Supplemental Materials

Molecular Biology of the Cell

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Supplementary Figure legends:

Figure S1. **MGRN1 overexpression rescues the death phenotype and loss of MGRN1 does not affect proteins of the intrinsic pathway.**
(A) HeLa cells treated with mock siRNA, MGRN1 siRNA1 or MGRN1siRNA2 were further transfected with empty vector or MGRN1-GFP and cleavage of CASP9 analysed. Note that exogenous expression of MGRN1-GFP compromised activation of CASP9. Blots for MGRN1 and GAPDH serve as loading controls.

(B) The immunoblots shown in panel A were analyzed for the fold change in CASP9 protein levels. Graphs show results from 3 independent experiments. *p≤0.05, **p≤0.01, NS not significant (p=0.5 and 0.07), using Student's *t*-test. Error bars, +SEM.

(C) HeLa cells treated with mock or MGRN1 siRNAs were lysed and immunoblotted to check for the levels of BAD, BAX, BCL-XL, MCL1 and cleaved CASP8 (Ac-CASP8) proteins. Level of β-tubulin serves as loading control and that of MGRN1 is used to verify the knockdown efficiency. No change was observed across samples.

(D) The immunoblots shown in panel C were analyzed for the fold change in the indicated protein levels. Graphs show results from 5 independent experiments. NS not significant (p=0.1, BAD; p=0.26, BAX; p=0.4, BCL-XL; p=0.13, MCL1) using Student's *t*-test. Error bars, +SEM.

Figure S2. **ER stress leads to ER expansion and activation of CASP4.**
(A) HeLa cells transiently transfected with ER-GFP construct were either left untreated or treated with 2μM thapsigargin (Tg) for 6h. Cells were imaged to observe changes in ER morphology under the indicated conditions. Note an expansion in ER area upon treatment with the ER stress-inducing drug thapsigargin. Scale bar, 15 μm. ~100 cells were imaged for each condition. Formula for calculating ER area is indicated.

(B) HeLa cells treated with mock siRNA, MGRN1 siRNA1 or MGRN1 siRNA2 were immunoblotted to check the levels of CASP4. Note activation of CASP4 upon MGRN1 depletion. Blots for MGRN1 and GAPDH serve as loading controls.

(C) Graphs are generated by analysing immunoblots in panel B from 3 different experiments. Mean±s.d. **p≤0.01, ***p≤0.001using Student's t-test.

(D) HeLa cells treated with indicated siRNAs were lysed and immunoblotted to check for the levels of BCL2 protein. GAPDH serves as the loading control; MGRN1 levels verify the knockdown efficiency. Graph on the right is generated using analyses of the immunoblots from 4 independent experiments. Mean±s.d, **p≤0.01using Student's t-test.

(E) HEK cells treated with the indicated concentrations of thapsigargin for 6h or left untreated were lysed and immunoblotted to check for the protein levels of BIP, CHOP and CASP9. Note activation of CASP9 is detected at higher concentrations of thapsigargin. GAPDH serves as the loading control.

(F) HeLa cells were similarly treated with the indicated concentrations of thapsigargin for 6h or left untreated. Cell lysates were immunoblotted to check for the indicated protein levels. Note activation of CASP9 is detected at 1μM and 2μM concentration of thapsigargin. GAPDH serves as the loading control.

Figure S3. **MGRN1 depletion causes increase in ER protein load.**
(A) Mock or MGRN1 siRNAs transfected cells were lysed in microsome isolation buffer [containing 0.3M sucrose and 10mM Hepes (pH-7.0)]. Proportion of lysate loaded is denoted in brackets by "X". The gel was stained with Coomassie Brilliant Blue R-250 solution. Markers for cytosol (β-tubulin), mitochondria (VDAC1) and microsome (TRAP-α) fractions are shown.

(B) The gel shown in panel A was analyzed for the fold change in the organellar fraction levels. Graph shows results from 3 independent experiments. Error bars, ±SEM.

Figure S4. **ALIX and ALG-2 are degraded by the autophagosomal-lysosomal system.**
(A) Untransfected HeLa cells were left untreated or treated with Tg (400nM, for 6h) alone, or with BAPTA (75μM, for 6h). Note immunoblot analysis shows that Tg treatment alone cannot elicit CASP9 activation.

(B) Graph plotted with data from panel A shows analyses from 3 independent experiments. NS not significant (p=0.4 and 0.6) using Student's *t*-test. Error bars, $\pm$SEM.

(C) HeLa cells transfected with mock or MGRN1 siRNAs were left untreated or treated with 300nM Bafilomycin A1 (vacuolar H+-ATPase inhibitor) for 6h. Cell lysates were analysed by Western blotting assays for the levels of ALIX and ALG-2 proteins. Knockdown of MGRN1 is known to block autophagosomal-lysosomal degradation. Note increased levels of ALIX and ALG-2 upon MGRN1 depletion. Treatment of cells with Bafilomycin A1 elevated the levels of these proteins. GAPDH serves as the loading control, efficiency of knockdown was checked using antibody against MGRN1. The efficacy of block in lysosomal degradation was verified by the levels of p62.

(D) Graph generated using results of panel C from 4 independent experiments. *$p \leq 0.05$, **$p \leq 0.01$ using Student's t-test. Error bars, $\pm$SEM.

(E) To further verify if the turn-over of ALIX and ALG-2 was regulated via the proteasomal degradation pathway, mock and MGRN1 depleted HeLa cells were either left untreated or treated with 25μM of MG132 (proteasome inhibitor) for 6h. Cell lysates were immunoblotted using antibodies against ALIX and ALG-2. Treatment of cells with MG132 did not stabilize either of the proteins. GAPDH serves as the loading control, efficiency of knockdown was checked using antibody against MGRN1. Rather, degradation of these proteins continued even after blocking the proteasome. Blocked proteasomal degradation was assayed by probing against β-catenin.

(F) Immunoblots from panel F were analyzed from 4 independent experiments. Error bars, $\pm$SEM.

Figure S5. **Ubiquitination of TSG101 by MGRN1 is crucial for cell survival.**
(A) HeLa cells transfected with mock siRNA, TSG101 siRNA1 or TSG101 siRNA2 were immunoblotted to check for the levels of CASP9 and CASP4 proteins. Levels of TSG101 show knockdown efficiency and GAPDH serve as loading control.

(B) Histograms plotted with data from panel A. Graphs show analyses from 3 independent experiments. *p≤0.05, **p≤0.01, using Student's *t*-test. Error bars, ±SEM.

(C) Total RNA was isolated from mock or MGRN1 or TSG101 siRNAs treated HeLa cells. Quantitative RT-PCR was performed using Syber Green and primers against TSG101 and GAPDH. Samples were present in triplicates. $2^{-\Delta\Delta Ct}$ values for TSG101 were calculated. Note that MGRN1 knockdown does not affect the expression of TSG101. Graph shows mean±s.d. from 3 independent experiments. *p≤0.05, N.S., not significant (p=0.11) using Student's t-test.

(D) Cells similarly treated as in panel C were lysed to check for the protein levels of TSG101 and MGRN1. Loss of MGRN1 does not affect the protein levels of TSG101; similarly MGRN1 levels remain unaltered when TSG101 is depleted. GAPDH levels serve as the loading control.

(E) HeLa cells treated with mock or MGRN1 siRNAs were transiently transfected with empty vector, ubiquitin (Ub) or Ub K0 and immunoblotted for the indicated antibodies. GAPDH and MGRN1 serve as loading controls. Note higher levels of cleaved CASP9, ALIX and ALG-2 are detected in MGRN1 knockdown cells transfected with mock vector; presence of Ub or Ub K0 rescues this phenotype.

(F) HeLa cells treated with mock or MGRN1 siRNAs transiently transfected with empty vector or HA-TSG101 were lysed in immunoprecipitation buffer and immunoprecipitated under denaturing condition with anti-HA or anti-TSG101 antibodies as indicated. Ubiquitinated TSG101was detected by immunoblotting with anti-Ub antibody. TSG101 and MGRN1 serve as loading controls. Note ubiquitination of TSG101 is restored in MGRN1 depleted cells upon overexpression of HA-TSG101. ◄HA-TSG101, ←endogenous TSG101.

(G) HeLa cells transiently co-transfected with FLAG-ALIX along with HA-TSG101 or HA-M95R-TSG101 were immunoprecipitated with anti-HA antibody. Western blot analysis with anti-FLAG antibody shows co-immunoprecipitation of ALIX with TSG101. However, note lack of similar interaction in presence of M95R-TSG101. Proportion of lysate loaded as input; expression of the transfected TSG101 constructs also verified by immunoblotting lysates with anti-HA antibody.

Figure S6. **ALIX-TSG101 interaction is independent of ALG-2.**
(A) HeLa cells transiently co-transfected with HA–TSG101 along with FLAG-ALIX or FLAG-ALIX-ΔALG-2 were immunoprecipitated with anti-HA antibody. Note that interaction between ALIX and TSG101 is detected with similar efficiency even when ALX-ΔALG-2 mutant is used. Proportion of lysate was checked for the expression of the transfected TSG101 across samples by immunoblotting lysates with anti-HA antibody. Input levels of ALIX in these lysates verified using anti-FLAG antibody. IgG(H) heavy chain. 5mM $CaCl_2$ was used in the buffer.

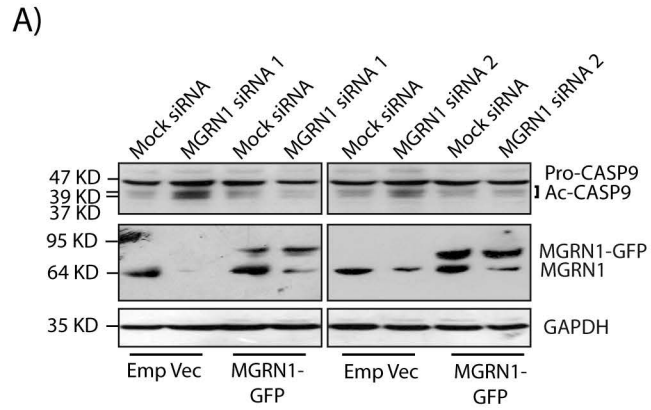
(B) HeLa cells transiently transfected with FLAG-ALIX or FLAG-ALIX-ΔALG-2 were immunoprecipitated with anti-FLAG antibody. Note that interaction between ALIX and TSG101 is detected with similar efficiency even when ALX-ΔALG-2 mutant is used. Input levels of ALIX in these lysates verified using anti-FLAG antibody. GAPDH serves as loading control. IgG(H) heavy chain.

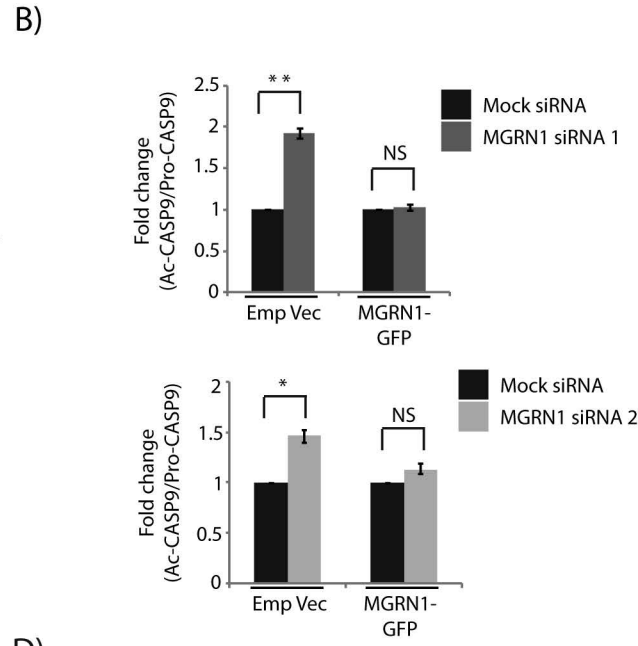
(C) HEK293T cells treated with mock or ALG-2 siRNAs were transiently transfected with FLAG–ALIX. Cells were lysed in 50μM Ca^{2+} buffer and immunoprecipitated with anti-FLAG (right panels). Western blot analyses show co-immunoprecipitation between ALIX and TSG101, even when ALG-2 is depleted in cells. Reverse coimmunoprecipitation with anti-TSG101 (left panels). Efficiency of knockdown was checked using anti-ALG-2 antibody. GAPDH levels serve as the loading control.

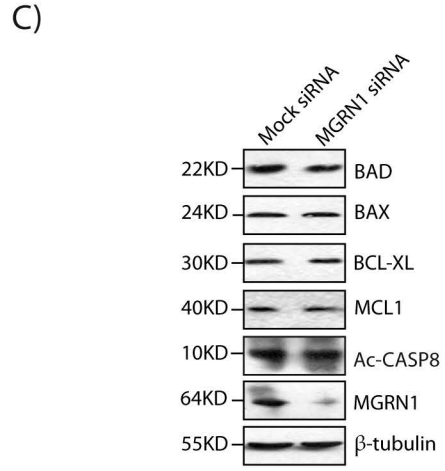
(D) Interaction between ALIX and TSG101 was verified in HeLa cells in a similar experiment as in panel C.

(E) Similar experiments as in panel C were performed in SHSY5Y cells.

Figure S1


A)
Mock siRNA
MGRN1 siRNA 1
Mock siRNA
MGRN1 siRNA 1
Mock siRNA
MGRN1 siRNA 2
Mock siRNA
MGRN1 siRNA 2
47 KD
39 KD
37 KD
Pro-CASP9
Ac-CASP9
95 KD
64 KD
MGRN1-GFP
MGRN1
35 KD
GAPDH
Emp Vec
MGRN1-GFP
Emp Vec
MGRN1-GFP


B)
Fold change (Ac-CASP9/Pro-CASP9)
**
NS
Mock siRNA
MGRN1 siRNA 1
Emp Vec
MGRN1-GFP
Fold change (Ac-CASP9/Pro-CASP9)
*
NS
Mock siRNA
MGRN1 siRNA 2
Emp Vec
MGRN1-GFP


C)
Mock siRNA
MGRN1 siRNA
22KD
BAD
24KD
BAX
30KD
BCL-XL
40KD
MCL1
10KD
Ac-CASP8
64KD
MGRN1
55KD
β-tubulin

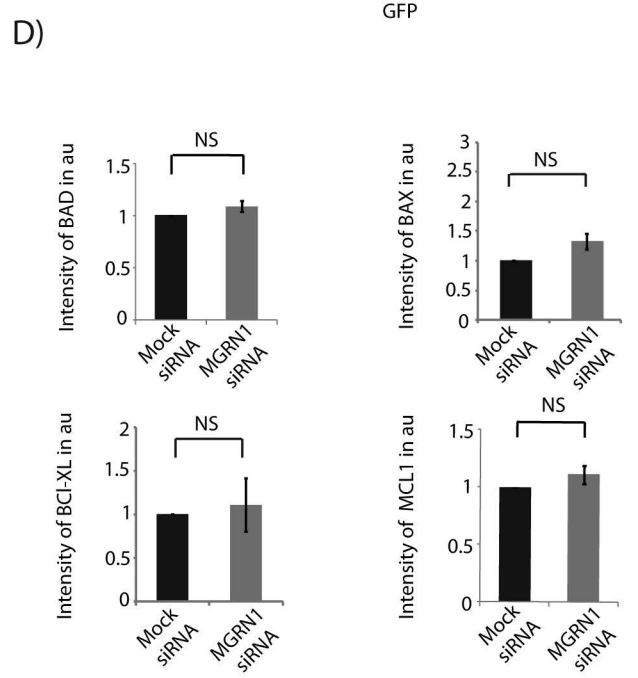

D)
Intensity of BAD in au
NS
Mock siRNA
MGRN1 siRNA
Intensity of BAX in au
NS
Mock siRNA
MGRN1 siRNA
Intensity of BCl-XL in au
NS
Mock siRNA
MGRN1 siRNA
Intensity of MCL1 in au
NS
Mock siRNA
MGRN1 siRNA

Figure S2

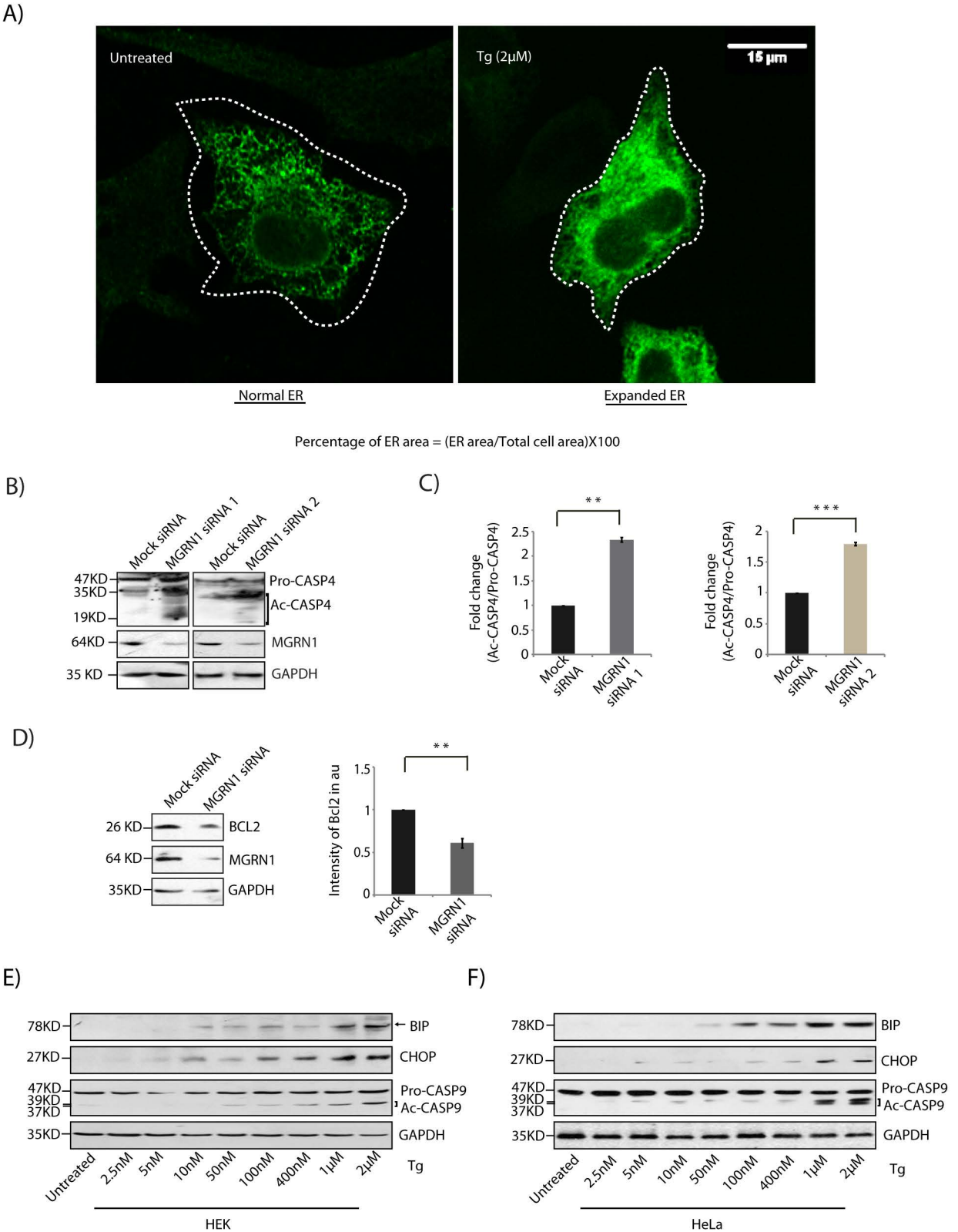
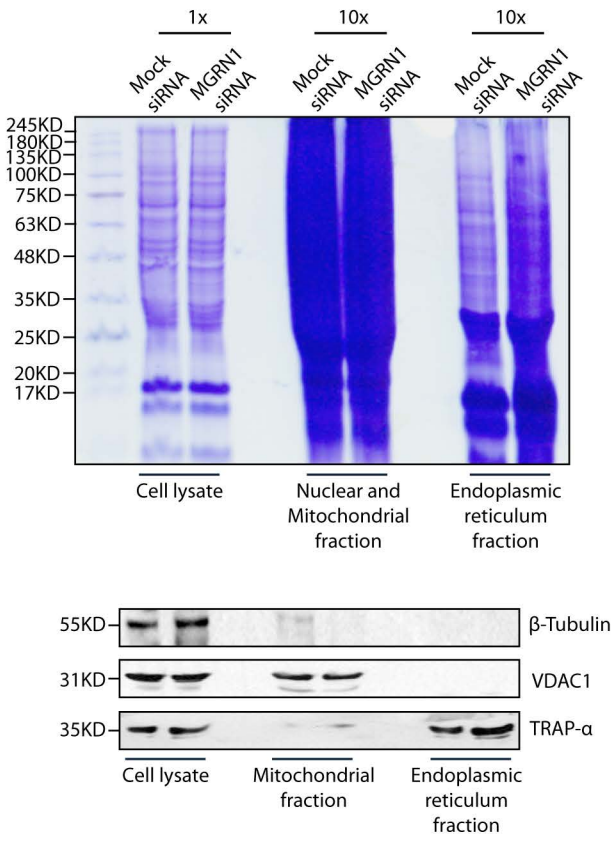

A)
Untreated
Tg (2μM)
15 μm
Normal ER
Expanded ER
Percentage of ER area = (ER area/Total cell area)X100
B)
Mock siRNA
MGRN1 siRNA 1
Mock siRNA
MGRN1 siRNA 2
47KD
35KD
19KD
Pro-CASP4
Ac-CASP4
64KD
MGRN1
35 KD
GAPDH
C)
Fold change (Ac-CASP4/Pro-CASP4)
**
Mock siRNA
MGRN1 siRNA 1
***
Mock siRNA
MGRN1 siRNA 2
D)
Mock siRNA
MGRN1 siRNA
26 KD
BCL2
64 KD
MGRN1
35KD
GAPDH
Intensity of Bcl2 in au
**
Mock siRNA
MGRN1 siRNA
E)
78KD
BIP
27KD
CHOP
47KD
39KD
37KD
Pro-CASP9
Ac-CASP9
35KD
GAPDH
Untreated
2.5nM
5nM
10nM
50nM
100nM
400nM
1μM
2μM
Tg
HEK
F)
78KD
BIP
27KD
CHOP
47KD
39KD
37KD
Pro-CASP9
Ac-CASP9
35KD
GAPDH
Untreated
2.5nM
5nM
10nM
50nM
100nM
400nM
1μM
2μM
Tg
HeLa

Figure S3

A)


1x
10x
10x
Mock siRNA
MGRN1 siRNA
Mock siRNA
MGRN1 siRNA
Mock siRNA
MGRN1 siRNA
245KD
180KD
135KD
100KD
75KD
63KD
48KD
35KD
25KD
20KD
17KD
Cell lysate
Nuclear and Mitochondrial fraction
Endoplasmic reticulum fraction
55KD
β-Tubulin
31KD
VDAC1
35KD
TRAP-α
Cell lysate
Mitochondrial fraction
Endoplasmic reticulum fraction

B)

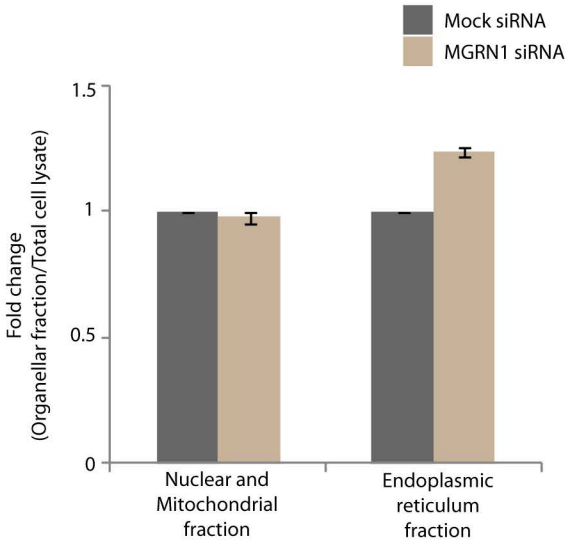

Mock siRNA
MGRN1 siRNA
Fold change
(Organellar fraction/Total cell lysate)
1.5
1
0.5
0
Nuclear and Mitochondrial fraction
Endoplasmic reticulum fraction

Figure S4

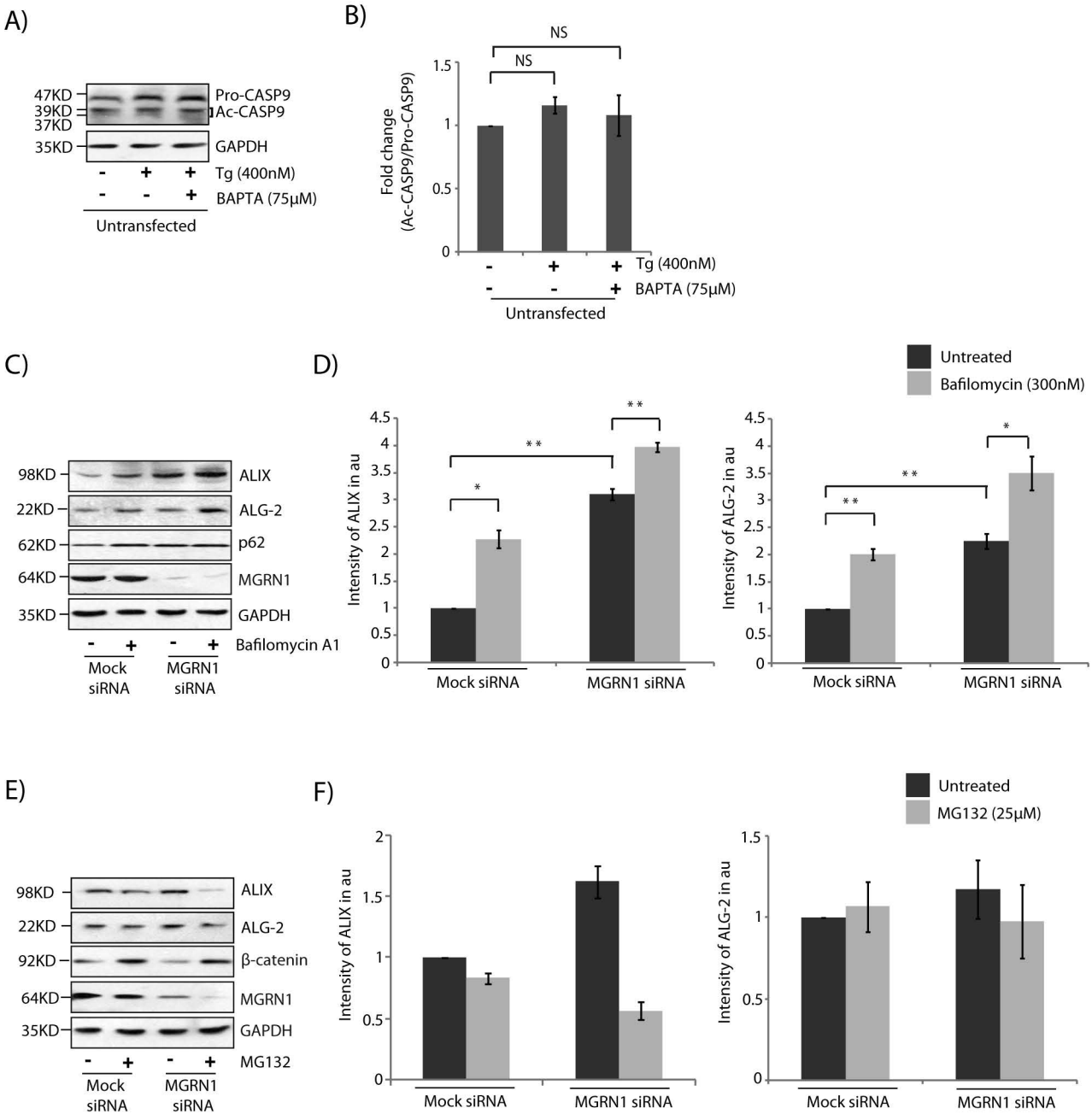

A)
47KD
39KD
37KD
35KD
Pro-CASP9
Ac-CASP9
GAPDH
- + + Tg (400nM)
- - + BAPTA (75μM)
Untransfected
B)
NS
NS
Fold change (Ac-CASP9/Pro-CASP9)
1.5
1
0.5
0
- + + Tg (400nM)
- - + BAPTA (75μM)
Untransfected
C)
98KD
22KD
62KD
64KD
35KD
ALIX
ALG-2
p62
MGRN1
GAPDH
- + - + Bafilomycin A1
Mock siRNA
MGRN1 siRNA
D)
Untreated
Bafilomycin (300nM)
Intensity of ALIX in au
Intensity of ALG-2 in au
Mock siRNA
MGRN1 siRNA
E)
98KD
22KD
92KD
64KD
35KD
ALIX
ALG-2
β-catenin
MGRN1
GAPDH
- + - + MG132
Mock siRNA
MGRN1 siRNA
F)
Untreated
MG132 (25μM)
Intensity of ALIX in au
Intensity of ALG-2 in au
Mock siRNA
MGRN1 siRNA

Figure S5

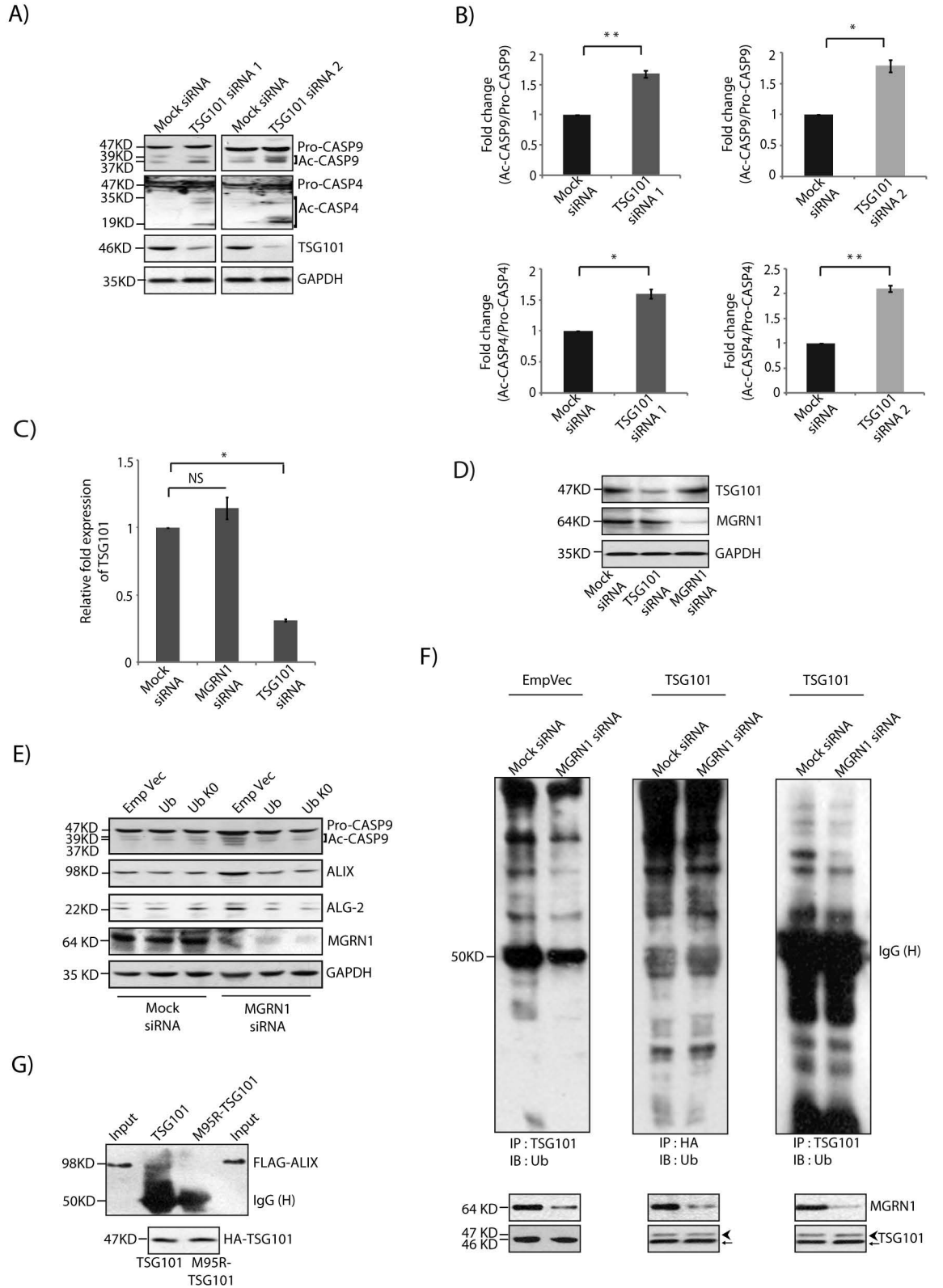
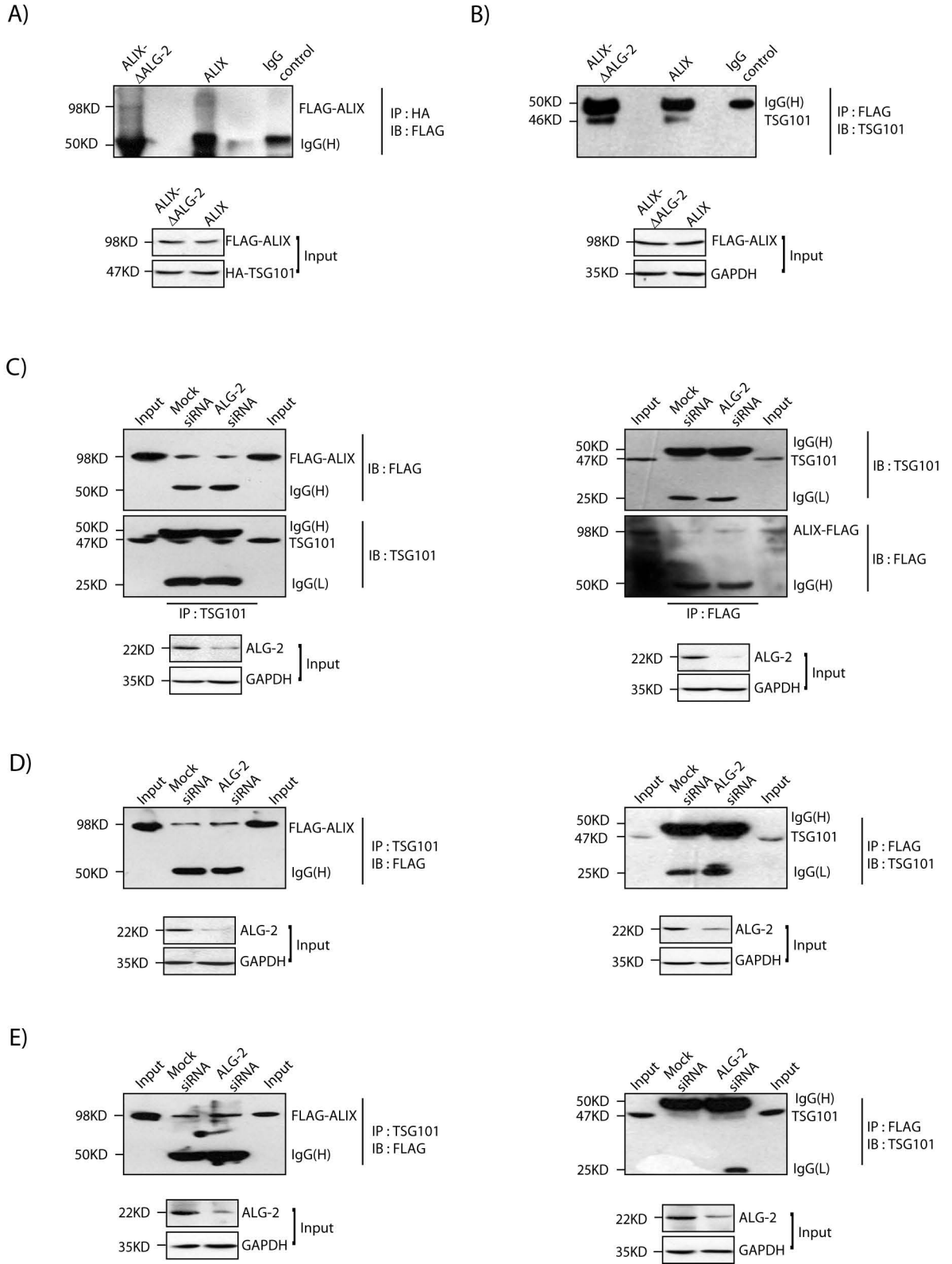

A)
Mock siRNA
TSG101 siRNA 1
Mock siRNA
TSG101 siRNA 2
47KD
39KD
37KD
Pro-CASP9
Ac-CASP9
47KD
35KD
19KD
Pro-CASP4
Ac-CASP4
46KD
TSG101
35KD
GAPDH
B)
Fold change (Ac-CASP9/Pro-CASP9)
Fold change (Ac-CASP4/Pro-CASP4)
Mock siRNA
TSG101 siRNA 1
TSG101 siRNA 2
C)
Relative fold expression of TSG101
NS
Mock siRNA
MGRN1 siRNA
TSG101 siRNA
D)
47KD
TSG101
64KD
MGRN1
35KD
GAPDH
Mock siRNA
TSG101 siRNA
MGRN1 siRNA
E)
Emp Vec
Ub
Ub K0
47KD
39KD
37KD
Pro-CASP9
Ac-CASP9
98KD
ALIX
22KD
ALG-2
64 KD
MGRN1
35 KD
GAPDH
Mock siRNA
MGRN1 siRNA
F)
EmpVec
TSG101
Mock siRNA
MGRN1 siRNA
50KD
IgG (H)
IP : TSG101
IB : Ub
IP : HA
IB : Ub
64 KD
47 KD
46 KD
MGRN1
TSG101
G)
Input
TSG101
M95R-TSG101
98KD
FLAG-ALIX
50KD
IgG (H)
47KD
HA-TSG101
TSG101
M95R-TSG101

Figure S6


A)
ALIX-ΔALG-2
ALIX
IgG control
98KD
50KD
FLAG-ALIX
IgG(H)
IP : HA
IB : FLAG
ALIX-ΔALG-2
ALIX
98KD
47KD
FLAG-ALIX
HA-TSG101
Input
B)
ALIX-ΔALG-2
ALIX
IgG control
50KD
46KD
IgG(H)
TSG101
IP : FLAG
IB : TSG101
ALIX-ΔALG-2
ALIX
98KD
35KD
FLAG-ALIX
GAPDH
Input
C)
Input
Mock siRNA
ALG-2 siRNA
Input
98KD
50KD
FLAG-ALIX
IgG(H)
IB : FLAG
50KD
47KD
25KD
IgG(H)
TSG101
IgG(L)
IB : TSG101
IP : TSG101
22KD
35KD
ALG-2
GAPDH
Input
Input
Mock siRNA
ALG-2 siRNA
Input
50KD
47KD
25KD
IgG(H)
TSG101
IgG(L)
IB : TSG101
98KD
50KD
ALIX-FLAG
IgG(H)
IB : FLAG
IP : FLAG
22KD
35KD
ALG-2
GAPDH
Input
D)
Input
Mock siRNA
ALG-2 siRNA
Input
98KD
50KD
FLAG-ALIX
IgG(H)
IP : TSG101
IB : FLAG
22KD
35KD
ALG-2
GAPDH
Input
Input
Mock siRNA
ALG-2 siRNA
Input
50KD
47KD
25KD
IgG(H)
TSG101
IgG(L)
IP : FLAG
IB : TSG101
22KD
35KD
ALG-2
GAPDH
Input
E)
Input
Mock siRNA
ALG-2 siRNA
Input
98KD
50KD
FLAG-ALIX
IgG(H)
IP : TSG101
IB : FLAG
22KD
35KD
ALG-2
GAPDH
Input
Input
Mock siRNA
ALG-2 siRNA
Input
50KD
47KD
25KD
IgG(H)
TSG101
IgG(L)
IP : FLAG
IB : TSG101
22KD
35KD
ALG-2
GAPDH
Input