Expanded View Figures

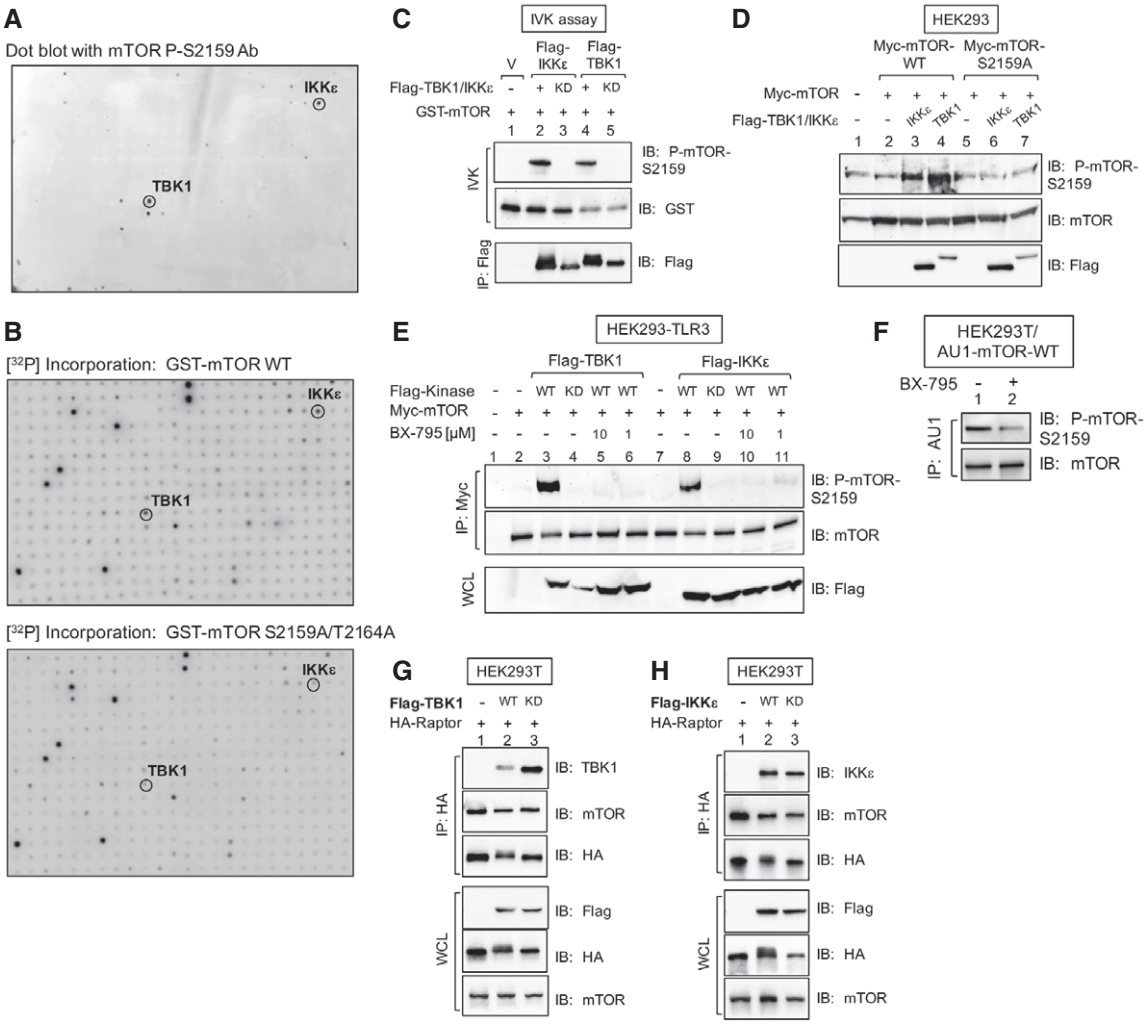

Figure EV1. An *in vitro* human kinome screen identified TBK1 and IKKε as mTOR S2159 kinases that interact with mTORC1 (related to Fig 1).

A *In vitro* kinome screen with recombinant GST-mTOR substrate and ~300 recombinant active kinases. Substrate phosphorylation was detected with mTOR P-S2159 antibodies.

B Similar to (A), except that GST-mTOR wild type (WT) or GST-mTOR S2159A/T2164A (AA) was used as substrate, and [γ-^{32}P]-ATP was included in the reactions. [^{32}P] incorporation was detected by autoradiography.

C TBK1 and IKKε immune complex *in vitro* kinase (IVK) assays. Flag-TBK1 or Flag-IKKε WT (+) or kinase dead (KD) was immunoprecipitated from transfected HEK293 cells and incubated with GST-mTOR substrate. IVK reactions were performed by incubating the Flag-TBK1 or Flag-IKKε immunoprecipitates (IP) with GST-mTOR substrate [200 ng] for 30 min at 30°C. Immunoprecipitates (IPs) were immunoblotted (IB) as indicated.

D Cellular overexpression of TBK1 and IKKε in cells increases mTOR P-S2159. HEK293 cells were co-transfected with Myc-mTOR (WT or S2159A) together with Flag-IKKε or Flag-TBK1 or plasmids. Whole-cell lysate (WCL) was immunoblotted as indicated.

E Overexpression of TBK1 and IKKε in cells increases mTOR P-S2159 in a BX-795-sensitive manner. HEK293-TLR3 cells were co-transfected with Myc-mTOR and Flag-TBK1 or Flag-IKKε wild type (+) or kinase dead (KD) and then treated with BX-795 [10 μM or 1 μM] (2 h). Myc-mTOR was immunoprecipitated, and IPs and WCL were immunoblotted as indicated.

F Cellular BX-795 treatment decreases mTOR S2159 phosphorylation. HEK293T cells stably expressing AU1-mTOR were pre-treated with BX-795 [10 μM] (2 h). AU1-mTOR was immunoprecipitated and immunoblotted as indicated.

G, H Flag-TBK1 and Flag-IKKε co-immunoprecipitate with HA-raptor and mTOR. HEK293T cells stably expressing AU1-mTOR were transfected with Flag-TBK1 (G) or Flag-IKKε (H) wild-type (+) or kinase-dead (KD) plasmids together with HA-raptor. HA-raptor was immunoprecipitated and immunoblotted as indicated.

Source data are available online for this figure.

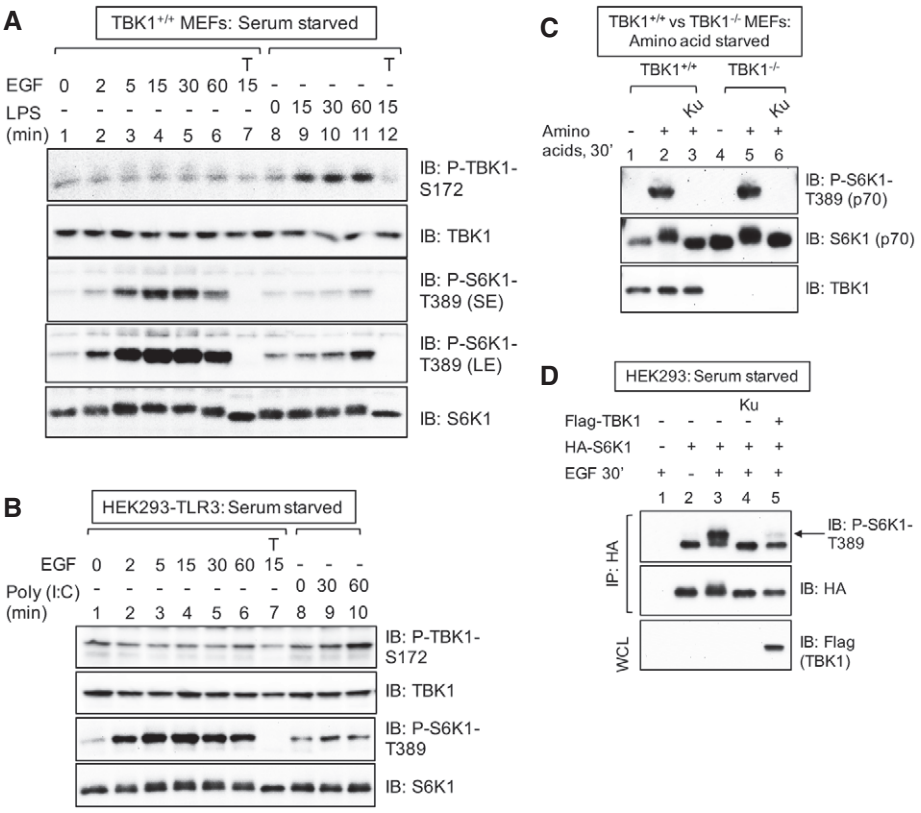

Figure EV2. LPS but not EGF increases TBK1 activation loop site phosphorylation in MEFs; TBK1 is not required for amino acid stimulated mTORC1 signaling; ectopic TBK1 overexpression suppresses mTORC1 signaling (related to Fig 2).

A EGF fails to increase TBK1 S172 phosphorylation in MEFs. Cells were serum deprived (20 h) and stimulated −/+ EGF [25 ng/ml] or LPS [100 ng/ml] for the times indicated. Whole-cell lysate (WCL) was immunoblotted as indicated. SE: short exposure; LE: long exposure.

B EGF fails to increase TBK1 S172 phosphorylation in HEK293/TLR3 cells. Cells were serum deprived (20 h) and stimulated −/+ EGF [25 ng/ml] or poly(I:C) [50 ng/ml] for the times indicated.

C TBK1 is not required for amino acid-stimulated mTORC1 signaling. $TBK1^{+/+}$ and $TBK1^{-/-}$ MEFs were deprived of amino acids by incubation in D-PBS + glucose + dialyzed FBS [10%] (60 min). Amino acids were added back by incubating the cells in DMEM/FBS [10%] (30 min).

D Cellular overexpression of TBK1 inhibits mTORC1 signaling. HEK293 cells were co-transfected with HA-S6K1 together with Flag-TBK1, serum-starved (20 h), pre-treated with Ku-0063794 [1 μM] (30 min), and stimulated −/+ EGF [25 ng/ml] (30 min). HA-S6K1 was immunoprecipitated, and IPs and WCL were immunoblotted (IB) as indicated.

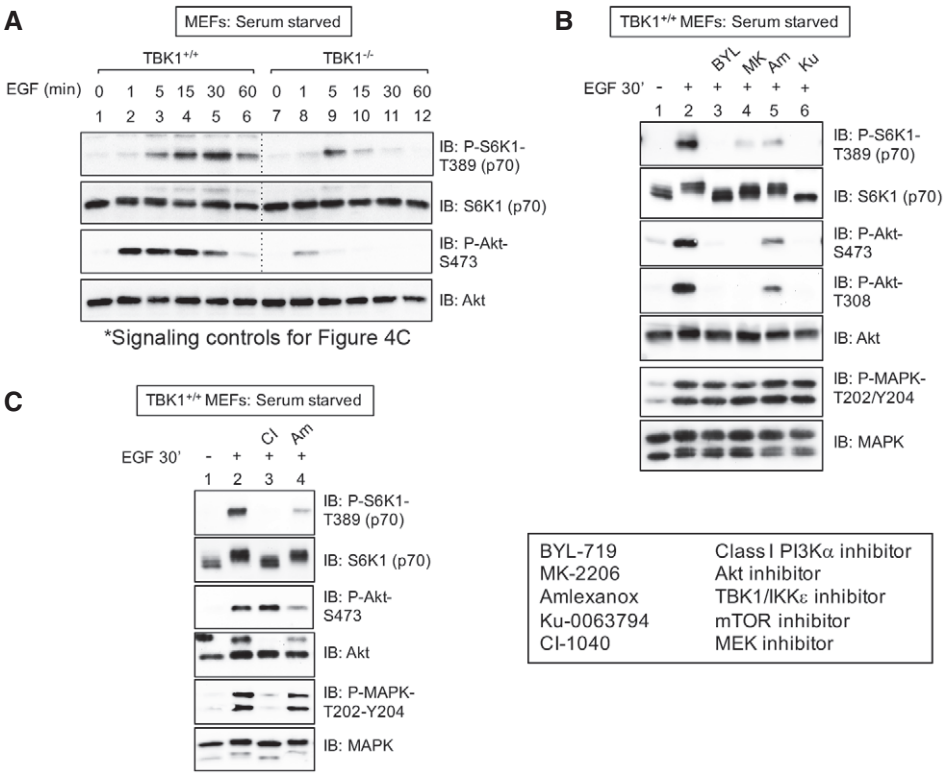

Figure EV3. PI3K, Akt, MAPK, and TBK1 activity is required for EGF-stimulated mTORC1 signaling (related to Fig 4).

A mTORC1 signaling controls for Fig 4C. TBK1$^{+/+}$ and TBK1$^{-/-}$ MEFs were serum-starved (20 h) and stimulated without (–) or with (+) EGF [25 ng/ml] for 0–60 min. WCL was immunoblotted as indicated.

B PI3Kα, Akt, and TBK1 activity is required for EGF-stimulated mTORC1 signaling. TBK1$^{+/+}$ MEFs were serum-starved (20 h), pre-treated with the PI3Kα class I inhibitor BYL-719 [10 μM] (30 min), the Akt inhibitor MK-2206 [10 μM] (30 min), amlexanox [100 μM] (2 h), or Ku-0063794 [1 μM] (30 min), and stimulated –/+ epidermal growth factor (EGF) [50 ng/ml] (30 min).

C MAPK and TBK1 activity is required for EGF-stimulated mTORC1 signaling: TBK1$^{+/+}$ MEFs were treated as above except they were pre-treated with the MEK inhibitor CI-1040 [10 μM] or amlexanox [100 μM] (30 min).

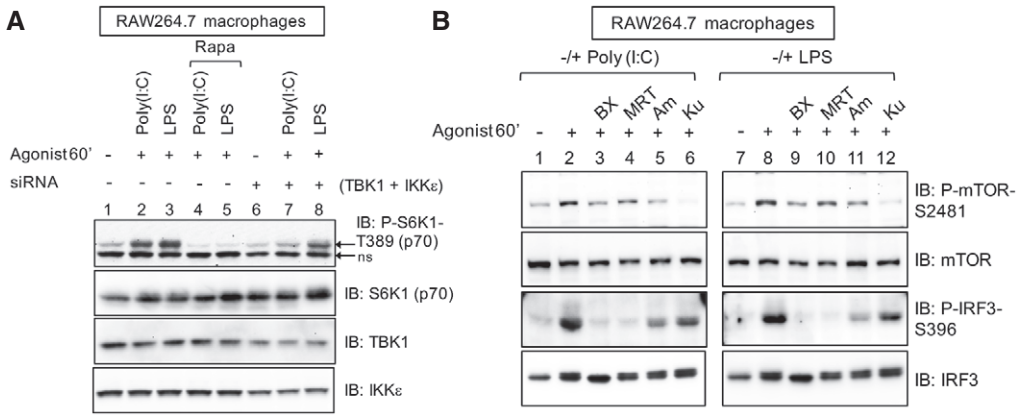

Figure EV4. TBK1 promotes mTORC1 signaling in RAW264.7 macrophages upon TLR3 and TLR4 activation (related to Figs 5 and 6).

A siRNA-mediated knockdown of TBK1/IKKε reduces TLR3/4-stimulated mTORC1 signaling. RAW264.7 macrophages were transfected with siRNAs targeting TBK1 and IKKε. After 5 days, cells were pre-treated with rapamycin [20 ng/ml] (30 min) and stimulated −/+ poly(I:C) [30 μg/ml] or LPS [100 ng/ml] (60 min). ns = non-specific band.

B Pharmacologic inhibition of TBK1 reduces mTOR catalytic activity as measured by mTOR S2481 auto-phosphorylation. RAW264.7 macrophages were pre-treated with the TBK1 inhibitors BX-795 [10 μM] (30 min), MRT-67307 [10 μM] (30 min), amlexanox [100 μM] (2 h) or with the mTOR inhibitor Ku-0063794 [1 μM] (30 min) and stimulated −/+ poly(I:C) [30 μg/ml] or LPS [100 ng/ml] (60 min). WCL was immunoblotted as indicated.

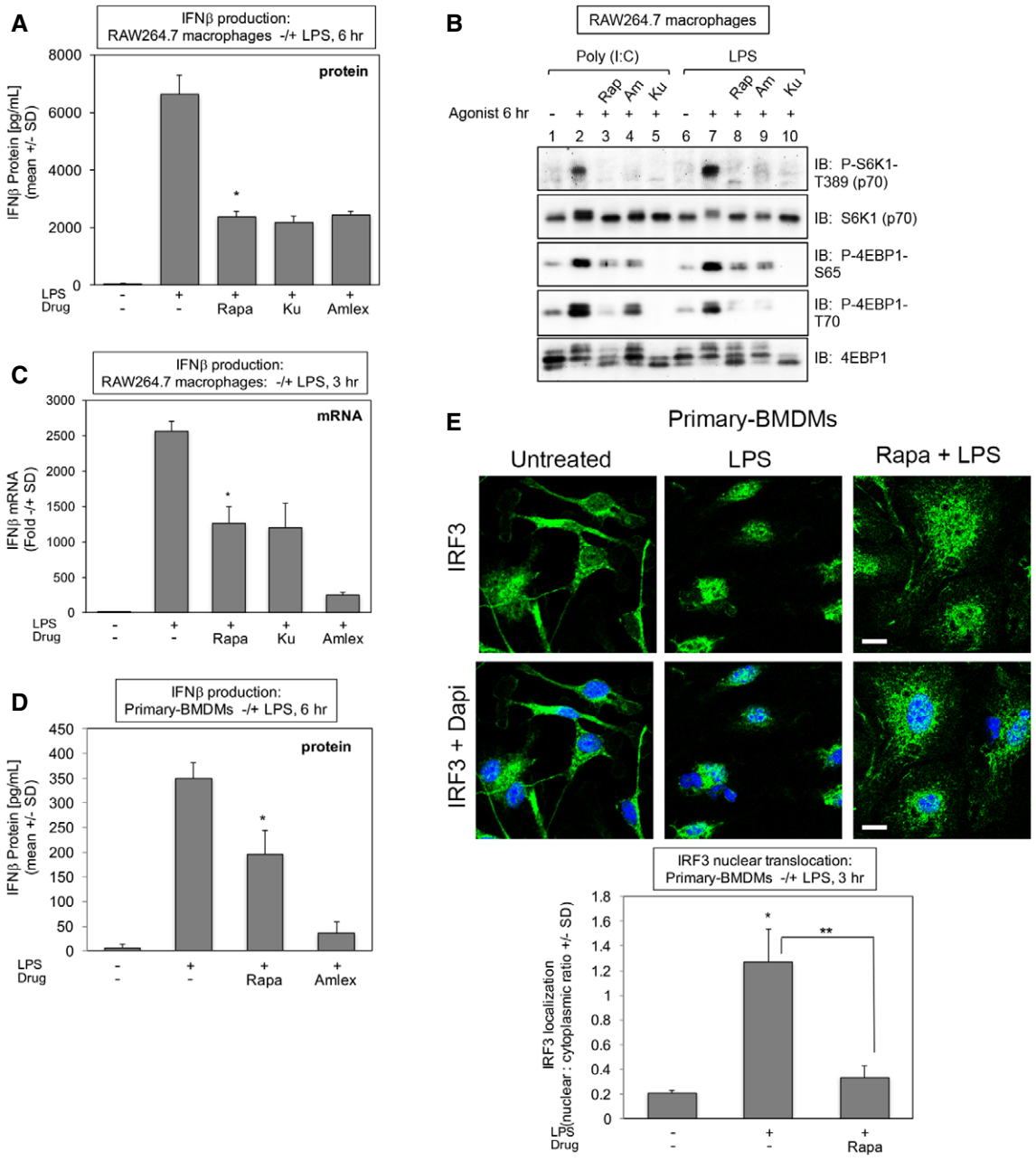

Figure EV5. mTORC1 function is required for IFN-β production and IRF3 nuclear translocation in RAW264.7 macrophages upon TLR4 activation (related to Fig 7).

A mTORC1 inhibition with rapamycin suppresses TLR4-induced IFN-β protein production in RAW264.7 macrophages. Cells were pre-treated with rapamycin [20 ng/ml] (30 min), Ku-0063794 [1 μM] (30 min), or amlexanox [50 μM] (2 h) and stimulated −/+ LPS [100 ng/ml] (6 h). The secretion of IFN-β into the culture media was measured by ELISA. Results represent the mean ± SD of quadruplicate samples from one experiment. *P = 0.007 relative to +LPS by paired t-test (two-tailed).

B Rapamycin maintains suppression of TLR3/4-induced mTORC1 signaling at 6 h of treatment in RAW264.7 macrophages. Cells were treated as in (A). WCL was immunoblotted as indicated.

C Rapamycin suppresses TLR4-induced IFN-β mRNA production in RAW264.7 macrophages. Cells were treated as in (A) but LPS treatment lasted 3 h. IFN-β gene expression was measured by qRT–PCR. Results represent the mean ± SD of triplicate samples from one experiment. *P = 0.002 relative to +LPS by paired t-test (two-tailed).

D Rapamycin suppresses TLR4-stimulated IFN-β protein production in primary macrophages. BMDMs were pre-treated with rapamycin or amlexanox and analyzed as in (A). Results represent the mean ± SD of triplicate samples from one experiment. *P = 0.02 relative to +LPS no rapamycin by paired t-test (two-tailed).

E Rapamycin suppresses TLR4-stimulated IRF3 nuclear translocation in primary macrophages. BMDMs were treated with LPS for 3 h as in (A), fixed, and processed for confocal immunofluorescence microscopy using an anti-IRF3-Alexa488 antibody and DAPI staining. The graph represents the mean ± SD of at least 95 cells from one experiment. *P = 0.002 relative to no LPS by paired t-test (two-tailed); **P = 0.005 relative to +LPS by paired t-test (two-tailed). Scale bar = 10 μm. Note: the control image is the same as the control image shown in Fig 7E.

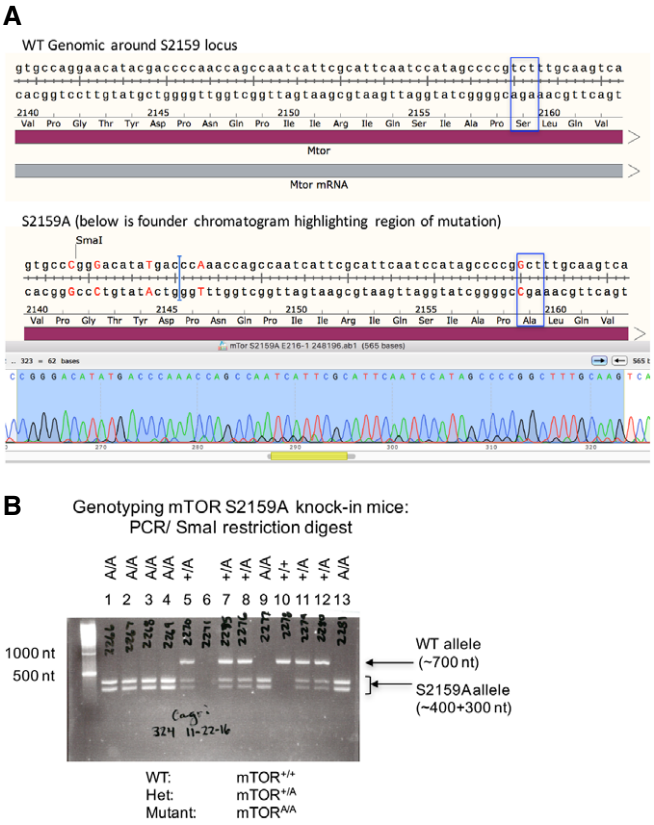

Figure EV6. Generation and genotyping mTOR$^{A/A}$ genome-edited mice (related to Fig 8).

A TOPO cloning and DNA sequencing of founder mTOR$^{A/A}$ mice. Sequence analysis confirmed the presence of an edited mTOR gene containing an Ala substitution at Ser2159. The blue box highlights the S2159A point mutation. Sequencing also confirmed the introduction of a new SmaI restriction site to facilitate genotyping and several silent mutations to prevent re-targeting of edited genomic DNA.

B Representative DNA agarose gel depicting genotyping of wild-type (mTOR$^{+/+}$), heterozygous (mTOR$^{+/A}$), and homozygous mutant S2159A knock-in mice (mTOR$^{A/A}$). A ~700 nt fragment of genomic DNA surrounding the mTOR S2159 locus was PCR amplified and digested with SmaI. Restriction products of ~400 nt and ~300 nt indicate the presence of the S2159A knock-in allele.