Xenoestrogen-induced Regulation of EZH2 and Histone Methylation via Non-Genomic Estrogen Receptor Signaling to PI3K/AKT

Tiffany G. Bredfeldt, Kristen L. Greathouse, Stephen H. Safe, Mien-Chie Hung, Mark T. Bedford, Cheryl L. Walker

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. E2-BSA does not activate the genomic ER pathway.

RNA was extracted from MCF-7 cells treated with E2-BSA (25 nM), DES (50 nM), or VEH for 6 h. DNase treated RNA was then reversed transcribed into cDNA. cDNA was then subjected to qPCR analysis. The calibrator for this qPCR experiment was a VEH sample. Fold change was calculated for each sample in comparison to the calibrator. Graph bars represent mean ± SEM. Statistical significance ($p \leq 0.05$) was determined by student's t-test.

Figure S2. MCF-7 cells stably transfected with CA-AKT have increased P-EZH2 and reduced H3K27Me3 levels.

A. Lysates were collected from MCF-7 cells or MCF-7 cells stably transfected with CA-AKT and analyzed by western blot to determine activity of AKT pathway. Increases in phospho-TSC2 and phospho-S6K demonstrate elevated AKT activity.

B. Increased AKT activity induces EZH2 phosphorylation. P-EZH2 was immunoprecipitated from MCF-7 cells or MCF-7 cells stably transfected with CA-AKT. Immunoprecipitates were resolved by SDS-PAGE and blotted for EZH2. EZH2 and AKT inputs are used as a loading control.

C. Constitutively active AKT (CA-AKT) causes reduction in H3K27Me3 levels in an estrogen-responsive cell line. Histone proteins were collected from MCF-7 or MCF-7 CA-AKT cells and analyzed by western blot.

Graphs show densitometry analysis of western blot data as represented by change in phosphorylation (P-EZH2/total EZH2) or methylation (H3K27Me3/total H3) relative to MCF-7 cells. Statistical significance was given to data with $p \leq 0.05$ as determined by student's t-test.

Figure S3. Growth factor-induced activation of the PI3K/AKT pathway and downstream EZH2 phosphorylation.

A. MCF-7 cells were starved for 48 h and then treated with IGF (20 ng/ml), which activated the PI3K/AKT pathway.
B. Serum deprived MCF-7 cells were treated with 20% serum for indicated times. Activation of the PI3K/AKT pathway was detected by western blot analysis.
C. Immunoprecipitation of phospho-EZH2 from MCF-7 cells treated with 20% serum demonstrates growth factor-induced EZH2 phosphorylation as determined by immunoprecipitation of phospho-EZH2.

Each panel displays a representative western blot from three independent experiments. Graphs display the increase in phospho-AKT relative to loading control (mean ± SEM), which has been normalized to VEH treated cells as determined by densitometry.

Figure S4. Xenoestrogen-induced activation of non-genomic signaling causes phosphorylation of EZH2.

MCF-7 cells were transfected using 6 μg of plasmid DNA (for a 10 cm plate), Opti-MEM, and Lipofectamine 2000 according to manufacturer's specifications (Invitrogen, Carlsbad, CA). Cells were

transfected with myc-EZH2 or mock in complete media. 12-24 h after transfection, complete media was replaced with phenol red-free, serum-free media for an additional 24 h prior to treatment. Following transfection and subsequent starvation, MCF-7 cells were treated with DES (50 nM) for indicated times and cell lysates were collected and subjected to immunoprecipitation followed by western blot analysis.

Figure S5. A low concentration of xenoestrogen activates the PI3K/AKT pathway to phosphorylate EZH2.

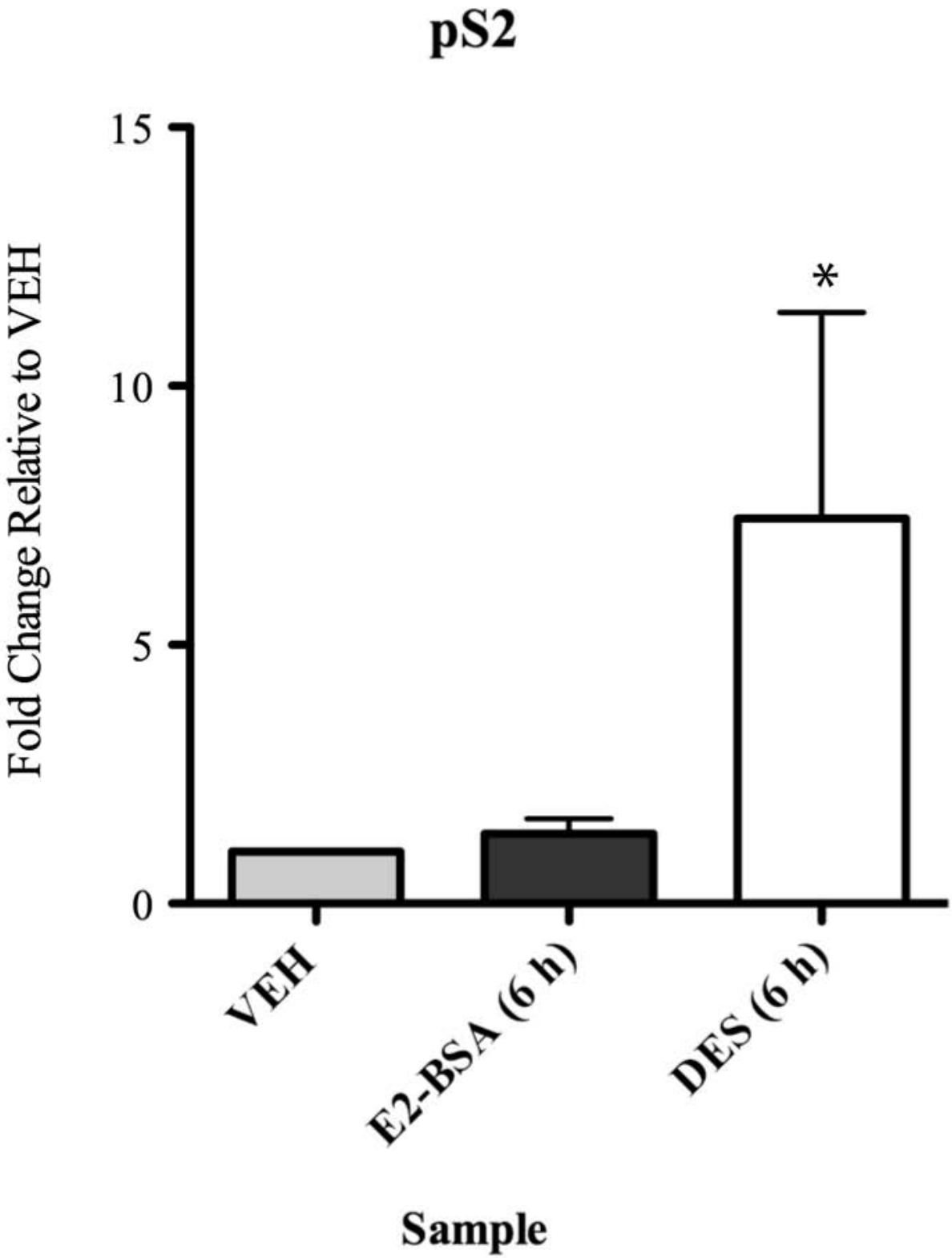
A. Lysates were collected from MCF-7 cells treated with 5 nM DES or VEH (ethanol) and analyzed by western blot for activation of the PI3K/AKT pathway, as detected by increases in phospho-AKT, phospho-S6K, and phospho-S6.
B. Immunoprecipitation of phospho-EZH2 from MCF-7 cells treated with 5 nM DES indicates that activation of rapid ER signaling by this treatment causes EZH2 phosphorylation.

   Graphs display densitometry analysis of western blots of phospho-AKT or phospho-EZH2 from independent experiments relative to VEH treated cells. Statistical significance ($p \leq 0.05$) was determined by student's t-test.

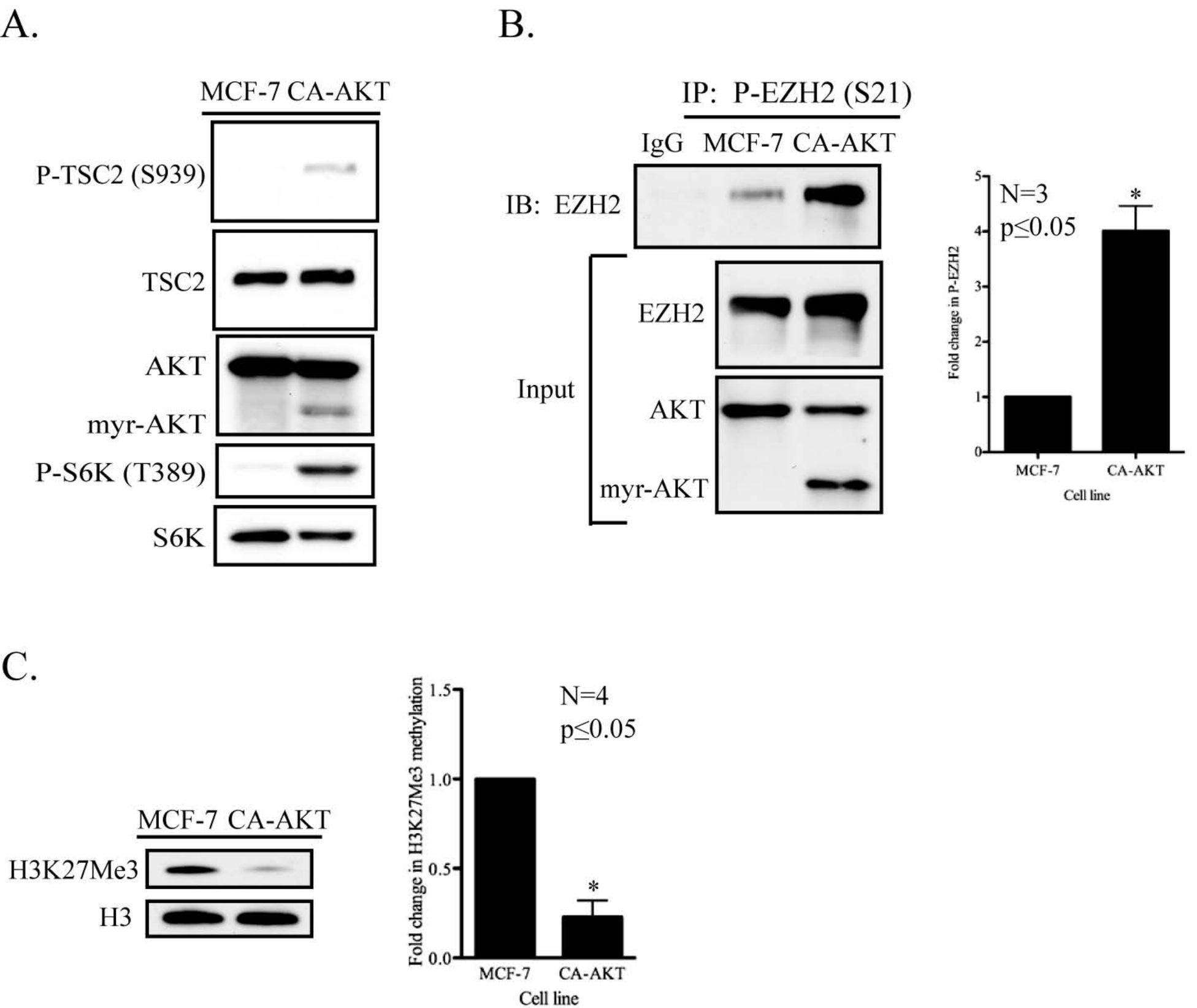
Figure S6. Scheme demonstrating estrogen-induced reprogramming of gene expression in uterine myometrial cells.

1. Cells are depleted of hormone and growth factors for 48 h.
2. Non-genomic signaling and EZH2 phosphorylation is stimulated by daily treatment with E2-BSA or DES to modulate H3K27Me3 levels and promote chromatin remodeling.
3. "Priming" estrogen treatments are removed and cells are washed with PBS to remove estrogen and maintained in serum- and hormone-free media for 48 h.
4. Cells are challenged with DES and the expression of estrogen-responsive genes is analyzed by qPCR.

Supplemental Figure 1

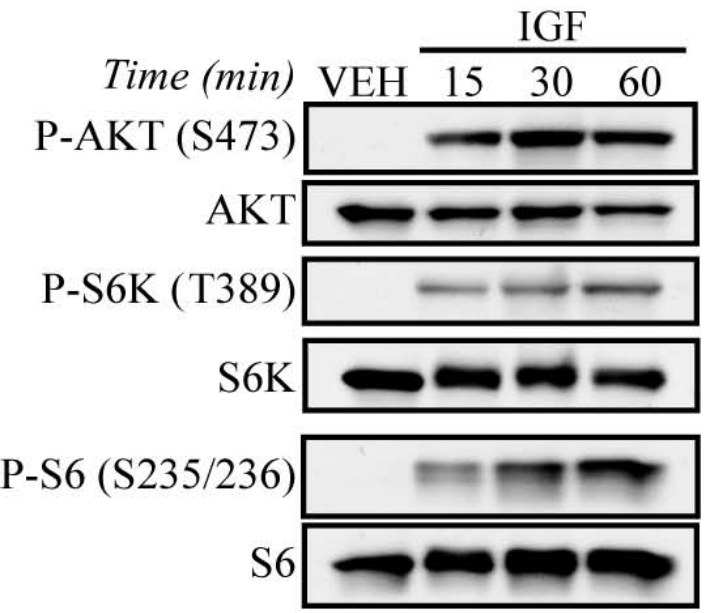


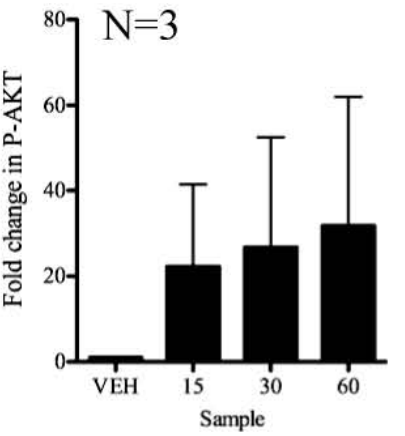
Supplemental Figure 2



Supplemental Figure 3

A.





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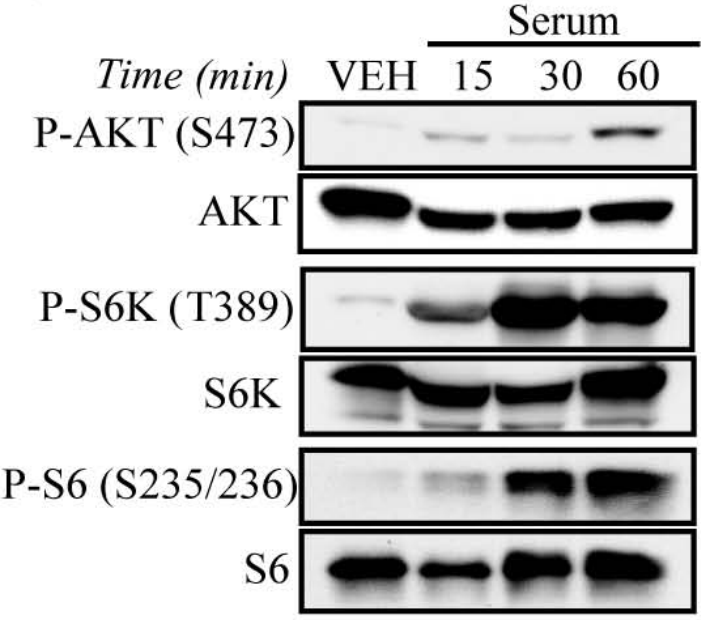

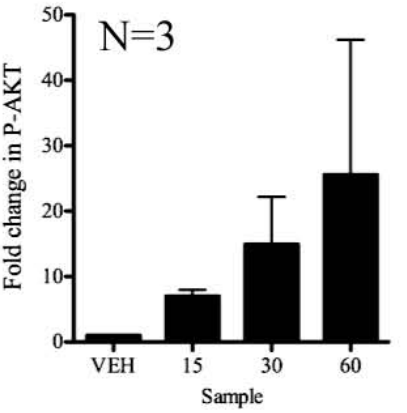

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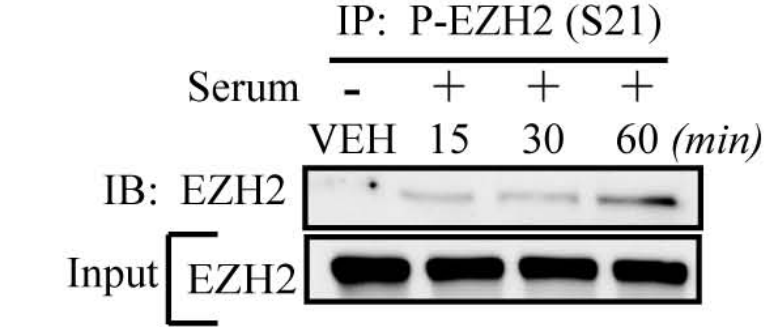
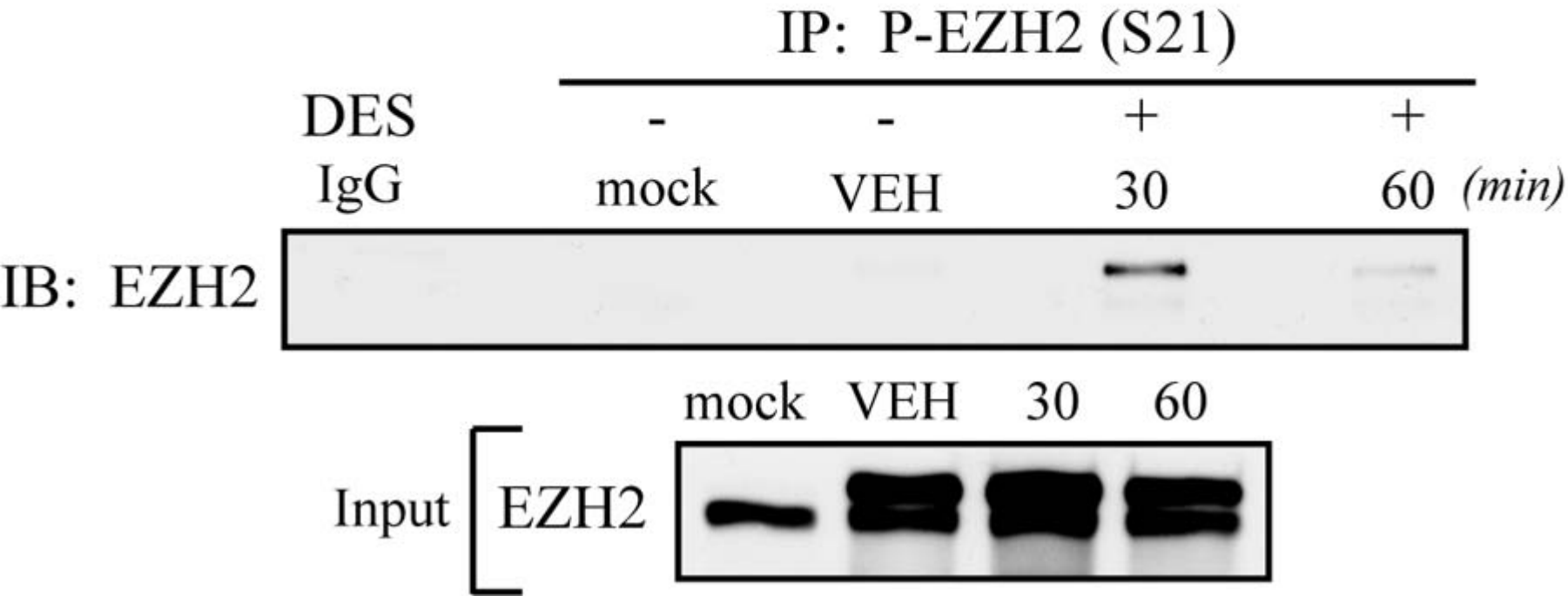
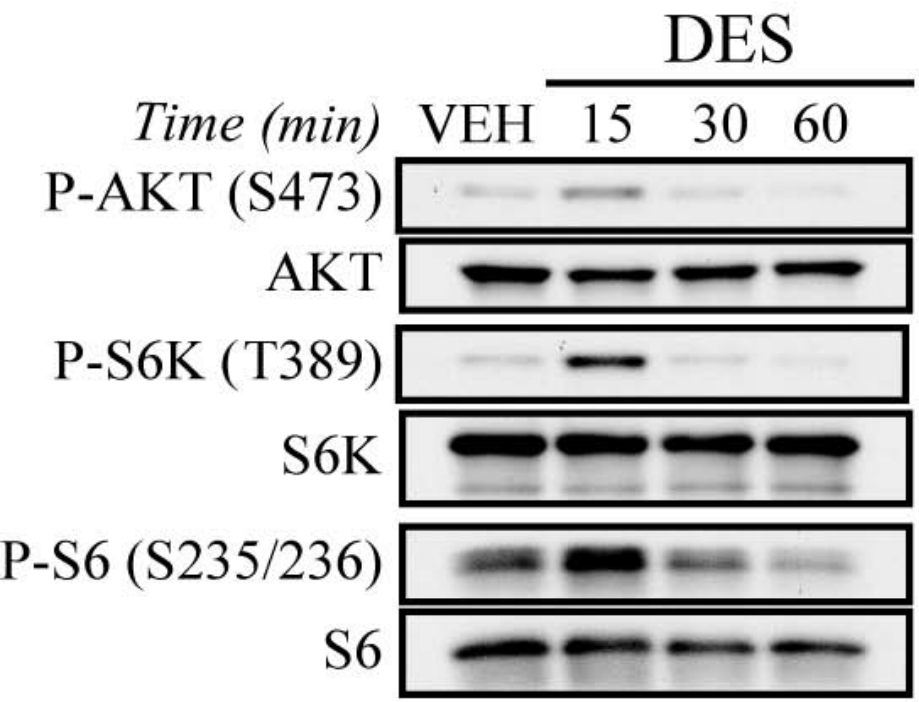
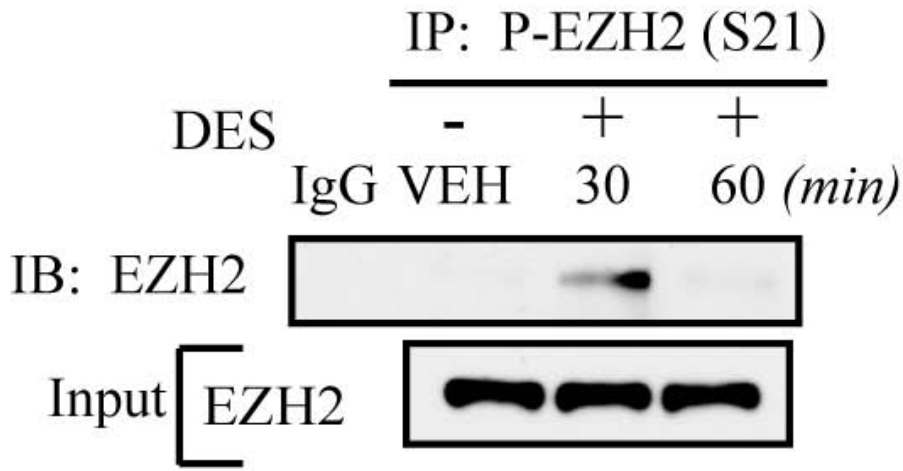


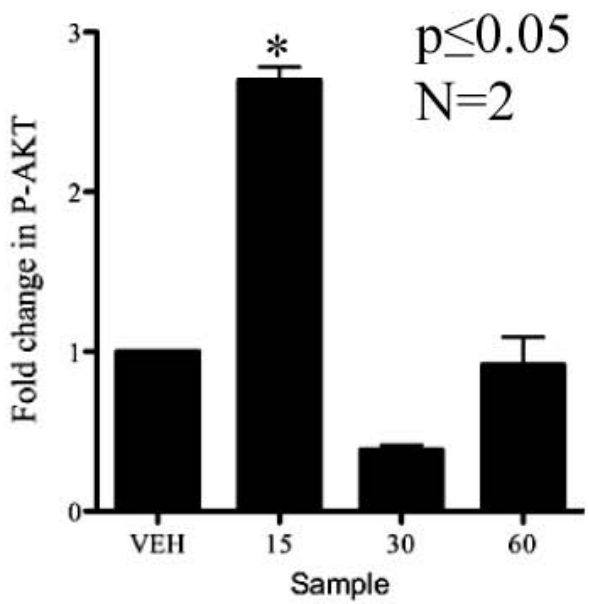
Supplemental Figure 4

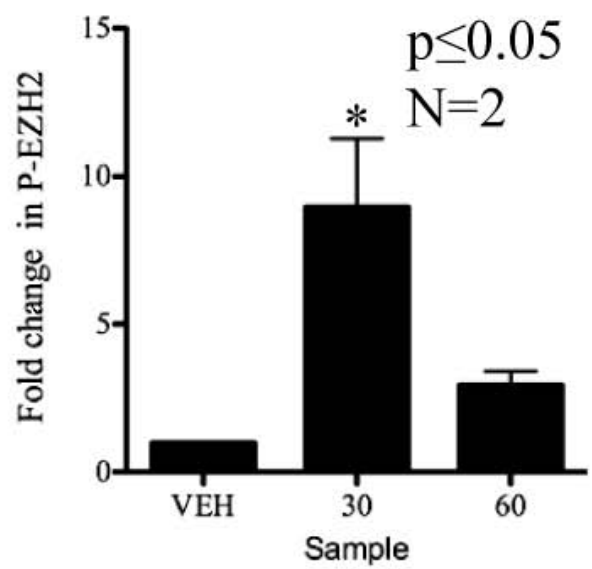


Supplemental Figure 5









Supplemental Figure 6