Supporting Information

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SI Materials and Methods

Antibodies and Other Reagents. Anti-myosin heavy chain (anti-MHC) (MF20) and anti-myogenin (F5D) were from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development, National Institutes of Health, and maintained by the University of Iowa, Department of Biological Sciences.Anti-tubulin antibody was from Abcam, anti-Myc clone 9E10 from Covance, anti-Flag M2 from Sigma, anti-GST (B-14) from Santa Cruz, and anti-raptor and anti-rictor from Bethyl Laboratories. All other primary antibodies were from Cell Signaling Technology. All secondary antibodies were from Jackson ImmunoResearch Laboratories, Inc. Inhibitor of the Ser/Thr kinase Akt (Akti-1/2) was from Calbiochem. Protein G-agarose and His-Akt were from Millipore. All other reagents were from Sigma-Aldrich.

Cell Culture. HEK293 and HeLa cells were maintained in DMEM containing 10% (vol/vol) FBS at 37 °C with 5% (vol/vol) CO_2. All transient transfections in HEK293 cells were performed with PolyFect (Qiagen) following manufacturer's protocols at 60–70% cell confluence. C2C12 myoblasts were maintained in DMEM containing 1 g/L glucose and 10% (vol/vol) FBS at 37 °C with 7.5% (vol/vol) CO_2. Transfection of C2C12 was performed using TransIT-LT1 (Mirus) following the manufacturer's recommendations, followed by selection in 1.0 mg/mL G418 for 2–3 d. Primary myoblasts were isolated from 2- to 5-d-old mice (FVB strain) as described previously (1) and maintained at low density on 1% (wt/vol) gelatin-coated tissue culture plates. All animal experiments in this study followed protocols approved by the Animal Care and Use Committee at the University of Illinois at Urbana–Champaign. Lentiviral infection of C2C12 and primary myoblasts was performed as previously described (2), with 3 μg/mL puromycin selection in C2C12 for 2 d and no drug selection for primary myoblasts.

Cell Lysis, Western Blotting, and Immunoprecipitation. Cells were lysed in ice-cold lysis buffer [40 mM Hepes (pH 7.2), 120 mM NaCl, 10 mM pyrophosphate, 50 mM NaF, 10 mM β-glycerophosphate, 2 mM EDTA, 1× Sigma protease inhibitor mixture, and 0.3% (wt/vol) 3-[(3-Cholamidopropyl)dimethylammonio] propanesulfonic acid (CHAPS)]. The supernatant after microcentrifugation at 13,000 × g for 10 min was collected and subjected to immunoprecipitation at 4 °C with various antibodies in the lysis buffer, followed by incubation with Protein G-agarose. The beads were washed with lysis buffer and then boiled in SDS sample buffer. Proteins were resolved on SDS/PAGE and transferred onto PVDF membrane (Millipore), followed by incubation with various antibodies according to the manufacturer's recommendations. Detection of horseradish peroxidase-conjugated secondary antibodies was performed with Western Lighting Chemiluminescence Reagent Plus (Perkin-Elmer, Inc.). Quantification of Western band intensities was performed by densitometry of X-ray film images using the software Image J.

Purification of GST-Fusion Proteins and GST Pull-Down Assays. GST and GST-XPLN (*e*x*change factor found in *p*latelets, *l*eukemic, and *n*euronal tissues) were expressed in *Escherichia coli* and purified using glutathione Sepharose beads (GE Healthcare) following the manufacturer's recommendations. Purified proteins (20 μg each) were preincubated with cell lysates for 30 min before incubation with glutathione Sepharose for 60 min, followed by washing with lysis buffer.

In Vitro Mammalian Target of Rapamycin Kinase Assays. Mammalian target of rapamycin complex 1 (mTORC1) and mTORC2 were immunoprecipitated using antiraptor and antirictor antibodies, respectively. The kinase assays were performed following procedures described by Ikenoue et al. (3). mTORC1 kinase assays were carried out at 30 °C for 30 min in 25 mM Hepes (pH 7.4), 50 mM KCl, 10 mM $MgCl_2$, and 250 μM ATP, with 100 ng GST-4EBP1 as the substrate. mTORC2 kinase assays were carried out at 37 °C for 30 min in 25 mM Hepes (pH 7.4), 100 mM potassium acetate, 1 mM $MgCl_2$, and 500 μM ATP, with 250 ng His-Akt as the substrate. Where applicable, purified GST proteins (1 μg each) were added to the immunocomplexes 30 min before initiation of the kinase assay by the addition of ATP. Reactions were stopped by the addition of SDS sample buffer and boiling.

Myoblast Differentiation. Myogenic differentiation of C2C12 cells and primary myoblasts were induced at 100% and 50–70% confluence, respectively, by switching to DMEM containing 2% (vol/vol) horse serum as previously described (2). Differentiated cells in 12-well plates were fixed and stained for MHC and DAPI as previously described (2). The stained cells were examined with a Leica DMI 4000B fluorescence microscope, and the fluorescent images were captured using a RETIGA EXi camera and analyzed with Q-capture Pro51 software (Q-Imaging). The fusion index was calculated as the percentage of nuclei in myocytes with ≥2 nuclei. Each data point was generated from scoring five randomly chosen microscopic fields.

Small GTPase RhoA Activity Assay. The amount of GTP-bound RhoA was measured using the method described by Ren and Schwartz (4). Briefly, cells were lysed in 50 mM Tris (pH 7.4), 10 mM $MgCl_2$, 500 mM NaCl, 1% (vol/vol) Triton X-100, 0.1% SDS, 0.5% deoxycholate, and 1× Protease Inhibitor Mixture. Cleared lysates were incubated with glutathione-Sepharose beads containing 30 μg of GST-RBD (GST fusion to the Rho-binding domain of Rhotekin protein), and the beads were washed in 50 mM Tris (pH 7.4), 10 mM $MgCl_2$, 150 mM NaCl, 1% (vol/vol) Triton X-100, and 1× Protease Inhibitor Mixture. Bound proteins and lysates were analyzed by Western blotting with anti-RhoA antibody.

shRNA. XPLN shRNAs were from the MISSION TRC library (Sigma-Aldrich). The clone identification numbers are the following: mouse XPLN #1, NM_027871.1–458s1c1; human XPLN #1, NM_019555.1–226s1c1; and mouse and human XPLN #2, NM_019555.1–578s1c1.

1. Ge Y, Sun Y, Chen J (2011) IGF-II is regulated by microRNA-125b in skeletal myogenesis. *J Cell Biol* 192(1):69–81.
2. Yoon MS, Chen J (2008) PLD regulates myoblast differentiation through the mTOR-IGF2 pathway. *J Cell Sci* 121(Pt 3):282–289.
3. Ikenoue T, Hong S, Inoki K (2009) Monitoring mammalian target of rapamycin (mTOR) activity. *Methods Enzymol* 452:165–180.
4. Ren XD, Schwartz MA (2000) Determination of GTP loading on Rho. *Methods Enzymol* 325:264–272.

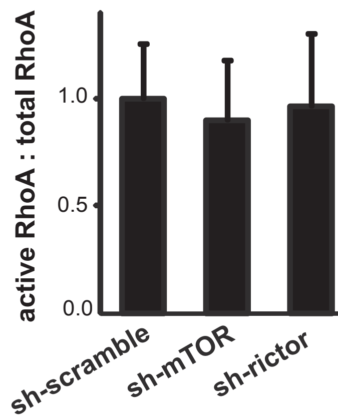

Fig. S1. Knockdown of mTORC2 does not affect RhoA GTP loading. HEK293 cells were infected with lentiviruses expressing shRNAs for mTOR, rictor, or a scrambled sequence as control. Cell lysates were subjected to GST-RBD pull-down assays followed by Western analysis. Active RhoA (RhoA pulled down with GST-RBD) and total RhoA (RhoA in cell lysates) were quantified by densitometry. The ratios of active RhoA versus total RhoA were calculated and normalized against the control (scramble). Each data point was compared with the control by paired *t* test, and no significant difference was found.

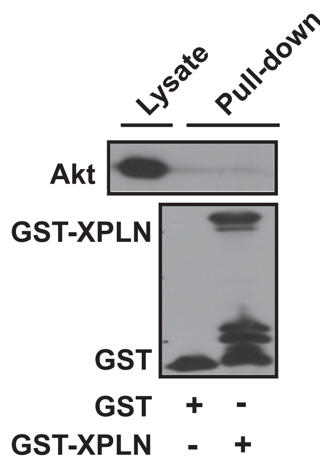

Fig. S2. There is no detectable interaction between XPLN and Akt. HEK293 cell lysates were subjected to pulldown assay with GST-XPLN or GST, and endogenous Akt was detected by Western.

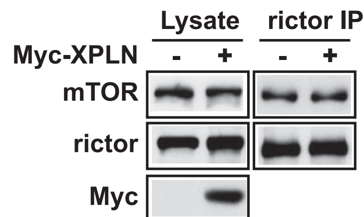

Fig. S3. Overexpression of XPLN does not affect mTOR–rictor interaction. HEK293 cells were transfected with Myc-XPLN. Cell lysates were subjected to endogenous rictor immunoprecipitation followed by Western analysis.

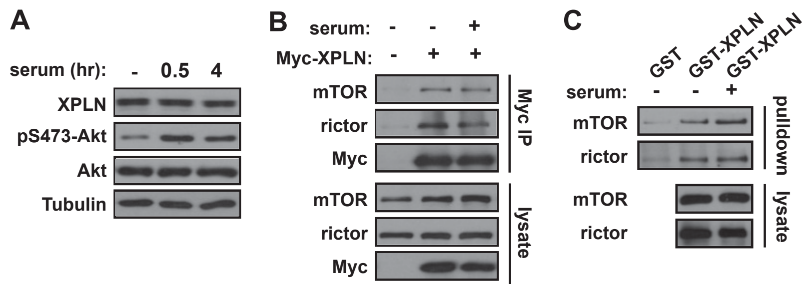

Fig. S4. Serum stimulation does not affect XPLN protein level or its interaction with mTORC2. (*A*) HEK293 cells were serum-starved overnight and then stimulated with 10% (vol/vol) FBS for 30 min or 4 h, followed by Western analysis. (*B*) HEK293 cells were transfected with Myc-XPLN and serum-starved overnight followed by stimulation with 10% (vol/vol) FBS for 30 min. Immunoprecipitation was performed with anti-Myc antibody. (*C*) HEK293 cells were serum-starved and then stimulated with 10% (vol/vol) FBS for 30 min, followed by GST pull-down assays.

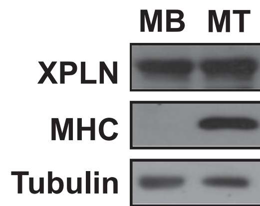

Fig. S5. XPLN protein level does not change during myoblast differentiation. C2C12 cells were lysed before (MB, myoblast) or after (MT, myotube) a 3-d differentiation and subjected to Western analysis.