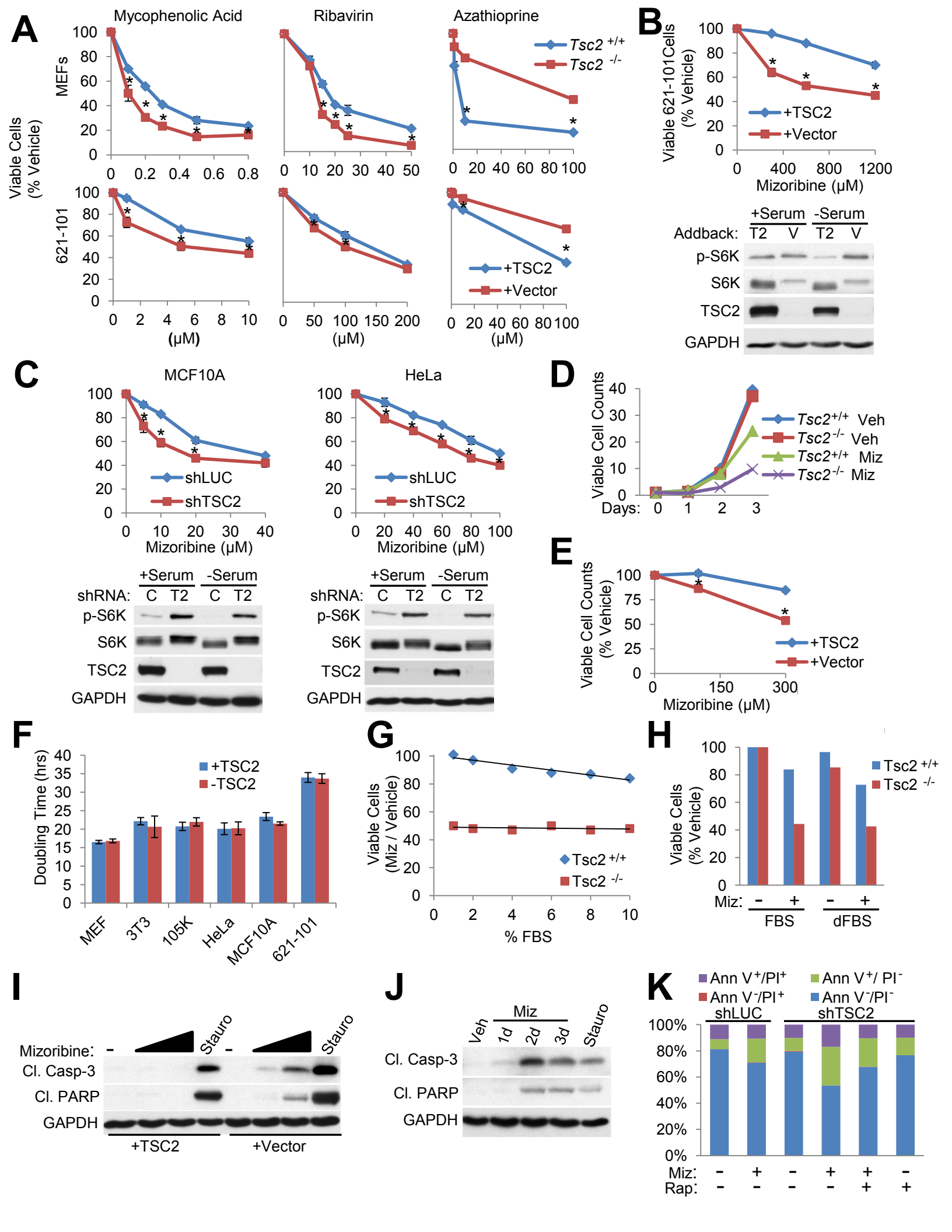
A
Mycophenolic Acid
Ribavirin
Azathioprine
Tsc2 +/+
Tsc2 -/-
MEFs
621-101
Viable Cells (% Vehicle)
(μM)
B
Viable 621-101Cells (% Vehicle)
+TSC2
+Vector
Mizoribine (μM)
+Serum
-Serum
Addback:
T2
V
p-S6K
S6K
TSC2
GAPDH
C
MCF10A
HeLa
shLUC
shTSC2
Mizoribine (μM)
shRNA:
C
T2
D
Viable Cell Counts
Tsc2+/+ Veh
Tsc2-/- Veh
Tsc2+/+ Miz
Tsc2-/- Miz
Days:
E
Viable Cell Counts (% Vehicle)
Mizoribine (μM)
F
Doubling Time (hrs)
+TSC2
-TSC2
MEF
3T3
105K
HeLa
MCF10A
621-101
G
Viable Cells (Miz / Vehicle)
% FBS
H
Viable Cells (% Vehicle)
Miz:
FBS
dFBS
I
Mizoribine:
Stauro
Cl. Casp-3
Cl. PARP
GAPDH
J
Veh
Miz
1d
2d
3d
K
Ann V+/PI+
Ann V+/ PI-
Ann V-/PI+
Ann V-/PI-
Rap:

Figure S1, related to Figure 1.

(A) *Tsc2*$^{+/+}$;*Trp53*$^{-/-}$ and *Tsc2*$^{-/-}$;*Trp53*$^{-/-}$ MEFs (top graphs) and 621-101 cells with stable reconstitution of TSC2 or empty vector (lower graphs) were treated with the indicated compounds at indicated concentrations for 72 hr and viable cells are graphed as a percentage of vehicle-treated cells. n=6 biological replicates

(B,C) 621-101 cells with stable reconstitution of TSC2 or empty vector and MCF10A or HeLa cells stably expressing shRNA targeting luciferase or TSC2 (C) were treated with indicated doses of mizoribine for 72 hr and viable cells are graphed as a percentage of vehicle-treated cells. Below, mTORC1 signaling was assessed by immunoblot in these cells grown overnight in the presence or absence of serum. n=6 biological replicates

(D) Proliferation curves of *Tsc2*$^{+/+}$;*Trp53*$^{-/-}$ and *Tsc2*$^{-/-}$;*Trp53*$^{-/-}$ MEFs treated with vehicle or mizoribine (2 μM) for the indicated times measured as viable cell counts by trypan blue exclusion and graphed relative to day 0. n=3 biological replicates

(E) Viable cell counts in 621-101 cells with stable reconstitution of TSC2 or empty vector treated with vehicle or indicated concentrations of mizoribine for 72 hr measured by trypan blue exclusion and graphed as percent of vehicle treated cells. n=4 biological replicates

(F) Doubling times of the indicated TSC2-expressing and TSC2-deficient cell pairs measured over a 72 hr time course. n=3 biological replicates

(G) *Tsc2*$^{+/+}$;*Trp53*$^{-/-}$ and *Tsc2*$^{-/-}$;*Trp53*$^{-/-}$ MEFs were cultured in media containing indicated concentrations of FBS for 72 hr with vehicle or mizoribine (2 μM) and viability of mizoribine treated cells is graphed relative to vehicle under each serum concentration. n=3 biological replicates

(H) *Tsc2*$^{+/+}$;*Trp53*$^{-/-}$ and *Tsc2*$^{-/-}$;*Trp53*$^{-/-}$ MEFs were cultured in media containing 10% fetal bovine serum (FBS) or dialyzed FBS (dFBS) for 72 hr with vehicle or mizoribine (2 μM) and cell viability is graphed as percent of vehicle treated cells in FBS. n=3 biological replicates

(I) *Tsc2*$^{-/-}$;*Trp53*$^{+/+}$ 3T3-immortalized MEFs with stable reconstitution of TSC2 or empty vector were treated with vehicle, mizoribine (2 μM or 5 μM, 48 hr), or staurosporine (5 μM, 4 hr) followed by immunoblot for indicated proteins.

(J) *Tsc2*$^{-/-}$;*Trp53*$^{-/-}$ MEFs were treated with mizoribine (2 μM) for the indicated times or staurosporine (5 μM) for 3 hr followed by immunoblot for indicated proteins.

(K) HeLa cells stably expressing shRNA targeting luciferase or TSC2 were treated as indicated with vehicle, mizoribine (35 μM), or rapamycin (20 nM) for 72 hr. Cell death was quantified by Annexin V (Ann V)/Propidium Iodide (PI) staining measured by flow cytometry, and graphed as the percentage of the total cell population. n=3 biological replicates

Graphical data are represented as mean of indicated replicates, error bars represent ± SEM. *$p<0.05$ by two-tailed Student's t test

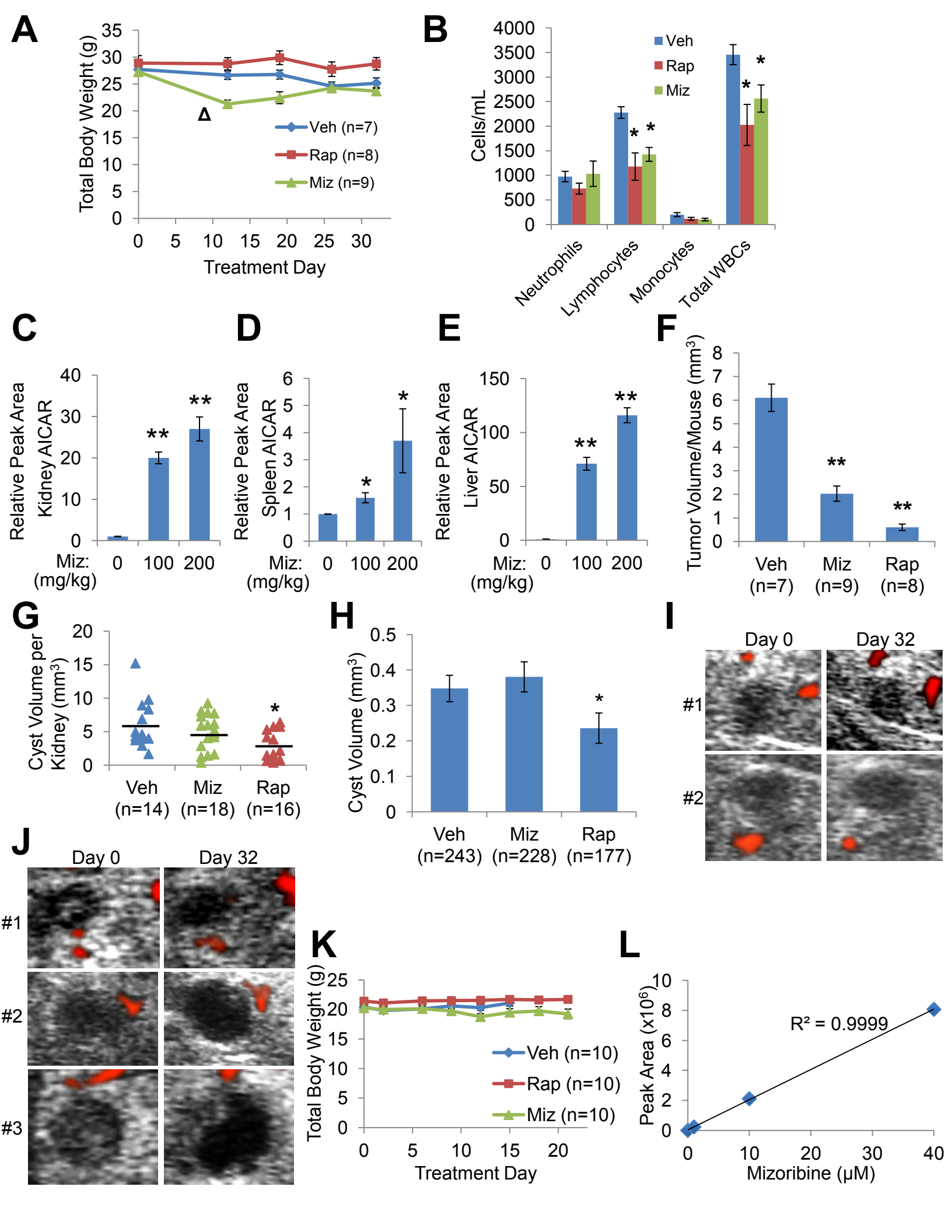
A
Total Body Weight (g)
Treatment Day
Δ
Veh (n=7)
Rap (n=8)
Miz (n=9)
B
Cells/mL
Veh
Rap
Miz
Neutrophils
Lymphocytes
Monocytes
Total WBCs
C
Relative Peak Area Kidney AICAR
Miz: (mg/kg)
D
Relative Peak Area Spleen AICAR
E
Relative Peak Area Liver AICAR
F
Tumor Volume/Mouse (mm3)
Veh (n=7)
Miz (n=9)
Rap (n=8)
G
Cyst Volume per Kidney (mm3)
Veh (n=14)
Miz (n=18)
Rap (n=16)
H
Cyst Volume (mm3)
Veh (n=243)
Miz (n=228)
Rap (n=177)
I
Day 0
Day 32
#1
#2
J
Day 0
Day 32
#1
#2
#3
K
Total Body Weight (g)
Treatment Day
Veh (n=10)
Rap (n=10)
Miz (n=10)
L
Peak Area (x10^6)
R² = 0.9999
Mizoribine (μM)

Figure S2, related to Figure 2.

(A) Mean body weight of *Tsc2*$^{+/-}$ mice in each treatment group from Figure 2 (Δ = last day of 200 mg/kg/day mizoribine before 5 day break followed by 100 mg/kg/day). n=mice/group

(B) White blood cell (WBC) counts from the mice in A measured 3 hr after the final treatment injection. n=7-9 mice/group

(C-E) Wild type A/J mice were injected daily with vehicle or indicated doses of mizoribine for 5 days and AICAR levels were measured by LC-MS/MS and graphed relative to vehicle in kidney (C), spleen (D), and liver (E) harvested 3 hr after the final treatment injection. n=4-5 mice/group

(F-H) From the mice in A at the completion of treatment: tumor and cyst measurements represented as total tumor volume per mouse (n=mouse number) (F), total cyst volume per kidney (n=kidney number) (G), or average volume per cyst (n=cyst number) (H).

(I,J) Ultrasound imaging of renal cystadenomas from 2 vehicle-treated (I) and 3 mizoribine-treated (J) *Tsc2*$^{+/-}$ mice before (Day 0) and after (Day 32) treatment. Individual cystadenomas were identified by their position within the kidney and by Doppler imaging of surrounding vasculature, shown in red.

(K) Total body weight of ELT3 xenograft tumor bearing mice from Figure 2 during the treatment period. n=mouse number

(L) Mizoribine standard curve used to calculate plasma mizoribine concentration in Figure 2I. Mizoribine was added into plasma from untreated mice at final concentrations of 0, 1, 10, and 40 μM (n=3). Metabolites were extracted from these standards alongside plasma from vehicle and mizoribine treated mice, and mizoribine was measured by LC-MS/MS.

Graphical data are represented as mean of indicated replicates, error bars represent ± SEM. *$p<0.05$, **$p<0.00002$ by two-tailed Student's t test

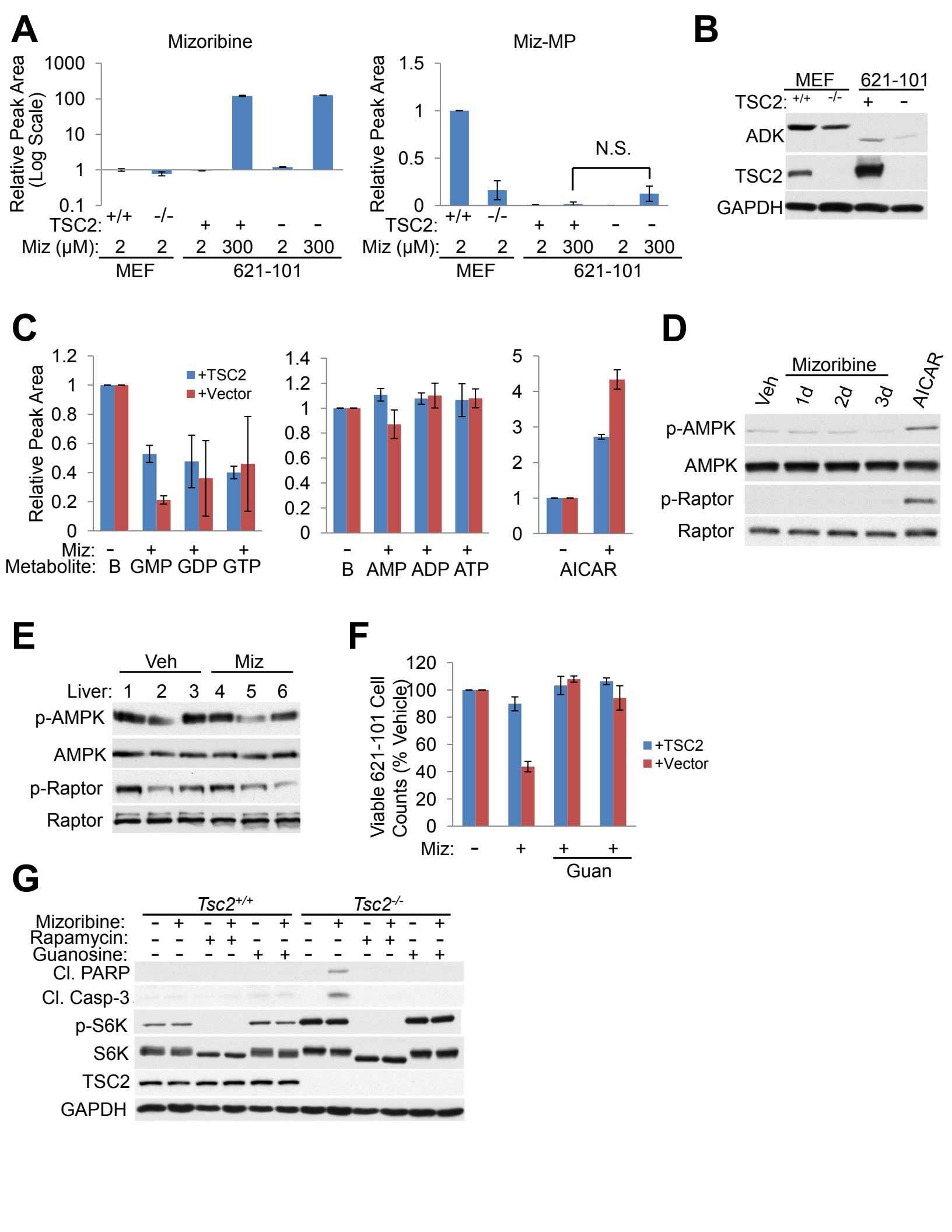
A
Mizoribine
Relative Peak Area (Log Scale)
1000
100
10
1
0.1
TSC2: +/+ -/- + + - -
Miz (μM): 2 2 2 300 2 300
MEF
621-101
Miz-MP
Relative Peak Area
1.5
1
0.5
0
N.S.
TSC2: +/+ -/- + + - -
Miz (μM): 2 2 2 300 2 300
MEF
621-101
B
MEF
621-101
TSC2: +/+ -/- + -
ADK
TSC2
GAPDH
C
Relative Peak Area
+TSC2
+Vector
1.2
1
0.8
0.6
0.4
0.2
0
Miz: - + + +
Metabolite: B GMP GDP GTP
1.4
1.2
1
0.8
0.6
0.4
0.2
0
- + + +
B AMP ADP ATP
5
4
3
2
1
0
- +
AICAR
D
Mizoribine
Veh
1d
2d
3d
AICAR
p-AMPK
AMPK
p-Raptor
Raptor
E
Veh
Miz
Liver: 1 2 3 4 5 6
p-AMPK
AMPK
p-Raptor
Raptor
F
Viable 621-101 Cell Counts (% Vehicle)
120
100
80
60
40
20
0
+TSC2
+Vector
Miz: - + + +
Guan
G
Tsc2+/+
Tsc2-/-
Mizoribine: - + - + - + - + - + - +
Rapamycin: - - + + - - - - + + - -
Guanosine: - - - - + + - - - - + +
Cl. PARP
Cl. Casp-3
p-S6K
S6K
TSC2
GAPDH

Figure S3, related to Figure 3.

(A) Intracellular mizoribine and mizoribine monophosphate (Miz-MP) levels measured by LC-MS/MS in *Tsc2*$^{+/+}$;*Trp53*$^{-/-}$ and *Tsc2*$^{-/-}$;*Trp53*$^{-/-}$ MEFs, and 621-101 cells with stable reconstitution of TSC2 or empty vector, treated with indicated mizoribine concentrations (18 hr). N.S. = not significant. Data are graphed relative to *Tsc2*$^{+/+}$;*Trp53*$^{-/-}$ MEFs treated with 2 μM mizoribine. n=3 biological replicates

(B) Immunoblots for indicated proteins in untreated cells from A.

(C) Relative abundance of the indicated metabolites, measured by LC-MS/MS and normalized to baseline, represented by left bars “B” (the abundance of each metabolite in vehicle-treated cells with values set to 1), from 621-101 cells with stable reconstitution of TSC2 or empty vector treated with vehicle or mizoribine (300 μM, 16 hr). n=3 biological replicates

(D) Immunoblots for indicated proteins from *Tsc2*$^{-/-}$;*Trp53*$^{-/-}$ MEFs treated for indicated times with vehicle, mizoribine (2 μM), or AICAR (250 μM).

(E) Immunoblots for indicated proteins from livers of vehicle or mizoribine treated *Tsc2*$^{+/-}$ mice from Figure 2.

(F) Viable 621-101 cell counts, measured by trypan blue exclusion, after treatment with mizoribine (300 μM, 96 hr) in the presence or absence of guanosine (10 μM), normalized to vehicle-treated cells. n=3 biological replicates

(G) Immunoblots for indicated proteins from *Tsc2*$^{+/+}$;*Trp53*$^{-/-}$ and *Tsc2*$^{-/-}$;*Trp53*$^{-/-}$ MEFs treated for 48 hr with vehicle, mizoribine (2 μM), rapamycin (20 nM), and/or guanosine (50 μM).

Graphical data are represented as mean ± SEM.

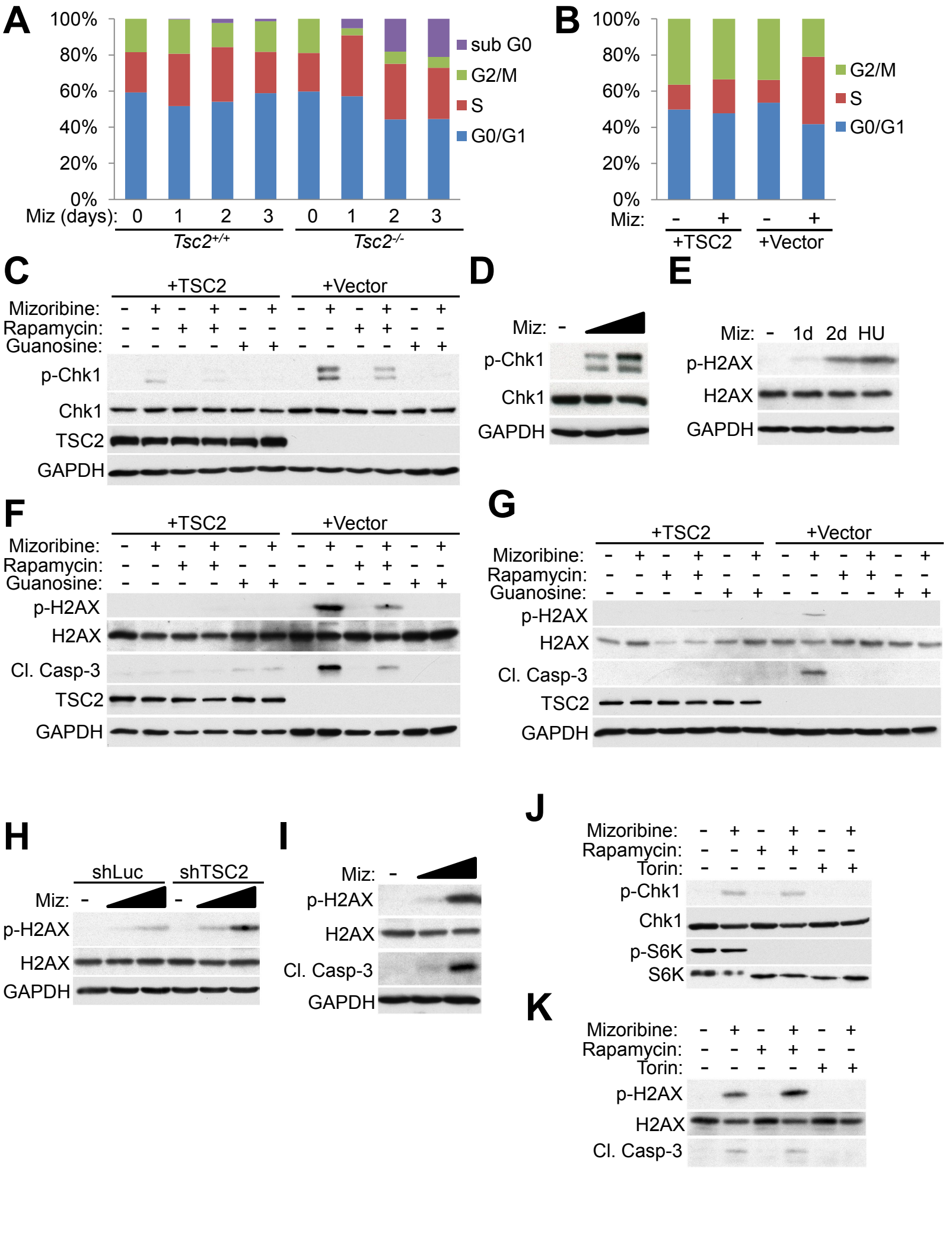
A
100%
80%
60%
40%
20%
0%
sub G0
G2/M
S
G0/G1
Miz (days): 0 1 2 3 0 1 2 3
Tsc2+/+
Tsc2-/-
B
100%
80%
60%
40%
20%
0%
G2/M
S
G0/G1
Miz: - + - +
+TSC2
+Vector
C
+TSC2
+Vector
Mizoribine: - + - + - + - + - + - +
Rapamycin: - - + + - - - - + + - -
Guanosine: - - - - + + - - - - + +
p-Chk1
Chk1
TSC2
GAPDH
D
Miz: -
p-Chk1
Chk1
GAPDH
E
Miz: - 1d 2d HU
p-H2AX
H2AX
GAPDH
F
+TSC2
+Vector
Mizoribine: - + - + - + - + - + - +
Rapamycin: - - + + - - - - + + - -
Guanosine: - - - - + + - - - - + +
p-H2AX
H2AX
Cl. Casp-3
TSC2
GAPDH
G
+TSC2
+Vector
Mizoribine: - + - + - + - + - + - +
Rapamycin: - - + + - - - - + + - -
Guanosine: - - - - + + - - - - + +
p-H2AX
H2AX
Cl. Casp-3
TSC2
GAPDH
H
shLuc
shTSC2
Miz: - -
p-H2AX
H2AX
GAPDH
I
Miz: -
p-H2AX
H2AX
Cl. Casp-3
GAPDH
J
Mizoribine: - + - + - +
Rapamycin: - - + + - -
Torin: - - - - + +
p-Chk1
Chk1
p-S6K
S6K
K
Mizoribine: - + - + - +
Rapamycin: - - + + - -
Torin: - - - - + +
p-H2AX
H2AX
Cl. Casp-3

Figure S4, related to Figure 4.

(A,B) Quantification of cell cycle profiles from *Tsc2*$^{+/+}$*;Trp53*$^{-/-}$ and *Tsc2*$^{-/-}$*;Trp53*$^{-/-}$ MEFs treated for indicated durations (A) or *Tsc2*$^{-/-}$*;Trp53*$^{+/+}$ 3T3 MEFs with stable reconstitution of TSC2 or empty vector treated for 48 hr (B) with vehicle or 2 μM mizoribine based on propidium iodide intensity and graphed as percent of the total population. Data are represented as mean of biological replicates (n=3).

(C) Immunoblots from *Tsc2*$^{-/-}$*;Trp53*$^{+/+}$ 3T3 MEFs with stable reconstitution of TSC2 or empty vector treated as indicated for 24 hr with vehicle, 3.5 μM mizoribine, 20 nM rapamycin, or 50 μM guanosine.

(D) Immunoblots from ELT3 cells treated with vehicle or mizoribine (2 μM or 5 μM, 24 hr).

(E) Immunoblots from *Tsc2*$^{-/-}$*;Trp53*$^{-/-}$ MEFs treated with 2 μM mizoribine for indicated times, or hydroxyurea (HU, 3 mM, 24 hr).

(F,G) Immunoblots from *Tsc2*$^{-/-}$*;Trp53*$^{+/+}$ 3T3 MEFs with stable reconstitution of TSC2 or empty vector (F) or 105K tumor cells with stable reconstitution of TSC2 or empty vector (G) treated as indicated for 48 hr with 3.5 μM mizoribine, 20 nM rapamycin, or 50μM guanosine.

(H) Immunoblots from HeLa cells stably expressing shRNAs targeting luciferase or TSC2 treated with vehicle or increasing doses of mizoribine (25 or 50 μM, 72 hr).

(I) Immunoblots from ELT3 cells treated with vehicle or increasing doses of mizoribine (2 or 5 μM, 48 hr).

(J,K) HCT116 cells treated for 24 hr (J) or 48 hr (K) with vehicle or 250 μM mizoribine alone or in combination with 20 nM rapamycin or 250 nM Torin1.

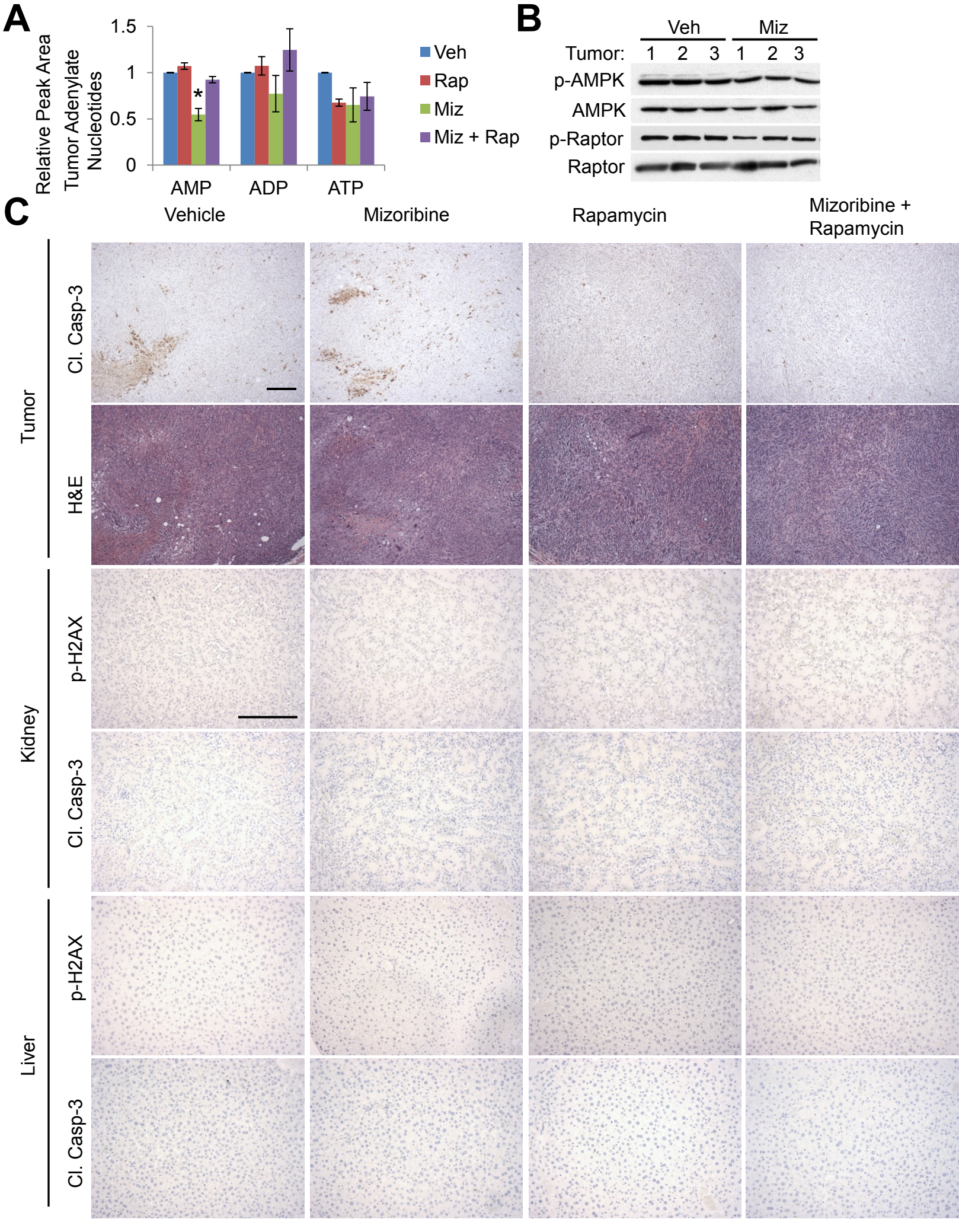
A
Relative Peak Area
Tumor Adenylate
Nucleotides
1.5
1
0.5
0
*
AMP
ADP
ATP
Veh
Rap
Miz
Miz + Rap
B
Veh
Miz
Tumor:
1 2 3 1 2 3
p-AMPK
AMPK
p-Raptor
Raptor
C
Vehicle
Mizoribine
Rapamycin
Mizoribine +
Rapamycin
Tumor
Cl. Casp-3
H&E
Kidney
p-H2AX
Cl. Casp-3
Liver
p-H2AX
Cl. Casp-3

Figure S5, related to Figure 5.

(A) Levels of adenylate nucleotides in ELT3 xenograft tumors measured by LC-MS/MS and graphed relative to vehicle-treated mice. n=3 mice/group. Data are presented as mean ± SEM. *p<0.04 by two-tailed Student's t test.

(B) Immunoblots on vehicle or mizoribine treated ELT3 xenograft tumors from Figure 5 using indicated antibodies.

(C) Immunohistochemical staining on ELT3 xenograft tumors showing necrotic and non-necrotic regions, or on kidneys and livers from the same mice using indicated antibodies. Scale bars = 0.2 mm

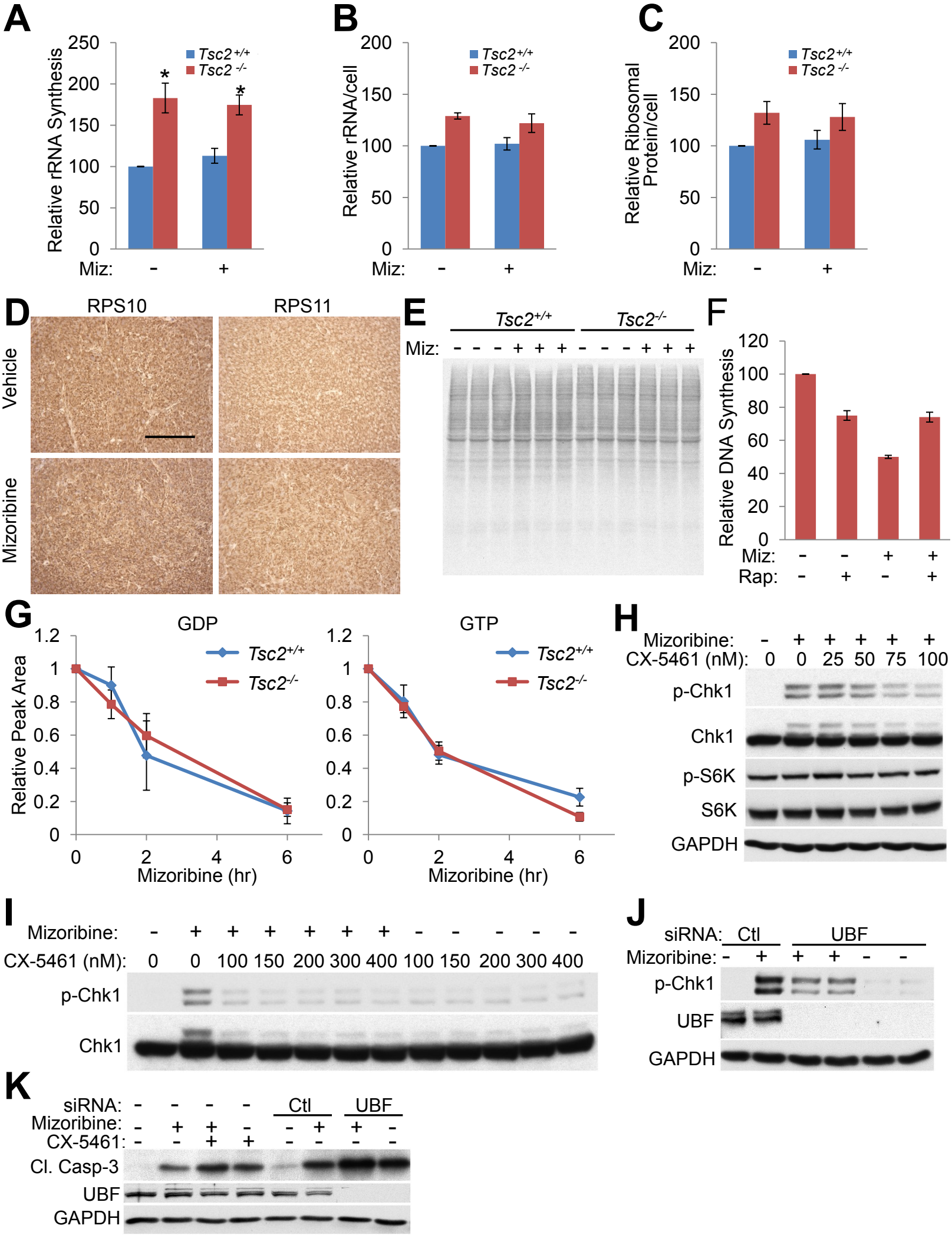
A
Relative rRNA Synthesis
250
200
150
100
50
0
Tsc2+/+
Tsc2 -/-
*
*
Miz: - +
B
Relative rRNA/cell
200
150
100
50
0
Tsc2+/+
Tsc2 -/-
Miz: - +
C
Relative Ribosomal Protein/cell
200
150
100
50
0
Tsc2+/+
Tsc2 -/-
Miz: - +
D
RPS10
RPS11
Vehicle
Mizoribine
E
Tsc2+/+
Tsc2-/-
Miz: - - - + + + - - - + + +
F
Relative DNA Synthesis
120
100
80
60
40
20
0
Miz: - - + +
Rap: - + - +
G
GDP
GTP
Relative Peak Area
1.2
1
0.8
0.6
0.4
0.2
0
Tsc2+/+
Tsc2-/-
0 2 4 6
Mizoribine (hr)
H
Mizoribine: - + + + + +
CX-5461 (nM): 0 0 25 50 75 100
p-Chk1
Chk1
p-S6K
S6K
GAPDH
I
Mizoribine: - + + + + + + - - - - -
CX-5461 (nM): 0 0 100 150 200 300 400 100 150 200 300 400
p-Chk1
Chk1
J
siRNA: Ctl UBF
Mizoribine: - + + + - -
p-Chk1
UBF
GAPDH
K
siRNA: - - - - Ctl UBF
Mizoribine: - + + - - + + -
CX-5461: - - + + - - - -
Cl. Casp-3
UBF
GAPDH

Figure S6, related to Figure 6.

(A) Ribosomal RNA (rRNA) synthesis was measured in *Tsc2*$^{+/+}$*;Trp53*$^{-/-}$ and *Tsc2*$^{-/-}$*;Trp53*$^{-/-}$ MEFs treated for 5 hr with vehicle or 2 μM mizoribine. Cells were labeled with 2,8-^{3}H-adenine for 1 hr. Ribosomes were purified and total ribosomal RNA was isolated. Radioactive counts per minute were normalized to total rRNA and graphed relative to vehicle-treated *Tsc2*$^{+/+}$ cells. n=3 biological replicates

(B,C) *Tsc2*$^{+/+}$*;Trp53*$^{-/-}$ and *Tsc2*$^{-/-}$*;Trp53*$^{-/-}$ MEFs were treated for 6 hr with vehicle or 2 μM mizoribine. Ribosomes were purified and total ribosomal RNA (B) or ribosomal protein (C) was isolated and quantified. Cells were counted in parallel plates treated under the same conditions and data are represented as rRNA or ribosomal protein per cell. n=3 biological replicates

(D) Immunohistochemical staining, using indicated antibodies, on ELT3 xenograft tumors from Figure 5. Scale bar = 0.2 mm

(E) Autoradiograph from *Tsc2*$^{+/+}$*;Trp53*$^{-/-}$ and *Tsc2*$^{-/-}$*;Trp53*$^{-/-}$ MEFs treated for 6 hr with vehicle or 2 μM mizoribine and labeled for 20 min with ^{35}S-Methionine before total protein was extracted and analyzed by SDS-PAGE.

(F) DNA synthesis was measured in *Tsc2*$^{-/-}$*;Trp53*$^{-/-}$ MEFs treated for 3 hr with vehicle, 20 nM rapamycin, or 2 μM mizoribine alone or in combination. Cells were labeled with 2,8-^{3}H-adenine for 1 hr. Total DNA was isolated and radioactive counts per minute were normalized to total DNA and graphed relative to vehicle-treated cells. n=3 biological replicates

(G) *Tsc2*$^{+/+}$*;Trp53*$^{-/-}$ and *Tsc2*$^{-/-}$*;Trp53*$^{-/-}$ MEFs were treated with 2 μM mizoribine for indicated times and GDP and GTP levels were measured by LC-MS/MS.

(H,I) *Tsc2*$^{-/-}$*;Trp53*$^{-/-}$ MEFs were treated for 16 hr with 2 μM mizoribine alone or in combination with indicated doses of CX-5461.

(J) *Tsc2*$^{-/-}$*;Trp53*$^{-/-}$ MEFs were transfected with control or UBF siRNAs for 24 hr then treated with vehicle or 2 μM mizoribine for 16 hr.

(K) *Tsc2*$^{-/-}$*;Trp53*$^{-/-}$ MEFs were treated for 48 hr with vehicle, 2 μM mizoribine, or 100 nM CX-5461 alone or in combination (lanes 1-4), or transfected with control or UBF siRNAs for 24 hr then treated for 48 hr with vehicle or 2 μM mizoribine (lanes 5-8).

Graphical data are represented as mean ± SEM. *p<0.05 by two-tailed Student's t test.