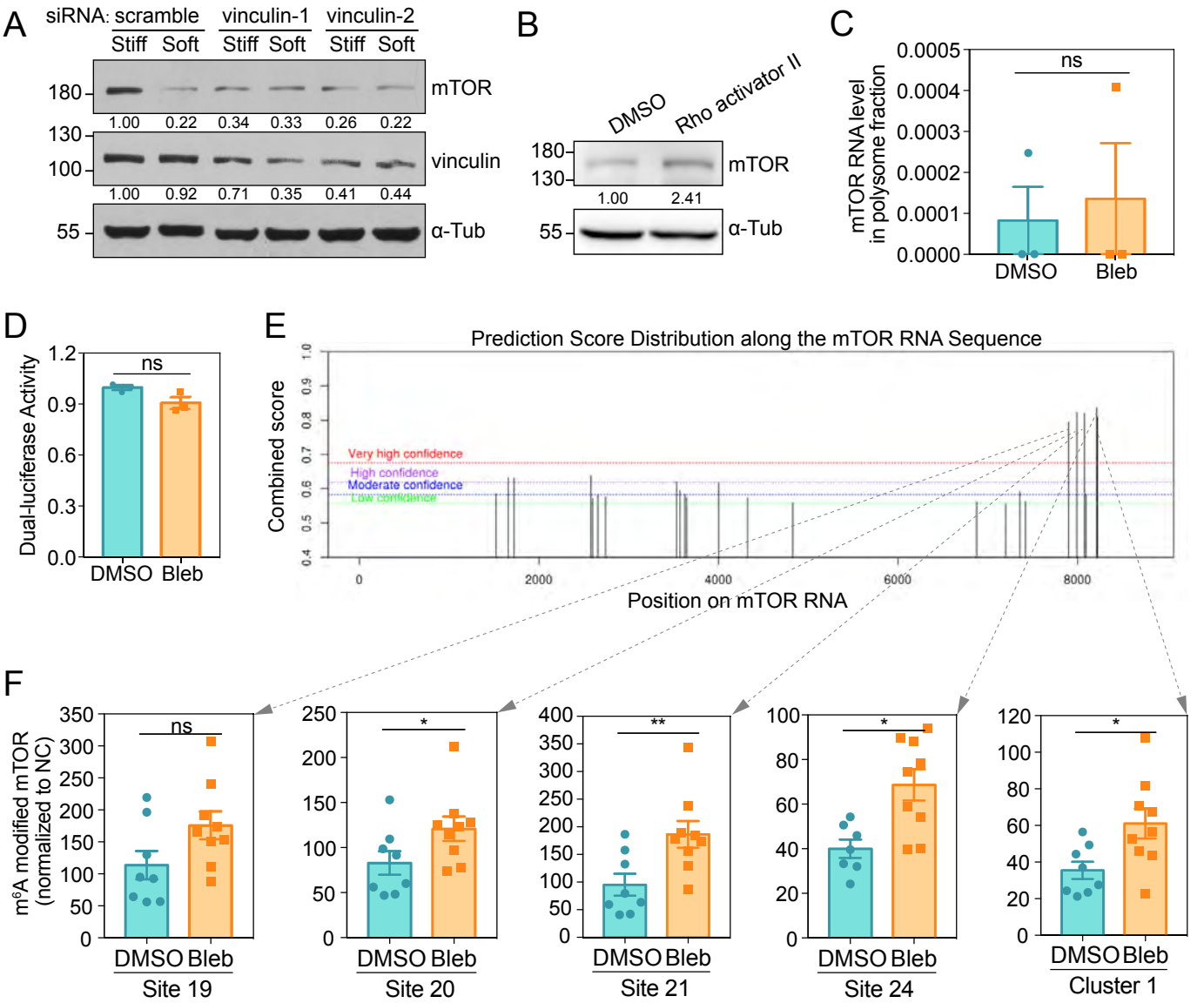
A
siRNA: scramble vinculin-1 vinculin-2
Stiff Soft Stiff Soft Stiff Soft
180
130
100
55
mTOR
vinculin
α-Tub
1.00 0.22 0.34 0.33 0.26 0.22
1.00 0.92 0.71 0.35 0.41 0.44
B
DMSO
Rho activator II
180
130
55
mTOR
α-Tub
1.00 2.41
C
mTOR RNA level in polysome fraction
0.0005
0.0004
0.0003
0.0002
0.0001
0.0000
ns
DMSO Bleb
D
Dual-luciferase Activity
1.2
0.9
0.6
0.3
0.0
ns
DMSO Bleb
E
Prediction Score Distribution along the mTOR RNA Sequence
Combined score
Very high confidence
High confidence
Moderate confidence
Low confidence
0
2000
4000
6000
8000
Position on mTOR RNA
F
m6A modified mTOR (normalized to NC)
ns
DMSO Bleb
Site 19
*
DMSO Bleb
Site 20
**
DMSO Bleb
Site 21
*
DMSO Bleb
Site 24
*
DMSO Bleb
Cluster 1

Fig. S3. m^{6}A modification of mTOR increases with reduced actomyosin contractility

(A) Western blot showing the mTOR and vinculin levels in scramble, si-vinculin-1, si-vinculin-2 MDA-MB-231 cells cultured on stiff or soft substrates. α-tubulin is used as loading control.

(B) Western blot showing the mTOR levels in cells treated with DMSO or Rho activator II. α-tubulin is used as loading control.

(C) The abundance of mTOR transcripts was measured by RT-qPCR in polysome fraction from the polysome profiling (error bar: mean with SEM, ns = 0.7542 by unpaired Student's *t* test).

(D) Dual-luciferase reporter assay showing transcriptional activity of mTOR mRNA in MDA-MB-231 cells treated with DMSO or Bleb ($N_{DMSO} = N_{Bleb} = 3$ independent experiments, error bar: mean with SEM, ns =0.0700 by unpaired Student's *t* test).

(E) Prediction by SRAMP showing the potential m^{6}A modification positions on mTOR mRNA sequence and its combined confidence score.

(F) The ratios of m^{6}A modified mTOR on site 19, site 20, site 21, site 24 and cluster 1 in DMSO and Bleb treatment groups ($N_{DMSO} = N_{Bleb} = 3$ independent experiments, error bar: mean with SEM, ns = 0.0564, $*p_{Site20} = 0.0267$, $**p_{Site\ 21} = 0.0038$, $*p_{Site24} = 0.0332$, $*p_{Cluster1} = 0.0176$ by paired Student's *t* test).

Figure S4

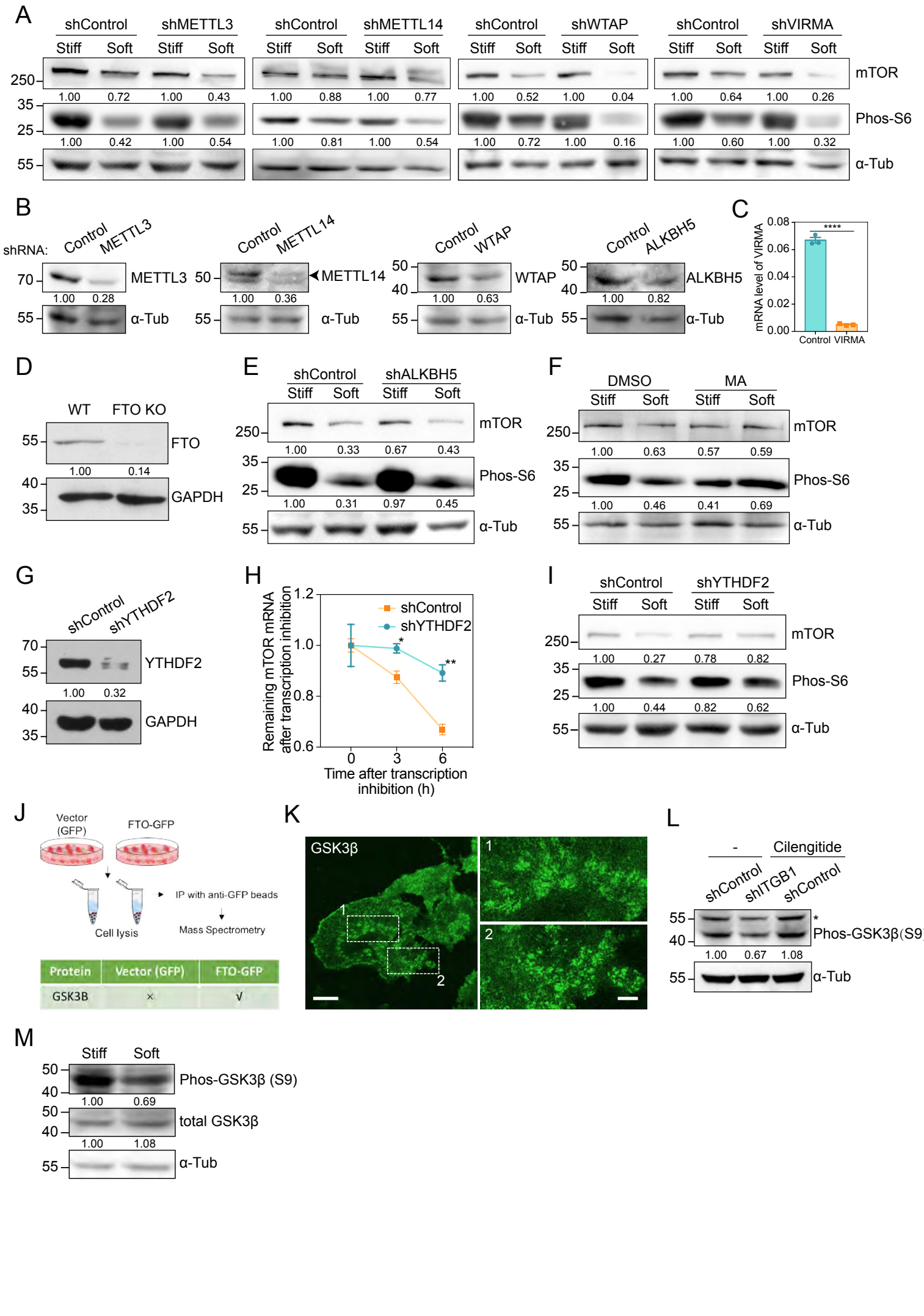
A
shControl
shMETTL3
shControl
shMETTL14
shControl
shWTAP
shControl
shVIRMA
Stiff
Soft
mTOR
Phos-S6
α-Tub
B
shRNA:
Control
METTL3
METTL14
WTAP
ALKBH5
C
mRNA level of VIRMA
Control
VIRMA
D
WT
FTO KO
FTO
GAPDH
E
F
DMSO
MA
G
shYTHDF2
YTHDF2
H
Remaining mTOR mRNA after transcription inhibition
Time after transcription inhibition (h)
I
J
Vector (GFP)
FTO-GFP
IP with anti-GFP beads
Cell lysis
Mass Spectrometry
Protein
GSK3B
K
GSK3β
L
Cilengitide
Phos-GSK3β(S9)
M
Phos-GSK3β (S9)
total GSK3β

Fig. S4. The stability of mTOR transcripts is regulated by YTHDF2

(A) Western blot showing the mTOR and Phos-S6 levels in shControl, shMETTL3, shMETTL14, shWTAP and shVIRMA cells cultured on stiff or soft substrates. α-tubulin is used as loading control.

(B) Western blot showing the levels of METTL3/METTL14/WTAP/ALKH5 in shControl and corresponding shRNA-targeted MDA-MB-231 cells. α-tubulin is used as loading control.

(C) The knocked down efficiency of VIRMA quantified by qPCR assay (error bar: mean with SEM, ****$p < 0.0001$ by unpaired Student's *t* test).

(D) Western blot showing the levels of FTO in WT and FTO KO MDA-MB-231 cells. GAPDH is used as loading control.

(E) Western blot showing the levels of mTOR and Phos-S6 in shControl and shALKBH5 cells cultured on stiff or soft substrates. α-tubulin is used as loading control.

(F) Western blot showing the levels of mTOR and Phos-S6 in MDA-MB-231 cells supplemented with DMSO or meclofenamic acid (MA) on stiff or soft substrates. α-tubulin is used as loading control.

(G) Western blot showing the successful silencing of YTHDF2 in MDA-MB-231 cells. GAPDH is used as loading control.

(H) qPCR assay showing the remaining mTOR RNA level in shControl and shYTHDF2 cells after transcription inhibition using Actinomycin D on stiff or soft substrates ($N_{soft} = N_{stiff} = 3$ independent experiments, error bar: mean with SEM, *$p = 0.0206$, **$p = 0.0044$ by unpaired Student's *t* test).

(I) Western blot showing the mTOR and Phos-S6 levels in shControl and shYTHDF2 cells on stiff or soft substrates. α-tubulin is used as loading control.

(J) Schematic diagram of IP-MS detecting the interaction between GSK3β and FTO.

(K) Representative images of overexpressed GSK3β-AcGFP in MDA-MB-231 cells indicating its localization at the ventral side of cells. The dashed boxes are zoomed in on the right. Scale bar, 20 μm and 5 μm.

(L) Western blot showing the Phos-GSK3β (S9) levels in WT and shIGTB1 cells supplemented with Cilengitide. The asterisk indicates a nonspecific band. α-tubulin is used as loading control.

(M) Western blot showing phos-GSK3β and total GSK3β levels in MDA-MB-231 cells plated on stiff or soft substrates. α-tubulin is used as loading control.

Figure S5

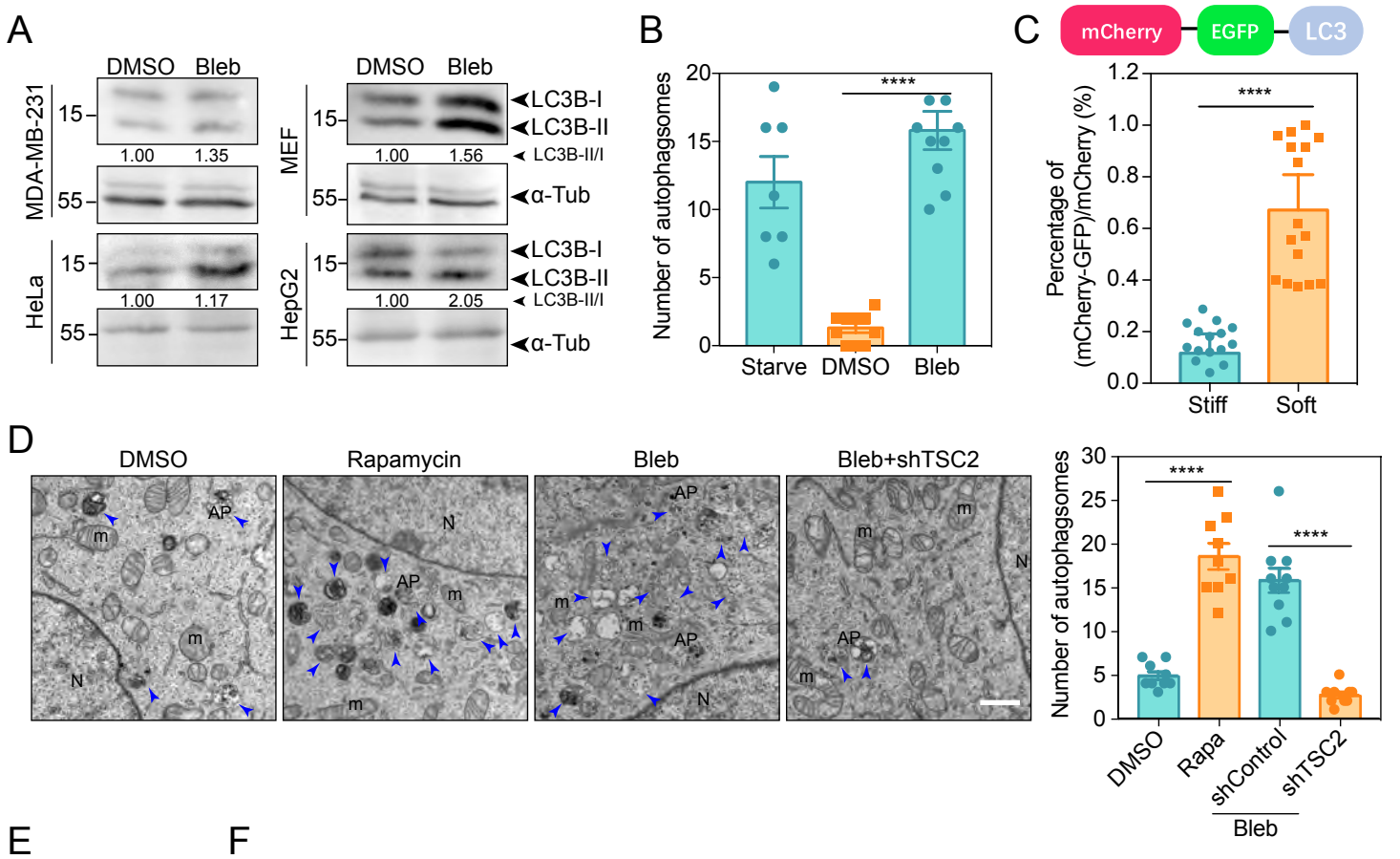
A
DMSO Bleb
MDA-MB-231
15
55
1.00 1.35
MEF
1.00 1.56
LC3B-I
LC3B-II
LC3B-II/I
α-Tub
HeLa
1.00 1.17
HepG2
1.00 2.05
B
Number of autophagsomes
****
Starve DMSO Bleb
C
mCherry
EGFP
LC3
Percentage of (mCherry-GFP)/mCherry (%)
Stiff Soft
D
DMSO
Rapamycin
Bleb
Bleb+shTSC2
AP
m
N
Number of autophagsomes
DMSO Rapa shControl shTSC2
Bleb
E
mRNA level of TSC2
shRNA: Control TSC2
F
LC3B
mTOR
G
Normalized mTOR intensity
adherent suspended
H
Normalized LC3B intensity
***
adherent suspended

Fig. S5. ECM detachment hampers mTORC1 pathway and elevates autophagy in animal models

(A) Western blot showing the autophagy maker LC3B levels in MDA-MB-231, HeLa, MEF and HepG2 cells with or without Bleb treatment. α-tubulin is used as loading control.

(B) Quantification of autophagosomes number in TEM images of MDA-MB-231 cells under starve condition or treated with DMSO or Bleb (N_{starve} = 7, N_{DMSO} = 10, N_{Bleb} = 9, error bar: mean with SEM, ****p < 0.0001 by unpaired Student's t test).

(C) Top: schematic diagram of fluorescent tandem mCherry-EGFP-LC3 probe. Bottom: quantification of intensity of (mCherry–GFP) / mCherry on stiff and soft substrates (N_{stiff} = 15, N_{soft} = 16, error bar: mean with SEM, ****p < 0.0001 by unpaired Student's t test).

(D) Left: representative TEM images of MDA-MB-231 cells or shTSC2 cells treated with DMSO, Rapamycin, Bleb by chemical-fixation. The blue arrow heads indicate autophagosomes. The letter "N" stands for the nucleus and "m" stands for mitochondria. Scale bar, 1 μm. Right: quantification of autophagosomes number in images on the left (N_{DMSO} = $N_{Rapamycin}$ = N_{Bleb} = $N_{Bleb+shTSC2}$ = 9, error bar: mean with SEM, ****p < 0.0001 by unpaired Student's t test).

(E) The knocked down efficiency of TSC2 quantified by qPCR assay (error bar: mean with SEM, ****p < 0.0001 by unpaired Student's t test).

(F) Immunohistochemistry images showing the detection of mTOR and LC3B in lactating mammary glands sections from BALB/c mice. The yellow dashed boxes are zoomed in on the right. The blue arrow heads indicate ECM-detached cells. Scale bar, 100 μm.

(G) Quantification of mTOR fluorescence intensity in flank (adherent) and cavity (suspended) tumor cells in the nude mouse model of malignant ascites ($N_{adherent}$ = 32, $N_{suspended}$ = 43 cells, error bar: mean with SEM, ****p < 0.0001 by unpaired Student's t test).

(H) Quantification of LC3B fluorescence intensity in flank (adherent) and cavity (suspended) tumor cells in the nude mouse model of malignant ascites ($N_{adherent}$ = 43, $N_{suspended}$ = 46 cells, error bar: mean with SEM, ***p = 0.0008 by unpaired Student's t test).