Trichoplein/Mitostatin regulates ER-mitochondria juxtaposition

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Supplementary online material

Supplementary online methods

Molecular Biology

Δ1-TpMs, Δ2-TpMs, Δ3-TpMs, Δ4-TpMs, Δ5-TpMs-GFP and TpMs-GFP were generated by amplifying the fragments of TpMs by PCR using the following primers : 5’- GGGGAATTCTACC ATGGCGCTCCCGACG CT- 3’ and 5’-GGGGGATCCTGCCTGTTTGGAGCTGCA GAT-3’ for Δ1-TpMs, 5’-AAAGGATCCCAAGTTCATGCTCAGCCTCAG-3’ for Δ2-TpMs, 5’-AAAGGATCCCTCCATCTCTCGAAGTTTCGG-3’ for Δ3-TpMs, 5'-AAAGGATCCGCCTGTCTGCCTCCAGCTCCT-3’ for Δ4-TpMs, 5'-AAAGGTACCGTTCCAAGCAATTTTTGGATG-3’ for Δ5-TpMs-GFP and 5'-AAAGGTACCGTTCCAAGCAATTTTTGGATG-3’ for TpMs-GFP. TpMs also was subcloned into pEGFP-N3, adding a codon stop to block the expression of the GFP, using the following primers : 5'-GGGGAATTCTACCATGGCGCTCCCGACGCT-3' and 5'-AAAGGTACCTCAGTTCCAAGCAATTTTTGG-3'. pEGFP-N3 was mutated to avoid expression of GFP by site directed mutagenesis using the following primers : 5'-TCCATCGCCACCTGAGTGAGCAAGGGCGAG-3' and 5'-CTCGCCCTTGCTCACTCAGGTGGCGATGGA-3'. To subclone TpMs into pEYFP-C1 (BD Biosciences), TpMs was amplified by PCR using the following primers : 5'-GGGAGATCTATGGCGCTCCCGACGCTGCCG-3' and 5'-AAAGGTACCTCAGTTCCAAGCAATTTTTGG-3'.

Cell culture

LnCap overexpressing stably TpMs-V5 (LnCaPB3A) were previously described (Vecchione *et al.*, 2009). These cells were cultured in Roswell Park Memorial Istitute (RPMI, Invitrogen) supplemented

with 10% foetal bovine serum, 2 mM L-glutamine, non-essential amino acid75 U/ml penicillin, 50 μg/ml streptomycin and 200 μg/ml geneticin (G-418, GIBCO) at 37°C in a 5% CO_2 incubator.

MEFs were transfected using Trasfectin (Biorad) according to the manufacturer's instructions. Transfection of HeLa cells and LnCapB3A with siRNAs and plasmid DNA was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. When indicated in the figure legend, HeLa cells were transfected with siRNA 24 hrs after seeding on glass coverslips and with mtRED/erYFP 48 hrs after plating.

HeLa cells stably overexpressing TpMs were generated by transfecting the cells with the plasmids pEGFP-N3 or TpMs-GFP in which GFP expression was blocked as described previously. Clones were selected by culturing cells in complete medium supplemented with 1 mg/mL G418 and single cell clones were generated by limiting dilution as previously described (Frezza *et al.*, 2006).

References

1. Cipolat S, de Brito OM, Dal Zilio B, and Scorrano L (2004) OPA1 requires mitofusin 1 to promote mitochondrial fusion. *Proc Natl Acad Sci U S A,* **101,** 15927-15932.

2. de Brito OM and Scorrano L (2008) Mitofusin 2 tethers endoplasmic reticulum to mitochondria. *Nature,* **456,** 605-610.

3. Frezza C, Cipolat S, Martins dB, Micaroni M, Beznoussenko GV, Rudka T, Bartoli D, Polishuck RS, Danial NN, De Strooper B, and Scorrano L (2006) OPA1 Controls Apoptotic Cristae Remodeling Independently from Mitochondrial Fusion. *Cell,* **126,** 177-189.

4. Vecchione A, Fassan M, Anesti V, Morrione A, Goldoni S, Baldassarre G, Byrne D, D'Arca D, Palazzo JP, Lloyd J, Scorrano L, Gomella LG, Iozzo RV, and Baffa R (2009) MITOSTATIN, a putative tumor suppressor on chromosome 12q24.1, is downregulated in human bladder and breast cancer. *Oncogene,* **28,** 257-269.

Legends to supplementary figures

Fig. S1 : **Mitochondrial targeting and fragmentation by TpMs does not require keratins.**

(**A**) HeLa cells were cotransfected with mtRFP and the indicated TpMs-GFP chimeras and after 24 hrs representative confocal images were acquired. Scale bar, 20 µm.

(**B**) HeLa cells were transfected with the indicated plasmids and after 24 hrs equal amounts (40 µg) of protein from mitochondrial, ER and cytosolic fractions were separated by SDS-PAGE and immunoblotted using the indicated antibodies. MnSOD, Mn-dependent superoxide dismutase; SERCA, sarco/endoplasmic reticulum Ca^{2+} ATPase; LDH, lactate dehydrogenase.

(**C**) Subcellular fractions were prepared from MEFs transfected with the indicated constructs and equal amounts (40 µg) of protein from the indicated fractions were separated by SDS-PAGE and immunoblotted using the indicated antibodies.

(**D**) Left : Representative confocal images of MEFs cotransfected with mtRFP and empty vector or TpMs-GFP. Scale bar, 20 µm. Right: Morphometric analysis of mitochondrial shape. 120 randomly selected images of mtRFP fluorescence were acquired, stored, and classified as described. Data represent mean ±SE of 3 independent experiments.

Fig. S2: **TpMs interacts with keratins.**

(**A**) Lysates from HeLa cells were prepared and equal amounts of protein (500 µg) dissolved in lysis buffer were immunoprecipitated with the indicated antibodies, separated by SDS-PAGE and immunoblotted using an anti-K8/K18 antibody. Input represents a 1:10 dilution of the total lysates.

(**B**) Pre-cleared lysates from HeLa cells were prepared and equal amounts of protein (500 µg) dissolved in CHAPS buffer were immunoprecipitated with the indicated antibodies (input represents a 1:10 dilution) and coprecipitated proteins were separated by SDS-PAGE and immunoblotted with the indicated antibodies.

(**C**) Representative images of HeLa cells transfected as indicated. Cells were fixed and immunostained with anti-cytokeratin8/18 (K8/K18), rhodamine-conjugated phalloidin or anti-tubulin antibody. Scale bar, 20 µm.

(**D**) Representative confocal images of HeLa cells cotransfected with mtCFP (cyan) and TpMs-V5. Cells were fixed and immunostained with FITC-conjugated anti-K8/K18 (green) and TRITC-conjugated anti-V5 antibodies (red). The merged image is also shown. The boxed areas are magnified 9 folds. Scale bar, 20 µm. The arrow represents a group of mitochondria where the blue color is clearlydistinguishable from violet color of the overlay of mitochondria and TpMs.

Fig. S3 : **Expression levels of TpMs.**

HeLa cells were transfected as indicated. After 24 h (**A**), or at the indicated times cells (**B**) cells were lysed and protein (20 µg) was analyzed by SDS-PAGE/immunoblotting.

(**C**) HeLa cells stably overexpressing empty vector or TpMs were lysed and equal amounts of protein (20 µg) were separated by SDS-PAGE and immunoblotted with the indicated antibodies.

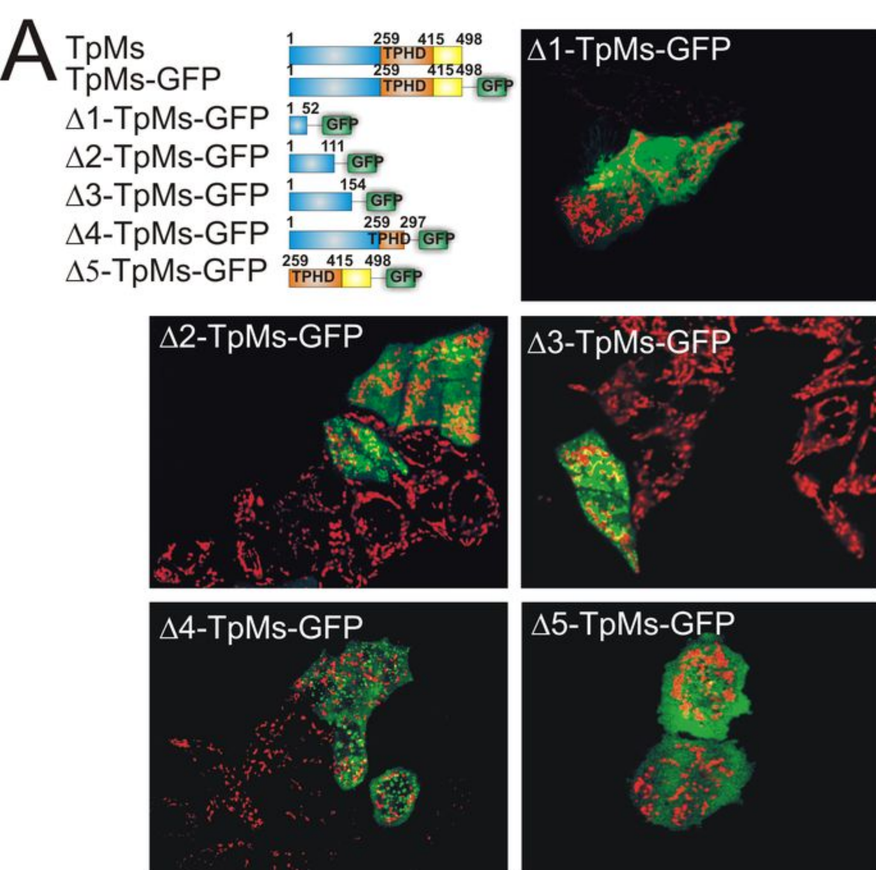
(**D**) HeLa cells were transfected as indicated Twenty-four (plasmids) or 48 hrs (siRNA) after the indicated transfections cells were lysed and protein (20 µg) was analyzed by SDS-PAGE/immunoblotting.

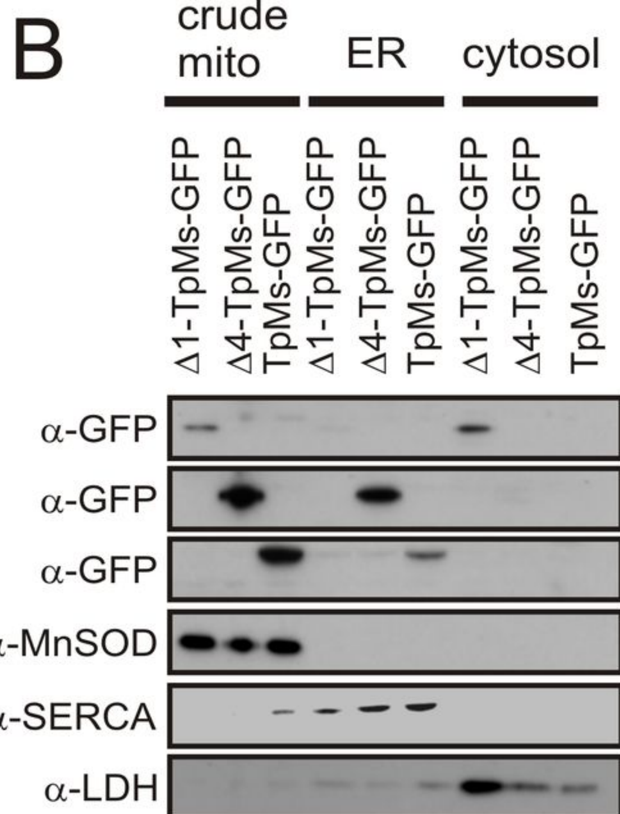
Fig. S4: **MEFs overexpressing TpMs are protected from hydrogen peroxide induced cell death.**

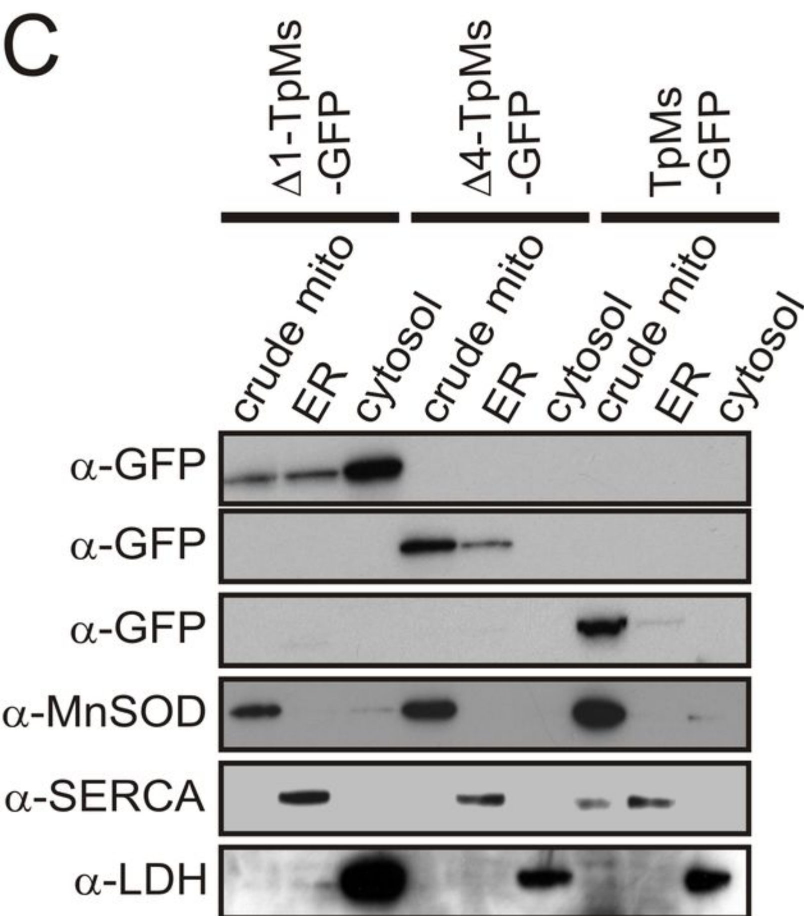
(**A**) MEFs transfected as indicated were treated with 2 µm STS for 6 hrs or 5 µm etoposide for 48 hrs. Cell death was determined cytofluorimetrically as the percentage of YFP-positive, annexin-V-Alexa568-positive cells. Data represent mean ±SE of 3 independent experiments.

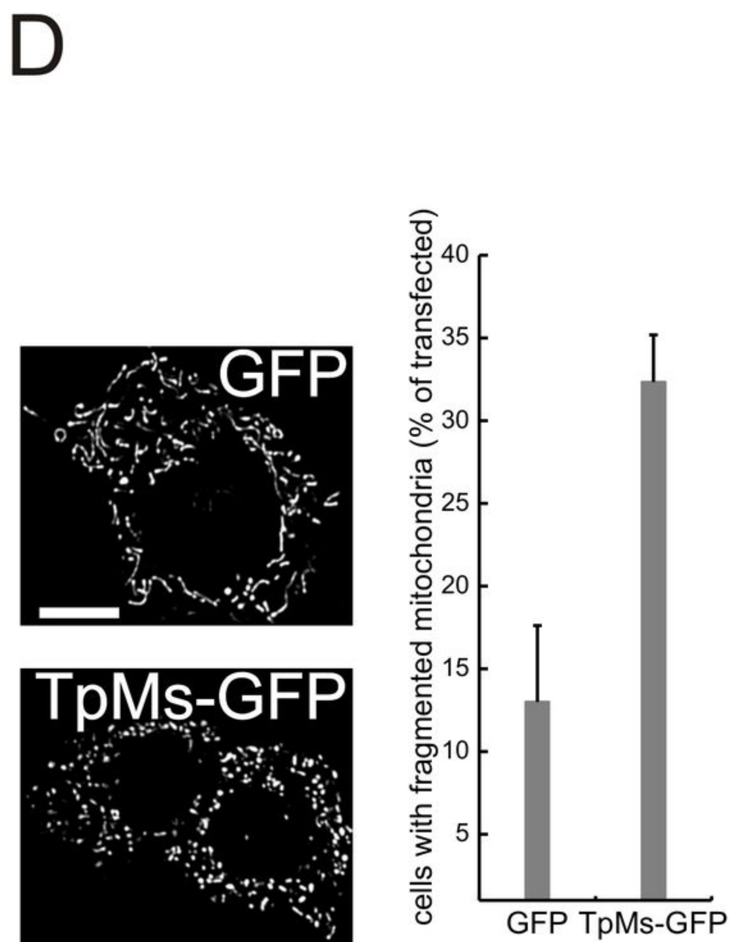
(**B**) MEFs were cotransfected with TpMs-V5 and pEGFP or tBID-GFP. After 48 hrs cell death was determined cytofluorimetrically as the percentage of GFP-positive, annexin-V-Alexa568-positive cells. Data represent mean ±SE of 3 independent experiments.

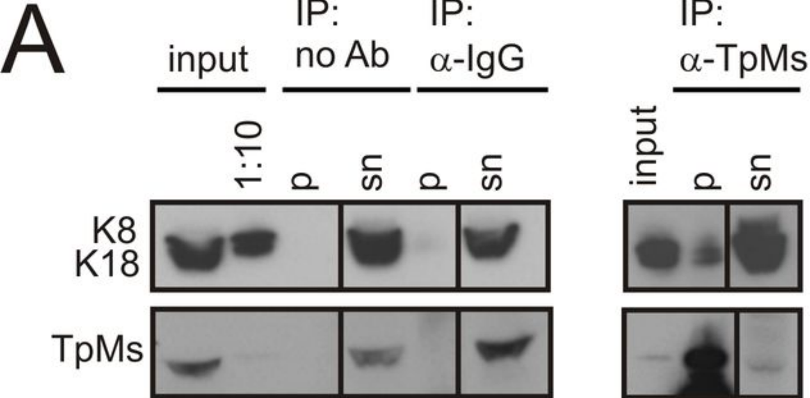
(**C**) MEFs transfected as indicated were treated with 1 mM H_2O_2. At the indicated times, viability was determined cytofluorimetrically as the percentage of YFP-positive, annexin-V-Alexa568-negative cells. Data represent mean ±SE of 3 independent experiments.

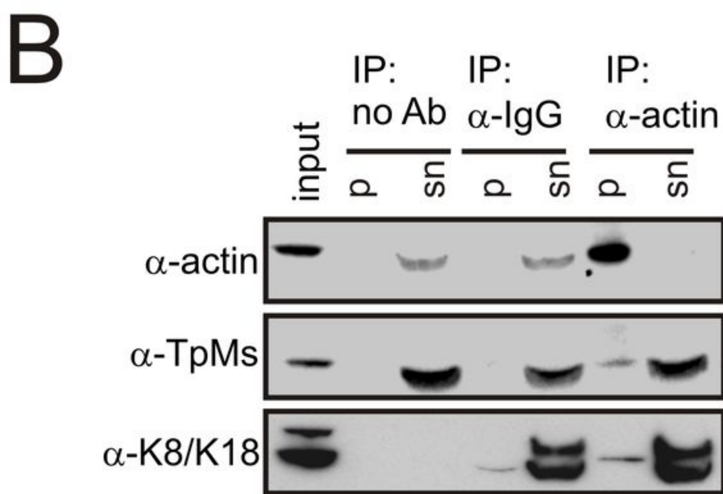

A
TpMs
TpMs-GFP
Δ1-TpMs-GFP
Δ2-TpMs-GFP
Δ3-TpMs-GFP
Δ4-TpMs-GFP
Δ5-TpMs-GFP
TPHD
GFP
1
259
415
498
52
111
154
297
Δ1-TpMs-GFP
Δ2-TpMs-GFP
Δ3-TpMs-GFP
Δ4-TpMs-GFP
Δ5-TpMs-GFP


B
crude mito
ER
cytosol
Δ1-TpMs-GFP
Δ4-TpMs-GFP
TpMs-GFP
α-GFP
α-GFP
α-GFP
α-MnSOD
α-SERCA
α-LDH


C
Δ1-TpMs -GFP
Δ4-TpMs -GFP
TpMs -GFP
crude mito
ER
cytosol
α-GFP
α-GFP
α-GFP
α-MnSOD
α-SERCA
α-LDH


D
GFP
TpMs-GFP
cells with fragmented mitochondria (% of transfected)
40
35
30
25
20
15
10
5
GFP
TpMs-GFP


A
input
IP:
no Ab
IP:
α-IgG
IP:
α-TpMs
1:10
p
sn
p
sn
input
p
sn
K8
K18
TpMs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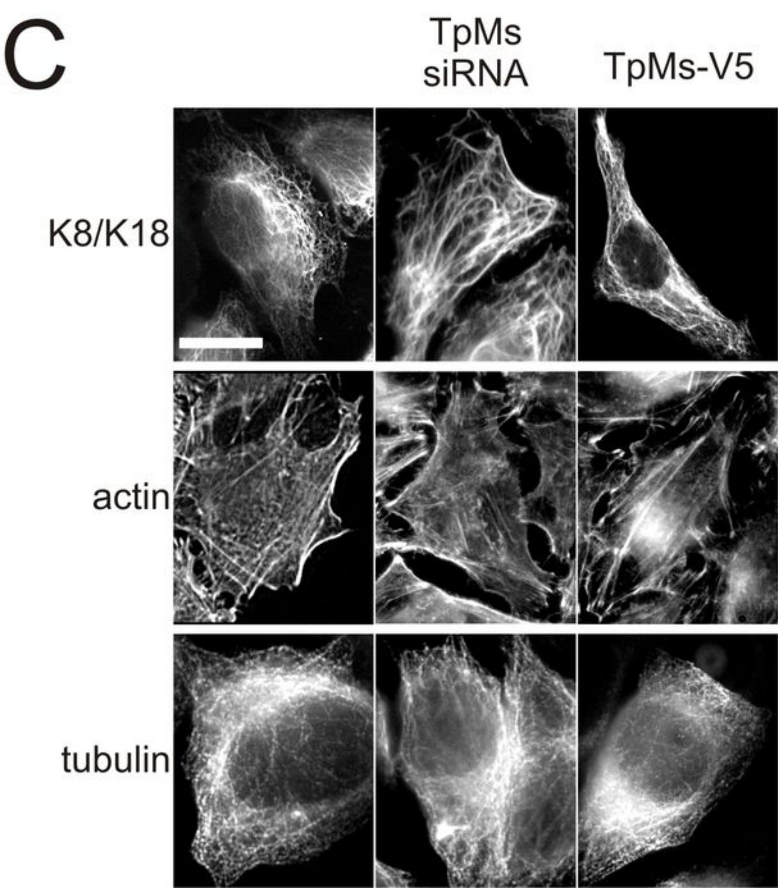
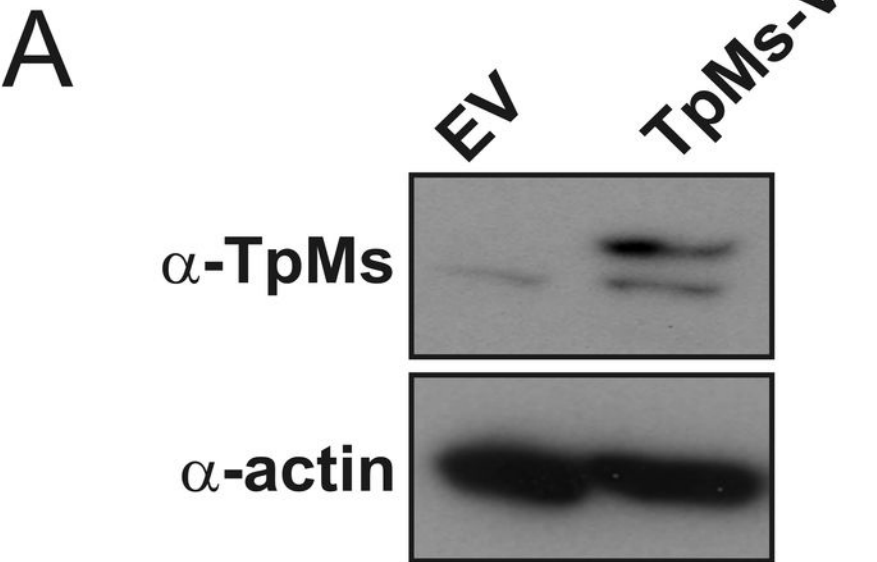
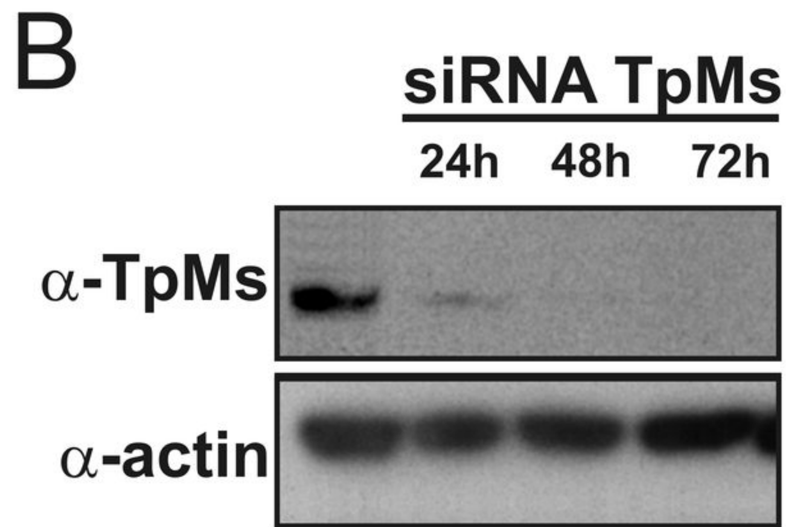
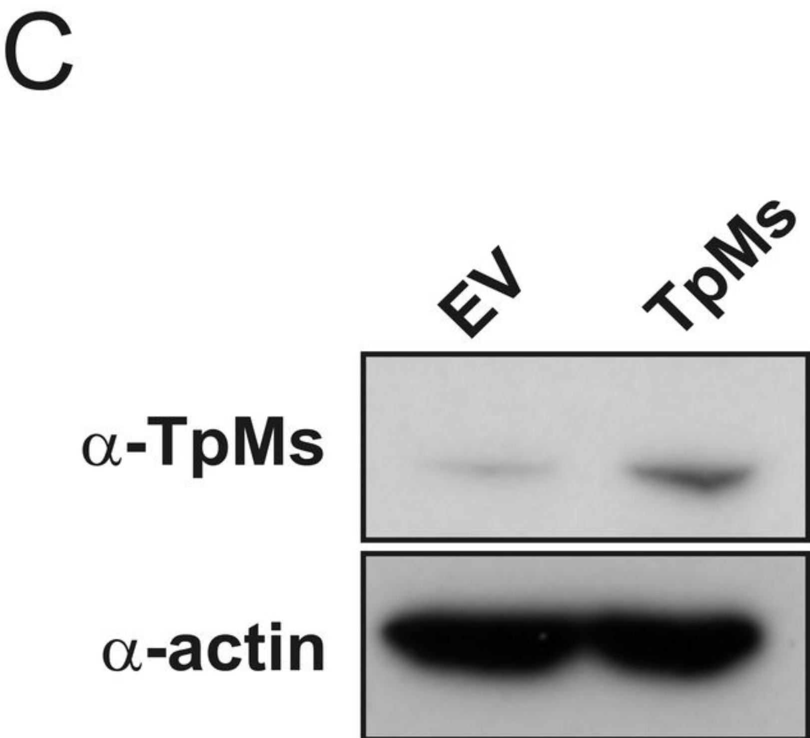
IP:
no Ab
IP:
α-IgG
IP:
α-actin
input
p
sn
p
sn
p
sn
α-actin
α-TpMs
α-K8/K18

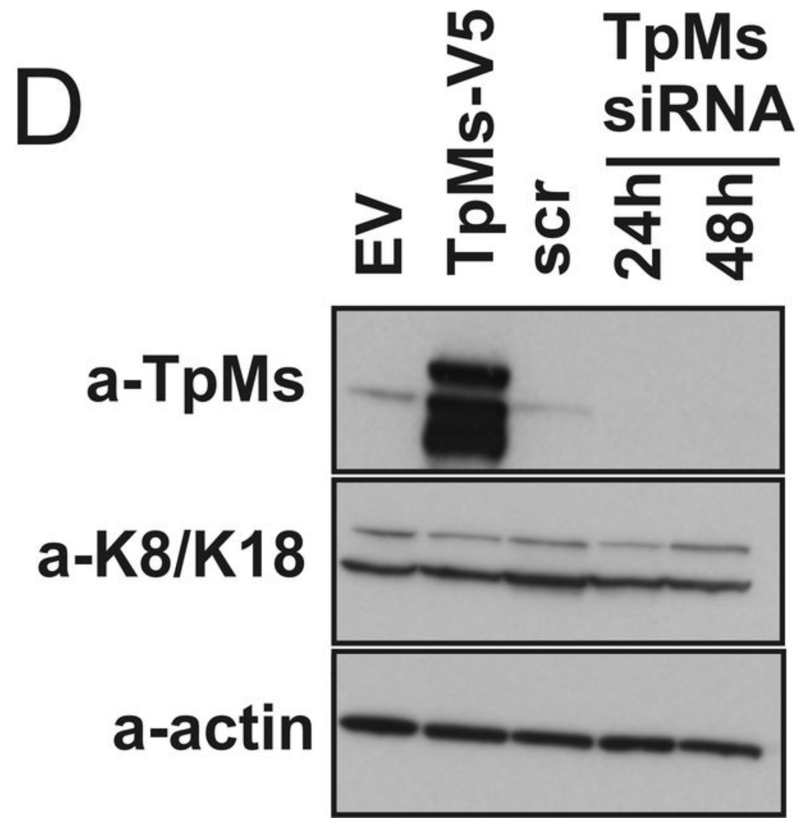

C
TpMs
siRNA
TpMs-V5
K8/K18
actin
tubulin

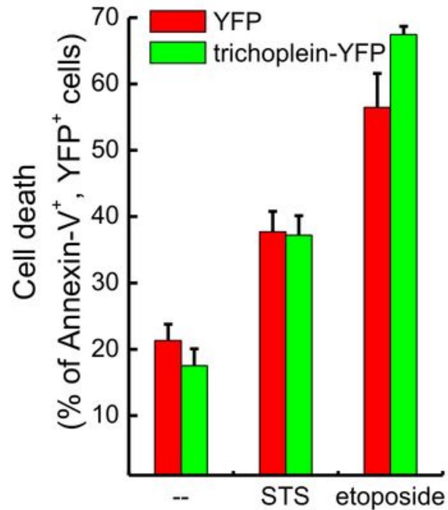
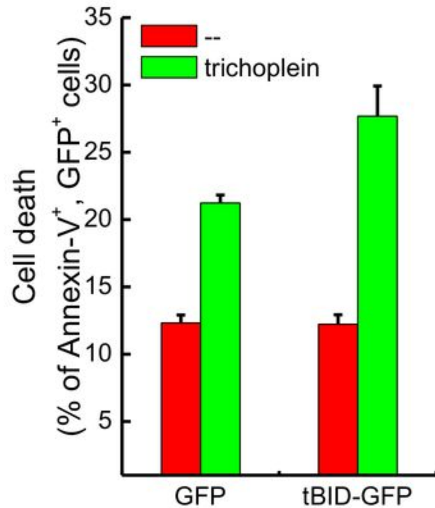
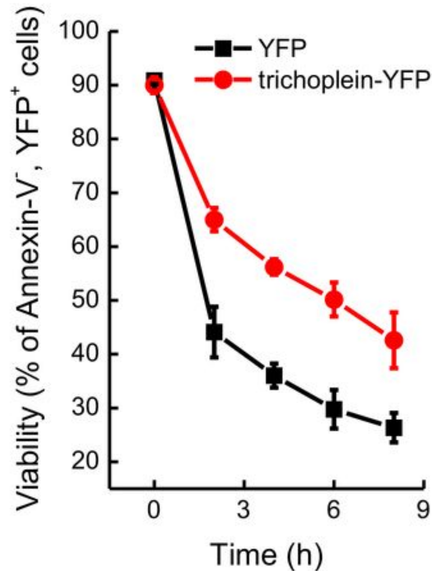
D
mtCFP
K8/K18
TpMs-V5
merge


A
EV
TpMs-V5
α-TpMs
α-actin


B
siRNA TpMs
24h
48h
72h
α-TpMs
α-actin


C
EV
TpMs
α-TpMs
α-actin


D
EV
TpMs-V5
scr
TpMs
siRNA
24h
48h
a-TpMs
a-K8/K18
a-actin


A
Cell death
(% of Annexin-V+, YFP+ cells)
YFP
trichoplein-YFP
70
60
50
40
30
20
10
--
STS
etoposide

B
Cell death
(% of Annexin-V+, GFP+ cells)
--
trichoplein
35
30
25
20
15
10
5
GFP
tBID-GFP

C
Viability (% of Annexin-V-, YFP+ cells)
YFP
trichoplein-YFP
100
90
80
70
60
50
40
30
20
0
3
6
9
Time (h)