Supporting Information:

Cooperative actions of Tbc1d1 and AS160/Tbc1d4 in GLUT4 trafficking activities

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List of the materials included:

Figure S1–S5

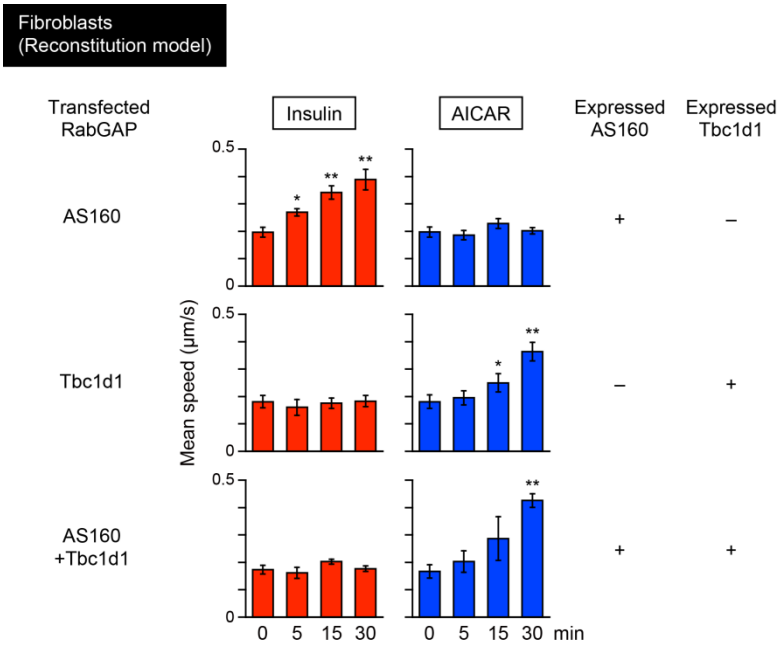

Figure S1. Time-dependent changes in mean speed of GLUT4 movement after insulin or AICAR stimulation in 3T3-L1 fibroblasts

Mean speed of GLUT4 movement was analyzed in 3T3-L1 fibroblasts exogenously expressing myc-GLUT4, HA-sortilin and both Tbc1d1 and AS160, or either one alone. The cells were stimulated with insulin (100 nM) or AICAR (1 mM) for 0, 5, 15, or 30 min. **P*<0.05, ***P*<0.01 by Dunnett's multiple comparison versus before stimulation (*n*=3).

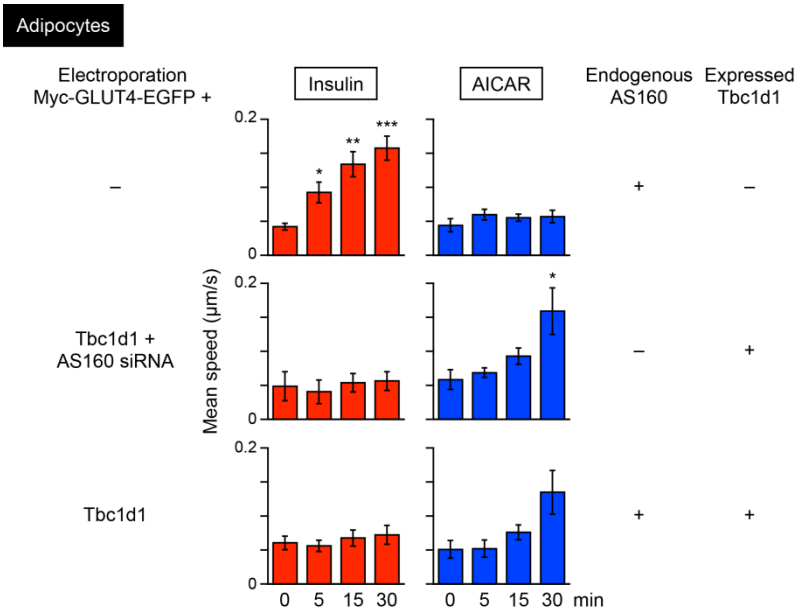

Figure S2. Time-dependent changes in mean speed of GLUT4 movement after insulin or AICAR stimulation in 3T3-L1 adipocytes

Mean speed of GLUT4 movement was analyzed in 3T3-L1 adipocytes electroporated with myc-GLUT4-EGFP (top), myc-GLUT4-EGFP + HaloTag-Tbc1d1 + AS160 siRNA (middle), and myc-GLUT4-EGFP + HaloTag-Tbc1d1 (bottom). The cells were stimulated with insulin (100 nM) or AICAR (1 mM) for 0, 5, 15, or 30 min. **P*<0.05, ***P*<0.01, ****P*<0.001 by Dunnett's multiple comparison versus before stimulation (*n*=4).

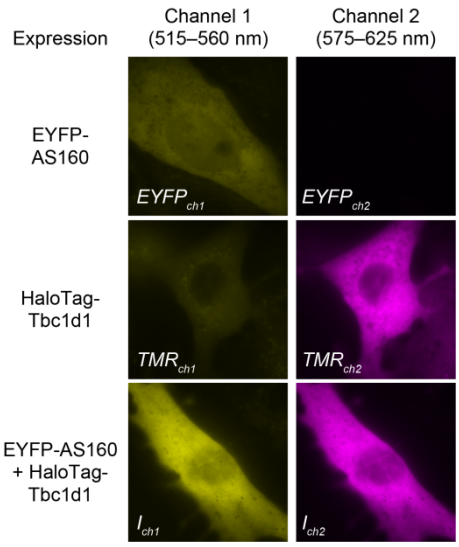

Figure S3. Schema for estimating relative abundance of exogenously-expressed AS160 and Tbc1d1

We utilized cells expressing both AS160 and Tbc1d1, or either one alone, fused to either EYFP or HaloTag. This figure shows representative epi-fluorescent images of the cells expressing AS160-EYFP (upper), HaloTag-Tbc1d1 (middle) and both AS160-EYFP and HaloTag-Tbc1d1 (lower). After staining of HaloTag-Tbc1d1 with HaloTag TMR ligand, the epi-fluorescence of the wavelengths at 515–560 nm (left, channel 1) and 575–625 nm (right, channel 2) was acquired by exciting the fluorescent molecules with 490–500 nm and 530–550 nm, respectively. Although EYFP and TMR fluorescence would mainly be collected by channel 1 and channel 2, respectively, there is non-negligible bleed-through fluorescence into the other channel. Therefore, fluorescent images of each channel derived from cells expressing both proteins are inevitably merged with the two fluorescent molecules. To correct the bleed-through, we calculated fractions of bleed-through of EYFP and TMR fluorescence based on the cells expressing either of these two proteins. This value allowed us to obtain precise fluorescence intensities of EYFP and TMR in cells expressing both proteins by using equation (4) in the Methods.

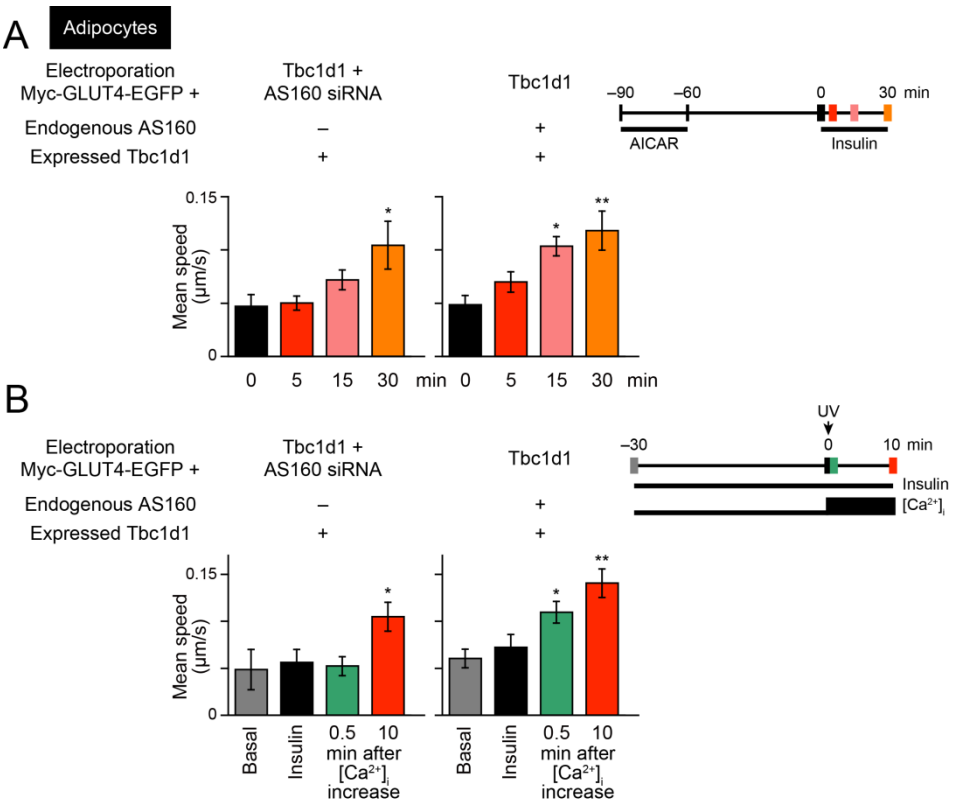

Figure S4. Accelerating potency of AS160 on insulin-responsive GLUT4 liberation in response to sequential/combined treatments in 3T3-L1 adipocytes

Responses to sequential treatment with AICAR and insulin (A) or combined treatment with insulin and Ca^{2+} (B) are shown. Mean speeds of GLUT4 movement in cells electroporated with myc-GLUT4-EGFP + HaloTag-Tbc1d1 + AS160 siRNA (left) or myc-GLUT4-EGFP + HaloTag-Tbc1d1 (right) are shown. Inset represents treatment and acquisition protocols. **P*<0.05, ***P*<0.01 by Dunnett's multiple comparison versus before stimulation (A) or versus insulin-stimulated states (just before photolysis) (B) (*n*=3).

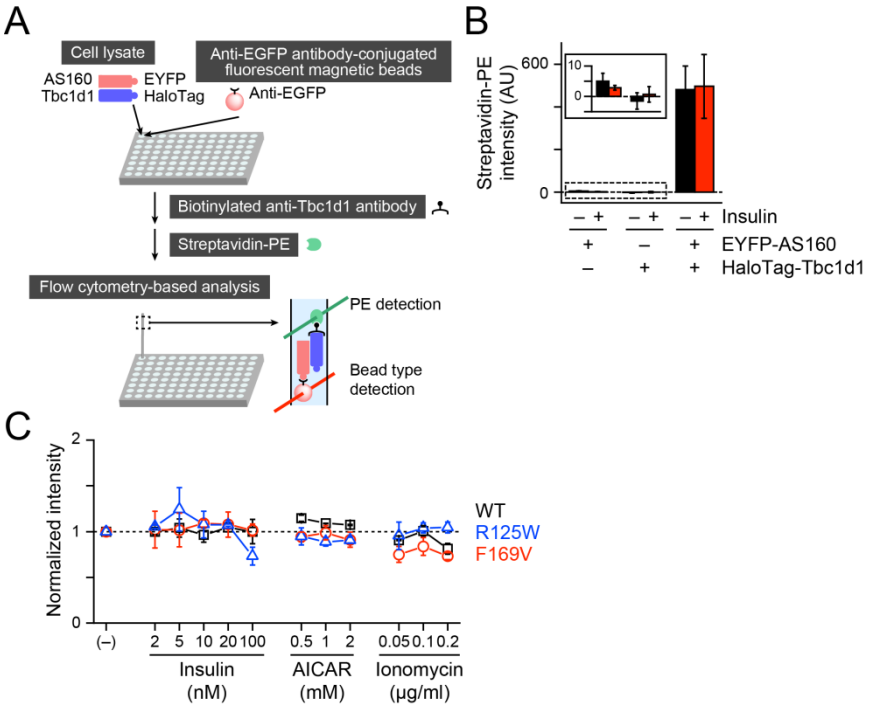

Figure S5. Detection of association between AS160 and Tbc1d1

(A) Schematic drawing for multiplex assays of heteromer formation. (B) Validation of the assay. In this experiment, cells were transfected with either EYFP-AS160 or HaloTag-Tbc1d1, or both. Intensity was obvious only when both EYFP-AS160 and HaloTag-Tbc1d1 were expressed. Inset shows magnification of the dotted region. (C) Effects of Tbc1d1 mutants on heteromer formation between AS160 and Tbc1d1. Cells expressing myc-GLUT4-mCherry, HA-sortilin, AS160-EYFP and WT or the indicated mutants of HaloTag-Tbc1d1 were stimulated with the indicated concentrations of insulin, AICAR or ionomycin. The values are shown as fold increases, as compared with untreated cells (n=3).