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Supplemental information

An mTORC1-GRASP55 signaling axis controls unconventional secretion to reshape the extracellular proteome upon stress

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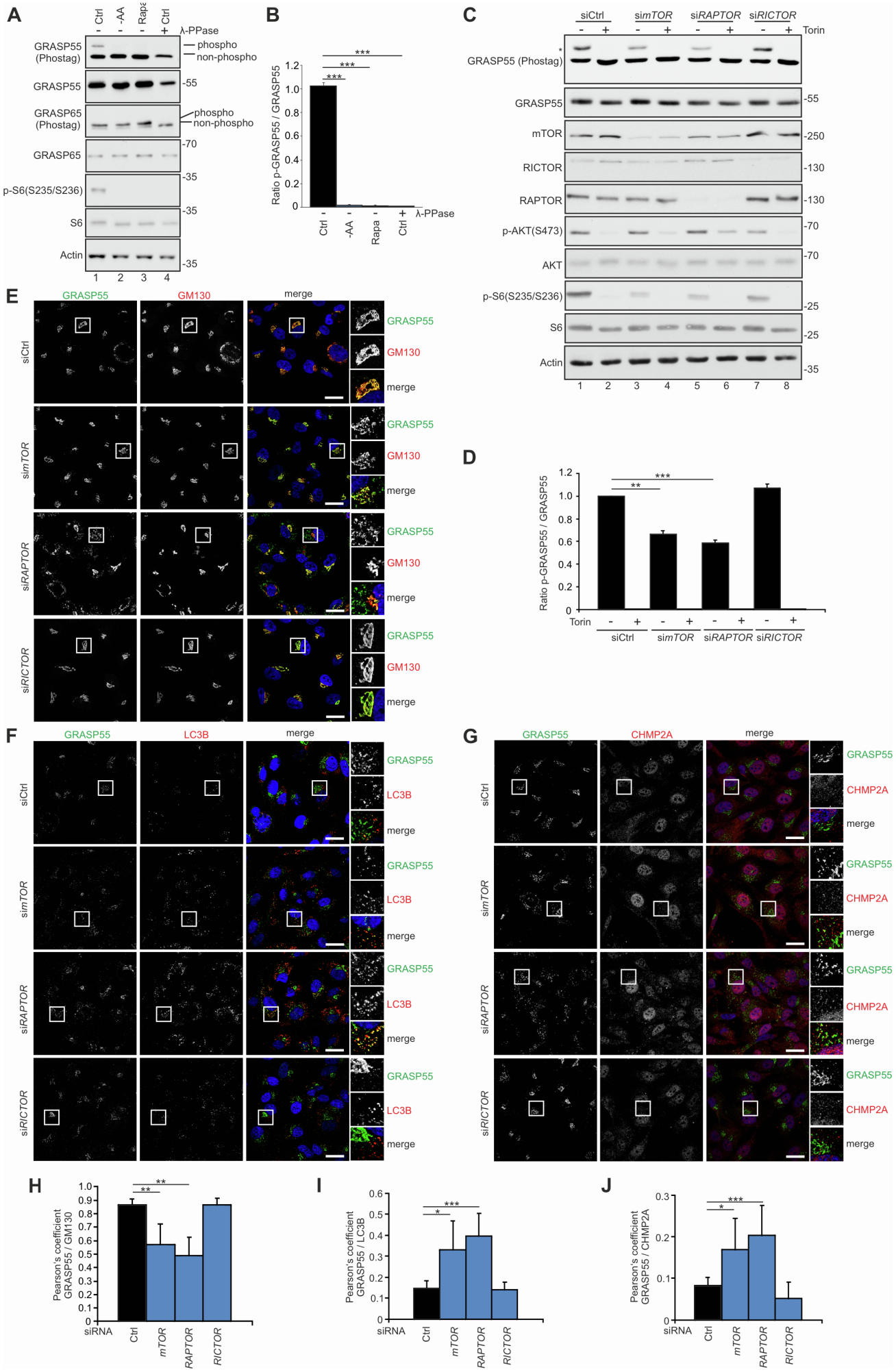
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Ctrl
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Rapa
Ctrl
λ-PPase
GRASP55 (Phostag)
phospho
non-phospho
GRASP55
GRASP65 (Phostag)
GRASP65
p-S6(S235/S236)
S6
Actin
-55
-70
-35
1 2 3 4
B
Ratio p-GRASP55 / GRASP55
C
siCtrl
simTOR
siRAPTOR
siRICTOR
Torin
GRASP55 (Phostag)
GRASP55
mTOR
RICTOR
RAPTOR
p-AKT(S473)
AKT
p-S6(S235/S236)
S6
Actin
-250
-130
-25
1 2 3 4 5 6 7 8
D
Ratio p-GRASP55 / GRASP55
E
GRASP55
GM130
merge
F
LC3B
G
CHMP2A
H
Pearson's coefficient GRASP55 / GM130
I
Pearson's coefficient GRASP55 / LC3B
J
Pearson's coefficient GRASP55 / CHMP2A
siRNA
Ctrl
mTOR
RAPTOR
RICTOR

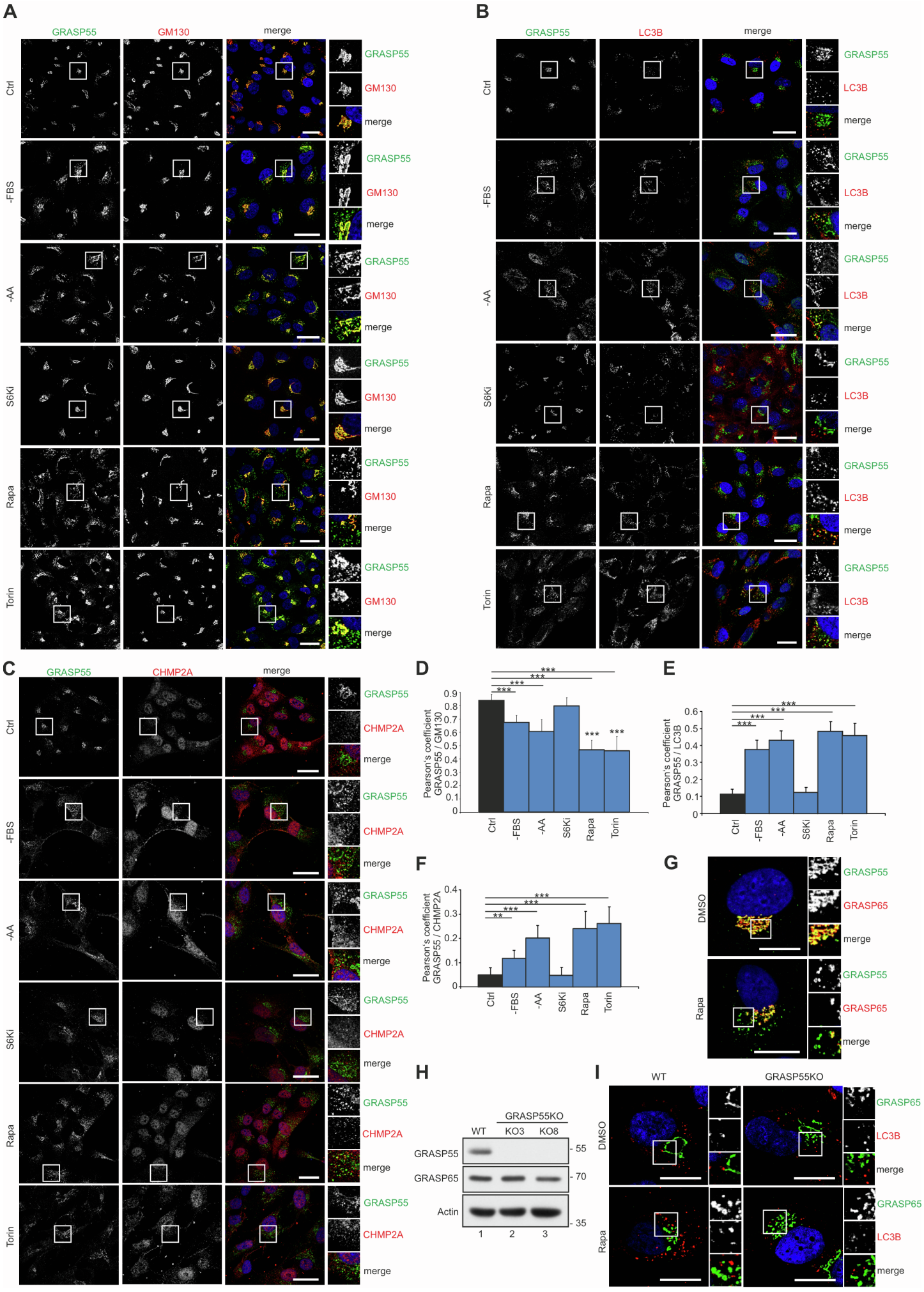
Figure S1. mTORC1, but not mTORC2, regulates GRASP55 phosphorylation and subcellular localization. Related to Figure 1.

(A-B) Migration of GRASP55 in Phos-tag gels reveals mTORC1-activity-dependent phosphorylation. Human diploid WI-26 fibroblasts treated with DMSO (Ctrl), starved with AA-free medium (-AA), or treated with Rapamycin (Rapa). GRASP55 and GRASP65 phosphorylation analyzed using Phos-tag gels and immunoblotting. Lambda-phosphatase treatment of DMSO lysates (compare lanes 1 and 4) confirms that the upper GRASP55 or GRASP65 bands correspond to phosphorylated GRASP55/GRASP65, whereas the lower bands represent non-phosphorylated forms. mTORC1 activity and the efficiency of the λ-phosphatase treatment assayed by S6 phosphorylation. Quantification of the p-GRASP55/GRASP55 ratio in (B).

(C-D) Knock-down of mTORC1 components compromises GRASP55 dephosphorylation. *mTOR*, *RAPTOR* or *RICTOR* genes knocked down in WI-26 cells using specific siRNAs. A scrambled, non-targeting siRNA was used as a negative control. Cells treated with Torin and mTORC1 and mTORC2 activity, as well as total protein levels, assayed by immunoblotting as indicated. GRASP55 phosphorylation analyzed using Phos-tag gels. Phosphorylated GRASP55 indicated by asterisk. Quantification of the p-GRASP55/GRASP55 ratio in (D).

(E-J) As in (C), but WI-26 cells analyzed for localization of endogenous GRASP55 by IF/confocal microscopy. *mTOR* or *RAPTOR*, but not *RICTOR*, knock-down decreases GRASP55 colocalization with the Golgi marker GM130 (E,H) and increases its colocalization with the autophagosome marker LC3B (F,I) and the MVB marker CHMP2A (G,J). Quantification of colocalization between GRASP55 and the organelle markers in (H-J). Representative magnified insets shown on the right side. Scale bars = 10 μm.

Data shown are representative of 3 replicate experiments. Data in (B, D, H-J) shown as mean ± SD. * $p<0.05$, ** $p<0.01$, *** $p<0.005$.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GRASP55
GM130
merge
Ctrl
-FBS
-AA
S6Ki
Rapa
Torin
B
GRASP55
LC3B
merge
C
GRASP55
CHMP2A
merge
D
Pearson's coefficient GRASP55 / GM130
E
Pearson's coefficient GRASP55 / LC3B
F
Pearson's coefficient GRASP55 / CHMP2A
G
DMSO
Rapa
GRASP65
H
GRASP55KO
WT
KO3
KO8
Actin
55
70
35
I
WT
GRASP55KO

Figure S2. GRASP55, but not GRASP65, relocalizes to secretory compartments in response to multiple stresses. Related to Figure 1.

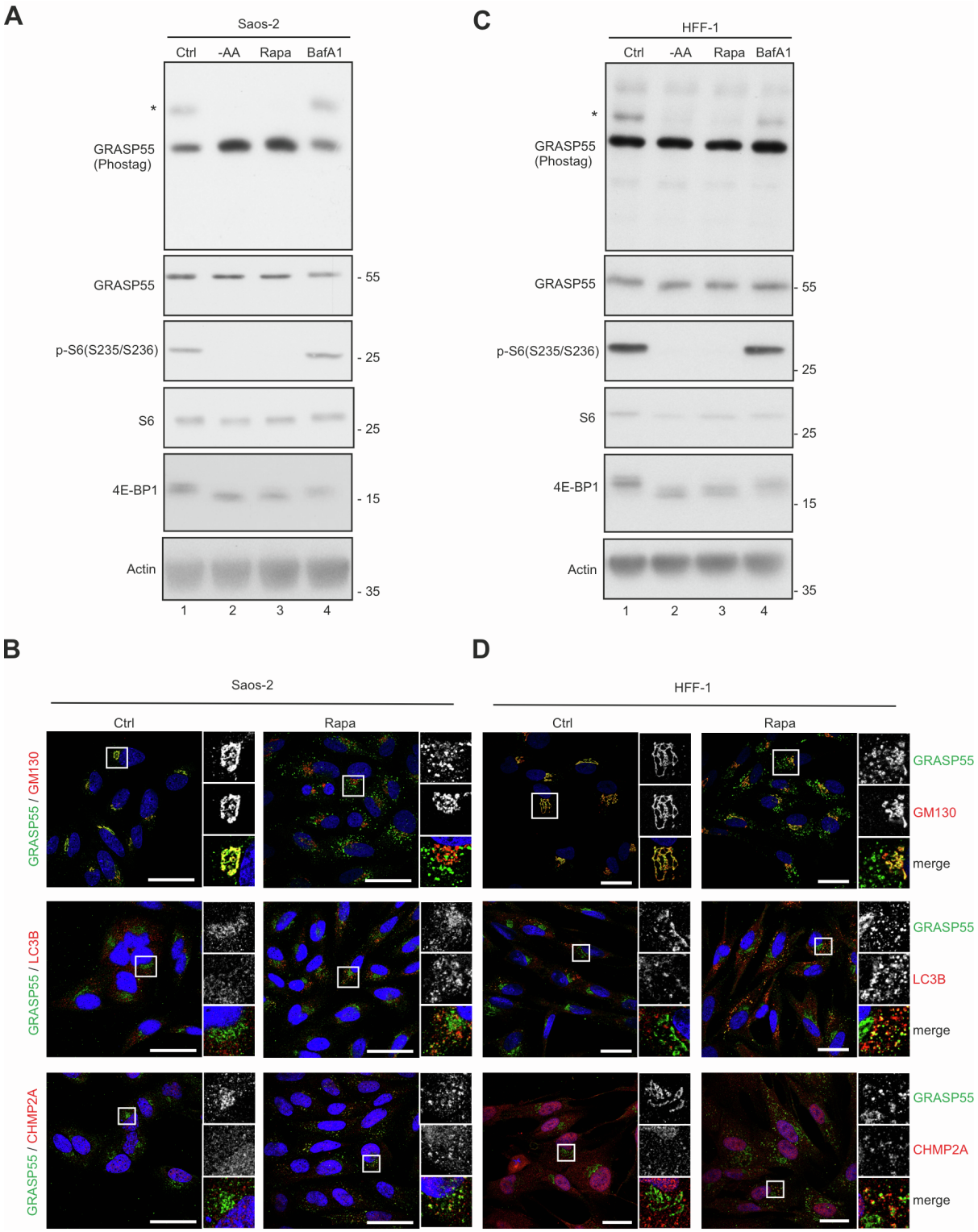
(A-F) WI-26 cells treated with media lacking serum (-FBS), amino acids (-AA), or containing Torin1 (Torin), Rapamycin (Rapa), or S6K inhibitor (S6Ki). Colocalization of GRASP55 with the Golgi (GM130) (A,D), autophagosomes (LC3B) (B,E) or MVBs (CHMP2A) (C,F) analyzed by IF/confocal microscopy. Quantification of colocalization between GRASP55 and the organelle markers in (D-F).

(G) GRASP55/GRASP65 colocalization analyzed in WT WI-26 cells treated with Rapamycin (Rapa) or DMSO, using IF/confocal microscopy. Note that, while the GRASP55 localization pattern changes in response to Rapamycin, GRASP65 maintains a Golgi-like pattern.

(H) Validation of GRASP55 KO cells. Immunoblotting using antibodies against GRASP55, GRASP65 and actin, with lysates from WT and two independent GRASP55 KO WI-26 clones (KO3, clone 3; KO8, clone 8).

(I) GRASP65 does not relocalize to autophagosomes in Rapamycin-treated cells. GRASP65/LC3B colocalization analyzed in WT or GRASP55 KO WI-26 cells as in (A).

For all microscopy panels, representative magnified insets shown on the right side. Scale bars = 10 μm. Images shown are representative of 3 replicate experiments. Data in (D-F) shown as mean ± SD. ** $p<0.01$, *** $p<0.005$.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Saos-2
Ctrl
-AA
Rapa
BafA1
*
GRASP55
(Phostag)
GRASP55
p-S6(S235/S236)
S6
4E-BP1
Actin
- 55
- 25
- 25
- 15
- 35
1
2
3
4
C
HFF-1
Ctrl
-AA
Rapa
BafA1
*
GRASP55
(Phostag)
GRASP55
p-S6(S235/S236)
S6
4E-BP1
Actin
- 55
- 25
- 25
- 15
- 35
1
2
3
4
B
Saos-2
Ctrl
Rapa
GRASP55 / GM130
GRASP55 / LC3B
GRASP55 / CHMP2A
GRASP55
GM130
merge
GRASP55
LC3B
merge
GRASP55
CHMP2A
merge
D
HFF-1
Ctrl
Rapa

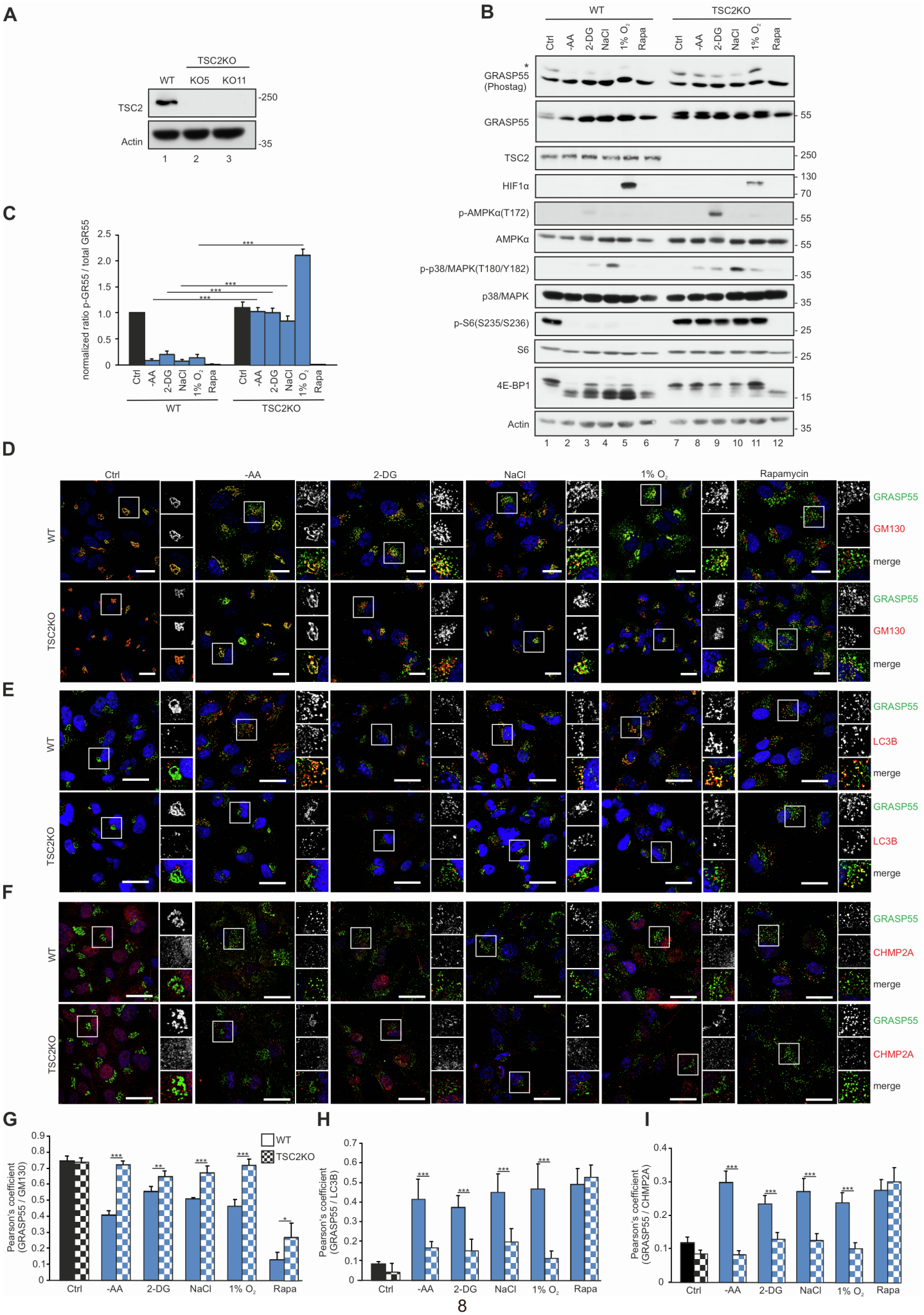
Figure S3. GRASP55 dephosphorylation and relocalization by mTORC1 inhibition occurs in diverse cell lines. Related to Figure 1.

(A) Phosphorylation of GRASP55 analyzed by Phos-tag gels and immunoblotting in control (Ctrl), AA-starved (-AA), Rapamycin-treated (Rapa) or Bafilomycin-A1-treated (BafA1) human bone osteosarcoma Saos-2 cells. mTORC1 activity assayed by immunoblotting with phospho-specific antibodies against mTORC1 downstream targets and antibodies against total proteins. Asterisk indicates the phosphorylated GRASP55 form.

(B) GRASP55 (GR55) colocalization with the Golgi (GM130), autophagosome (LC3B) and MVB (CHMP2A) markers assayed in control (Ctrl) or Rapamycin-treated (Rapa) Saos-2 cells. Representative magnified insets shown on the right side. Scale bars = 10 μm.

(C-D) As in (A-B), but using human foreskin fibroblasts (HFF-1).

Data shown are representative of 3 replicate experiments.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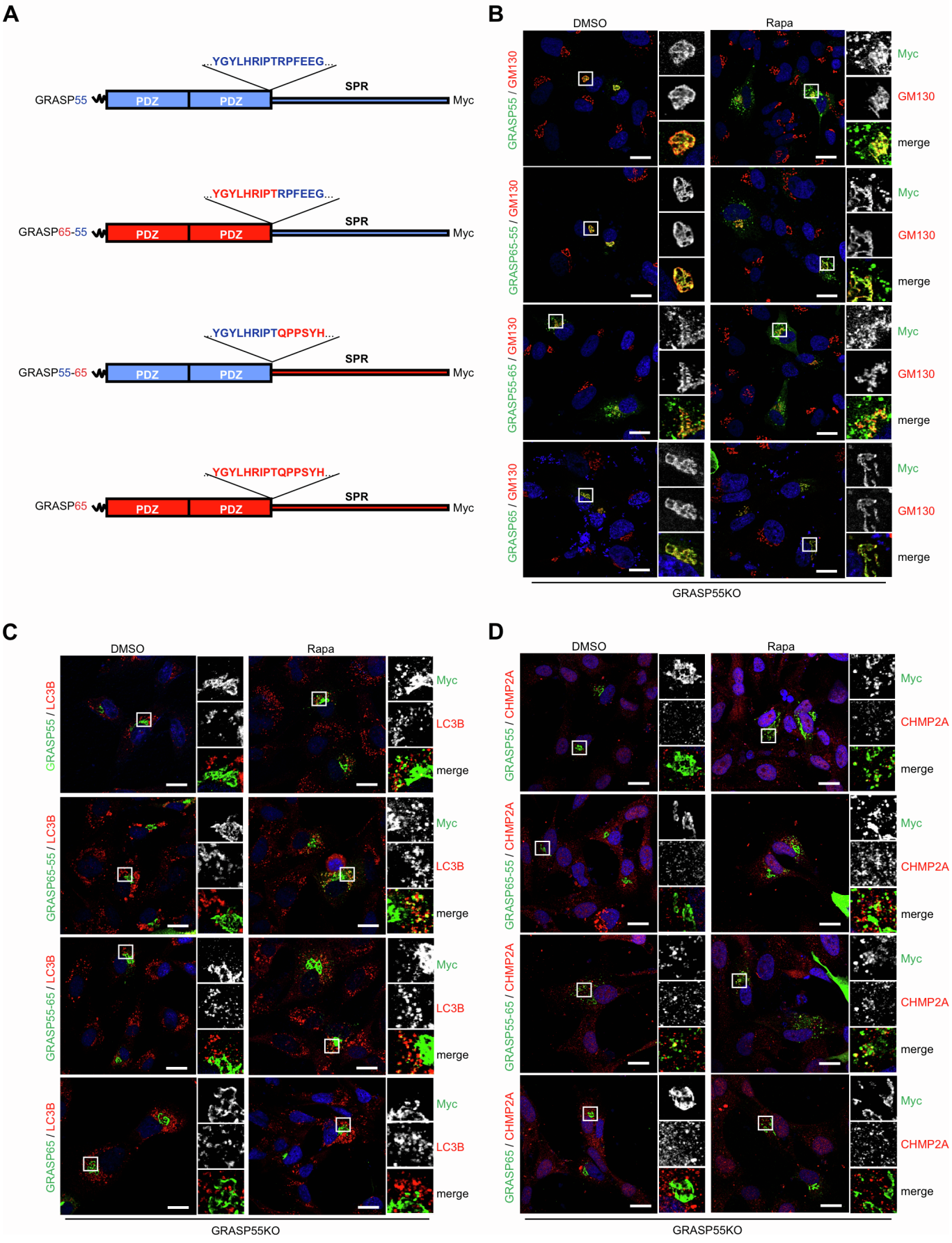
TSC2KO
WT
KO5
KO11
TSC2
-250
Actin
-35
1
2
3
B
WT
TSC2KO
Ctrl
-AA
2-DG
NaCl
1% O2
Rapa
GRASP55 (Phostag)
GRASP55
TSC2
HIF1α
p-AMPKα(T172)
AMPKα
p-p38/MAPK(T180/Y182)
p38/MAPK
p-S6(S235/S236)
S6
4E-BP1
Actin
250
130
70
55
35
25
15
1 2 3 4 5 6 7 8 9 10 11 12
C
normalized ratio p-GR55 / total GR55
WT
TSC2KO
D
Ctrl
-AA
2-DG
NaCl
1% O2
Rapamycin
WT
TSC2KO
GRASP55
GM130
merge
E
LC3B
F
CHMP2A
G
Pearson's coefficient (GRASP55 / GM130)
H
Pearson's coefficient (GRASP55 / LC3B)
I
Pearson's coefficient (GRASP55 / CHMP2A)

Figure S4. GRASP55 phosphorylation and localization do not respond to cellular stress in TSC2-null cells. Related to Figure 2.

(A) Validation of TSC2 KO cells. Immunoblotting using antibodies against TSC2 and actin, with lysates from WT and two independent TSC2 KO WI-26 clones (KO5, clone 5; KO11, clone 11).

(B-C) GRASP55 phosphorylation analyzed by Phos-tag gels and immunoblotting with lysates from WT or TSC2 KO (clone 11) WI-26 cells cultured in control conditions (Ctrl), starved for AA (-AA), treated with 2-DG to induce energetic stress, treated with NaCl to induce hyperosmotic stress, incubated in hypoxic conditions (1% O_2), or treated with Rapamycin to inhibit mTORC1 (Rapa). Phosphorylation and total protein levels of various signaling proteins assayed by immunoblotting using specific antibodies (B). Asterisk indicates phosphorylated GRASP55. Quantification of the p-GRASP55/GRASP55 ratio in the various conditions in (C).

(D-I) WI-26 cells treated as in (B) and samples analyzed for GRASP55 localization. Colocalization of endogenous GRASP55 with the Golgi (GM130) (D,G), autophagosome (LC3B) (E,H) and MVB (CHMP2A) (F,I) markers assayed in WT or TSC2 KO WI-26 cells by IF/confocal microscopy. Quantification of colocalization between GRASP55 and the organelle markers in (G-I). Representative magnified insets shown on the right side. Scale bars = 10 μm. Data shown are representative of at least 3 replicate experiments. Data in (C, G-I) shown as mean ± SD. * $p<0.05$, ** $p<0.01$, *** $p<0.005$.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PDZ
PDZ
Myc
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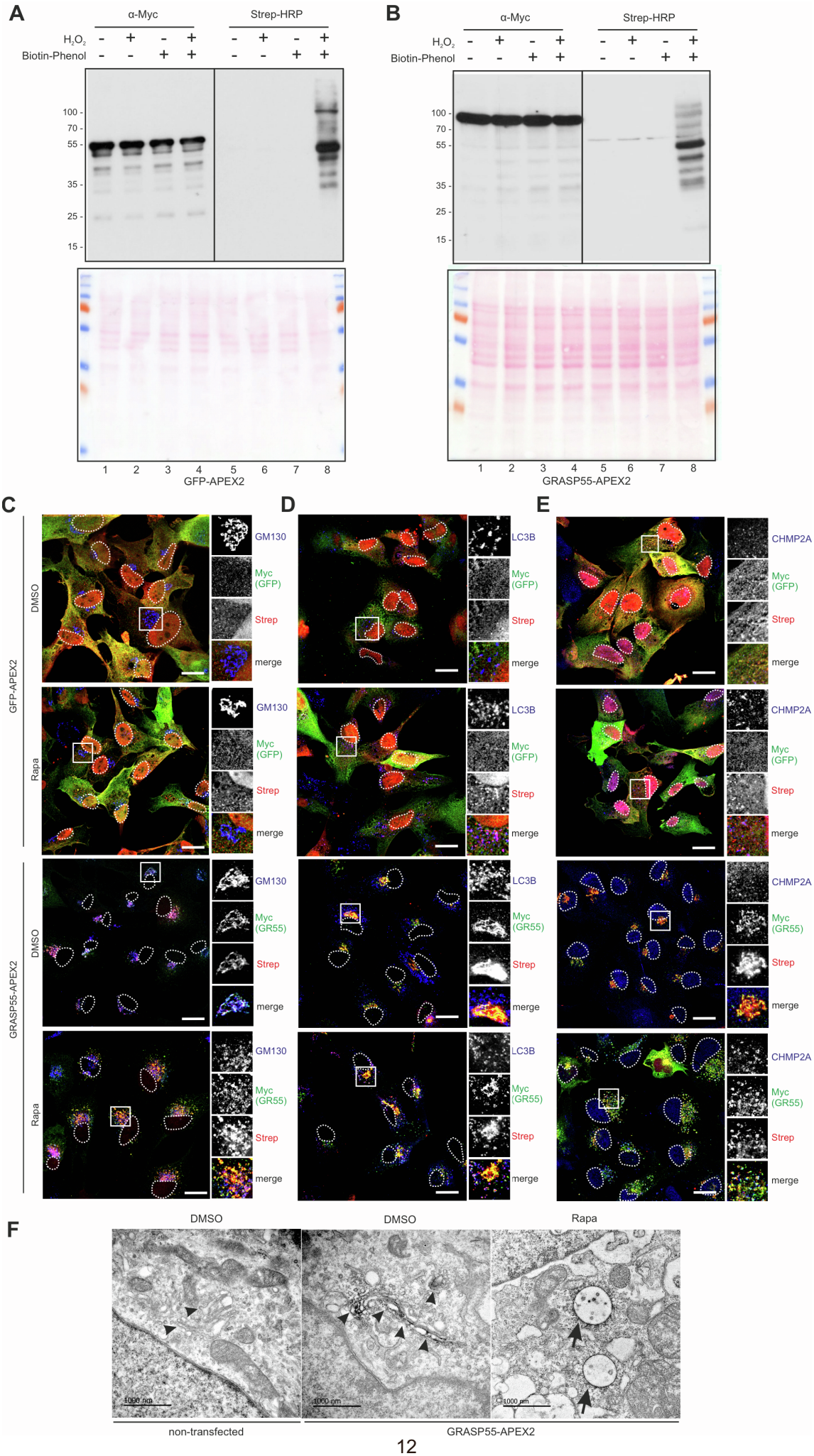
PDZ
PDZ
Myc
...YGYLHRIPTQPPSYH...
SPR
GRASP55-65
PDZ
PDZ
Myc
...YGYLHRIPTQPPSYH...
SPR
GRASP65
PDZ
PDZ
Myc
B
DMSO
Rapa
GRASP55 / GM130
GRASP65-55 / GM130
GRASP55-65 / GM130
GRASP65 / GM130
Myc
GM130
merge
GRASP55KO
C
DMSO
Rapa
GRASP55 / LC3B
GRASP65-55 / LC3B
GRASP55-65 / LC3B
GRASP65 / LC3B
Myc
LC3B
merge
GRASP55KO
D
DMSO
Rapa
GRASP55 / CHMP2A
GRASP65-55 / CHMP2A
GRASP55-65 / CHMP2A
GRASP65 / CHMP2A
Myc
CHMP2A
merge
GRASP55KO

Figure S5. Importance of the GRASP55 SPR region for its subcellular localization. Related to Figure 3.

(A) Schematic representation of Myc-tagged WT and GRASP55/GRASP65 chimeric proteins (55-65 or 65-55) indicating the main protein regions (PDZ, SPR) and the junction AA sequence. GRASP55 elements shown in blue, GRASP65 elements shown in red.

(B-D) GRASP55 phosphorylation by mTORC1 at its SPR region controls its subcellular localization. Colocalization of the proteins shown in (A) with GM130 (B), LC3B (C) and CHMP2A (D) markers, in control (DMSO) or Rapamycin-treated (Rapa) WI-26 cells, analyzed by IF/confocal microscopy in reconstituted GRASP55 KO WI-26 cells. Scale bars = 10 μm. Data shown are representative of 3 replicate experiments.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α-Myc
Strep-HRP
H2O2
Biotin-Phenol
100
70
55
35
25
15
1 2 3 4 5 6 7 8
GFP-APEX2
B
α-Myc
Strep-HRP
H2O2
Biotin-Phenol
100
70
55
35
25
15
1 2 3 4 5 6 7 8
GRASP55-APEX2
C
D
E
GFP-APEX2
GRASP55-APEX2
DMSO
Rapa
GM130
LC3B
CHMP2A
Myc (GFP)
Myc (GR55)
Strep
merge
F
DMSO
DMSO
Rapa
1000 nm
non-transfected
GRASP55-APEX2

Figure S6. Validation of the APEX2 proximity biotinylation system. Related to Figure 4.

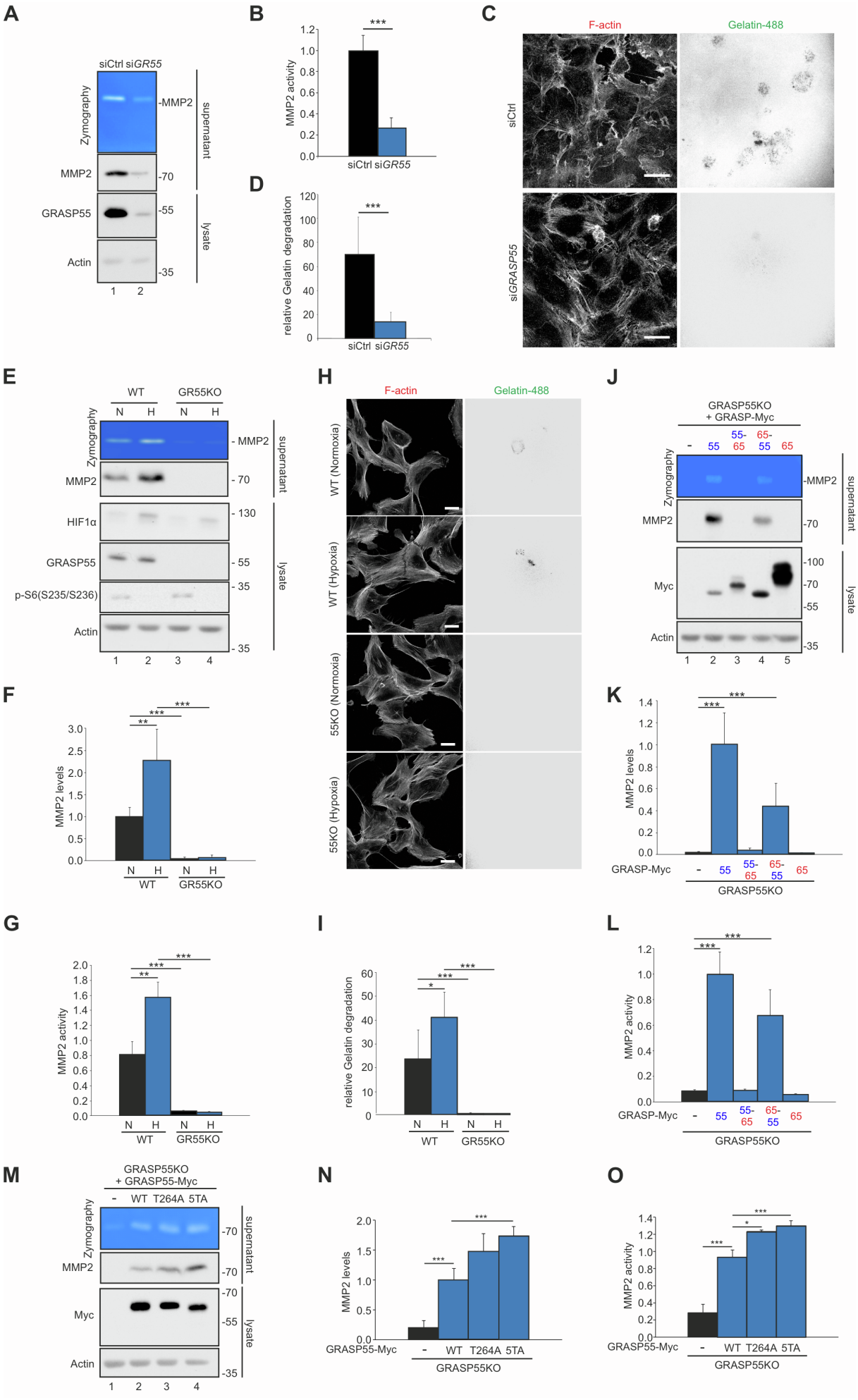
(A) Immunoblot analysis of Myc-GFP-APEX2-expressing WI-26 cells and activation of the biotinylation reaction with single components (Biotin-Phenol, H_2O_2) or in combination using an anti-Myc antibody and Streptavidin-HRP (Strep-HRP). Equal protein loading confirmed by Ponceau S staining.

(B) As in (A), but for Myc-GRASP55-APEX2-expressing WI-26 cells.

(C-E) Localization of exogenously expressed GRASP55-APEX2 resembles that of endogenous GRASP55. Immunofluorescence analysis of DMSO- or Rapamycin-treated (Rapa) WI-26 cells stably expressing Myc-tagged GRASP55-APEX2 (or GFP-APEX2 as control), co-stained with Streptavidin (Strep) and GM130 (C), LC3B (D) or CHMP2A (E), following the initiation of the biotinylation reaction by adding biotin-phenol and H_2O_2 to the cells. Nuclei outlined with a dotted line. Representative magnified insets shown on the right side. Scale bars = 10 μm.

(F) DAB staining in non-transfected or GRASP55-APEX2-expressing control (DMSO) or Rapamycin-treated (Rapa) WI-26 cells. Samples visualized with electron microscopy. Black dots show the localization of GRASP55-APEX2. Arrowheads indicate the Golgi, arrows indicate MVBs. Scale bars = 1 μm.

Data shown are representative of 3 replicate experiments.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Zymography
MMP2
GRASP55
Actin
-MMP2
-70
-55
-35
supernatant
lysate
1 2
B
MMP2 activity
siCtrl siGR55
***
D
relative Gelatin degradation
siCtrl siGR55
***
C
F-actin
Gelatin-488
siCtrl
siGRASP55
E
WT
GR55KO
N H N H
Zymography
MMP2
HIF1α
GRASP55
p-S6(S235/S236)
Actin
- MMP2
- 70
- 130
- 55
- 35
- 35
supernatant
lysate
1 2 3 4
F
MMP2 levels
N H N H
WT GR55KO
H
F-actin
Gelatin-488
WT (Normoxia)
WT (Hypoxia)
55KO (Normoxia)
55KO (Hypoxia)
J
GRASP55KO
+ GRASP-Myc
- 55 55-65 65-55 65
Zymography
MMP2
Myc
Actin
-MMP2
-70
-100
-70
-55
-35
supernatant
lysate
1 2 3 4 5
K
MMP2 levels
GRASP-Myc
- 55 55-65 65-55 65
GRASP55KO
G
MMP2 activity
N H N H
WT GR55KO
I
relative Gelatin degradation
N H N H
WT GR55KO
L
MMP2 activity
GRASP-Myc
- 55 55-65 65-55 65
GRASP55KO
M
GRASP55KO
+ GRASP55-Myc
- WT T264A 5TA
Zymography
MMP2
Myc
Actin
-70
-70
-70
-55
-35
supernatant
lysate
1 2 3 4
N
MMP2 levels
GRASP55-Myc
- WT T264A 5TA
GRASP55KO
O
MMP2 activity
GRASP55-Myc
- WT T264A 5TA
GRASP55KO

Figure S7. Non-phosphorylated GRASP55 induces MMP2 secretion and activity at the extracellular space upon stress. Related to Figure 7.

(A-B) MMP2 secretion and activity at the extracellular space is severely compromised in *GRASP55* knock-down cells. Zymography assay for MMP2 activity in the supernatant of control (siCtrl) and *GRASP55* knock-down (si*GR55*) WI-26 cells. Levels of secreted MMP2 protein analyzed by immunoblotting. The efficiency of the *GRASP55* knock-down assessed by blotting cell lysates with antibodies against GRASP55 and actin. Quantification of the secreted MMP2 levels in (B).

(C-D) Fluorescent gelatin degradation assay using control (siCtrl) and *GRASP55* knock-down (si*GR55*) WI-26 cells. Degraded gelatin shown as black spots. F-actin staining used as a cytoskeleton marker. Quantification of the relative gelatin degradation in (D). Scale bars = 10 μm.

(E-G) Hypoxia regulates MMP2 secretion, activity and function at the extracellular space via GRASP55. Zymography assay for MMP2 activity and immunoblotting for MMP2 levels in the supernatant of WT and GRASP55 KO (GR55KO) WI-26 cells, cultured under normoxic (N) or hypoxic (H) conditions. Intracellular MMP2, HIF1α, GRASP55, p-S6, and actin used as controls (E). Quantification of MMP2 secretion in (F) and MMP2 activity in (G).

(H-I) WT and GRASP55 KO WI-26 cells treated as in (E) and assayed by fluorescent gelatin degradation assays. Degraded gelatin shown as black spots. F-actin staining used as a cytoskeleton marker. Quantification of relative gelatin degradation in (I). Scale bars = 10 μm.

(J-L) The C-terminal SPR region of GRASP55 is responsible for inducing MMP2 secretion. Zymography assay for MMP2 activity and immunoblotting for MMP2 levels in the supernatant of WI-26 cells expressing the WT and chimeric proteins shown in Fig. S5A. Expression of the GRASP55 constructs and actin levels analyzed as controls (J). Quantification of MMP2 secretion in (K) and MMP2 activity in (L).

(M-O) Expression of non-phosphorylatable GRASP55 mutants enhances MMP2 secretion and activity at the extracellular space. Zymography assay for MMP2 activity and immunoblotting

for MMP2 levels in the supernatant of GRASP55 KO WI-26 cells stably expressing WT, T264A or 5TA GRASP55. Quantification of MMP2 secretion in (N) and MMP2 activity in (O).
Data shown are representative of 3 replicate experiments. Data in all bar plots shown as mean ± SD. * $p<0.05$, ** $p<0.01$, *** $p<0.005$.