Supplementary information

Rapamycin-upregulated miR-29b promotes mTORC1-hyperactive cell growth in TSC2-deficienct cells by downregulating tumor suppressor retinoic acid receptor β (RARβ)

Heng-Jia Liu[1], Hilaire C. Lam[1], Christian V. Baglini[1], Julie Nijmeh[1], Alischer A. Cottrill[2], Stephen Y. Chan[2], Elizabeth P. Henske[1,*]

Supplementary Materials and Methods

Cell lines and cell cultures

$Tsc2^{+/+}p53^{-/-}$ and $Tsc2^{-/-}p53^{-/-}$ mouse embryonic fibroblasts (MEFs) were provided by Dr. David Kwiatkowski (Brigham and Women's Hospital, Boston MA). These cells were obtained in December 2005, banked at passage 8 and subsequently thawed in 2015 where experiments were performed between passage 10-20. Tsc2 WT and Tsc2 KO MEFs were generated from $Tsc2^{flox/flox}$-Rosa26-CreERT2 embryos at E13-E14 as described previously [1], and were used for experiments between passage 15-25 following the initial generation. Tsc2-deficient ELT3 cells, provided by Cheryl Walker, were originally isolated from an Eker rat uterine leiomyoma [2] into which a luciferase cell reporter was introduced to generate the ERL4 cell line [3], which was used for experiments between passage 10-21.

$Tsc2^{+/+}p53^{-/-}$, $Tsc2^{-/-}p53^{-/-}$, ERL4, Tsc2 WT and Tsc2 KO were cultured at 37°C in 5% CO_2 in DMEM supplemented with 10% FBS, 4.5 g/L glucose, 110 mg/ml pyruvate and 2 mM glutamine, 100 units/ml penicillin, 1% (v/v) streptomycin. 105K EV and 105K TSC2 add-back were maintained as described above with addition of 100 μg/ml of hygromycin B. Testing for mycoplasma was performed every two months; all cells were confirmed to be mycoplasma-free before and during experiments.

Plasmids

Lentiviral mouse microRNA constructs expressing Ctrl-ZIP, miR-29b-ZIP, miR-Ctrl, miR-29b-1 or miR-29b-2 were acquired from System Biosciences, USA. Plasmids

encoding the mouse RARβ2 gene (pCMV6-RARβ-Myc-DDK, Cat# MR207145) and the mouse ING4 gene (pCMV6-ING4, Cat# MC200711) were purchased from Origene, USA.

Western blot analysis

Cells were lysed using 1% Triton in TBS containing protease and phosphatase inhibitors. Tumors were lysed using 1 x RIPA buffer containing protease and phosphatase inhibitors. Equal amounts of total protein were separated by electrophoresis on a 4-12% Bis-Tris gel and transferred to a PVDF membrane. Blots were blocked in 5% BSA, then incubated with primary antibodies (See Table S3) overnight at 4°C. The membranes were washed and then incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 hour. Bands were detected using SuperSignal West Pico Plus chemiluminescence substrate (Thermo Fisher Scientific, USA) and photographed with G:BOX blot imaging system (Syngene, UK).

RNA sequencing and Gene-set enrichment analysis

RNA isolated from $Tsc2^{-/-}$ MEFs expressing Ctrl-ZIP or miR-29b-ZIP treated with rapamycin was sequenced at the Harvard Medical School Biopolymers Facility. The quantity and quality of mRNA were examined using an Agilent 2200 TapeStation instrument and by SYBR qRT-PCR assay. Libraries were prepared from 10 ng total RNA per sample using the Integen X Apollo 324 system (IntegenX, USA) and NuGEN SPIA reagents (Tecan, Switzerland). Sequencing was performed on an Illumina HiSeq2000 instrument (Illumina, USA). Differential gene expression analysis on three biological samples was performed using the R package DESeq2 [4] by the Harvard Chan School of

Public Health Bioinformatics Core. Differentially regulated genes were subjected to DAVID bioinformatics resources to identify Gene Ontology (GO) terms.

Network analysis

Using computational gene network methodology that we previously described [5], genes differentially expressed between Ctrl-ZIP versus miR-29b-ZIP (fold-change > 1.25, $P < 0.05$) treated with rapamycin were mapped using compiled data from a consolidated interactome (CI) including data from BioGRID [6], CORUM [7], DIP [8], InnateDB [9], IntAct [10], MatrixDB [11] and MINT [12], and in total contains 12,469 genes and 113,689 interactions. The direct targets of miR-29b were identified on the network using publicly available miRNA target prediction databases, including Diana microT, TargetScan and PicTar.

Supplementary Table 3. Antibodies and their working concentration used in this study.

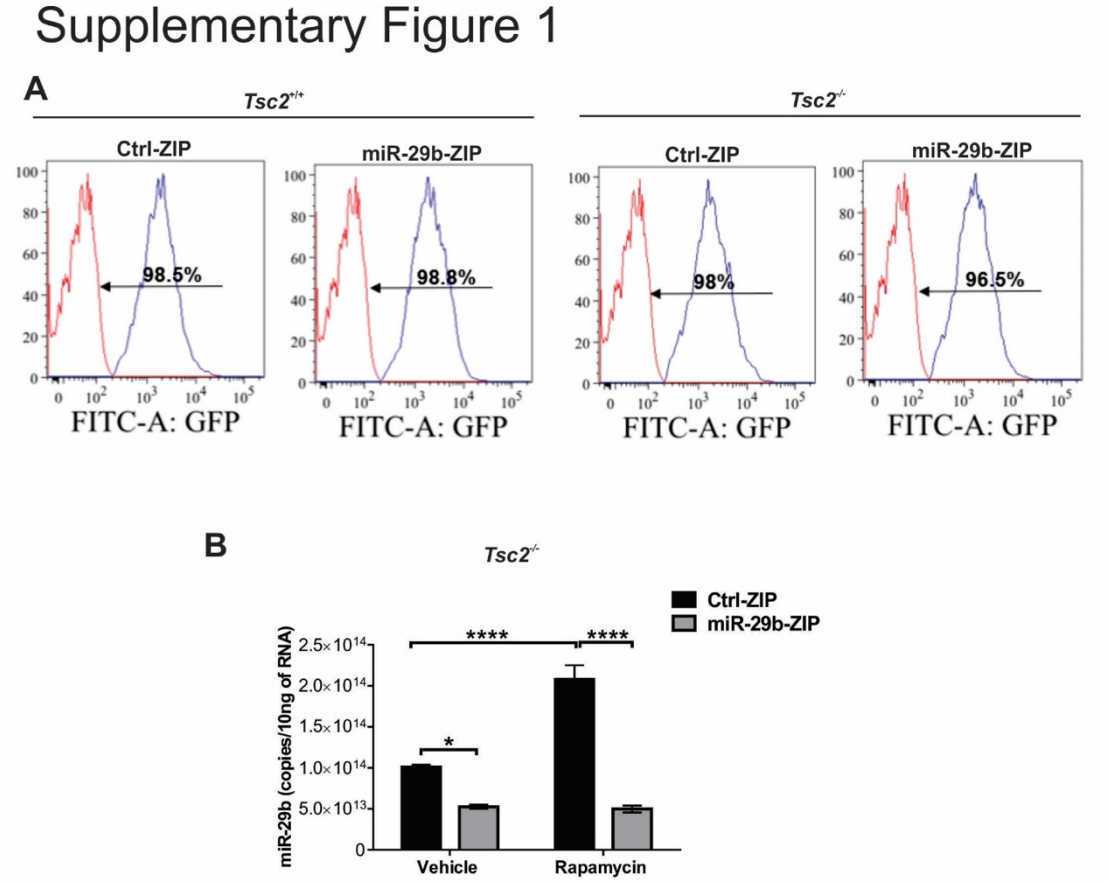
Antibody	Catalog number	Application	Dilution
RARβ	Santa Cruz, #Sc-552	Western blot, Co-IP	1:500, 5 µg
ING4	Thermo Fisher Scientific, #40-7700	Western blot, Co-IP	1:1000, 5 µg
S6	Cell Signaling, #2217	Western blot	1:1000
p-S6	Cell Signaling, #4858	Western blot	1:1000
Vinculin	Cell Signaling, #13901	Western blot	1:1000
Myc	Cell Signaling, #2276	Western blot	1:1000

Supplementary Table 4. qRT-PCR primers

qRT-PCR primers
RARB Assay ID: Mm01319677_m1 (ThermoFisher Scientific)
ING4 Assay ID: Mm00460097_m1 (ThermoFisher Scientific)
β-actin Assay ID: 4352341E (ThermoFisher Scientific)
miR-29b Assay ID: 478369 (ThermoFisher Scientific)
miR-320a Assay ID: 478594 (ThermoFisher Scientific)
cel-miR-39 Assay ID: 478293 (ThermoFisher Scientific)

Suplementary Figures

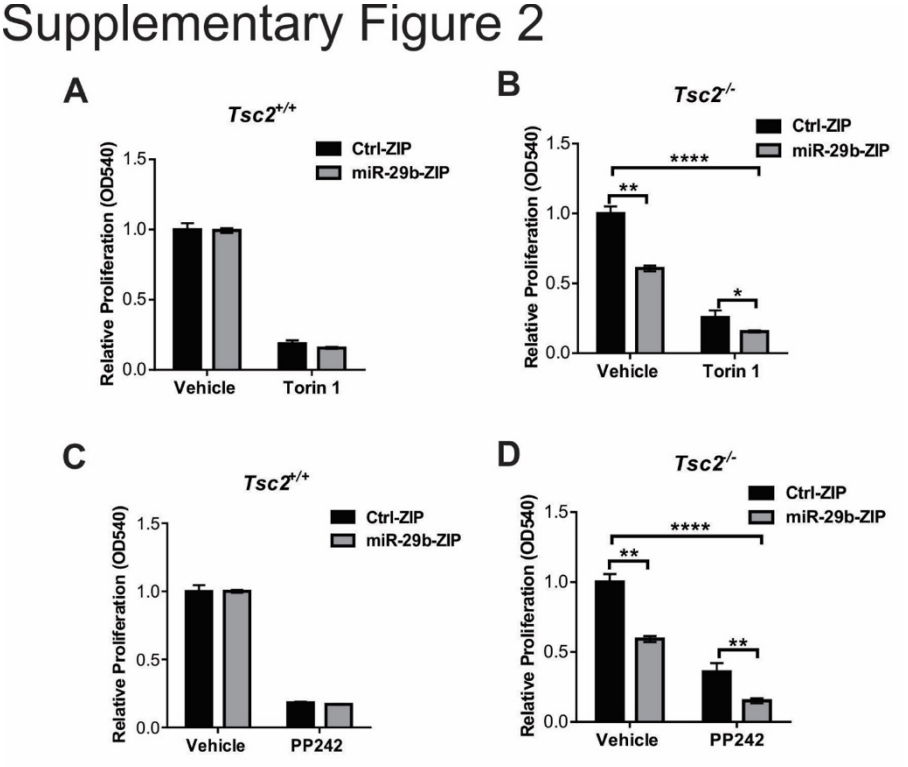
Supplementary Figure. 1



Supplementary Figure. 1. GFP expression in Tsc2$^{+/+}$ and Tsc2$^{-/-}$ MEFs transduced with lentivirus carrying Ctrl-ZIP or miR-29b-ZIP and absolute quantification of miR-29b expression.

a, GFP, an expression marker encoded by the miR-ZIP vector. The panel shows GFP-expression of stable populations of Tsc2$^{+/+}$ and Tsc2$^{-/-}$ MEFs expressing Ctrl-ZIP or miR-29b-ZIP after FACS. **b**, Absolute amount of miR-29b in Tsc2$^{-/-}$ MEFs expressing Ctrl-ZIP or miR-29b-ZIP treated with vehicle or rapamycin (20 nM) for 24 hours. Results are from *n* = 3 biological replicates. Data are presented as mean ± SD. Data for bar graphs were calculated using two-way ANOVA followed by Bonferroni's post-test for multiple comparisons. *: $P < 0.05$; ****: $P < 0.0001$

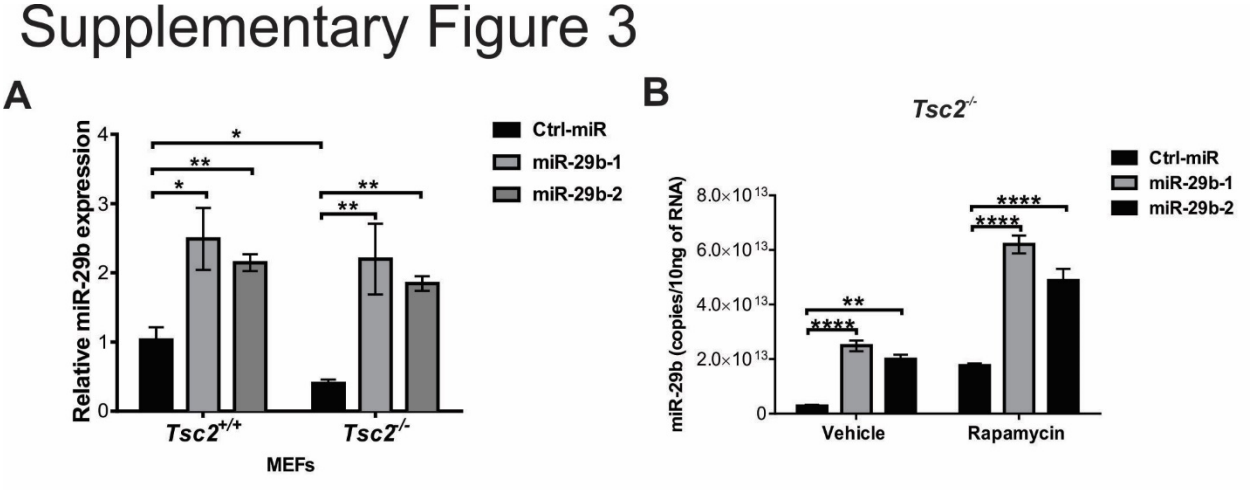
Supplementary Figure. 2



Supplementary Figure. 2. miR-29b further suppressed cell proliferation following Torin 1 or PP242 treatment in Tsc2-deficient, but not Tsc2 wild-type cells.

a-b, Cell proliferation was assessed by crystal violet staining in $Tsc2^{+/+}$ (**a**) or $Tsc2^{-/-}$ (**b**) MEFs stably expressing miR-29b-ZIP or Ctrl-ZIP following treatment with Torin 1 (100 nM) or vehicle (DMSO) for 72 hours. Data are presented as mean-fold change in OD 540 ± SD relative to Ctrl-ZIP cells treated with vehicle of three independent experiments. **c-d,** Cell proliferation was assessed by crystal violet staining in $Tsc2^{+/+}$ (**c**) or $Tsc2^{-/-}$ (**d**) MEFs stably expressing miR-29b-ZIP or Ctrl-ZIP following treatment with PP242 (200 nM) or vehicle (DMSO) for 72 hours. Data are from *n* = 3 biological replicates and presented as mean-fold change in OD 540 ± SD relative to Ctrl-ZIP cells treated with vehicle. Data for bar graphs were calculated using two-way ANOVA followed by Bonferroni's post-test for multiple comparisons. *: $P < 0.05$; **: $P < 0.01$; ****: $P < 0.0001$.

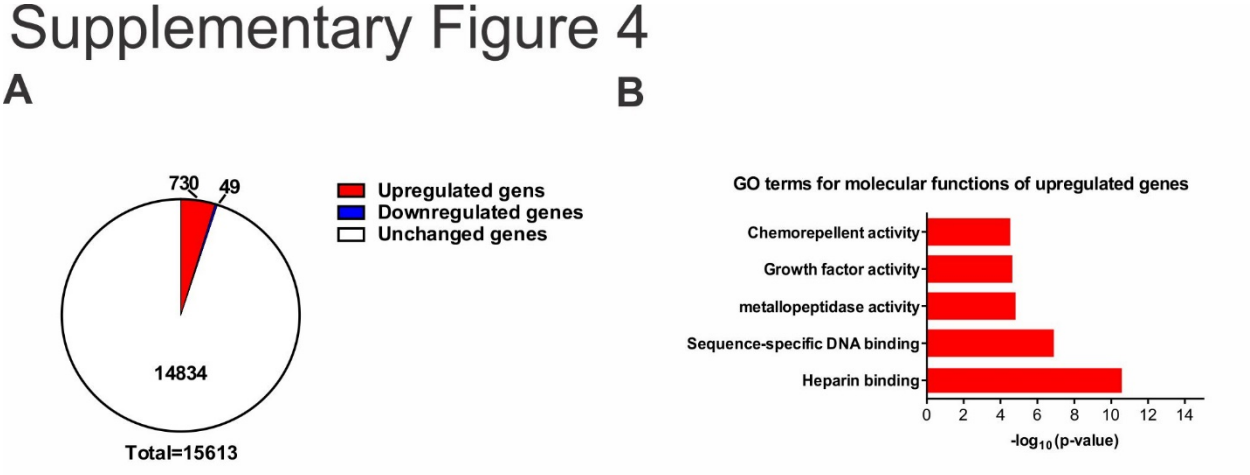
Supplementary Figure. 3



Supplementary Figure. 3. qPCR analysis of miR-29b overexpression in Tsc2$^{+/+}$ and Tsc2$^{-/-}$ MEFs.

a, Tsc2$^{+/+}$ and Tsc2$^{-/-}$ MEFs were transduced with Ctrl-miR or the two precursors of miR-29b (miR-29b-1 or miR-29b-2) lentivirus. miRNA was isolated and qPCR using Taqman assays for miR-29b or miR-320a (endogenous control) was performed. Data are presented as mean-fold change in miR-29b expression ± S.E. relative to Tsc2$^{+/+}$ MEFs transduced with Ctrl-miR. **b,** Absolute levels of miR-29b in Tsc2$^{-/-}$ MEFs expressing Ctrl-miR, miR-29b-1 or miR-29b-2 treated with vehicle or rapamycin (20 nM) for 24 hours from *n* = 3 biological replicates. Data for bar graphs were calculated using two-way ANOVA followed by Bonferroni's post-test for multiple comparisons *: $P < 0.05$; **: $P < 0.01$; ****: $P < 0.0001$

Supplementary Figure. 4



Supplementary Figure. 4. RNA-sequencing identifies the number of differentially regulated genes by miR-29b following rapamycin treatment in Tsc2$^{-/-}$ MEFs.

a, Pie chart representing the number of genes that are upregulated (fold change > 1.3, P < 0.05), downregulated (fold change < 0.5, P < 0.05) or unchanged in rapamycin-treated miR-29b knockdown cells compared to controls. **b,** Gene ontology (GO) analysis of 730 upregulated transcripts.

Supplementary References

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