Supplemental Material

Materials and Methods

S6K1 and GSK3 IP Kinase Assays

S6K1 immunoprecipitation (IP) kinase assays (Figures 4B and C) were performed on cell lysates from ~80%-confluent 10 cm dishes. Lysates were incubated at 4°C with 2 μl rabbit polyclonal S6K1 antibodies (Cell Signaling Technologies) for 2 h followed by 20 μl protein A/G agarose beads (Pierce) for 1 h. Immune complexes were washed three times with lysis buffer and twice with kinase reaction buffer (KRB; 20 mM HEPES, 10 mM $MgCl_2$, 0.5 mM EGTA, pH 7.4) containing phosphatase inhibitors (50 mM NaF, 50 mM β-glycerophosphate) and protease inhibitors (1:500 of Sigma cocktail). Kinase reactions were initiated by resuspending the immune complexes in 20 μl KRB containing 100 μM ATP, 10 mM DTT, and 100 ng recombinant rat GSK3β purified from *E. coli* (Upstate). Assays were terminated after 15 min at 30°C by boiling in SDS sample buffer, and the entire contents of the reactions were subjected to immunoblot analyses as indicated.

GSK3 IP kinase assays (Figure 5A) were performed on cell lysates obtained from ~80%-confluent 15 cm dishes. 2.5 mg of total cellular protein in lysis buffer was incubated at 4°C with 4 μl rabbit monoclonal GSK3α/β antibodies (Upstate), or rabbit IgG for control samples, for 1 h followed by 20 μl protein A/G agarose beads for 1.5 h. Immune complexes were washed four times with KRB containing 1% Igepal CA-630, 150 mM NaCl, 1 mM DTT, phosphatase inhibitors (10 mM NaF, 10 mM β-glycerophosphate, 1 mM $NaVO_3$) and protease inhibitors, then once with the same lacking detergent and NaCl. Kinase reactions were initiated by resuspending immune complexes in 20 μl KRB containing phosphatase inhibitors, 25 μM ATP, 5 μCi [γ-^{32}P]-ATP, and 20 mM phospho-glycogen synthase peptide-2 (Upstate), and samples were

incubated for 15 min at 30°C. The 20 μl supernatants from each reaction were spotted onto p81 paper, air dried, and washed five times with 175 mM orthophosphoric acid. Relative amounts of radiolabeled peptide were determined with a Beckman scintillation counter. The background counts obtained from the IgG control sample were subtracted from the anti-GSK3 reactions.

Figure Legends

Figure S1. Supplemental data related to Figure 2.

(A) TSC2 protein levels are similar in the *Tsc2*$^{-/-}$-TSC2-addback MEFs as in the *Tsc2*$^{+/+}$ MEFs. Equal concentrations of protein from *Tsc2*$^{+/+}$ MEFs and *Tsc2*$^{-/-}$ MEFs stably infected with either vector or TSC2-expressing retrovirus were subjected to immunoblot with antibodies to TSC2 and actin, as a loading control.

(B) GSK3 is phosphorylated in a growth factor-independent rapamycin-sensitive manner in TSC2 knockdown HeLa cells. 48 hours post-transfection with 80 nM control or TSC2 siRNAs, HeLa cells were serum starved for 24 h and treated for 15 min with 20 nM rapamycin, where indicated.

Figure S2. Supplemental data related to Figure 4.

S6K1, but not S6K2, is responsible for the constitutive phosphorylation of GSK3 in *Tsc2*$^{-/-}$ cells. 24 hours post-transfection with the indicated doses of control, S6K1, S6K2, or S6K1 plus S6K2 targetting siRNAs, *Tsc2*$^{-/-}$ MEFs were serum starved for 16 hours.

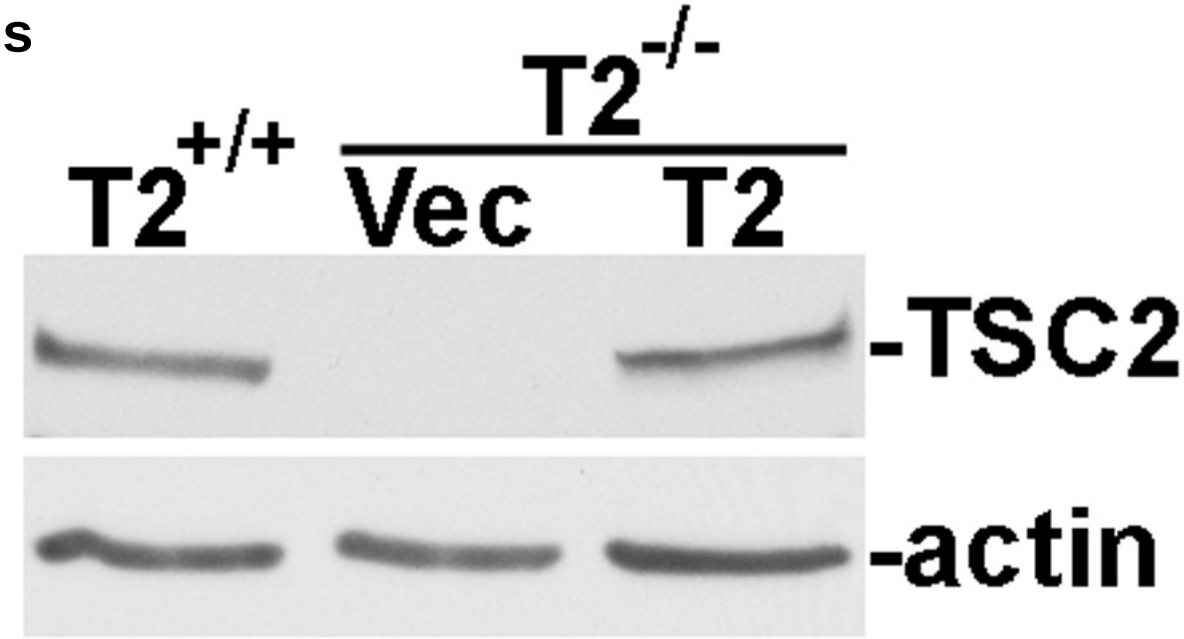
Note: Knockdown of S6K1, but not S6K2, decreases GSK3 phosphorylation, while knockdown of S6K2, but not S6K1, decreases S6 phosphorylation in these cells.

Figure S3. Supplemental data related to Figure 6.

(A) *Tsc1-/-* MEFs proliferate in low serum, and their sensitivity to rapamycin is partially rescued by inhibition of GSK3. 2X10^5 littermate-derived *Tsc1*$^{+/+}$ (WT) or *Tsc1*$^{-/-}$ (KO) MEFs were cultured in low serum (2%) with fresh nutrients and serum provided daily in the presence of 0.1% DMSO (vehicle control), 20 nM rapamycin (Rap), or Rap plus 10 μM SB216763, where indicated. Data are presented as mean ± SEM. Note: Unlike WT cells and *Tsc2*$^{-/-}$ cells, rapamycin is cytotoxic to these *Tsc1-/-* MEFs. However, the GSK3 inhibitor reverses this effect, suggesting that rapamycin-induced GSK3 activation contributes to *Tsc1*$^{-/-}$ cell death.

(B) GSK3 inhibitors do not rescue wild-type MEFs from growth arrest. *Tsc2*$^{+/+}$ MEFs were treated identically to *Tsc2*$^{-/-}$ MEFs (shown in Figure 6C) for 48 hours in the absence of serum. Data are presented as the mean ± SEM percentage of cells present relative to vehicle control. Note: As shown in Figure 6B, these cells do not proliferate under these conditions, and therefore, the original cell number plated was not subtracted, as it was in Figure 6C. GSK3 inhibitors do not significantly affect the proliferation of these cells, either in the presence of vehicle (not shown) or rapamycin.

A



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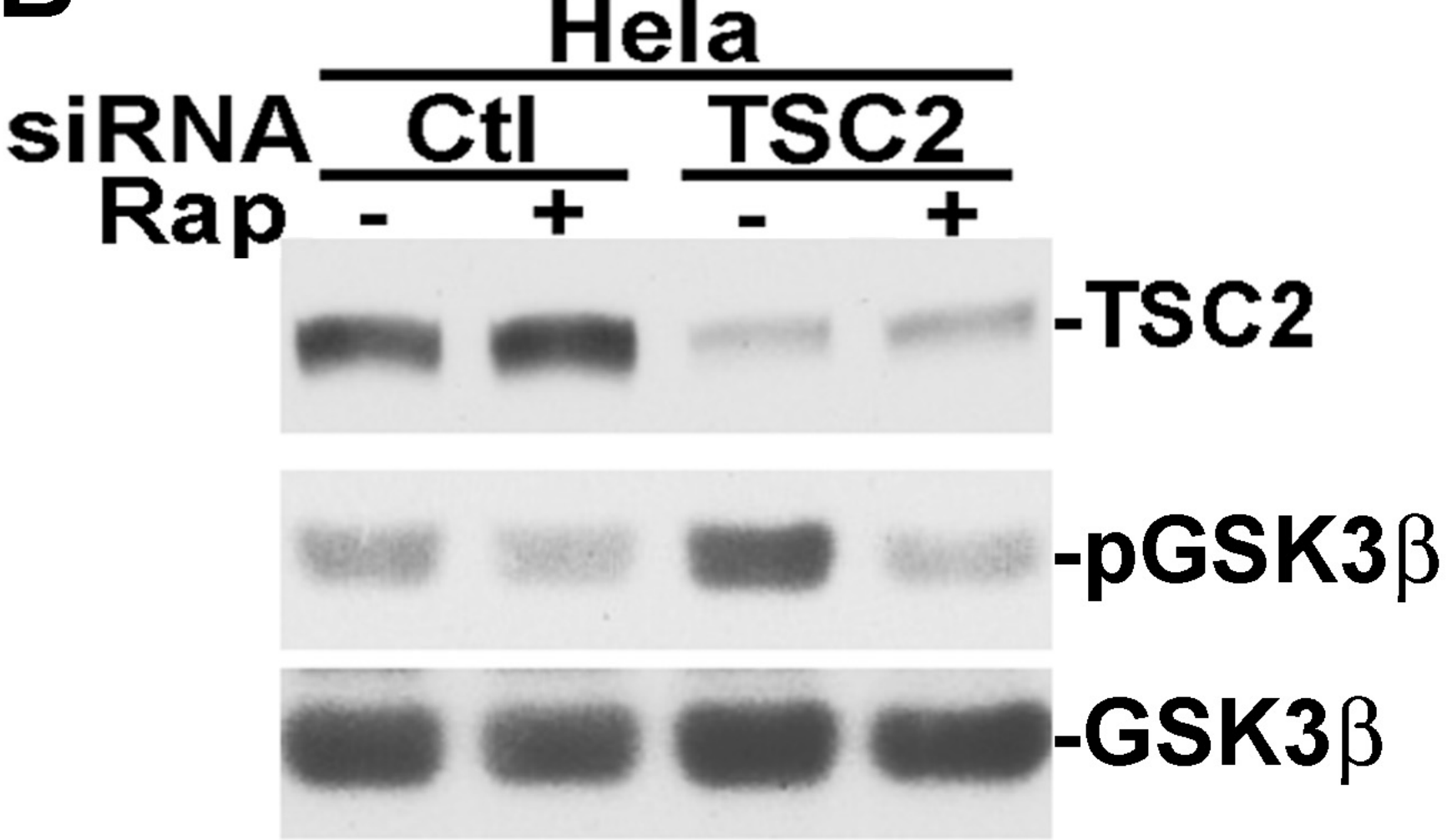
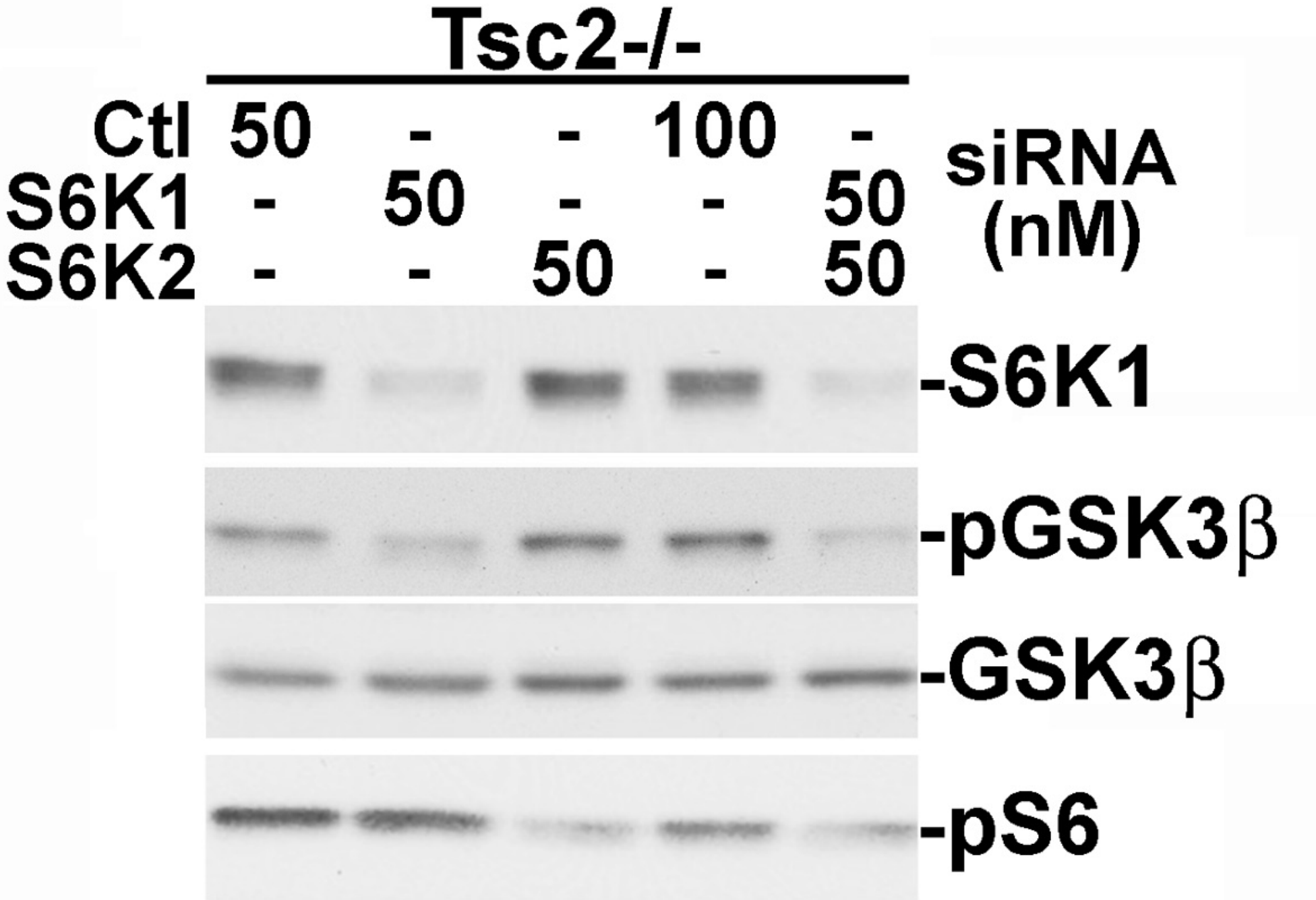
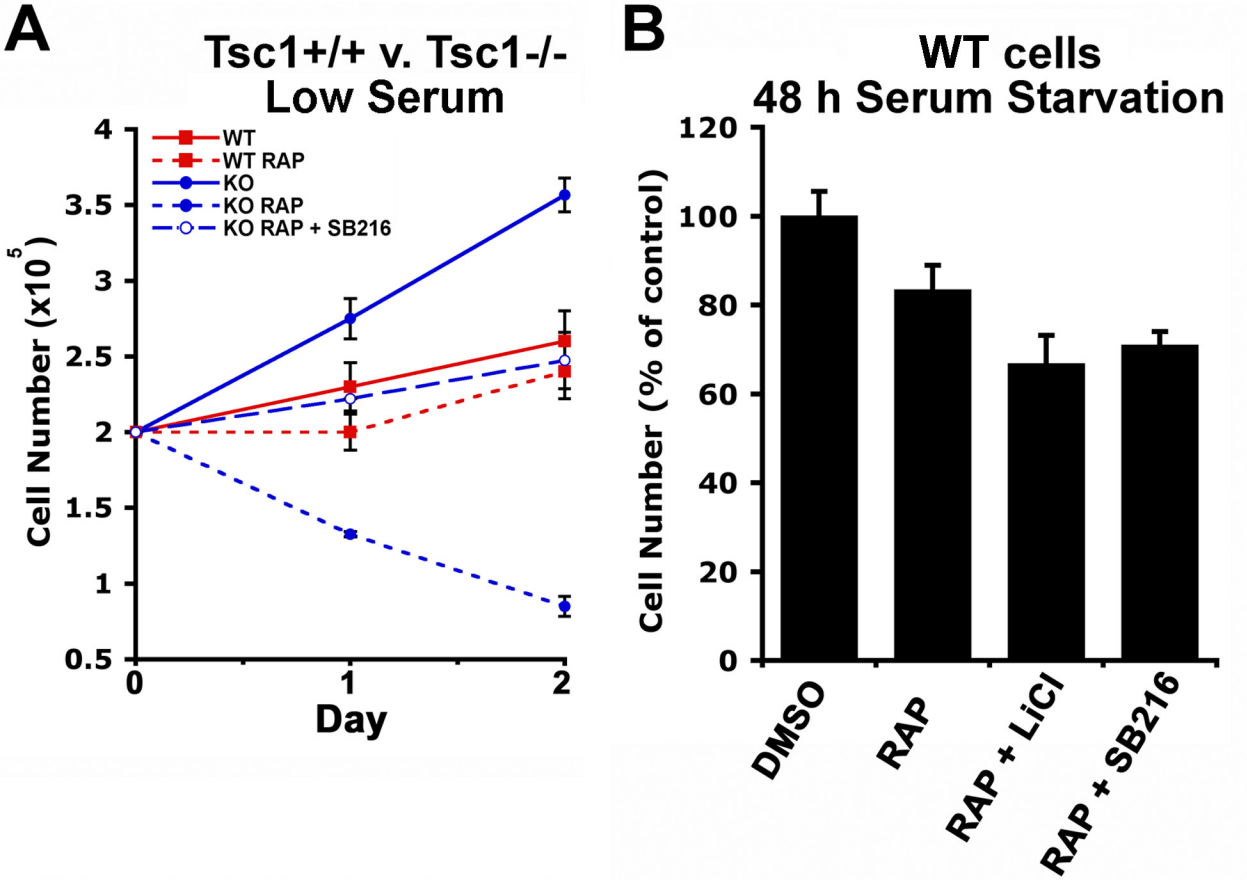


Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3