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Supplementary Materials for

BSTA Promotes mTORC2-Mediated Phosphorylation of Akt1 to Suppress Expression of *FoxC2* and Stimulate Adipocyte Differentiation

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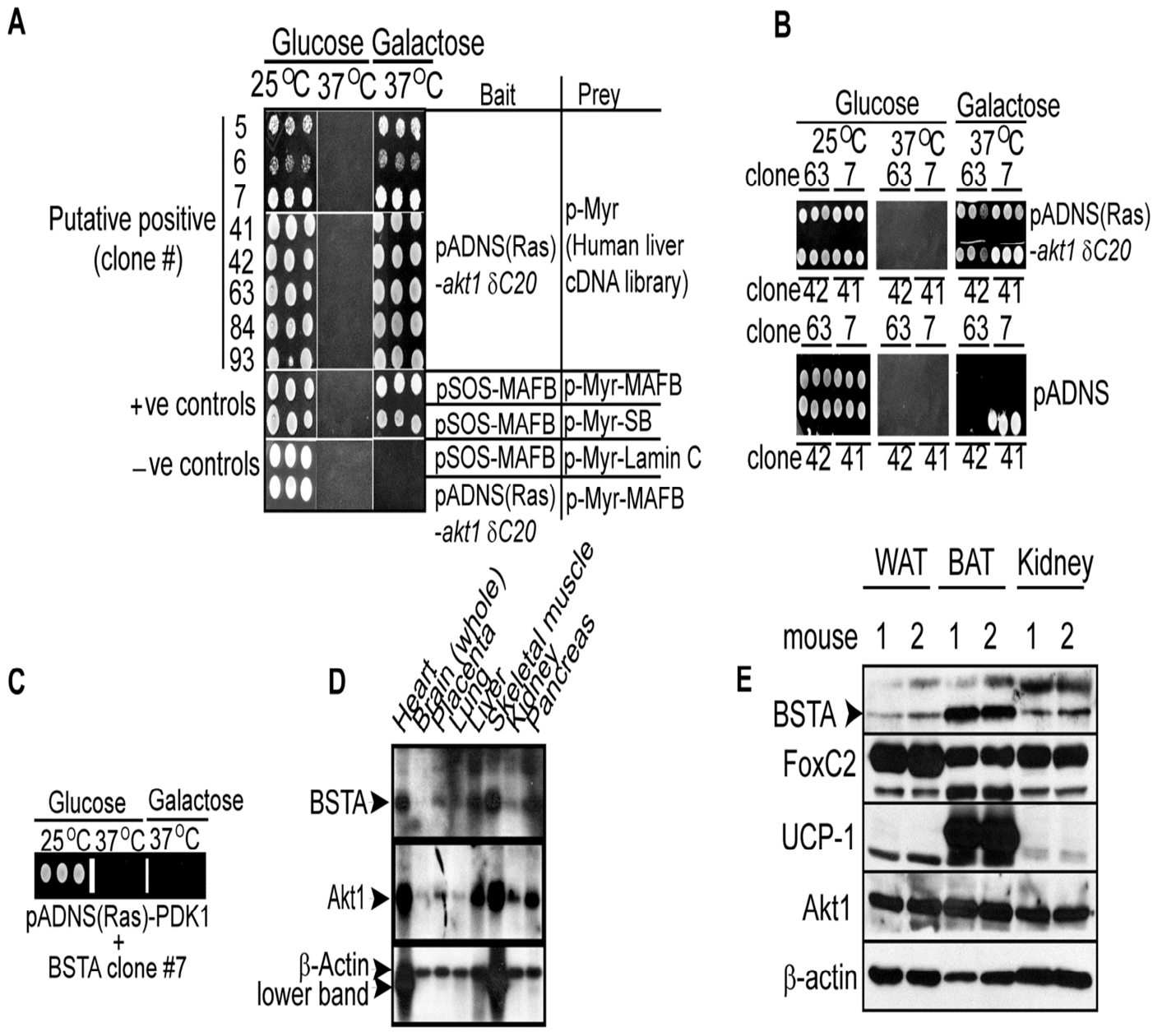

Figure S1. Identification of an Akt1-interacting protein. (**A**) A modified yeast two-hybrid system (RRS) was used to screen a human liver cDNA library for Akt1-interacting proteins. Briefly, 2 x 10^6 primary transformants from a human liver cDNA library were screened using pADNS(Ras)-*akt1δC20* or Akt1 (1-460 aa) as bait. A total of 12 positive transformants were identified (8 are shown in triplicate spotting), from which library plasmids were extracted and sequenced. Whereas clone 41 was Son of Sevenless (SOS) and picked up as a "false-positive" in this system, three were identical (clones 7, 42, and 63) and truncated (173-352 aa) cDNA clones of a gene of unknown function known as *syap1* that we referred to as *bsta*. N=3 experiments. (**B**) Yeast cells were retransformed with the positive *bsta* clones 7, 41, 42, and 63 and either the empty or bait-containing vector pADNS. N=3 experiments. (**C**) Yeast cells were also co-transformed with BSTA clone 7 and pADNS(Ras)-PDK1. N=2 experiments. (**D**) A premade human adult tissue blot (MTN Blot; Clontech) containing about 2 μg of purified poly A^+ RNA in each lane was probed wit *bsta* (1 **x** 10^7 cpm), *akt1*, and *β-actin* cDNA according to the manufacturer's instructions. Radiolabeled bands were visualized using autoradiography. N=2 experiments. (**E**) Three murine tissues (WAT: white adipose tissue, BAT: brown adipose tissue, and kidney) were probed by WB. N=2 experiments.

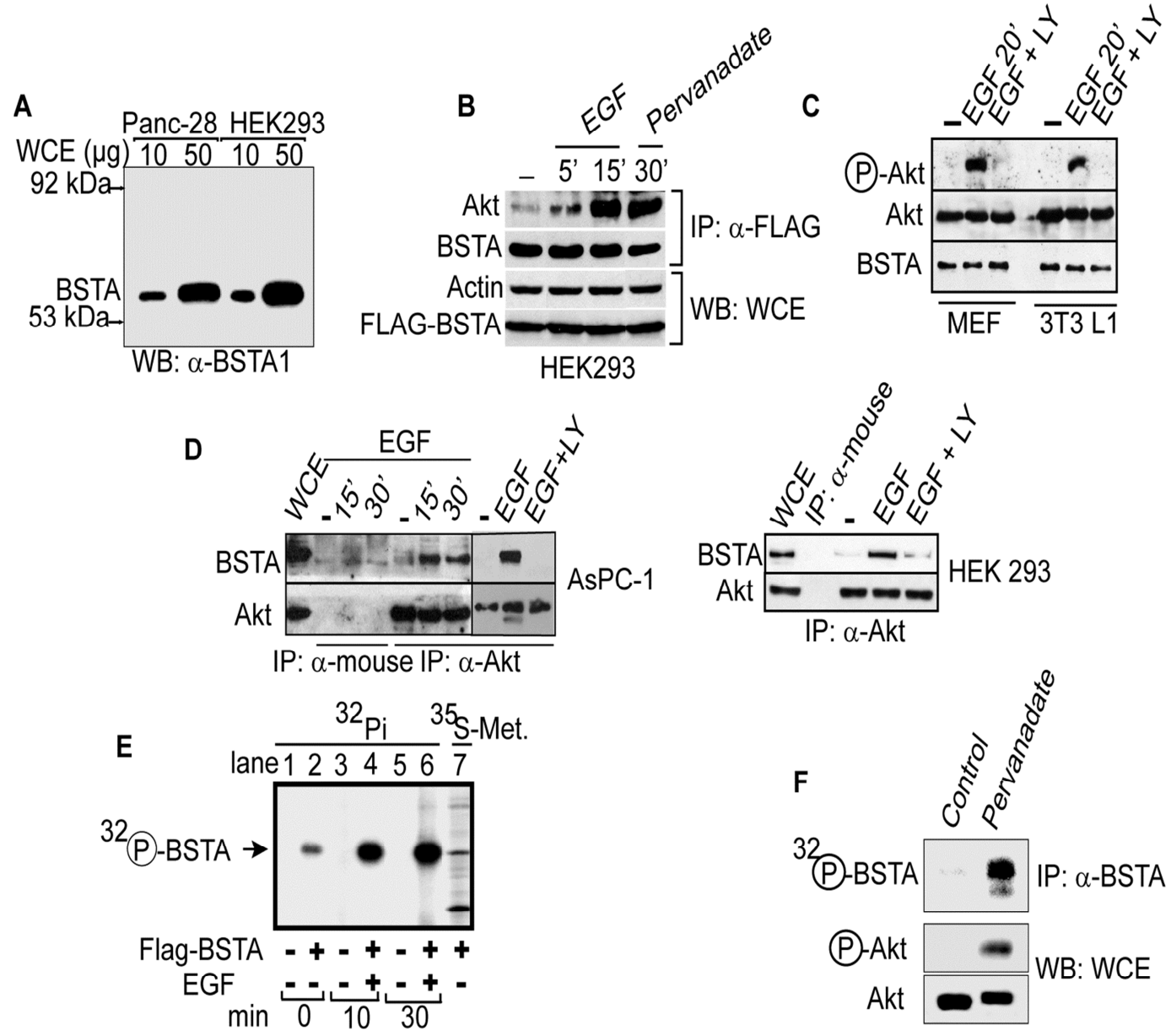

Figure S2. BSTA (M_r 55,000) interacts with Akt and is phosphorylated in cells. (**A**) Different amounts of Panc-28 and HEK 293 whole cell extracts (WCEs) were immunoblotted with anti-BSTA-1 antibodies to detect a polypeptide of M_r 55,000. N=5 experiments. (**B**) FLAG immunoprecipitates from HEK293 cells transiently expressing pCMV-FLAG-BSTA treated with EGF or pervanadate were immunoblotted as indicated. N=2 experiments. (**C**) WCEs used for IP in Fig. 1A were immunoblotted as indicated. N=2. (**D**) Endogenous Akt and BSTA interact with each other in AsPC-1 and HEK293 cells as demonstrated by coimmunoprecipitation. N=3 experiments. (**E**) FLAG immunoprecipitates from radiolabeled (^{32}Pi or ^{35}S-methionine) pCMV-FLAG2-BSTA-expressing NIH 3T3 cells were resolved using SDS-PAGE. Radiolabeled bands were visualized using autoradiography and the position of BSTA was determined by immunoblotting in parallel. N=2 experiments. (**F**) BSTA immunoprecipitates from pervanadate-stimulated (15 min), radiolabeled Cos-7 cells were visualized using autoradiography. WCEs were probed for phosphorylated Akt (at Ser^{473}) and total Akt as shown in the lower two panels. N=2 experiments.

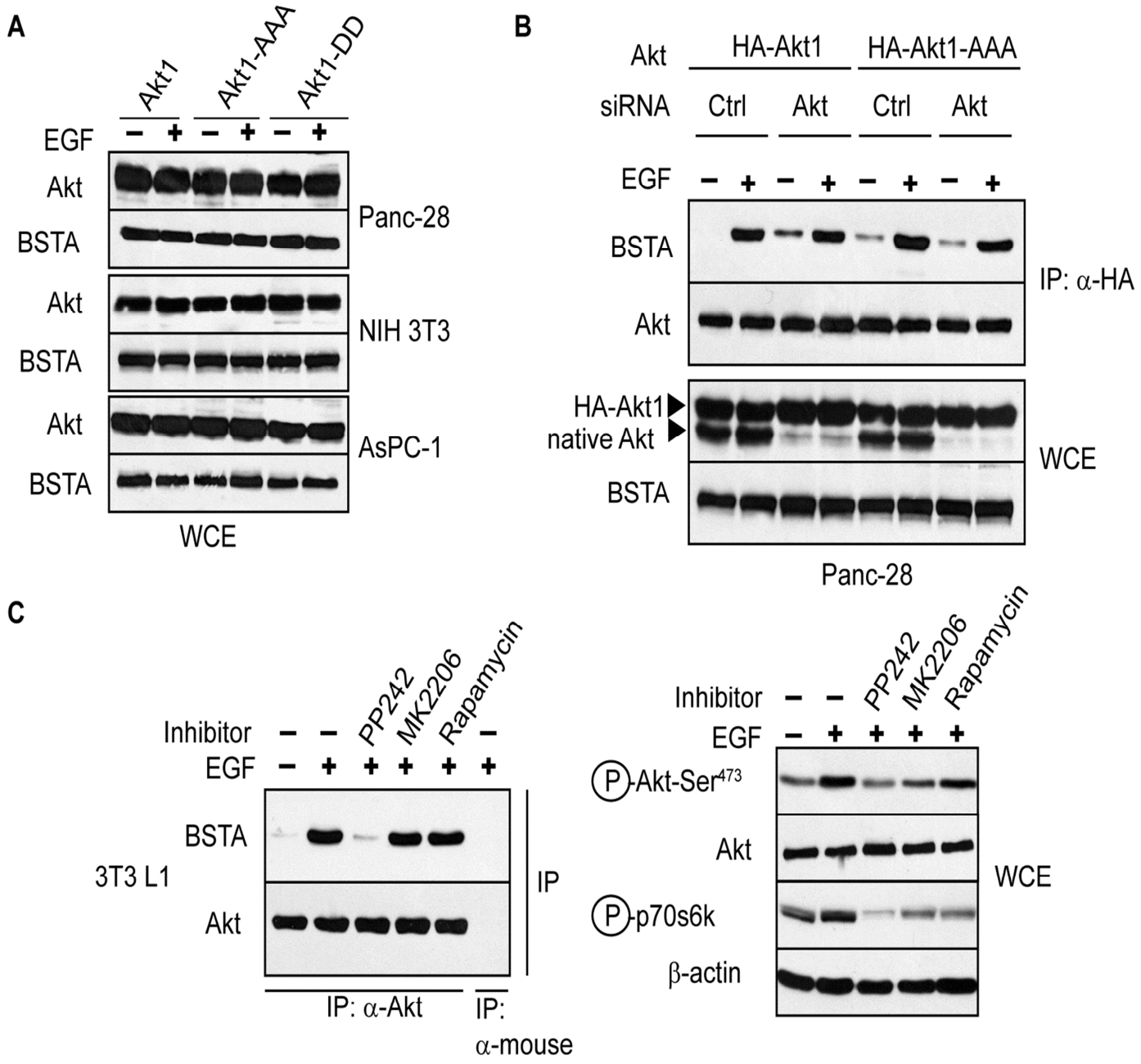

Figure S3. Akt activity is dispensable for the BSTA-Akt interaction. (**A**) WCEs used for immunoprecipitation in Fig. 1C were probed for total Akt and BSTA. N=2 experiments. (**B**) WCEs from HA-Akt1–, and HA-Akt1-AAA–expressing stable cells (Panc-28) treated with control or *akt* UTR-directed (*akt* 1 and 2, 100 nM) siRNAs were used for immunoprecipitation or directly immunoblotted. N=2 experiments. (**C**) Serum-starved 3T3 L1 cells were treated with vehicle, PP242 (mTOR inhibitor), MK2206 (Akt inhibitor), or rapamycin (mTORC1 inhibitor) for 4 hours before EGF stimulation (20 min). WCEs were subjected to immunoprecipitation or directly immunoblotted. N=2 experiments.

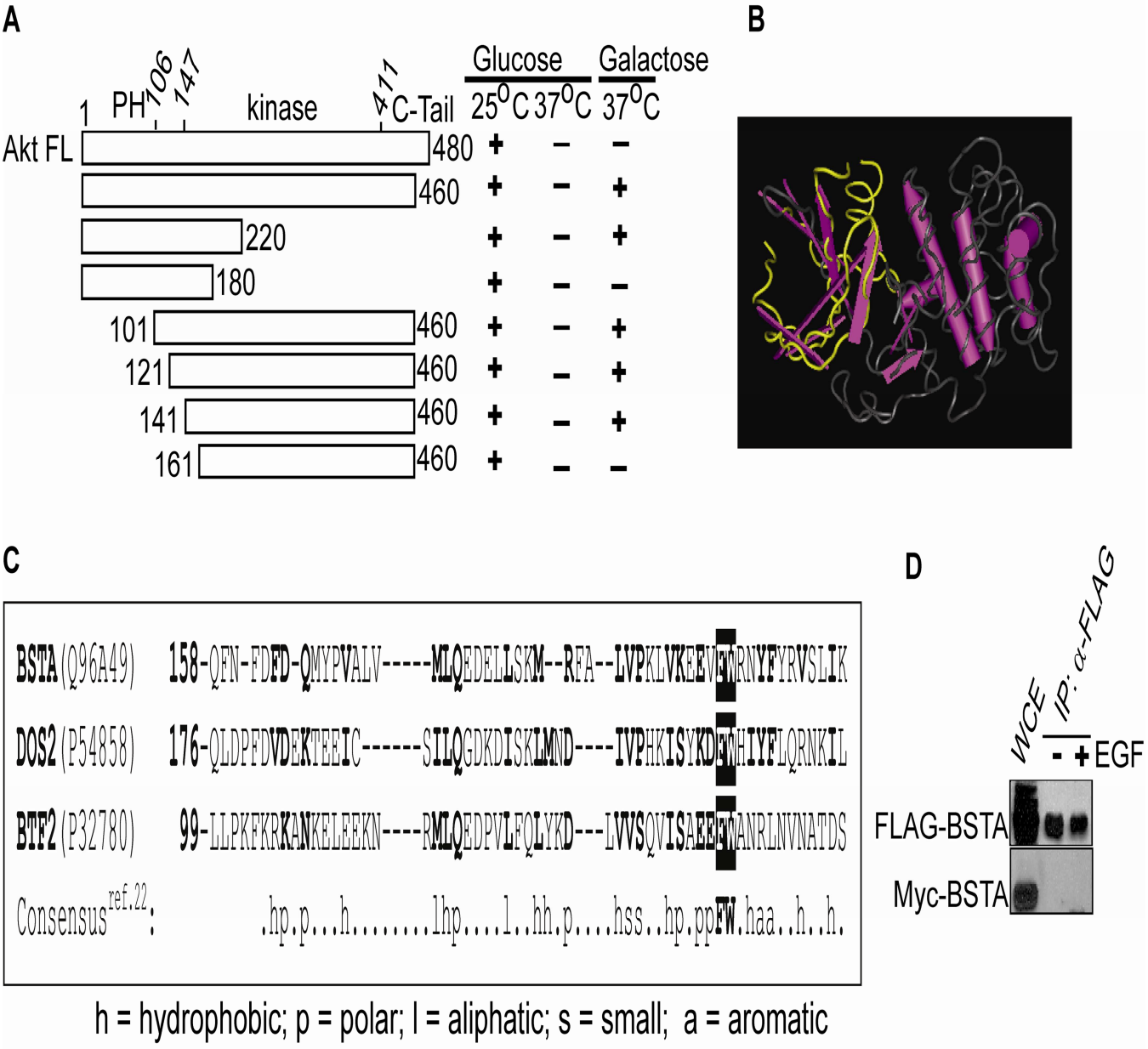

Figure S4. Identification of a putative BSTA-binding sequence in Akt1. (**A**) Full-length *akt1* (Akt FL) and its deletion mutants were generated in the pADNS vector and tested for interaction with truncated pMyr-BSTA (clone 7 in Fig. S1A) in yeast. N=3 experiments. (**B**) The crystal structure of Akt1 (MMDB ID: 22887; PDB ID: 1GZO) with the putative BSTA-binding sequence (shown in yellow) containing the β1-β4 strands of the β sheet and the α-B and α-C helices of the N-lobe. (**C**) The BSD domains of BSTA and two other proteins are aligned. Amino acids that fit the BSD domain consensus sequence (*22*) are shown in bold. (**D**) Twenty-four hours after transfection, Myc-BSTA- and FLAG-BSTA-expressing HEK293 cells were serum-starved (16 hours) and treated with or without EGF (10 min). FLAG immunoprecipitates were immunoblotted with anti-Myc antibodies. N=2 experiments.

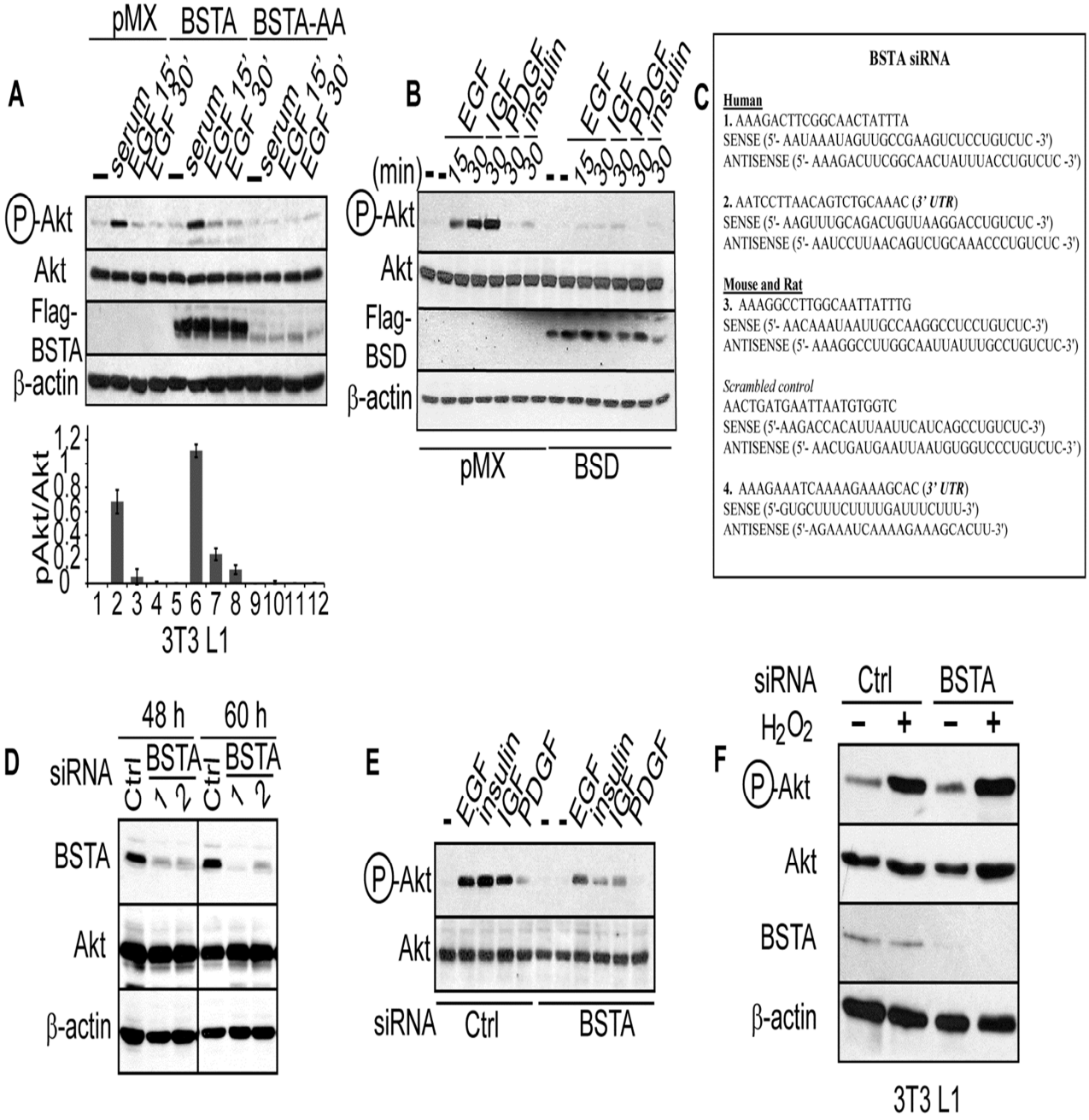

Figure S5. Effect of BSTA mutants and siRNA on the phosphorylation status of Akt. (**A**) 3T3 L1 cells stably transfected with vector, pMX-BSTA, or pMX-BSTA-AA were serum-starved and stimulated with 10% serum or EGF. Densitometric data (N=3 experiments) showing pAkt/Akt ratios (means ± SD) are presented below the representative blot. (**B**) Control and BSD-expressing BxPC-3 cells were stimulated and immunoblotted as indicated. N=2 experiments. (**C**) List of the siRNAs used. UTR, untranslated region. (**D**) Panc-28 cells transfected for 48 and 60 hours with two different siRNAs (directed against human BSTA) were immunoblotted for the indicated proteins. N=5 experiments. (**E**) siRNA-transfected Panc-28 cells were stimulated with various growth factors or insulin and immunoblotted as indicated. N=2 experiments. (**F**) siRNA transfected 3T3 L1 cells were serum-starved before treatment with or without 750 μM H_2O_2 (30 min), then immunoblotted as indicated. N=2 experiments.

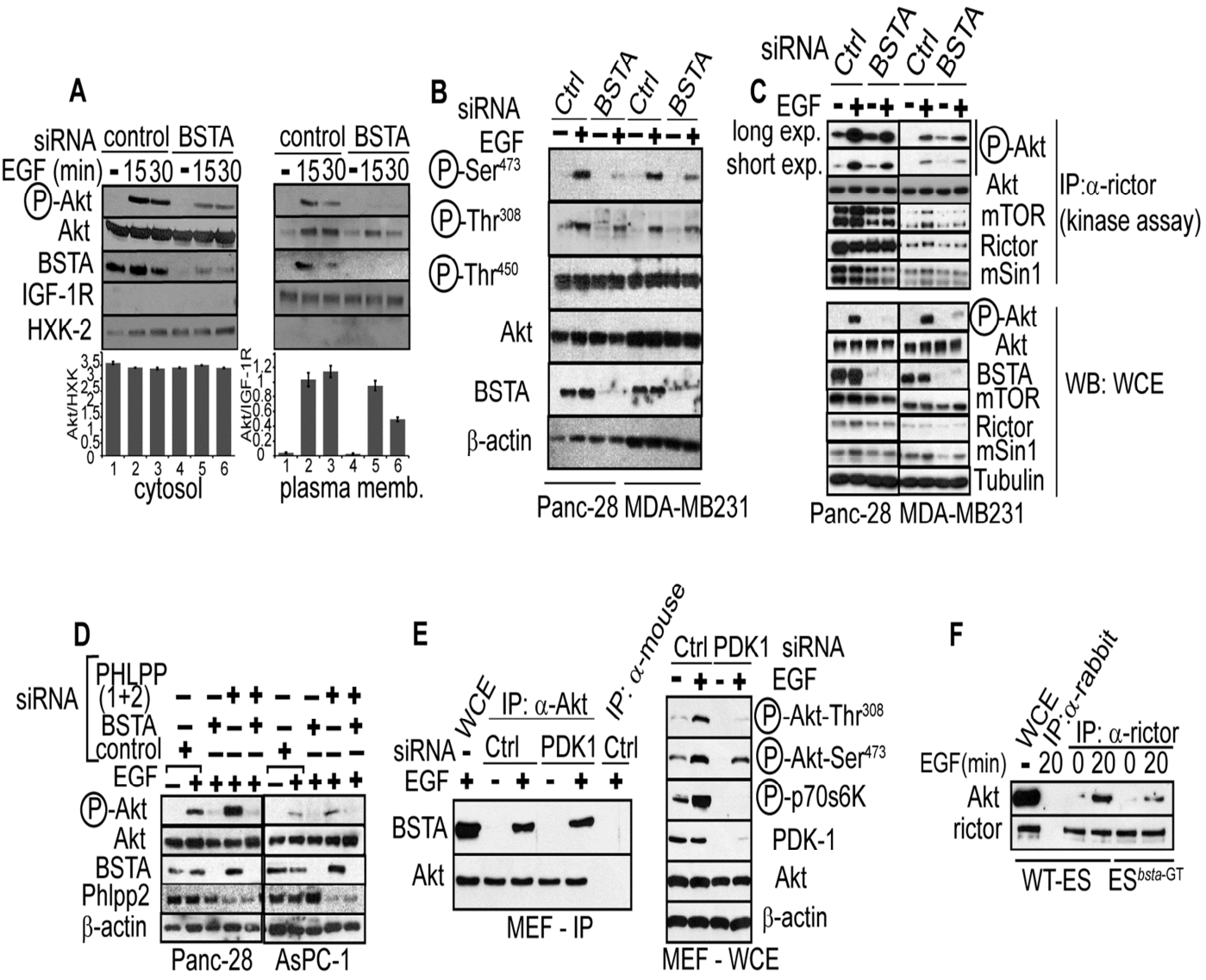

Figure S6. Mechanism of BSTA-mediated phosphorylation of Akt. (**A**) 48 hours after siRNA transfection, overnight serum starvation, and EGF treatment, Panc-28 cells were resolved into cytosol and plasma membrane (PM) fractions using differential centrifugation for Western blot analysis as described previously (*59*). The IGF receptor (IGF-1R) and hexokinase-2 (HXK-2) were analyzed as PM and cytosol markers, respectively. Densitometric data (N=3 experiments) showing pAkt/Akt ratios (means ± SD) are also presented below each representative blot. (**B**) WCEs from siRNA-transfected Panc-28 and MDA-MB231 cells were probed for phosphorylation of Akt (Ser^{473}, Thr^{308}, and Thr^{450}). N=2 experiments. (**C**) rictor immunoprecipitates from various Panc-28 and MDA-MB 231 protein extracts were assayed for mTORC2 activity by immunoblotting. WCEs were immunoblotted as indicated. N=3 experiments. (**D**) Panc-28 and AsPC-1 cells transfected with control, BSTA, or PHLPP1/2 siRNAs as indicated were serum-starved for 16 hours, treated with EGF (20 ng/ml) for 20 min, and immunoblotted. N=2 experiments. (**E**) WCEs from MEFs transfected with control or PDK1 siRNA (100 nM) were stimulated with EGF and either used for immunoprecipitations (left) or directly immunoblotted (right). N=2 experiments. (**F**) rictor immunoprecipitates from control and EGF-stimulated WT-ES and ES$^{bsta\text{-}GT}$ cells were immunoblotted to probe for the mTORC2-Akt interaction. N=2 experiments.

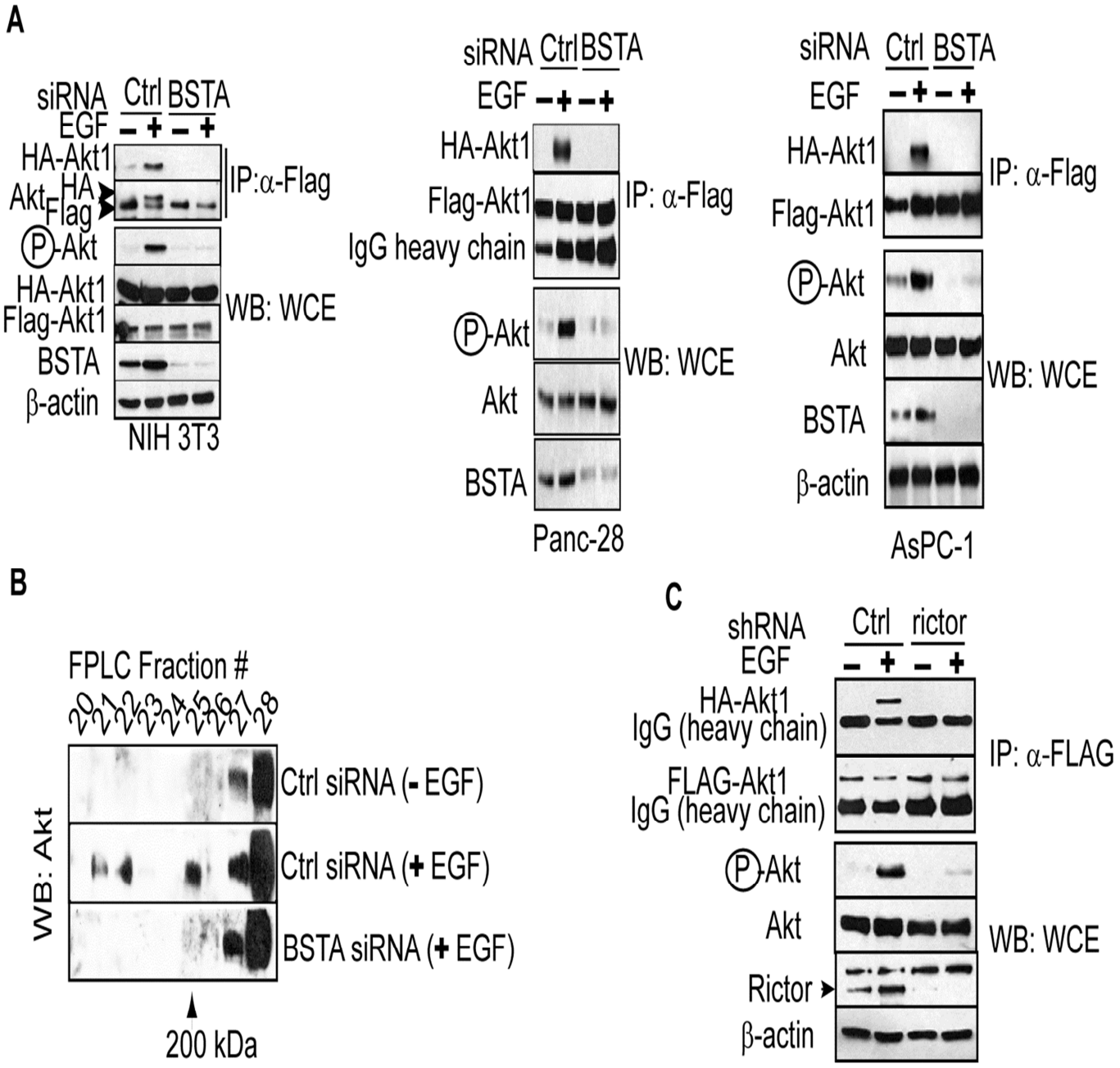

Figure S7. Akt multimerization requires BSTA and mTORC2. (**A**) Akt1 multimerization was examined in NIH 3T3, Panc-28, and AsPC-1 cells expressing HA-Akt1 and FLAG-Akt1 using immunoprecipitation and immunoblotting. N=2 experiments. (**B**) Panc-28 cells transfected with control or BSTA siRNAs were serum-starved (16 hours) and treated with or without EGF (20 ng/ml) for 20 min. WCEs were subjected to size-exclusion chromatography. The results for Akt (fractions 20-28) are shown here. N=2 experiments. (**C**) The requirement for mTORC2 in Akt1 dimerization was examined in HA-Akt1 and FLAG-Akt1 expressing Panc-28 cells tranfected with rector shRNA. N=2 experiments.

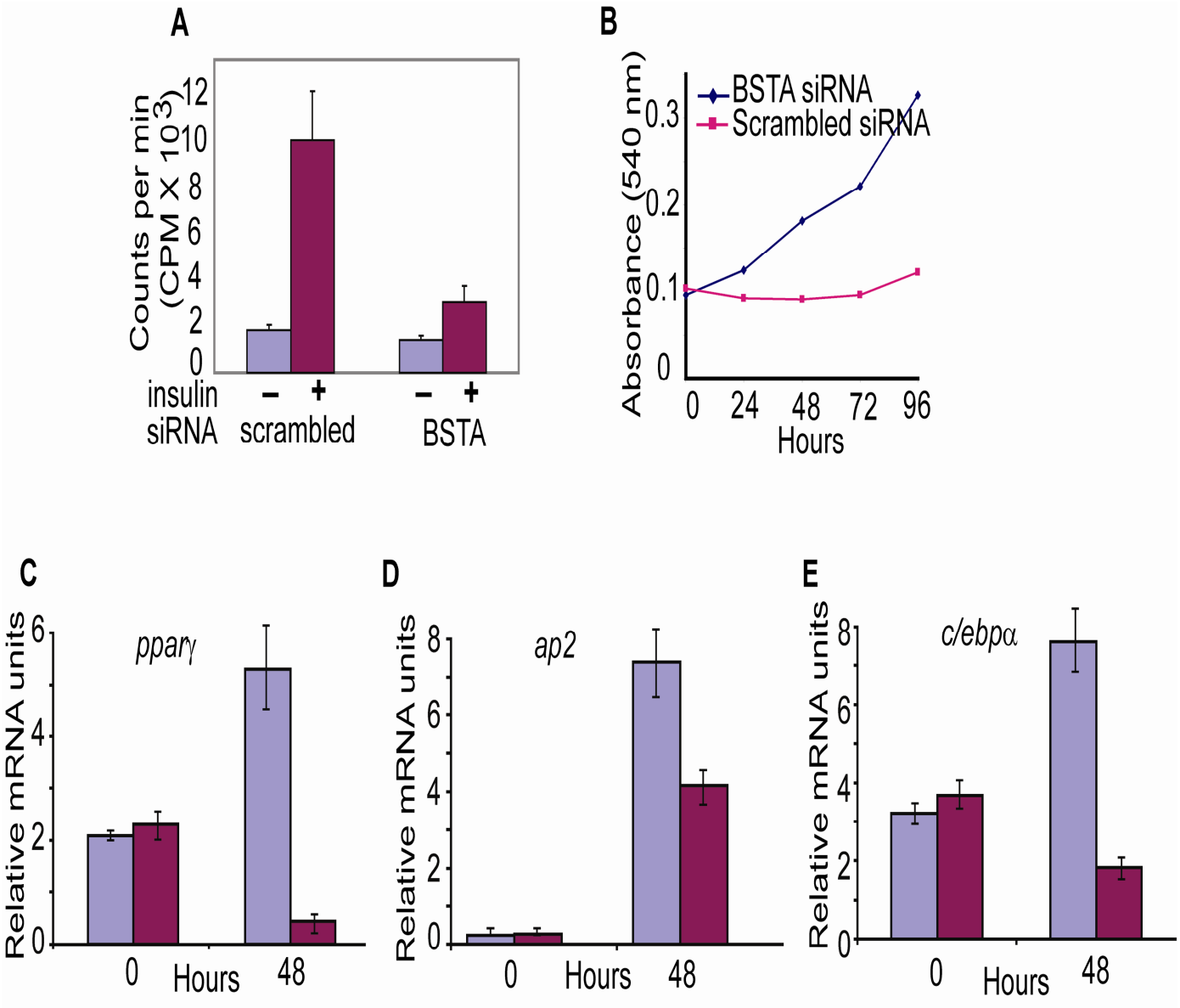

Figure S8. BSTA promotes glucose uptake and adipocyte differentiation. (**A**) 3T3 L1 adipocytes were electroporated with scr or BSTA siRNA and assayed for glucose uptake as described previously (*60*). N=3 experiments. (**B**) 5 days after differentiation was initiated, siRNA-transfected 3T3 L1 cells were trypsinized, cultured in fresh serum-containing medium from 1-4 days in 96-well plates (5 x 10^3 cells/well), and subjected to crystal violet assays as described previously (*54*). N=5 experiments. (**C**-**E**) 3T3 L1 cells electroporated with scr (purple bars) or BSTA siRNA (maroon bars) were induced to differentiate for 48 hours. Total RNA was isolated from the cells and analyzed for *pparγ*, *c/ebpα*, and *ap2* using qPCR. Averages and standard deviations obtained from three independent experiments are shown.

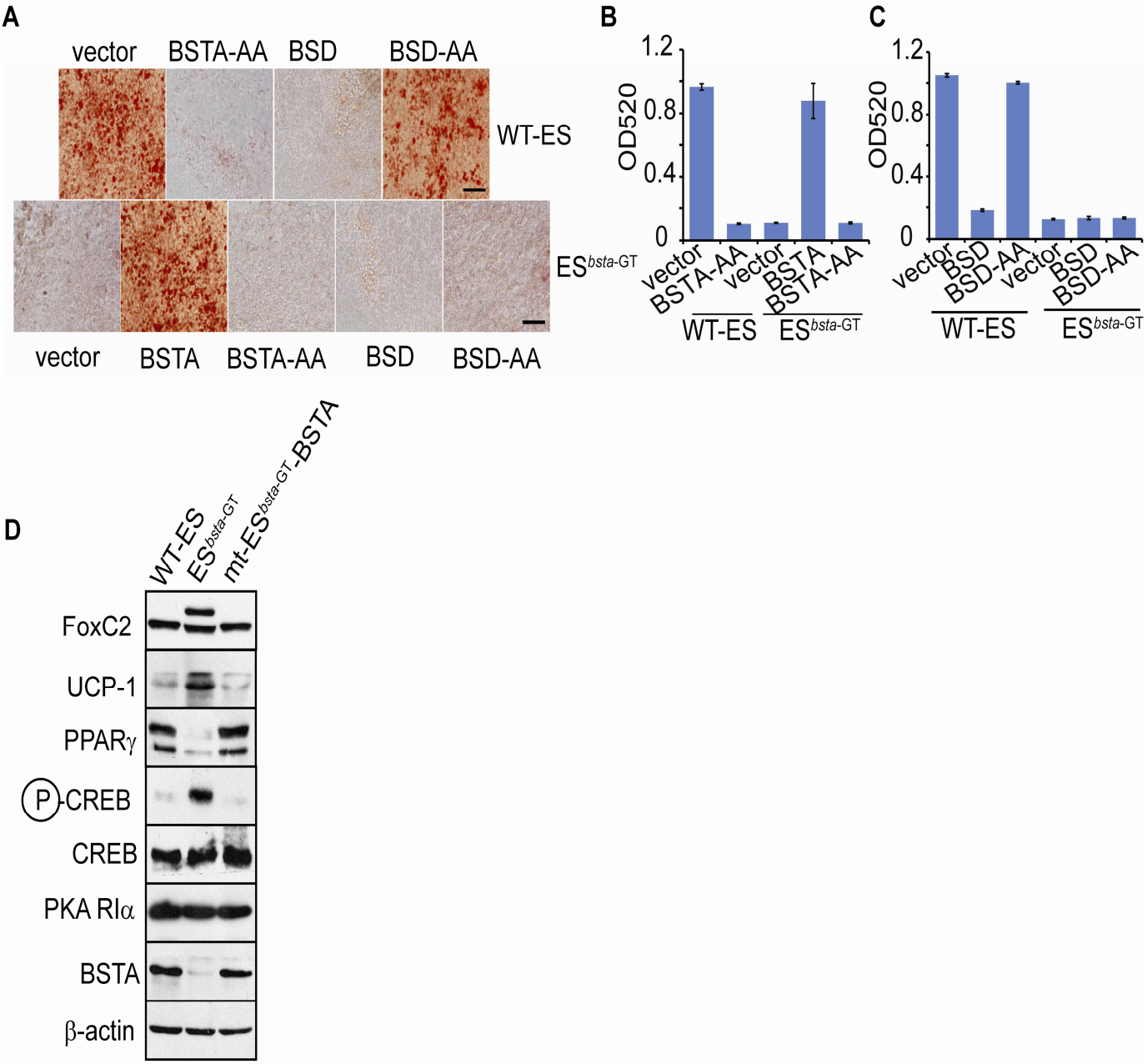

Figure S9. Effects of BSTA, BSTA-AA, BSD, and BSD-AA on adipocyte differentiation. (**A**) WT- and ES$^{bsta\text{-}GT}$ cells containing control vector, or expressing BSTA, BSTA-AA, BSD or BSD-AA were induced to differentiate (insulin+T_3) and stained with oil Red O. Scale bar, 100 μm. oil Red O staining was quantitated by measuring the absorbance at 520 nm (**B** and **C**). Averages and standard deviations obtained from three independent experiments are shown. (**D**) ES cells were immunoblotted for various proteins including the brown adipose tissue-specific marker UCP-1. N=2 experiments.

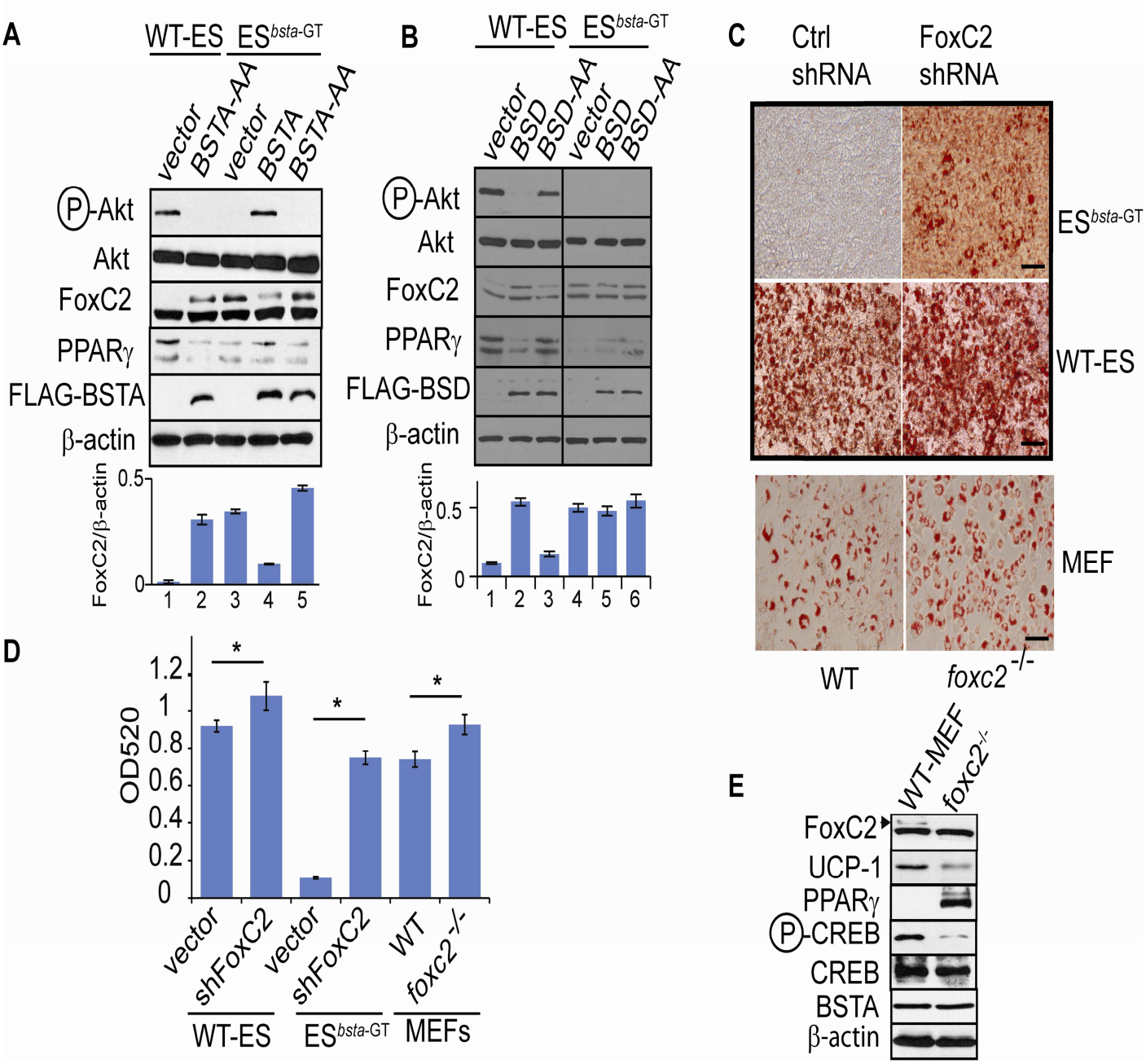

Figure S10. BSTA, Ser^{473} in Akt, and FoxC2 regulate adipocyte differentiation. (**A** and **B**) ES cells expressing BSTA, BSD and their mutants were immunoblotted as indicated. Densitometric data (N=3 experiments) showing FoxC2/β-actin ratios (means ± SD) is presented below each representative blot. (**C** and **D**) WT-ES and ES$^{bsta\text{-}GT}$ cells transduced with control or FoxC2 shRNAs (boxed panels), as well as WT and *foxc2*$^{-/-}$ MEFs, were induced to differentiate (insulin/IBMX/Dex), stained with oil Red O (**C**), and quantitated for oil Red O staining at 520 nm. Scale bar, 100 μm. Data from three independent experiments was analyzed (means ± SD; **P* < 0.001 obtained from one-way ANOVA followed by two-tailed t test.) (**D**). (**E**) WCEs from WT and *foxc2*$^{-/-}$ MEFs were analysed by WB. N=2 experiments.