Developmental Cell *11*

Supplemental Data

Ablation in Mice of the mTORC Components *raptor*, *rictor*, or *mLST8* Reveals that mTORC2 Is Required for Signaling to Akt-FOXO and PKCα, but Not S6K1

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Supplemental Experimental Procedures

Antibodies & Sources-Ki-67, PECAM-1, and Tie2 (BD Pharmingen); rictor (Sarbassov et al., 2004); mLST8 (Kim et al., 2003); FOXO3 T32 (gift from Anne Brunet, Stanford University); mTOR, total PKCα (Santa Cruz); phospho-S6K1^{T229} (R&D Systems); raptor, Akt, phospo-Akt^{S473}, phospho-Akt^{T308}, S6K, phospo-S6K^{T389}, phospho-4E-BP1$^{T37/46}$, phospho-4E-BP1^{S65}, total 4E-BP1, phospho-TSC2^{T1462}, phospho-TSC2^{S939}, total TSC2, total FOXO3a, phospho-GSKβ^{S9}, total GSK3β, phospho-PKC (pan) (βII Ser660), total PKCα (Cell Signaling).

Imaging-Blastocysts were viewed with a Zeiss Axiovert 40 CFL and imaged using a Canon Power Shot G5. Other embryos were imaged with a Leica MZ12 using Spot Advanced software. Processed histological specimens were analyzed with a Zeiss Axioskop microscope equipped with a 10X eyepiece and an Axiocam HRC and images were captured with Axiovision software release 4.4. TUNEL stained sections were imaged using a Zeiss Axiovert 200M with a 10X eyepiece and Axiovision 4.4 software.

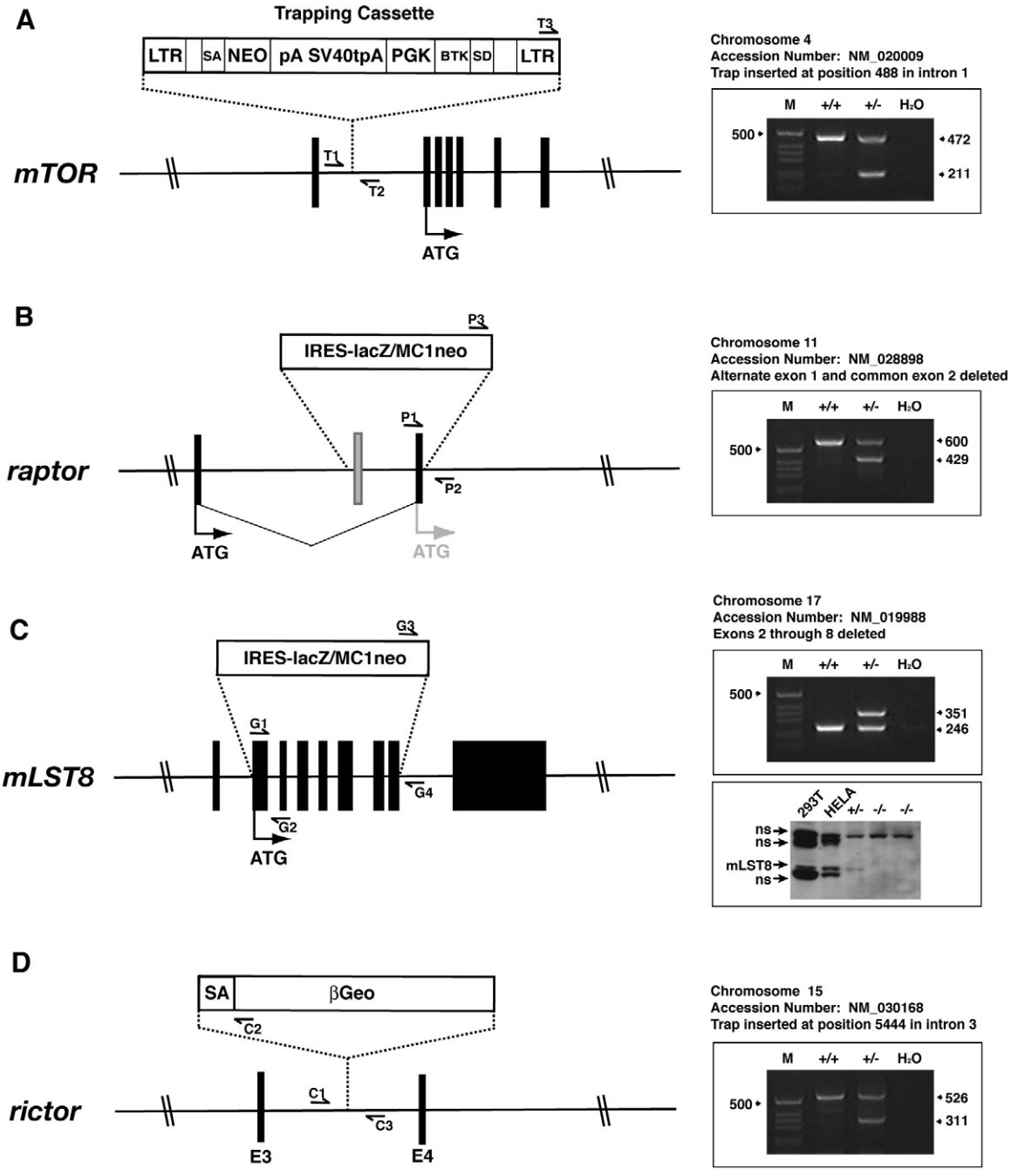
A
Trapping Cassette
T3
LTR
SA
NEO
pA SV40tpA
PGK
BTK
SD
LTR
mTOR
T1
T2
ATG
Chromosome 4
Accession Number: NM_020009
Trap inserted at position 488 in intron 1
M
+/+
+/-
H2O
500
472
211
B
P3
IRES-lacZ/MC1neo
P1
raptor
P2
ATG
ATG
Chromosome 11
Accession Number: NM_028898
Alternate exon 1 and common exon 2 deleted
M
+/+
+/-
H2O
500
600
429
C
G3
IRES-lacZ/MC1neo
G1
mLST8
G4
G2
ATG
Chromosome 17
Accession Number: NM_019988
Exons 2 through 8 deleted
M
+/+
+/-
H2O
500
351
246
293T
HELA
+/-
-/-
-/-
ns
ns
mLST8
ns
D
SA
βGeo
C2
C1
C3
rictor
E3
E4
Chromosome 15
Accession Number: NM_030168
Trap inserted at position 5444 in intron 3
M
+/+
+/-
H2O
500
526
311

Figure S1. Description of *mTOR, raptor, mLST8*, and *rictor* Targeted Alleles

Targeted ES cells of 129S5 origin were used to produce chimeric mice by blastocyst injection. Colonies were maintained on a mixed C57B6/129S5 background. *mTOR, raptor*, and *mLST8* were generated in collaboration with Bristol Myers Squibb (BMS). ES cells of 129P2 origin with a gene trap in *RCTR* were obtained from BayGenomics (http://baygenomics.ucsf.edu/) and used to produce chimeric mice by blastocyst injection. Colonies were maintained on a mixed Balbc/C57B6/129P2 background. Targeting constructs (left) and corresponding examples of PCR genotyping results (right) are shown. Sequences of primers used for genotyping appear in Table S1. The gene trap insertion was mapped to intron 3 of the *rictor* gene by 5' RACE. We subsequently used the following PCR-based strategy to map the exact site of insertion: Forward primers spaced 5 Kb apart that span intron 3, and a reverse primer that recognizes a short sequence in the 5' end of the trapping cassette, were generated. Using the Elongase® Amplification System (Invitrogen) we screened to find a forward and reverse primer pair that would amplify a DNA fragment, marking the rough location of the insert (i.e. to within 5KB). Once we localized the insertion site, we moved the forward primer closer to the reverse primer until we obtained a DNA fragment small enough for direct sequencing. All of the primers described in this report were generated by Integrated DNA Technologies. Included in the description of mLST8 is a Western blot analysis of total mLST8 protein. Non-specific (ns) bands are indicated. Rictor Western blot analysis is included in the main text. Because *mTOR* and *raptor* deficient embryos do not grow to a stage where sufficient protein can be recovered, we could not Western blots.

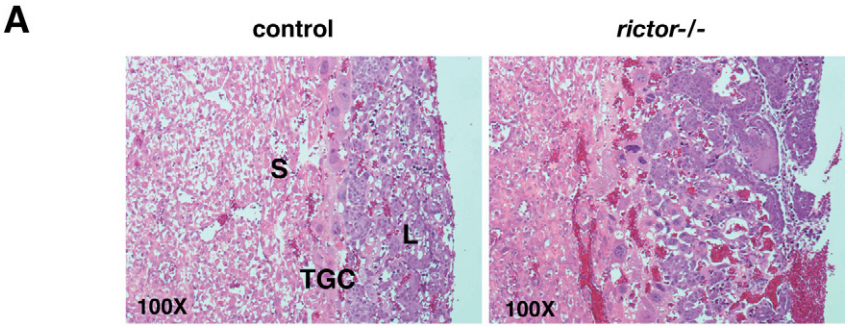

Figure S2. Hisological Analysis of *rictor*$^{-/-}$ Placenta

H&E stains of placenta showing the spongiotrophoblast (S), trophoblast giant cell (TGC), and labyrinth (L) layers. Placenta from *rictor* deficient embryos, like those from *mLST8* deficient embryos are disorganized and the labyrinth layer is enlarged.

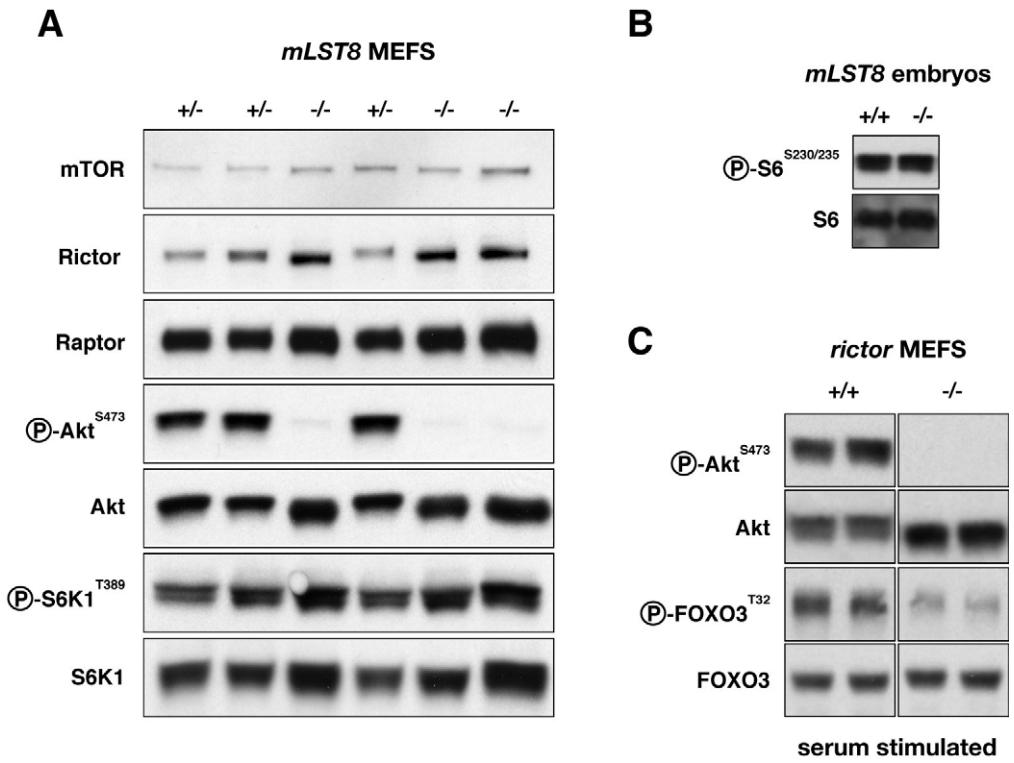

Figure S3. Additional Biochemical Evidence to Support the Text

(A) Additional immunoblots of *mLST8* control and three more knockout MEF lines showing that Akt/PKB S473 phosphorylation, but not S6K1 T389 phosphorylation, is ablated in the *mLST8* knockout MEFs. (B) Phosphorylation of S6 at S230/235, a commonly used surrogate marker for mTORC1 activity, is also unimpaired in *mLST8*$^{-/-}$ embryos. (C) Immunoblot of Akt/PKB S473 and FOXO3 T32 phosphorylation in control (+/+) and *rictor* knockout (-/-) MEFS following serum stimulation consistently shows T32 phosphorylation is impaired in the knockout lines.

Table S1. Sequences of Primers Used for Genotyping

Symbol in Figure S1	Alternative name (for laboratory use)	Sequence (5'-3')
T1	FWD Primer	TTC CGT GGA GCA TTT AGG TCT C
T2	REV Primer	AAG GCT TTC AAA CGC TAC ACT ACA G
T3	LTR2	AAA TGG CGT TAC TTA AGC TAG CCT GC
P1	Primer 7	GAG GCA GTC TTT TGA TCA GTT G
P2	Primer 8	ATG AGC TTT GCT TAA GAT ATC G
P3	Primer Neo3A	GCA GCG CAT CGC CTT CTA TC
G1	Primer 25	ACC TGC TGA GCA TGT TAA GG
G2	Primer 26	GCA ACT GCA GGC TAT GAC CAC
G3	Primer 13	CAG CAC ACT GTC ATT GAA GG
G4	Primer Neo3A	GCA GCG CAT CGC CTT CTA TC
C1	RRR347_F_01	AGA TAG CCA GAA GTG GGC ATC GGA CCC
C2	RRR347_R_01	CAT CTA GGA CTT CTC TCT GCA ACA GAT GCA GAT CAC
C3	RRR347_Trap_R_01	TGG GGT TCG TGT CCT ACA ACA CAC ACT CC