14-3-3 proteins protect AMPK-phosphorylated ten-eleven translocation-2 (TET2) from PP2A-mediated dephosphorylation

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Running title: *Dynamic regulation of TET2 by 14-3-3*

Supplementary Figure Legends.

Fig. S1. Antibody validation. (A) Validation of 14-3-3-θ and 14-3-3-ε specific antibodies by si-RNA knockdown in HEK293T cells. **(B)** TET2 antibody validation by overexpression of cDNA in HEK293T cells. **(C)** Sequence alignment demonstrating presence of S99 in reported isoforms of TET2. **(D**) WT and S99A TET2 were immunoprecipitated from HEK293T and analyzed by IB with phosphoserine 14-3-3 binding motif antibody. Data are biologic replicate corresponding to Fig. 1F.

Fig. S2. Impact of TET2 S99 phosphorylation on protein stability. (A) Biologic replicate corresponding to Fig. 2A-B. HEK293T cells were transfected with Myc-TET2 WT or Myc-TET2 S99A. After 36 hrs, cells were incubated with cycloheximide (CHX). Whole cell extracts were collected at the indicated time points and analyzed by IB. Ratios of Myc-band intensities normalized to actin are shown on the top. **(B)** Biologic replicate corresponding to Fig. 2C. HEK293T cells were transfected with WT and S99A TET2. After 24h hours, transfected cells were then treated with either vehicle (untreated), 50 mM calpeptin, or 100 nM bortezomib for 24 hrs followed by immunoblotting of whole cell extracts.

Fig. S3. Role of AMPK in TET2 phosphorylation. (A) Myc-TET2 WT and Myc-TET2 S99A, Flag-AMPK-WT and Flag-AMPK-KD constructs were transfected into HEK293T cells in different combinations as displayed in figure. Myc-immunoprecipitates were analyzed by immunoblot. Middle lane represents marker lane (M). **(B)** HEK293T cells were transfected with Myc-TET2 followed by treatment +/-metformin for 2 hours. Myc-TET2 was immunoprecipitated and analyzed by IB with p-14-3-3 binding motif antibody. The black line shows the border between two splices of a blot. **(C)** Whole cell lysates from AMPK WT (+/+) and AMPK KO (-/-) MEFs were collected after treatment with metformin +/- compound C (24 hrs treatment) and analyzed by IB. DMSO treated cells were set as control. Ratios of Myc-band intensities normalized to actin-band intensities are shown on the top. **(D)** Global 5-hmC levels of genomic DNA extracted from

HEK293T cells transfected with WT (8 ug) or S99A mutant TET2 (10 ug) plasmids. Methylene blue (MB) blots are included for loading control. **(E)** Global 5-hmC levels of genomic DNA extracted from AMPK +/+ MEF cells treated with DMSO (control) or AICAR for 24 hrs. Ratios of 5hmC dot intensities are shown below the blots.

Fig. S4. 14-3-3 interaction promotes TET2 stability. (A) Endogenous TET2 levels in HEK293T cells transfected with control vector (8 ug) or difopein construct (8 ug). **(B, C)** Stabilities of Myc-TET2 WT and Myc-TET2 S99A in the presence and absence of difopein. Quantification from three biological replicates is provided in panel C. **(D)** HEK293T were transfected with either CV or difopein construct. After 24 hours, cells were treated with CHX (50 ug/ml) and harvested at the indicated time points followed by analysis of endogenous TET2. **(E)** HEK293T cells (one 10 cm plate) were co-transfected with either (left panel) WT Myc-TET2 (8 ug) and CV (10 ug) or (right panel) Myc-TET2 WT (10 ug) and difopein (10 ug) constructs. After 24 hours, cells were treated with CHX (50 ug/ml) and harvested at the indicated time point followed by IB using anti-Myc antibody. **(F)** HEK293T cells (one 10 cm plate) were co-transfected with either (left panel) Myc-TET2 S99A (8 ug) and CV (10 ug) or (right panel) Myc-TET2 S99A (10 ug) and difopein (10 ug) constructs. After 24 hours, cells were treated with CHX (50 ug/ml) and harvested at the indicated time point followed by IB using anti-Myc antibody.

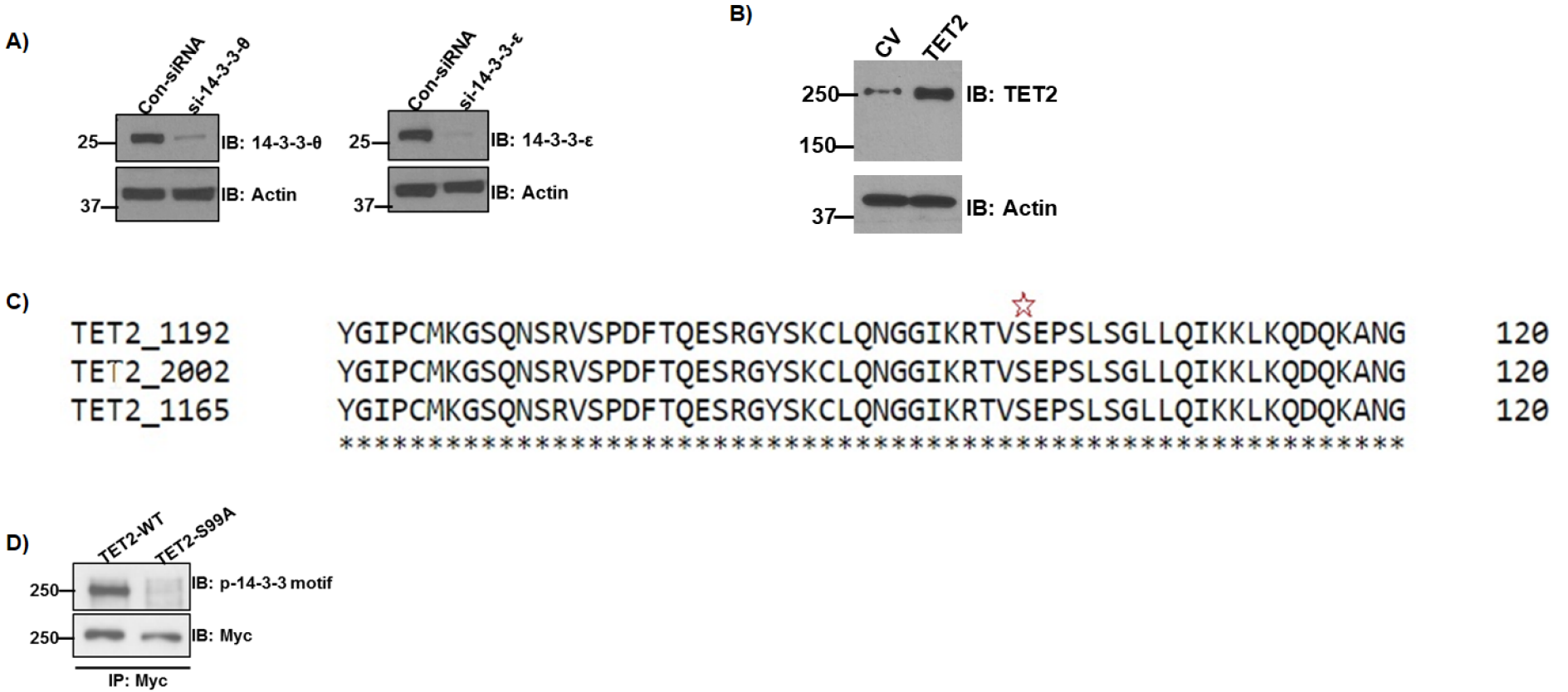
Figure S5. 14-3-3 protects S99 phosphorylation of TET2 *in vitro*.

Figure S6. Endogenous TET2 levels in HEK293T cells treated with siPP2A-Bα (construct #1).

Fig. S7. 14-3-3 protects TET2 S99 phosphorylation from PP2A. (A) HEK293T cells were transfected with Myc-TET2. 24 hours following transfection, cells were treated +/- PP2A inhibitor okadic acid (OKA) (two hour treatment). Immunoprecipitates were then analyzed by immunoblot with the indicated antibodies. **(B)** HEK293T cells were cotransfected with Myc-TET2 and difopein constructs. Forty-eight hours after transfection, cells were treated with the indicated concentrations of okadic acid (OKA) for 2 hours. Anti-Myc immunoprecipitates were assessed for S99 phosphorylation with p-serine 14-3-3 binding motif antibody. **(C)** HEK293T cells were transfected with the indicated Myc-TET2 construct followed by the indicated treatment. Myc IPs were then analyzed for S99 phosphorylation using the phosphoserine 14-3-3 binding motif antibody. **(D)** Endogenous TET2 levels were analyzed after the indicated treatments for 24 hrs.

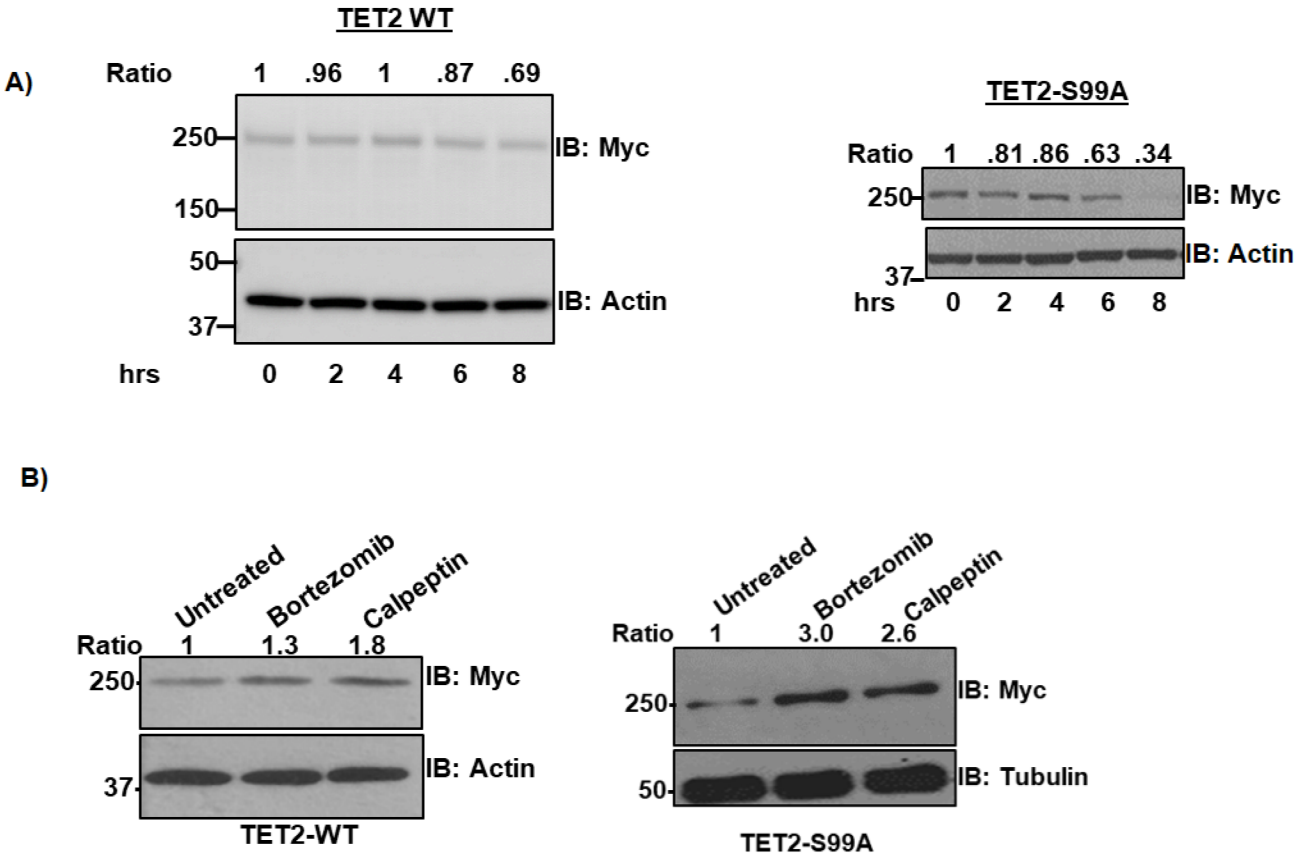
Supplementary Figures

Figure S1



Supplementary Figures

Figure S2



Supplementary Figures

Figure S3

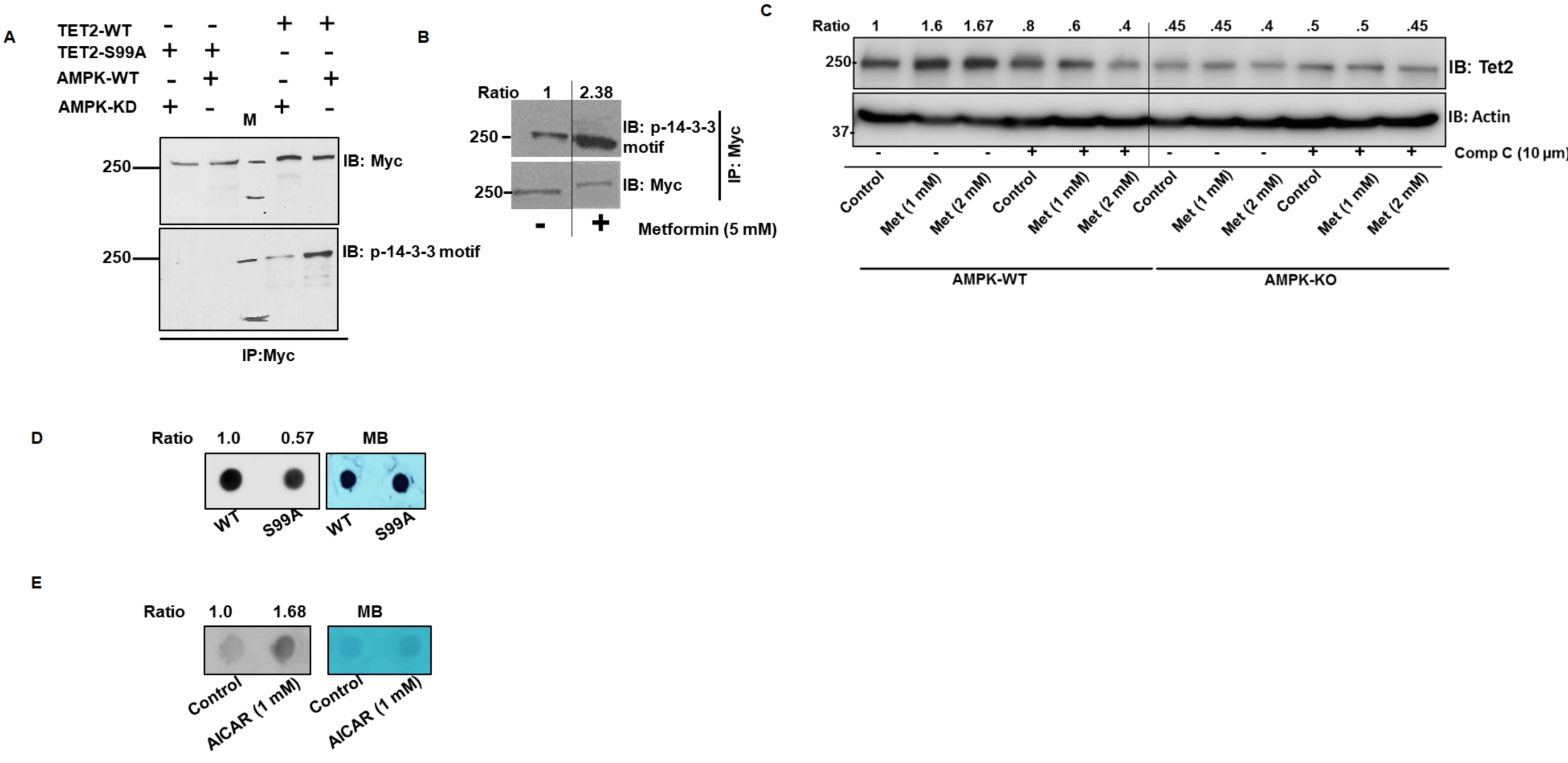
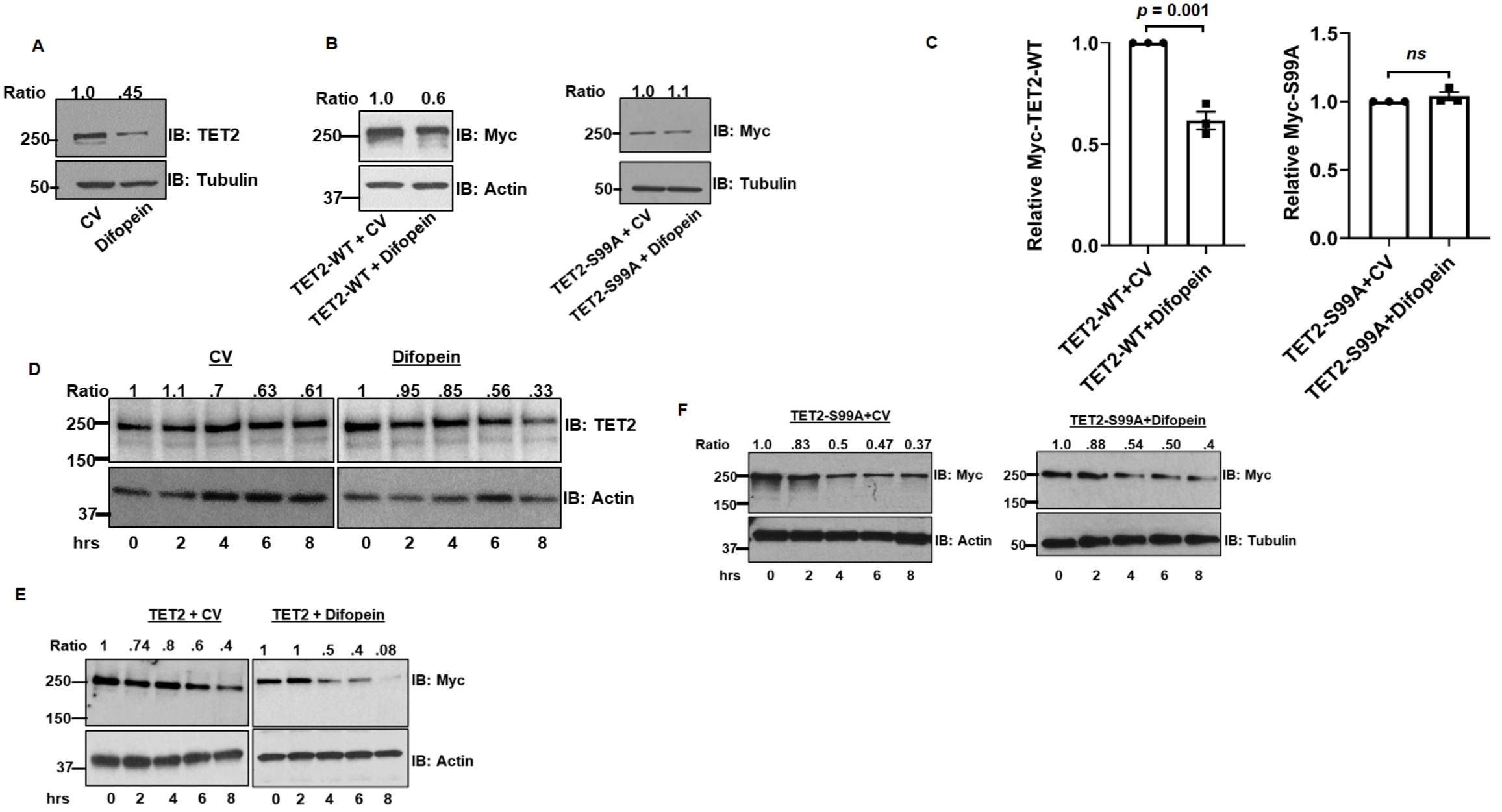
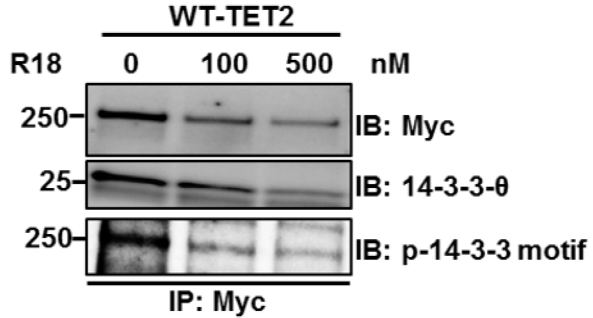


Figure S4


A
Ratio 1.0 .45
250
IB: TET2
50
IB: Tubulin
CV
Difopein
B
Ratio 1.0 0.6
250
IB: Myc
37
IB: Actin
TET2-WT + CV
TET2-WT + Difopein
Ratio 1.0 1.1
250
IB: Myc
50
IB: Tubulin
TET2-S99A + CV
TET2-S99A + Difopein
C
p = 0.001
Relative Myc-TET2-WT
1.0
0.5
0.0
TET2-WT+CV
TET2-WT+Difopein
ns
Relative Myc-S99A
1.5
1.0
0.5
0.0
TET2-S99A+CV
TET2-S99A+Difopein
D
CV
Difopein
Ratio 1 1.1 .7 .63 .61 1 .95 .85 .56 .33
250
150
37
IB: TET2
IB: Actin
hrs 0 2 4 6 8 0 2 4 6 8
E
TET2 + CV
TET2 + Difopein
Ratio 1 .74 .8 .6 .4 1 1 .5 .4 .08
250
150
37
IB: Myc
IB: Actin
hrs 0 2 4 6 8 0 2 4 6 8
F
TET2-S99A+CV
Ratio 1.0 .83 0.5 0.47 0.37
250
150
37
IB: Myc
IB: Actin
hrs 0 2 4 6 8
TET2-S99A+Difopein
1.0 .88 .54 .50 .4
250
150
50
IB: Myc
IB: Tubulin
0 2 4 6 8

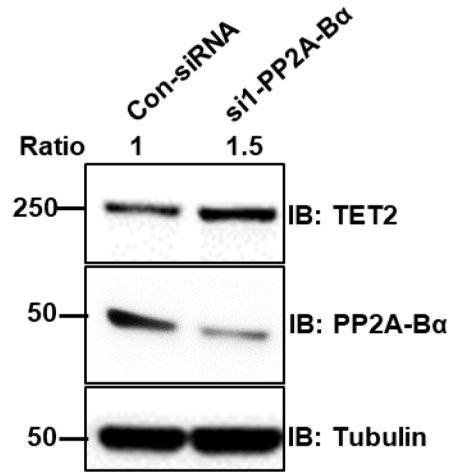
Supplementary Figures

Figure S5



Supplementary Figures

Figure S6



Supplementary Figures

Figure S7