Supplemental Information

Regulatory Mode Shift of Tbc1d1 is Required for Acquisition of Insulin-Responsive GLUT4 Trafficking Activity

Hiroyasu Hatakeyama and Makoto Kanzaki

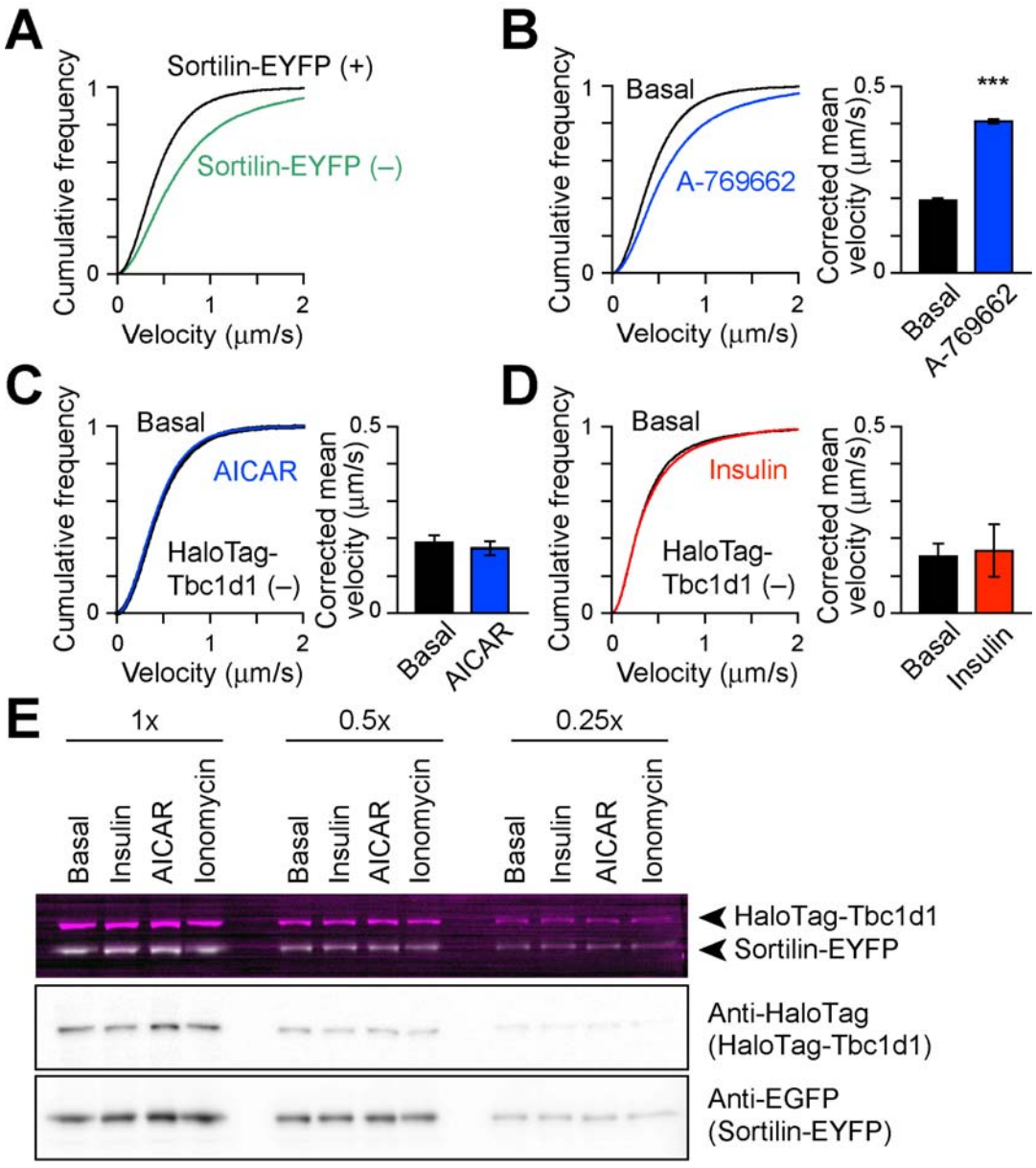

Figure S1. Tbc1d1 mediates AICAR-dependent GLUT4 liberation

(A) Critical roles of sortilin in generating static GLUT4 movement. Velocity distributions of GLUT4 movement in cells expressing mycGLUT4-ECFP and HaloTag-Tbc1d1/WT in the presence (black) or absence (green) of sortilin-EYFP are shown. Corrected mean velocities were shown in Figure 1F. (B) Velocity distributions (left) and corrected mean velocities (right) of GLUT4 movement in cells stimulated without (black, same as in Figure 2B) or with (blue) A-769662 (10 μM, 30 min, n=7). ****P*<0.001. (C) Effects of AICAR on GLUT4 movement in cells expressing only mycGLUT4ECFP and Sortilin-EYFP. Velocity distributions (left)

and corrected mean velocities (right) in cells stimulated without (black, n=7) or with (blue, n=5) AICAR are shown. (D) Effects of insulin on GLUT4 movement in cells expressing only mycGLUT4ECFP and Sortilin-EYFP. Velocity distributions (left) and corrected mean velocities (right) in cells before (black) or after (red) insulin stimulation (100 nM, 30 min, n=4) are shown. (E) Expression of exogenous proteins detected by their fluorescence (upper) and western blotting (middle and lower). The samples were the same as shown in Figure 2E and loaded in series dilutions. HaloTag-Tbc1d1 was fluorescently labeled by HaloTag Ligand TMR, and the fluorescence obtained from excitation wavelength at 488 nm (green) and 546 nm (magenta) were merged.

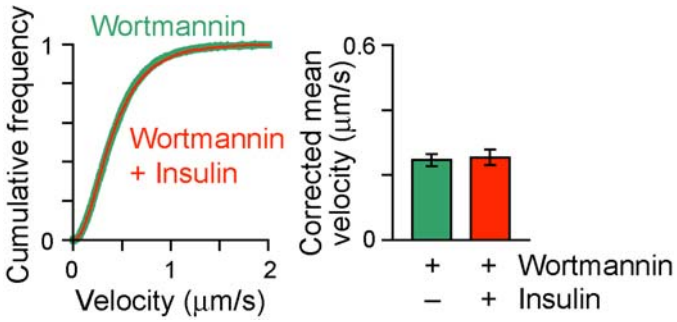

Figure S2. PI3K inhibition abolished insulin responsiveness of Tbc1d1 endowed by AICAR pretreatment

Cells were first stimulated with AICAR (1 mM, 30 min), washed thoroughly for 30 min and continuously incubated with wortmannin (100 nM, 30 min), a PI3K inhibitor. The cells were then stimulated with insulin (100 nM, 30 min). Velocity distributions (left) and corrected mean velocities (right) in cells before (green) and after (red) the addition of insulin, obtained from 6 cells, are shown.

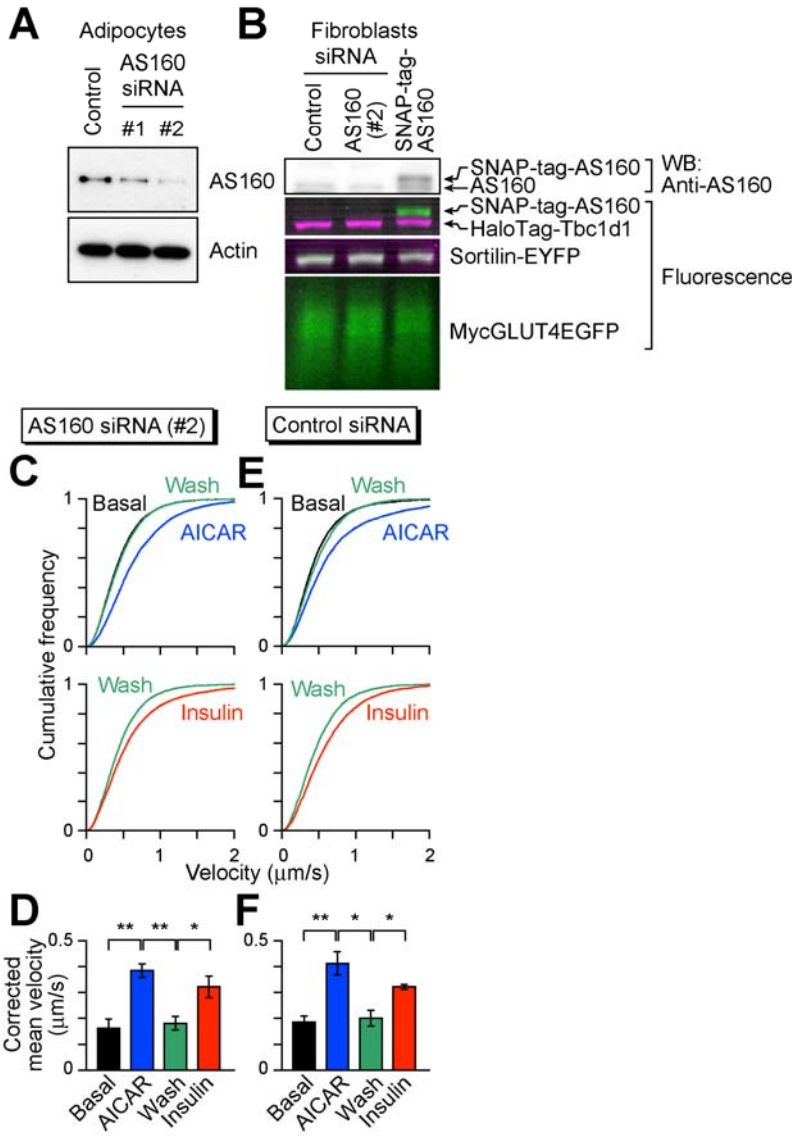

Figure S3. AS160 siRNA did not affect GLUT4 behavior

(A) Effects of AS160 siRNA in 3T3L1 adipocytes. Control or two distinct sets of AS160 siRNAs (#1:5'-GCUCUGCGCCCGUAGACUA-3', 5'-CGCUGAAGCACGGACUUUA-3', 5'-GUGACAACCAGCAUGUCUA-3', #2: 5'-CCUAUGAGGUAGAAUAUCA-3') were transfected by electroporation for validating AS160 siRNAs. Forty-eight hours after electroporation, cell lysates were subjected to western blotting analysis using anti-AS160 antibody (Abcam: AB62487). We used AS160 siRNA #2 in the following analyses. (B) Western blotting of endogenous AS160 and detection of exogenously expressed proteins in undifferentiated 3T3L1 fibroblasts. Twenty-four hours after transfection, SNAP-

tag-AS160 and HaloTag-Tbc1d1 in whole cell lysates were fluorescently labeled by SNAP-Vista Green and HaloTag Ligand TMR, respectively. The fluorescent images of the SDS-PAGE gel obtained from excitation wavelength at 488 nm (green) and 532 nm (magenta) were merged. (C–E) Velocity distributions (B and D) and corrected mean velocities (C and E) of GLUT4 movement in cells transfected with AS160 (B and C) or control (D and E) siRNA. Data were obtained from at least 3 cells. *P<0.05, **P<0.01.

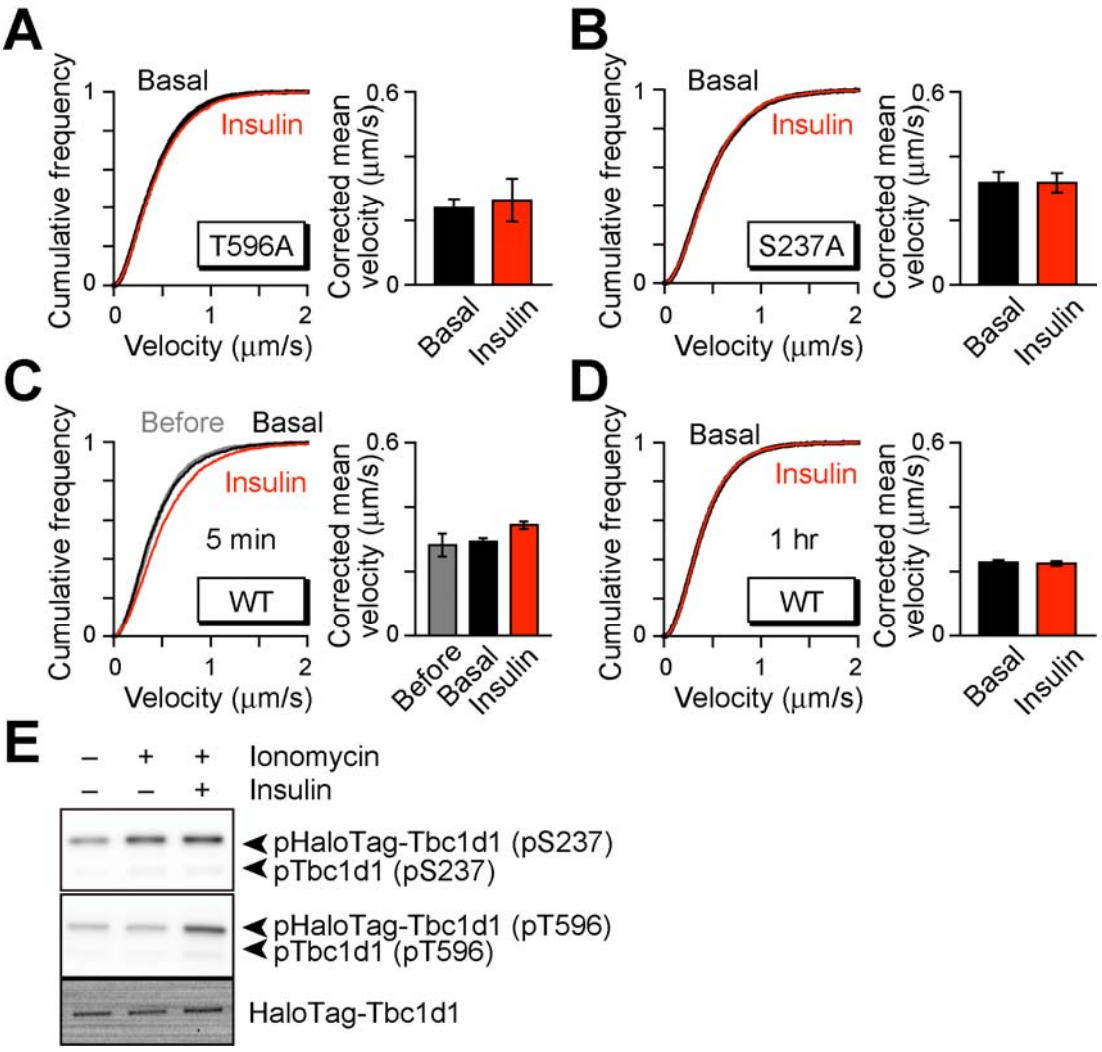

Figure S4. Acquisition of insulin responsiveness by combined stimulation with Ca^{2+} and insulin was short-lived

(A and B) Effects of combined stimulation with insulin and Ca^{2+} on GLUT4 movement in cells expressing T596A (A) or S237A (B) mutants of Tbc1d1. Stimulation protocols were the same as in Figure 5D. Data were obtained from 4 cells each. (C) Velocity distributions (left) and corrected mean velocities (right) of GLUT4 movement in cells before (gray), 5 min after (black) photolysis of NPE and after subsequent insulin stimulation (100 nM, 30 min, red, n=4). (D) Velocity distributions (left) and corrected mean velocities (right) of GLUT4 movement in cells before (black) and after (red) insulin stimulation (100 nM, 30 min, n=3). Insulin stimulation was performed 1 hr after NPE photolysis. (E) Tbc1d1 phosphorylation induced by inomycin or ionomycin + insulin.

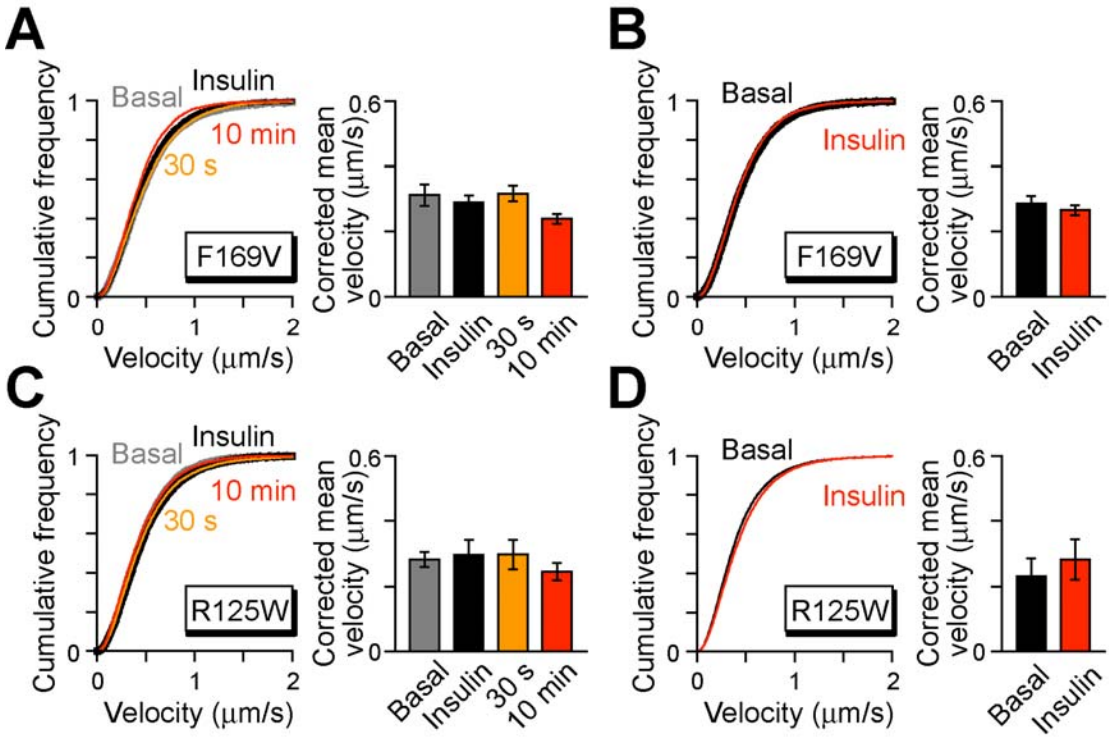

Figure S5. PTB1 Domain Essential for Ca^{2+}/Insulin-dependent GLUT4 Liberation

(A–D) Combined stimulation with Ca^{2+} and insulin in cells expressing Tbc1d1/F169V (A and B) and Tbc1d1/R125W (C and D). Results obtained by sequential stimulation (A and C, corresponding to Figure 5C) or simultaneous stimulation (B and D, corresponding to Figure 5D) are shown. Data were obtained from 3 or 4 cells.