



Supplemental Fig. S4. Delta-like 1 homolog (DLK1) to suppress a linkage between activin receptor type 2 B (ACVR2B) and its ligand, myostatin. (A) Binding of sDLK1 to ACVR2B, determined using co-immunoprecipitation in HEK293E cells overexpressing DLK1 and ACVR2B. (B) Co-immunoprecipitation of sDLK1 and ACVR2B, in C2C12 cells. (C) Competitive binding of sDLK1 and myostatin to ACVR2B, determined by competition assay (ELISA). IP, immunoprecipitation; IB, immunoblot analysis.

Immunoprecipitation assay: DLK1 and ACVR2B were transfected in 293E cells using polyethylenimine. Cells were harvested and lysed with modified radio-immunoprecipitation assay (RIPA) buffer. After being precleared with normal human immunoglobulin G (IgG) and protein G beads (GE), DLK1 or ACVR2B protein was immunoprecipitated with human DLK1 or ACVR2B antibody and protein G beads. Then beads were washed three times with the RIPA buffer and resuspended in 5 \times sodium dodecyl sulfate (SDS) sample buffer. After being boiled for 5 minutes, the supernatant was separated onto 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto to polyvinylidene fluoride (PVDF) membranes (GE). After being blocked with milk, the membranes were incubated with mouse anti-DLK1 monoclonal antibody (R&D) or goat anti-ACVR2B affinity purified polyclonal antibody (R&D) overnight at 4 $^{\circ}$ C. Anti-lamin B antibody (Santa Cruz) or anti- α -tubulin antibody (Santa Cruz) was used as unrelated negative control. The membranes were washed with TRIS-buffered saline with Tween (TBST) followed by incubation with secondary antibody, goat anti-mouse or anti-goat IgG conjugated with horseradish peroxidase (HRP, Santa Cruz) respectively. The signals were detected by chemiluminescence. Myoblast cell line C2C12 cells were harvested. All procedures were followed the same method with 293E.

Competition assay: To coat 96 well immunoplate, 0.2 μ g/mL of recombinant myostatin (R&D systems) was added into each well and incubated overnight at 4 $^{\circ}$ C. ACVR2B-Fc (kindly provided by Y-Biologics, Inc.) diluted by one half from 10 to 0.075 μ g/mL in phosphate buffered saline (PBS) was added to each well and incubated for 1 hour at room temperature. After washing with phosphate-buffered saline with Tween (PBST), anti-human IgG-HRP (1:5,000; Jackson ImmunoResearch) was added. Color development was performed with 3,3', 5,5' tetramethylbenzidine dihydrochloride (TMB) solution. For the competition assay, ACVR2B-Fc (1.25 μ g/mL) and FLAG-DLK1 (0, 10, or 50 μ g/mL) were co-incubated for an hour at room temperature and subsequently added into myostatin-coated immunoplate.