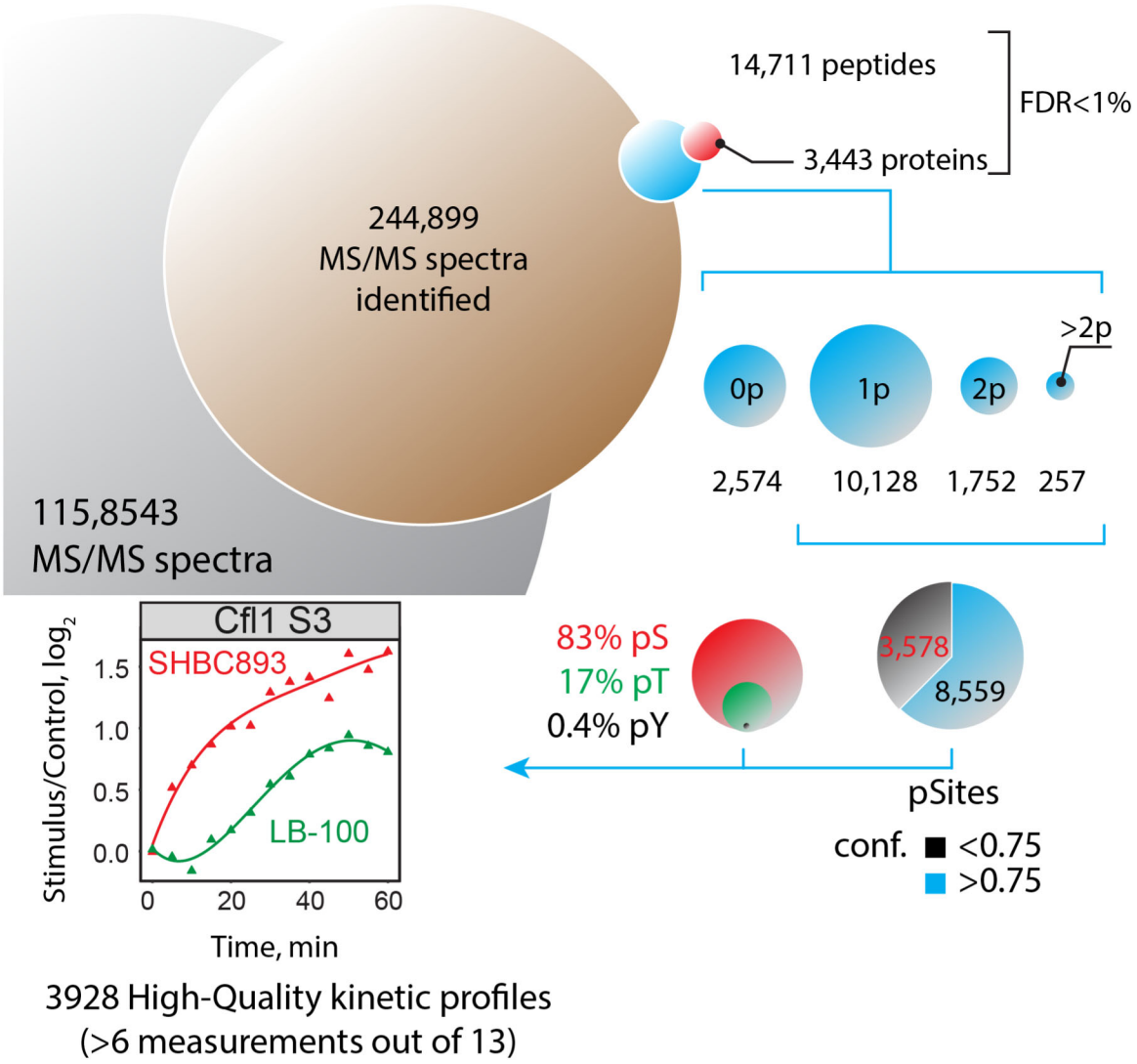
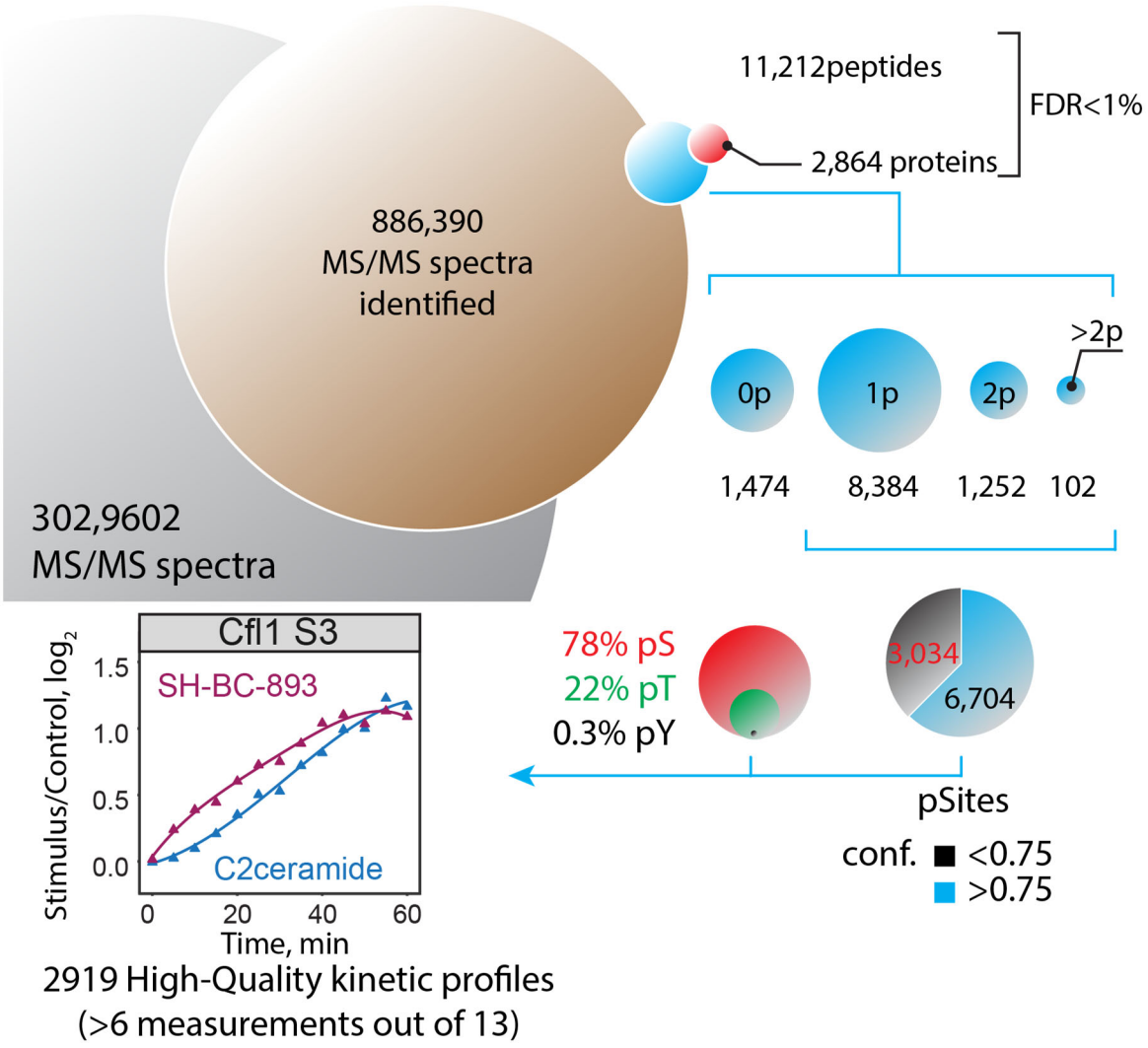
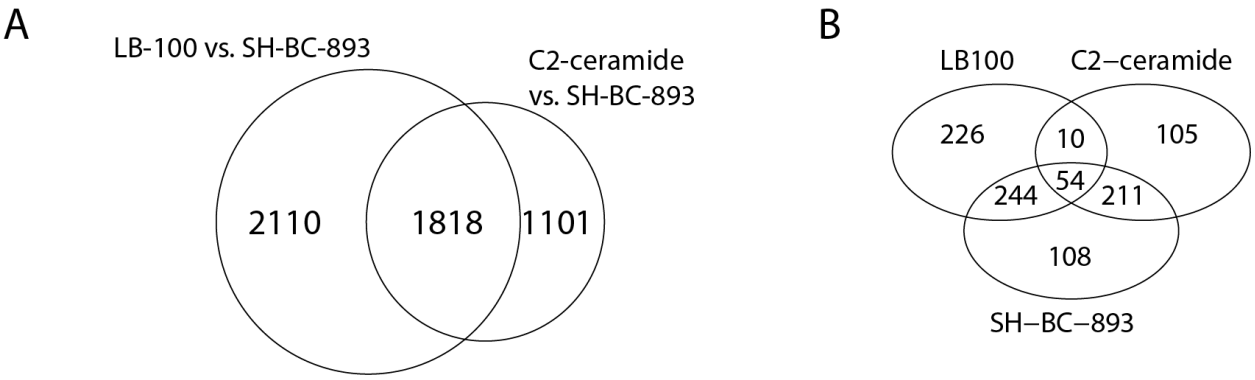


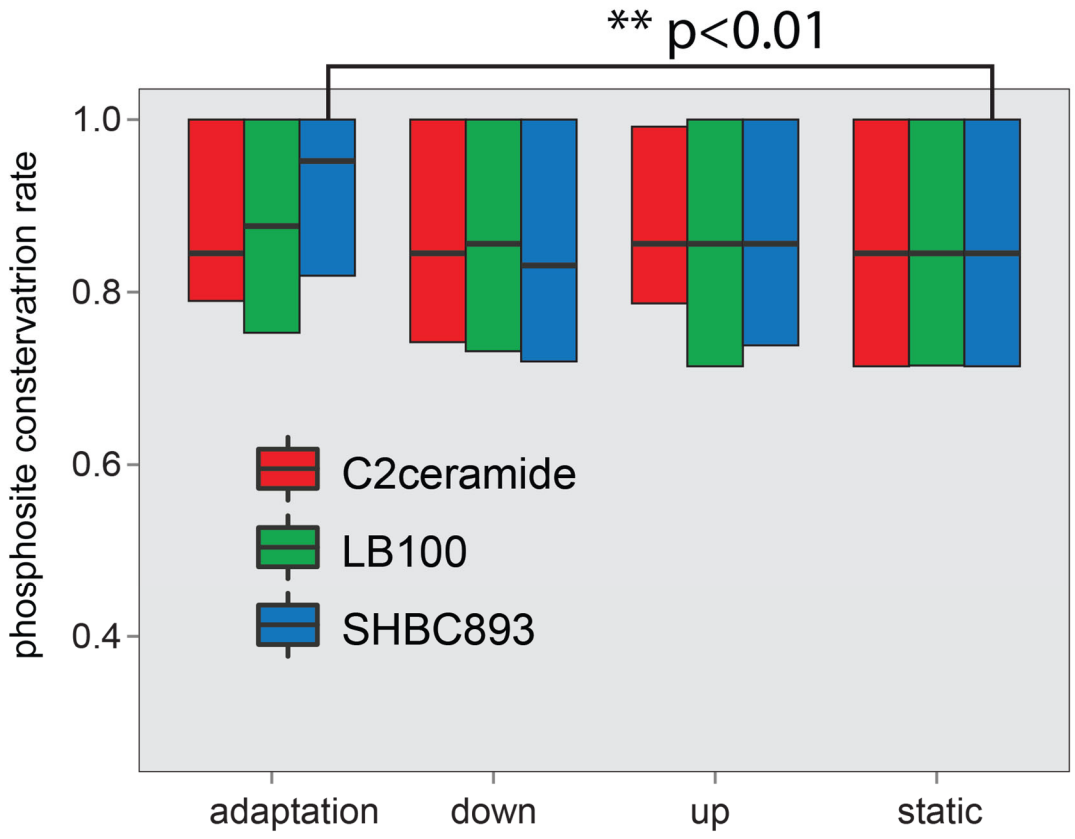
Supplementary Figure 1. Summary of statistics of triple SILAC phosphoproteomics experiment comparing LB-100 and SH-BC-893-treated FL5.12 cells.



Supplementary Figure 2. Summary of statistics of triple SILAC phosphoproteomics experiment comparing C2-ceramide and SH-BC-893-treated FL5.12 cells.

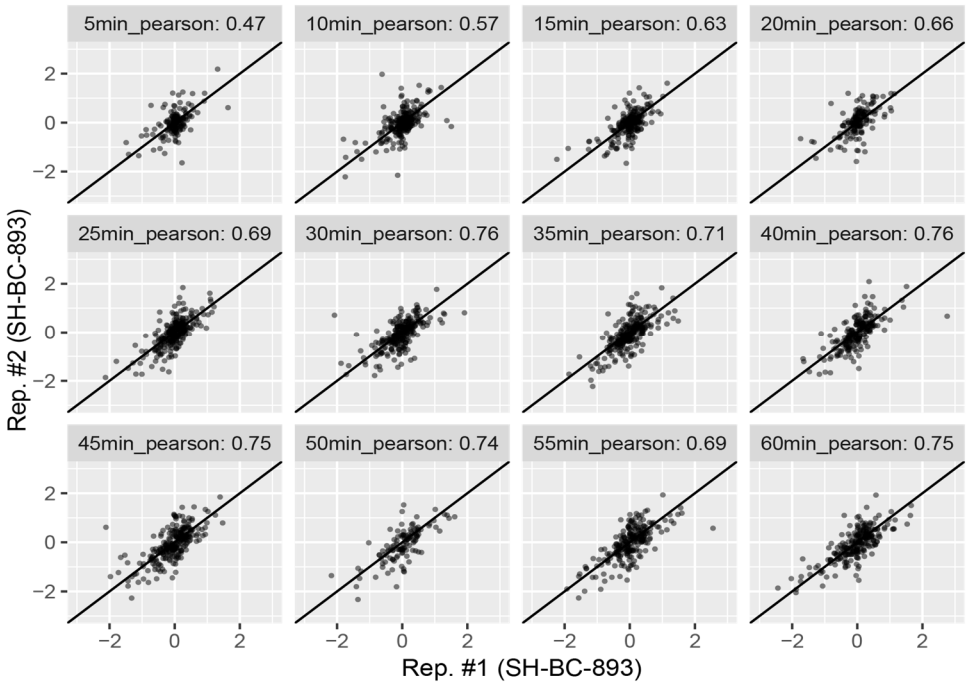


Supplementary Figure 3. Comparison of compounds affecting PP2A activity revealed different protein subsets affecting cell signaling. A) Overlap of high quality kinetic profiles obtained from both triple SILAC experiments comparing either SH-BC-893 and LB-100 or SH-BC-893 and C2-ceramide. 1860 profiles were common to both experiments. B) Overlap of dynamically regulated phosphosites.

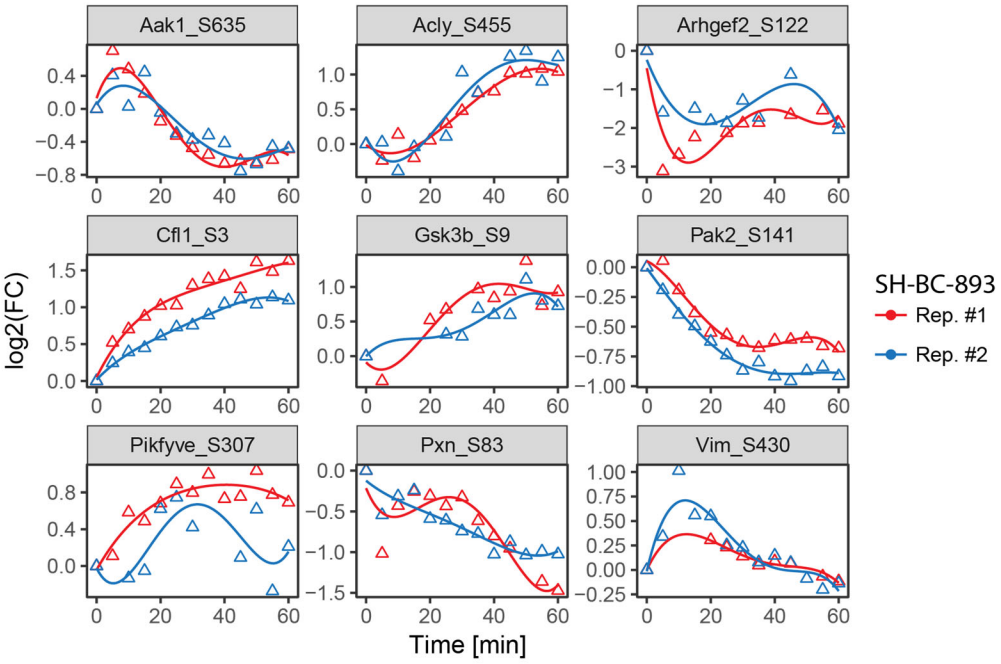


Supplementary Figure 4. Conservation rate analysis. Conservation of phosphorylation sites was compared based on grouping of the kinetic profiles observed for different treatments. Phosphorylation sites showing adaptation-like behavior upon treatment with SH-BC-893 are more conserved across species.

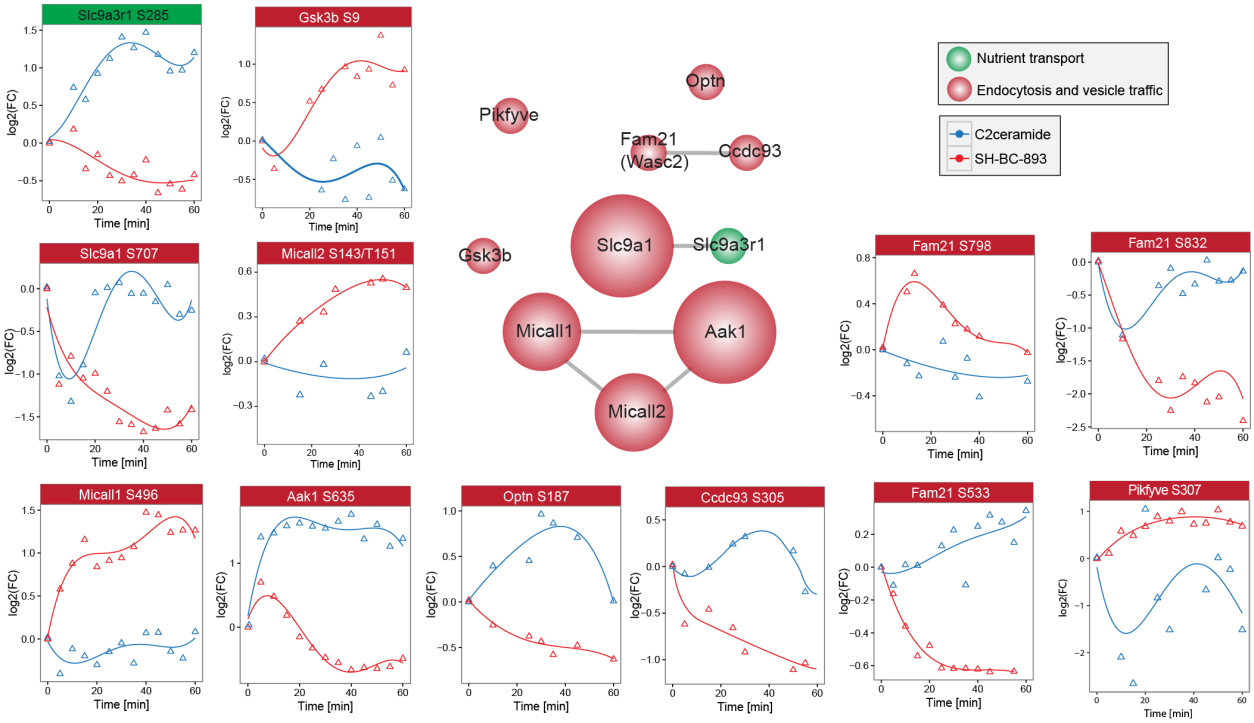
A)



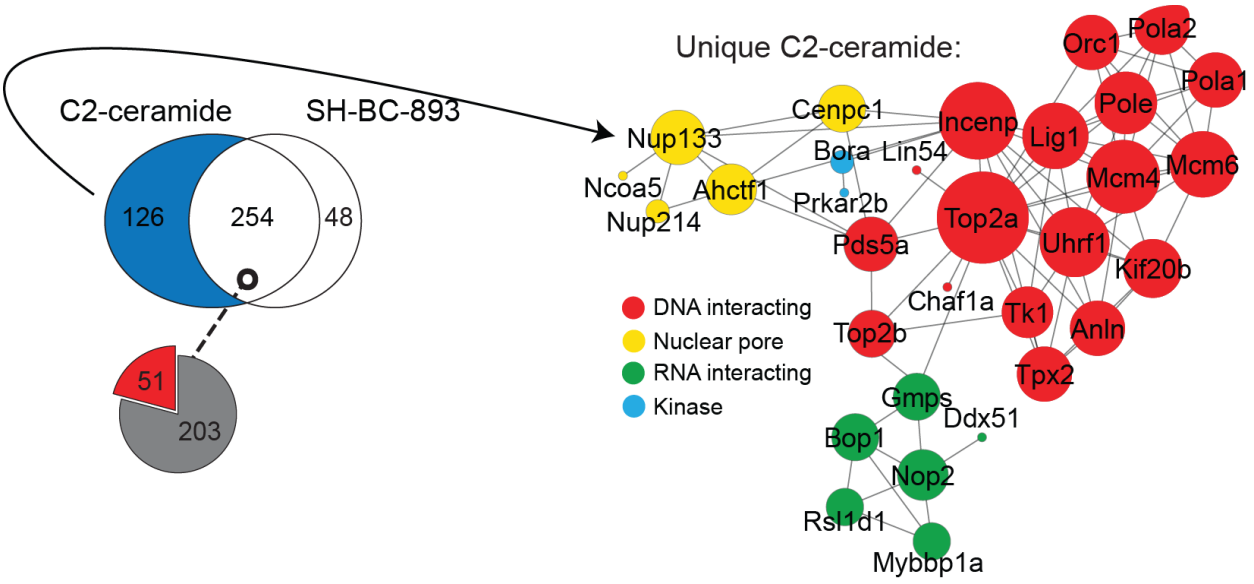
B)



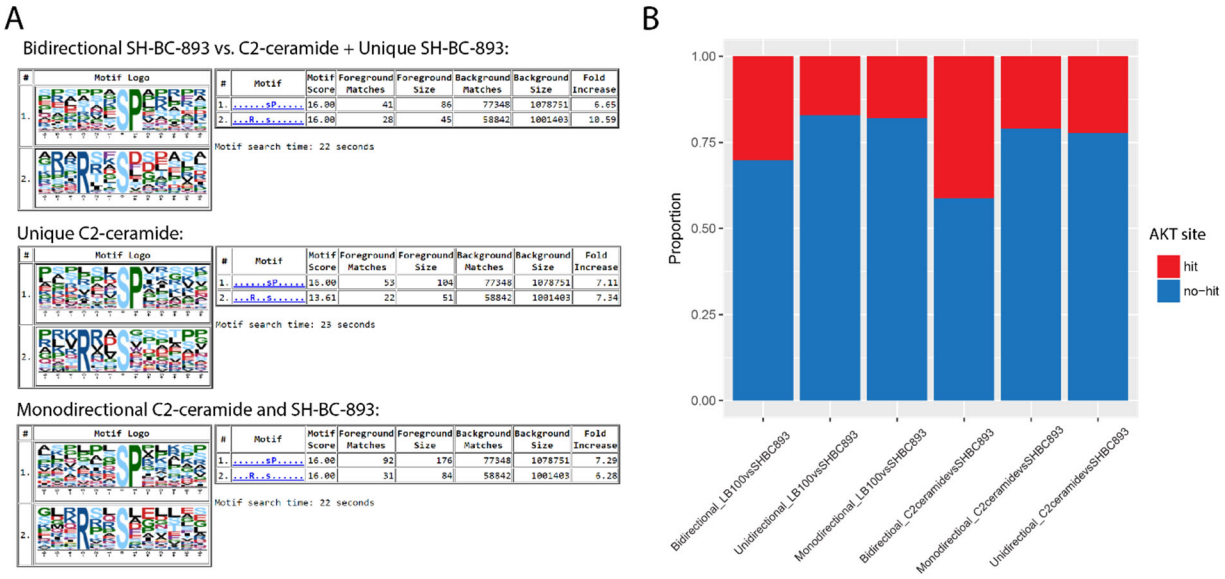
Supplementary Figure 5. A) Reproducibility of fold change measurements for dynamic phosphoproteomic profiles taken at different time points following incubation of FL5.11 cells with SH-BC-893. Replicates 1 and 2 correspond to experiments described in Figure 3C and Figure 6B, respectively. Pearson correlation coefficients are displayed for each time point. B) Comparison of dynamic profiles for 9 phosphopeptides obtained from two separate time course experiments (R1 and R2; SHBC893 treatments).



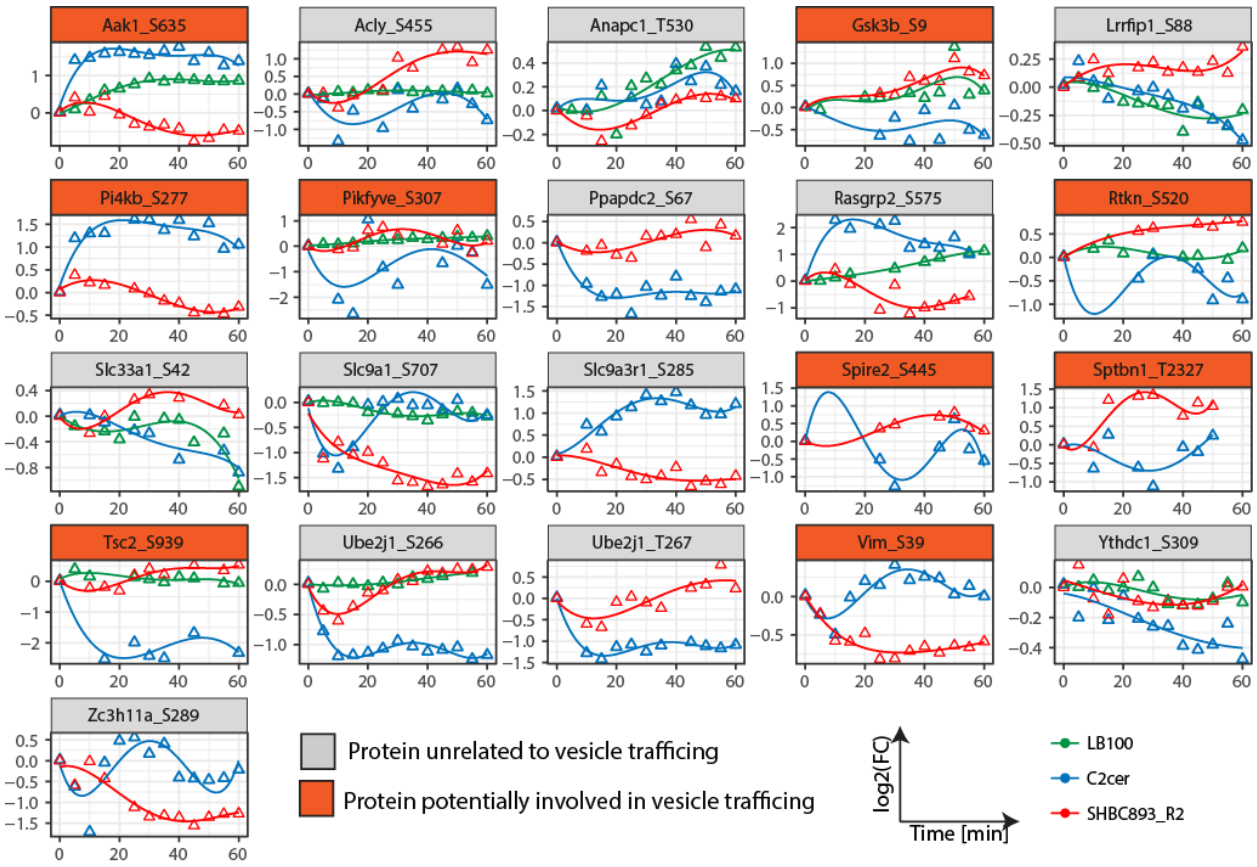
Supplementary Figure 6. Proteins from endosomes, late endosomes or intracellular membrane-bound organelles are differentially phosphorylated upon treatment with sphingolipids. Partial network and kinetic profiles of phosphorylation from proteins following cell treatment with SH-BC-893 and C2-ceramide.



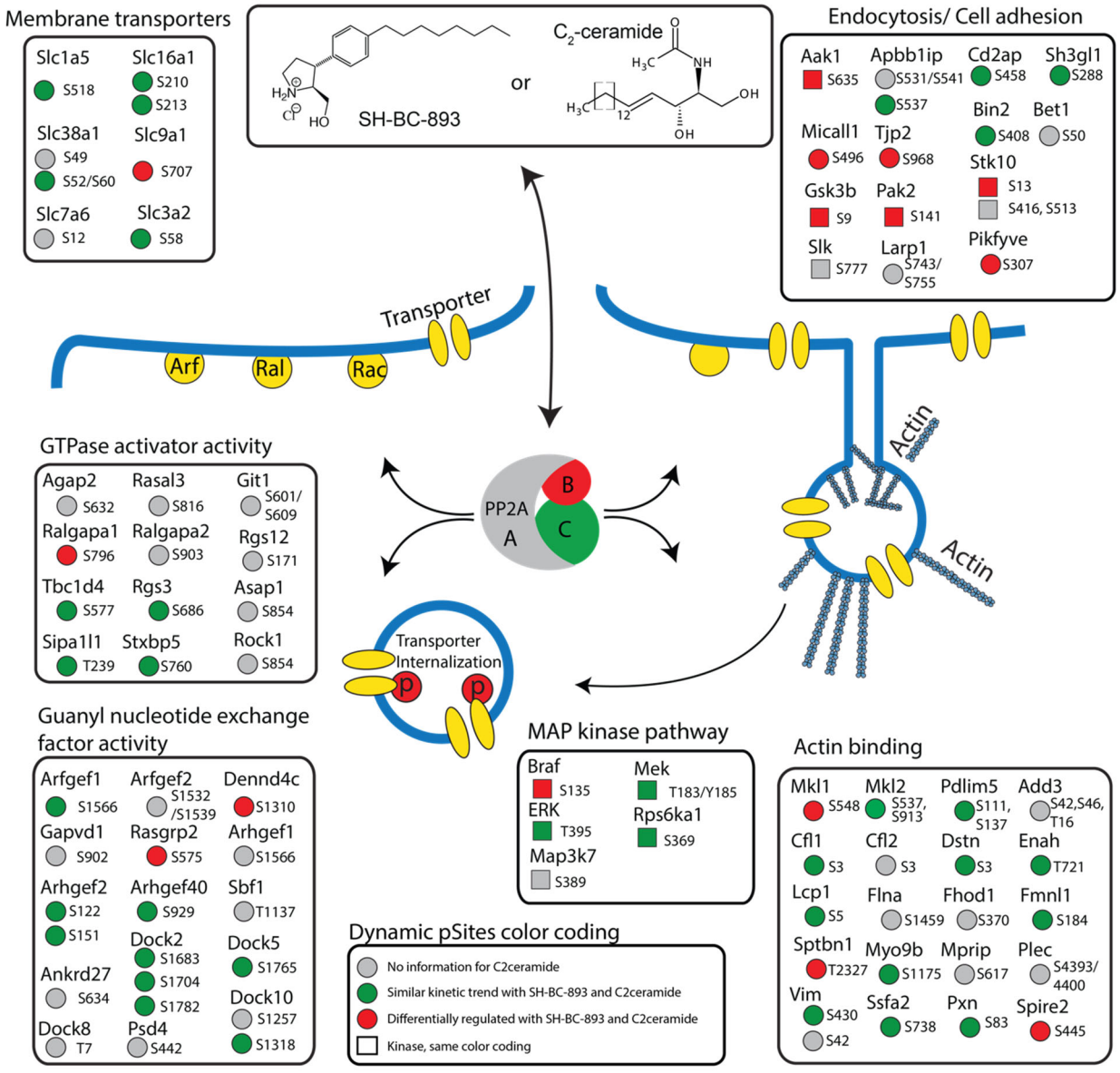
Supplementary Figure 7. Network of proteins that are uniquely phosphorylated upon C2-ceramide treatment.



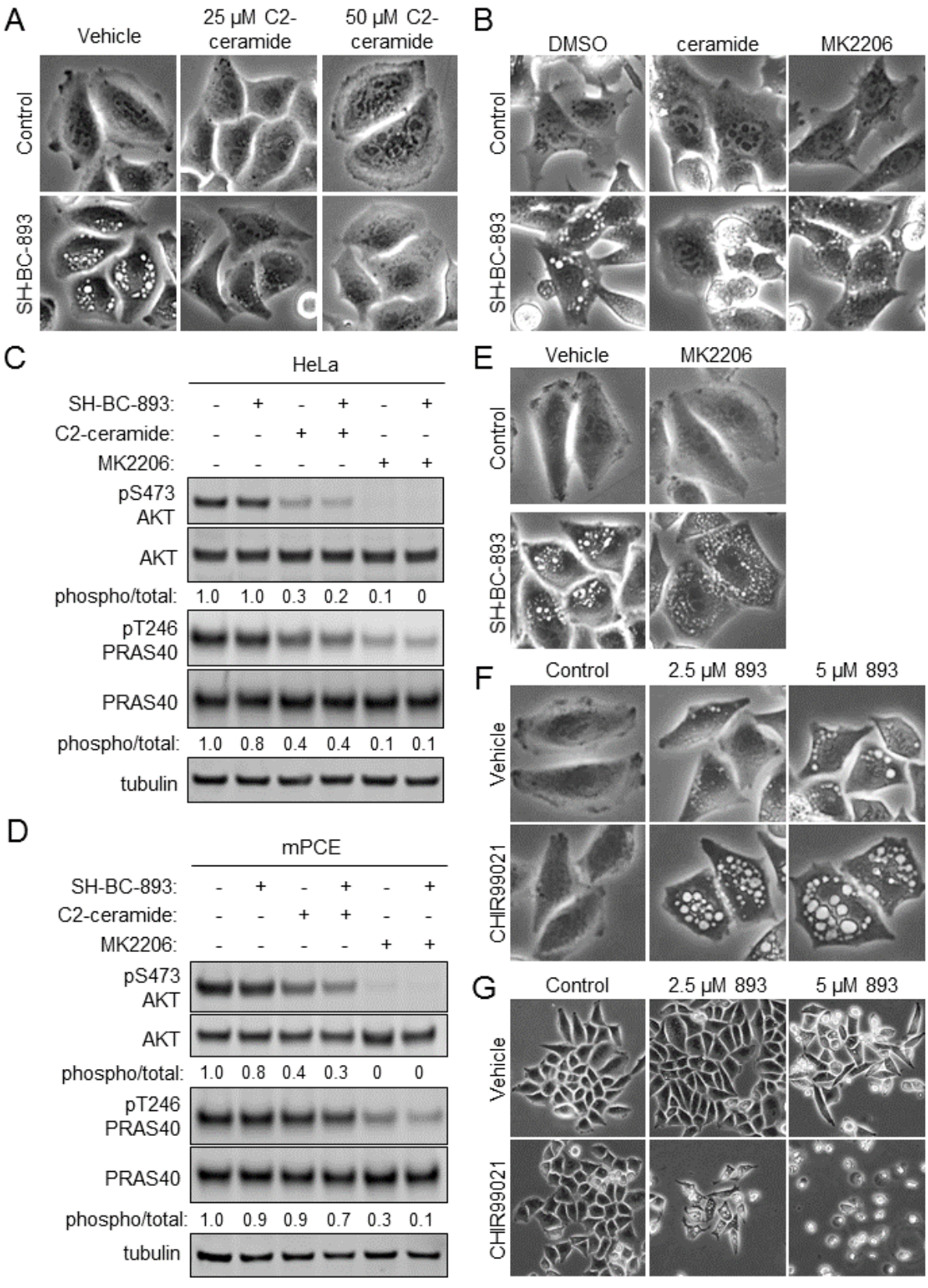
Supplementary Figure 8. Consensus motif of phosphorylation sites observed in dynamic profiles from C2-ceramide and SH-BC-893 phosphoproteomic experiments. A) Motif-X analyses of phosphorylation sites from three groups of dynamic profiles. B) Relative proportion of Akt sites within each group predicted from GPS 3.0 (http://gps.biocuckoo.org/).



Supplementary Figure 9. Dynamic profiles of putative Akt substrates associated with vesicle trafficking.



Supplementary Figure 10. Pictogram of proteins that are dynamically phosphorylated upon SH-BC-893 and C2-ceramide treatment. Only proteins associated to the main GO term molecular functions (Membrane transport, GTPase and GEF regulation, Actin binding, Endocytosis) are shown. Color coding indicates differences and similarities observed between C2-ceramide and SH-BC-893 treatments.


A
Vehicle
25 μM C2-ceramide
50 μM C2-ceramide
Control
SH-BC-893
B
DMSO
ceramide
MK2206
Control
SH-BC-893
C
HeLa
SH-BC-893: - + - + - +
C2-ceramide: - - + + - -
MK2206: - - - - + +
pS473 AKT
AKT
phospho/total: 1.0 1.0 0.3 0.2 0.1 0
pT246 PRAS40
PRAS40
phospho/total: 1.0 0.8 0.4 0.4 0.1 0.1
tubulin
D
mPCE
SH-BC-893: - + - + - +
C2-ceramide: - - + + - -
MK2206: - - - - + +
pS473 AKT
AKT
phospho/total: 1.0 0.8 0.4 0.3 0 0
pT246 PRAS40
PRAS40
phospho/total: 1.0 0.9 0.9 0.7 0.3 0.1
tubulin
E
Vehicle
MK2206
Control
SH-BC-893
F
Control
2.5 μM 893
5 μM 893
Vehicle
CHIR99021
G
Control
2.5 μM 893
5 μM 893
Vehicle
CHIR99021

Supplemental Figure 11: Ceramide inhibits vacuolation in multiple cell lines by reducing Akt activity. (A) HeLa cells treated for 3 h with SH-BC-893 (5 µM) and the indicated concentrations of C2-ceramide were imaged by phase contrast microscopy to visualize vacuoles. (B) Murine prostate cancer epithelial (mPCE) cells co-treated with SH-BC-893 (5 µM) and C2-ceramide (50 µM) or the Akt inhibitor MK2206 (1 µM) and imaged as in A. (C) Western blot for AKT (total and phospho-Ser473), PRAS40 (total and phospho-Thr246) and tubulin (loading control) in HeLa cells treated for 30 min with SH-BC-893 (5 µM), C2-ceramide (50 µM), and/or MK2206 (1 µM). (D) As in (B), except with mouse prostate cancer epithelial cells (mPCE). (E) HeLa cells treated for 6 h with SH-BC-893 (5 µM) and/or the AKT inhibitor MK2206 (1 µM) were visualized as in (A). (F) HeLa cells were treated for 6 h with indicated concentrations of SH-BC-893 or C2-ceramide and/or CHIR99021 (10 µM) as indicated and visualized as in (A). (G) As in E, except at 24 h.