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Supplemental Material to:

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LAMP2A overexpression in breast tumors promotes cancer cell survival via chaperone-mediated autophagy

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Supplementary Methods:

Lysosome isolation: Lysosome isolation was performed according to manufacturer instructions using Lysosome Enrichment Kit for Tissue and Cultured cells from Pierce (89839). Briefly 150 mg (collected from ~1 x 10^8 cells) were pelleted in a microcentrifuge tube at 850xg for 2 min. The pellet was then suspended in lysosome enrichment buffer A supplemented with inhibitors, and then lysed the cells with 120 strokes of Dounce Homogenizer. The lysed cells were then mixed with lysosome enrichment buffer 2 supplemented with inhibitors followed by centrifugation at 500xg for 10 min and collection of the supernatant. The supernatant was then applied to a discontinuous density gradient (30%, 27%, 23%, 20% and 17% with Optiprep media), and centrifuged at 60,000 rpm for 4 h at 4^{o}C. The lysosomal band was carefully removed, (which was located on the top of the gradient) and diluted further with PBS. The diluted lysosomes were then centrifuged at 13,000 rpm for 1 h at 4^{o}C. The supernatant was removed, and the lysosome pellet was kept on ice for downstream processing.

Immunofluorescence: Cells were first plated in a Fluorodish cell culture dish (WPI, Inc., FD35-100) 24 hours prior to transfection with LAMP2A-GFP plasmid. 48 hours post-transfection, cells were washed with 1X PBS followed by fixation with 4% paraformaldehyde for 5 min. The fixed cells were then incubated with 100% cold methanol for 10 minutes. After two times wash with PBS, cells were blocked with 3% BSA/0.01% Tween 20 for 30 minutes, which was then followed by staining with primary antibody (1:100, HSPA8 antibody) for 2 h. Cells were then incubated with fluorescent secondary antibody (Alexa fluoro 594 (1:400) (Invitrogen, A24921) for one hour. Finally, cells were washed with PBS and then photographed with Zeiss 510 LSM microscope (Microscopy facility, Lombardi Comprehensive Cancer Center).

NFKB1 assay: Luciferase assay for NFKB1 was performed according to the manufacturer instructions using Ready-To-Glow pNFKB1-MetLuc Vector Kit (Clontech, 631742). Briefly, cells were transiently transfected with either LAMP2A or *LAMP2A* siRNA along with pNFKB1-MetLuc2 reporter vector. 24 h post-transfection cells were spitted into 48 wells tissue culture plate, and were allowed to grow and secrete Metridia Luciferase into the complete growth medium for another 24 h. 50 µl of the culture medium from each sample in triplicate was transferred to 96-well tissue culture plate, where 5 µl of 1X substrate/reaction buffer was added to each well. The solution was mixed, transferred in a plate to a luminometer to record the light signals immediately.

Supplementary figure legends:

Figure S1: **Effect of LAMP2A expression on the CMA substrate, GAPDH, in MCF-7 cells**. Proliferating MCF-7 cells were either transfected with LAMP2A, or empty pcDNA3 vector for 48 h as well as transfected with *LAMP2A* siRNA or control siRNA for 72 hours. (A) shows a typical example of transfection with LAMP2A-GFP and the control background GFP plasmid in MCF-7 cells. (B and C) Cells from the indicated samples were harvested and lysates were fractionated on a 4-20% Tris glycine gel and immunoblotted with indicated antibodies. Densitometric analyses normalized to ACTB from three independent experiments are shown on the right of each panel. Error bars are SEM and asterisk represents a statistically significant difference (*$p < 0.05$, ANOVA; Tukey test). (D–F) LAMP2A-downregulated MCF-7 cells were either treated with proteasome inhibitor, MG132 (D) or incubated with lysosomal inhibitors, Bafilomycin A_1 (BAF A_1) (E) and 3-methyladenine (3MA) (F) as described under methods, followed by SDS PAGE and immunoblotting with the indicated antibodies. DMF, DMSO, and H_2O were the solvents for the inhibitors, and thus served as control.

Figure S2: **Isolated lysosomes from LAMP2A-overexpressed cells and GAPDH degradation**. T47D and MCF-7 cells were overexpressed by LAMP2A expression vector and then intact lysosomes were isolated from the cells as described under methods. Purified GAPDH protein was then incubated with the intact lysosomes isolated from the above samples, and then harvested, fractionated and immunoblotted with indicated antibodies.

Figure S3: **Effect of LAMP2A expression on the halflife of GAPDH**. Proliferating T47D cells were transfected with LAMP2A or pcDNA3 for 24 h (A), and similarly proliferating MCF-7 cells were transfected with *LAMP2A* siRNA or control siRNA (C). Representative autoradiogram of ^{35}[S]-Methionine labeled GAPDH at the indicated time points from T47D (B) and MCF-7 (D) cells are shown following immunoprecipitation with anti-GAPDH antibody in a pulse-chase experiment (see Methods). Lanes are labeled according to treatments, and the duration of hours after removal of ^{35}[S]-methionine (chase period). '0' represents initial labeling. Quantifications of the labeled bands from three independent experiments were performed using Image J software and are shown in (B) (T47D) and (D) (MCF-7) with error bars (SD). Linear trend lines were drawn through the time points, and the equations of the lines are shown on the graph.

Figure S4: **Analysis of the expression of indicated proteins in breast cancer and adjacent normal tissue samples**. The expression patterns of the indicated proteins that were obtained from breast cancer OncoPair INSTA-Blot™ was analyzed with respect to tubulin, which corroborates with the results obtained with ACTB. (A–F) show densitometric analysis of LAMP2A, HSPA8, LAMP2B, LAMP1, GAPDH and PKM in breast tumor versus normal adjacent tissue samples and are expressed in arbitrary units. The values are means ± SEMs of at least three independent experiments, asterisk represents a statistically significant difference between samples (*$p < 0.05$).

Figure S5: **Specificity of the LAMP2A antibody**. The specificity of the LAMP2A antibody used in this study was determined using the specific LAMP2A peptide that was used to generate the antibody. The sequence of this peptide is unique for LAMP2A and does not overlap with LAMP2B and LAMP1. Immunoblots were treated in pairs; (A) LAMP2A antibody and LAMP2A antibody + LAMP2A peptide; (B) LAMP2B antibody and LAMP2B antibody + LAMP2A peptide; (C) LAMP1 antibody and LAMP1 antibody + LAMP2A peptide. LAMP2A peptide diminishes LAMP2A signal (A; right) but not LAMP2B (B; right) and LAMP1 (C; right).

Figure S6: **LAMP2A inhibited protein carbonyl content (PCC) formation in MCF-7 cells**. Proliferating MCF-7 cells were transiently transfected with LAMP2A or empty pcDNA3 vector (A) for 48 h, as well as transfected with *LAMP2A* siRNA or control siRNA (B) for 72 h. Cytosolic extracts were prepared, and aliquots from each sample were tested for their PCC using STA-318 colorimetric kit from Cell Biolabs. Quantitative estimation of PCC, expressed as means ± SEMs of three independent experiments is shown. Significant changes in the PCC contents are indicated by an asterisk (*$p < 0.05$ ANOVA; Tukey test). Western blots showing the protein levels of LAMP2A and ACTB in different transfection conditions are provided in (C and D).

Figure S7: **Effect of LAMP2A expression on NFKB1 signaling pathway**. (A and B) Proliferating MCF-7 cells were transiently transfected with LAMP2A or empty pcDNA3 vector (A) for 48 h, as well as transfected with *LAMP2A* siRNA or control siRNA (B) for 72 h. Total cellular extracts were prepared from different samples, and aliquots were fractionated in SDS PAGE, and immunoblotted with RELA-NFKB1 and NFKB1A antibodies. Furthermore, subcellular fractionation was performed on LAMP2A-overexpressed MCF-7 (C) and T47D (D) cells. The samples were then run on a SDS PAGE and immunoblotted with indicated antibodies. T47D cells were either overexpressed (E) or underexpressed (F) for LAMP2A, and then

subjected to luciferase activity assessment, using pNFKB1 MetLuc Vector kit from Clontech (see Methods).

Figure S8: **Overexpression of LAMP2A suppressed AKT1**. Proliferating MCF-7 cells were transfected overnight with LAMP2A, or empty pcDNA3 vector. Lysates were prepared and fractionated in SDS PAGE gels. (A) Western blots analysis of total AKT1 and phospho AKT1 (Ser473). (B) AKT1 kinase activity using AKT1 substrate GSK3 fusion protein as a measure of AKT1 activation. Densitometric analyses from three independent experiments for pAKT1 and pGSK3 expression are given in (C and D) respectively. Data are mean ± SEMs, and significant differences are indicated by an asterisk (*$p < 0.05$ ANOVA; Tukey test).

Figure S9: **Overexpression of LAMP2A suppressed generation of ROS**. Confocal microscopic images of the live T47D cells transfected with either LAMP2A expression vector for 48 h or with *LAMP2A* siRNA for 72 h were shown in this figure. The modified cells were stained with carboxy-H_2DCFDA to detect ROS, and simultaneously with MitoTracker Red CMXRos to detect mitochondria. DIC image (phase) and the merged images with ROS and mitochondria staining are also shown. The intensities of the green stains between samples were analyzed, and represented as bar diagrams in Figure 7C and D.

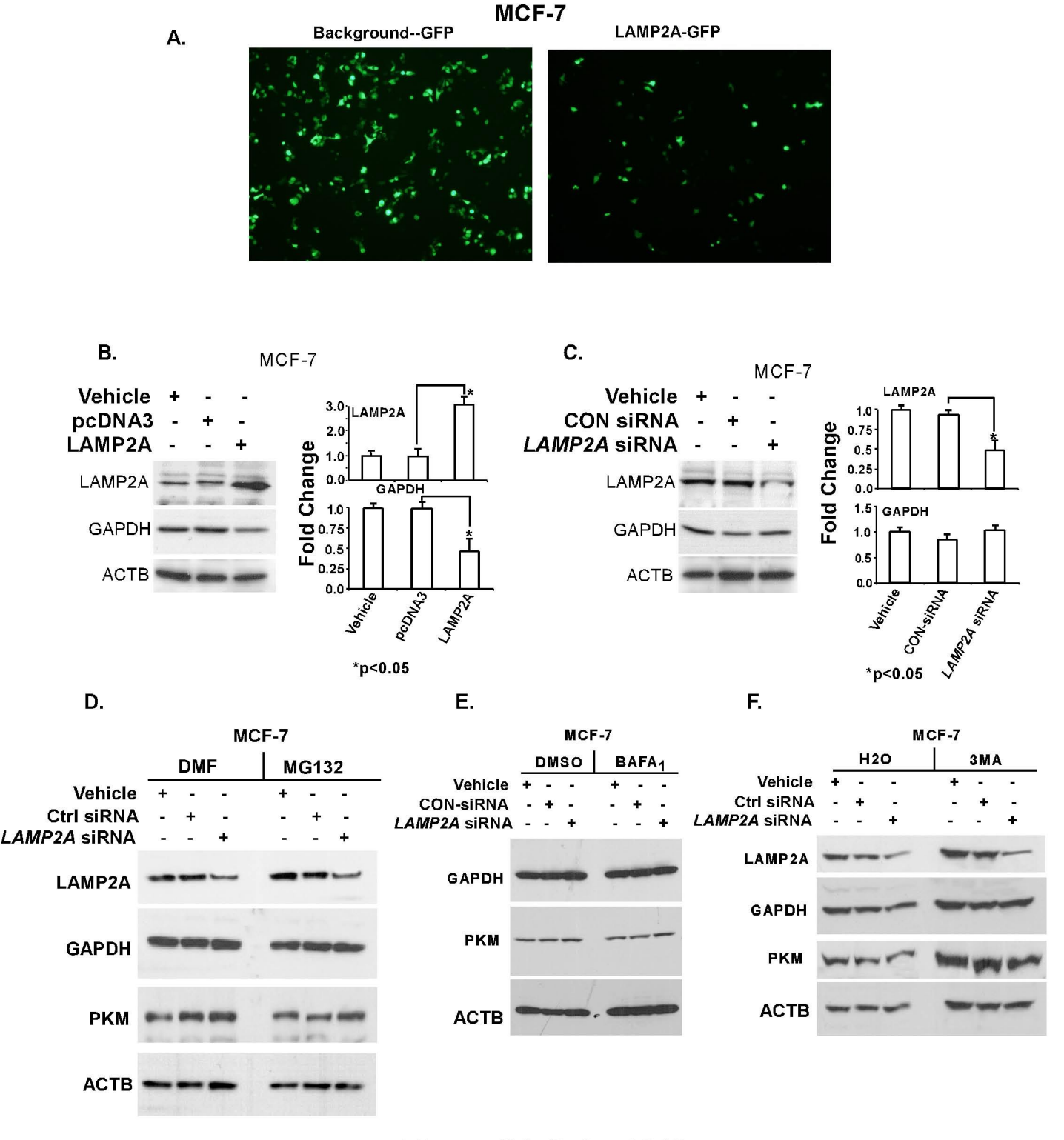


Figure S1. Saha 2012

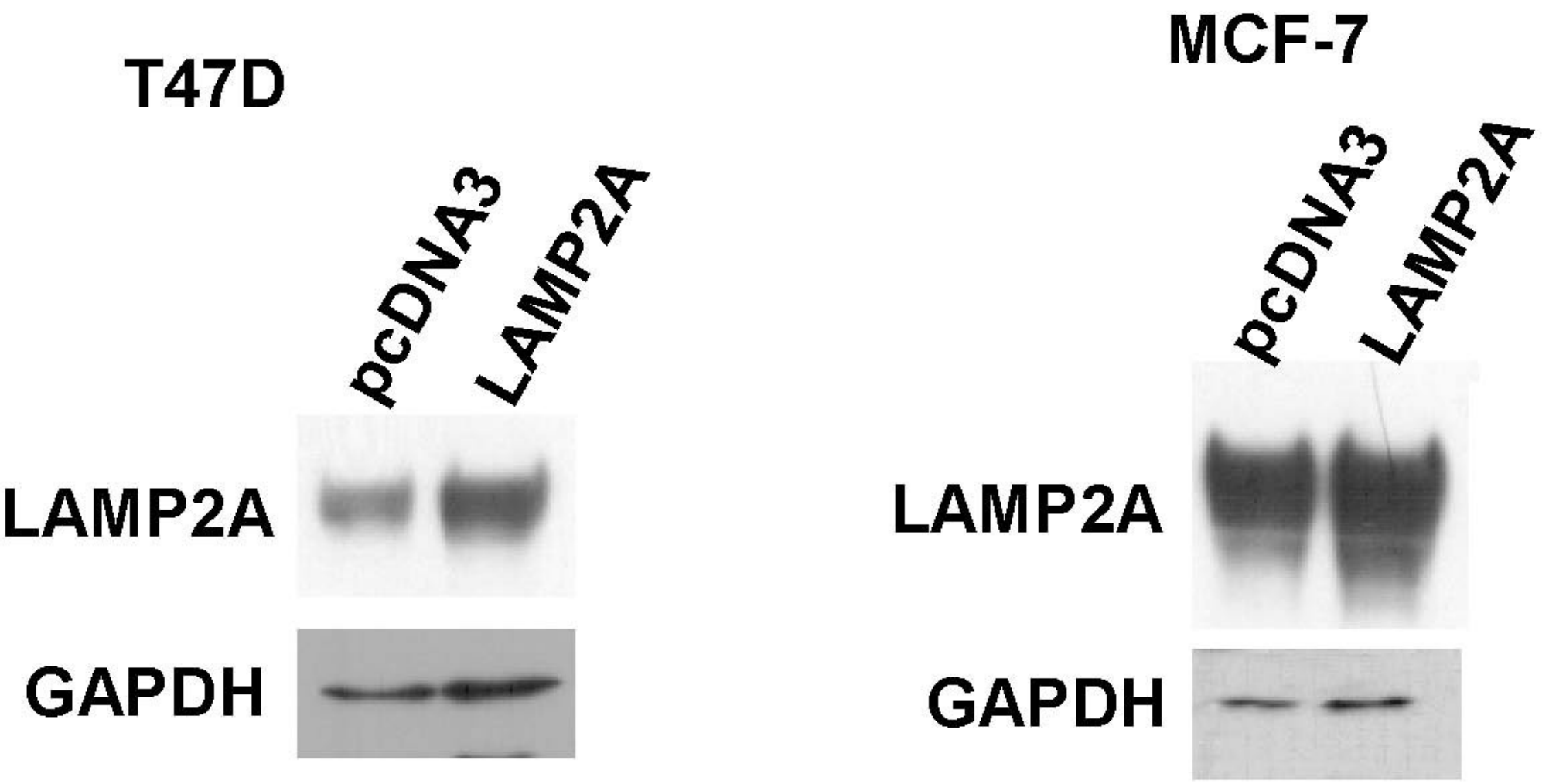


Figure S2. Saha CMA 2012

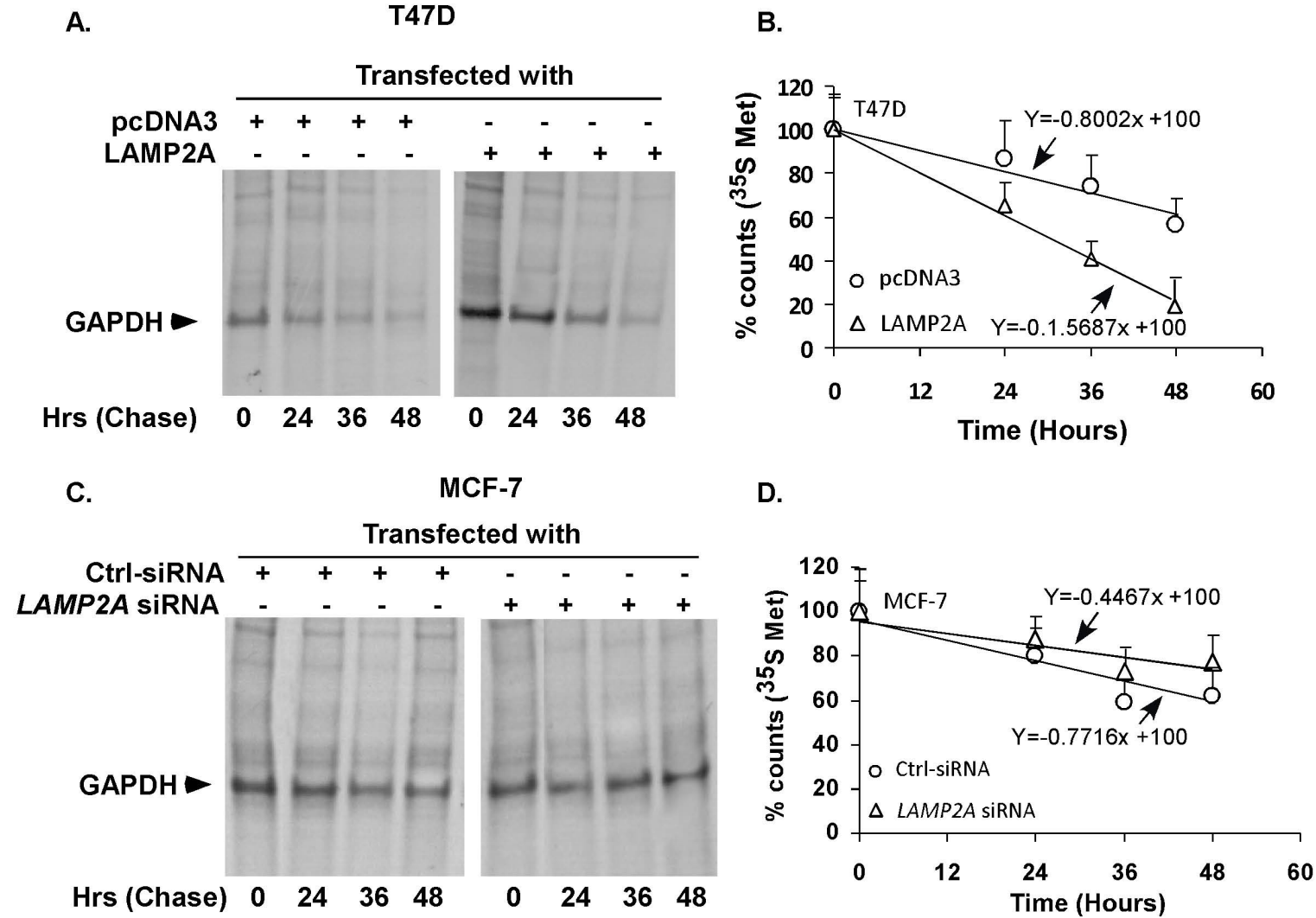
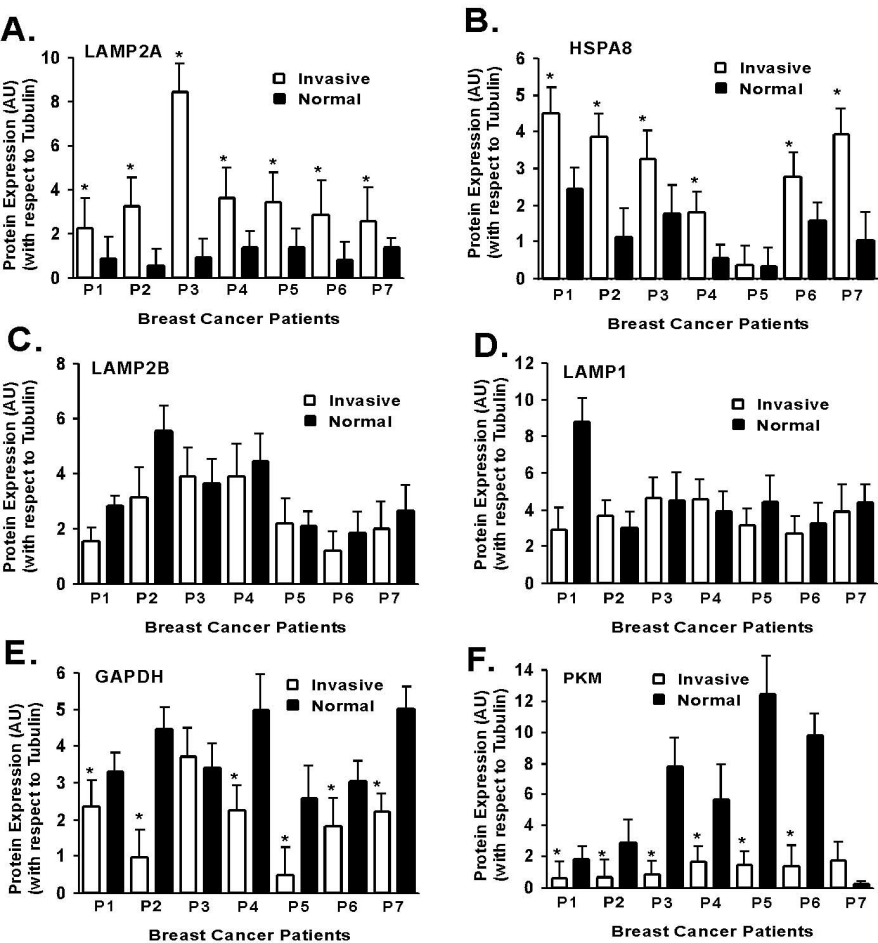


Figure S3. CMA Saha 2012



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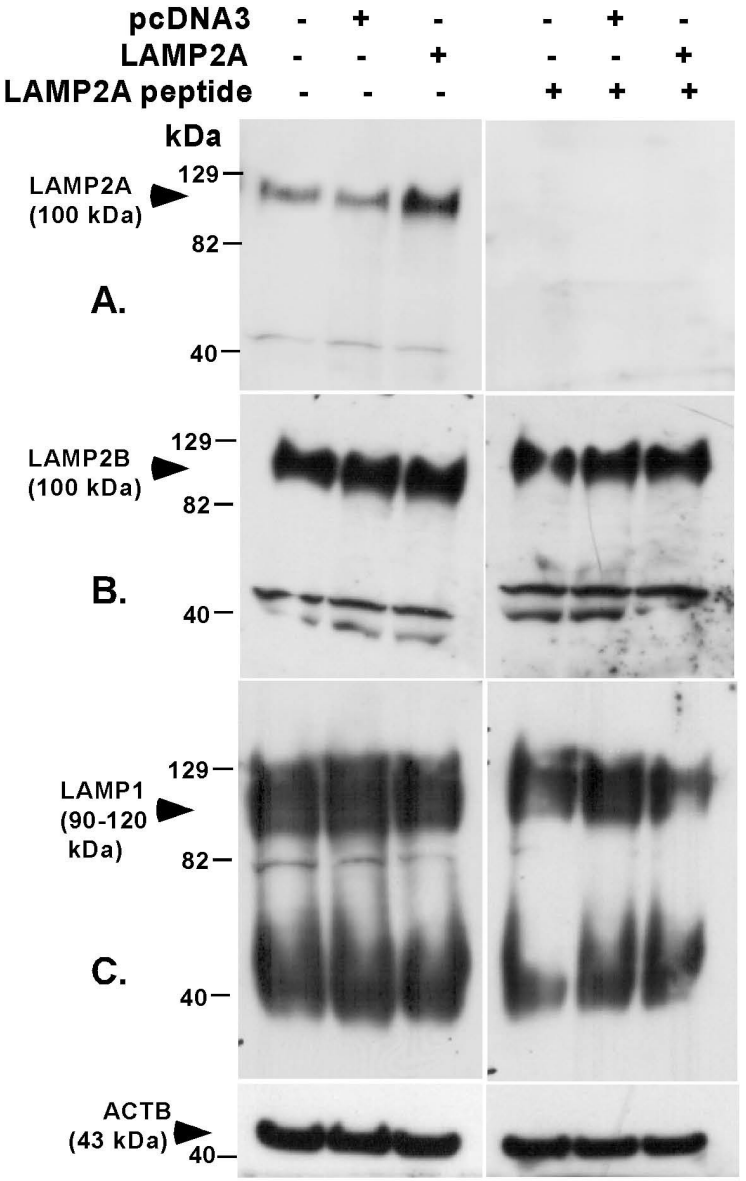

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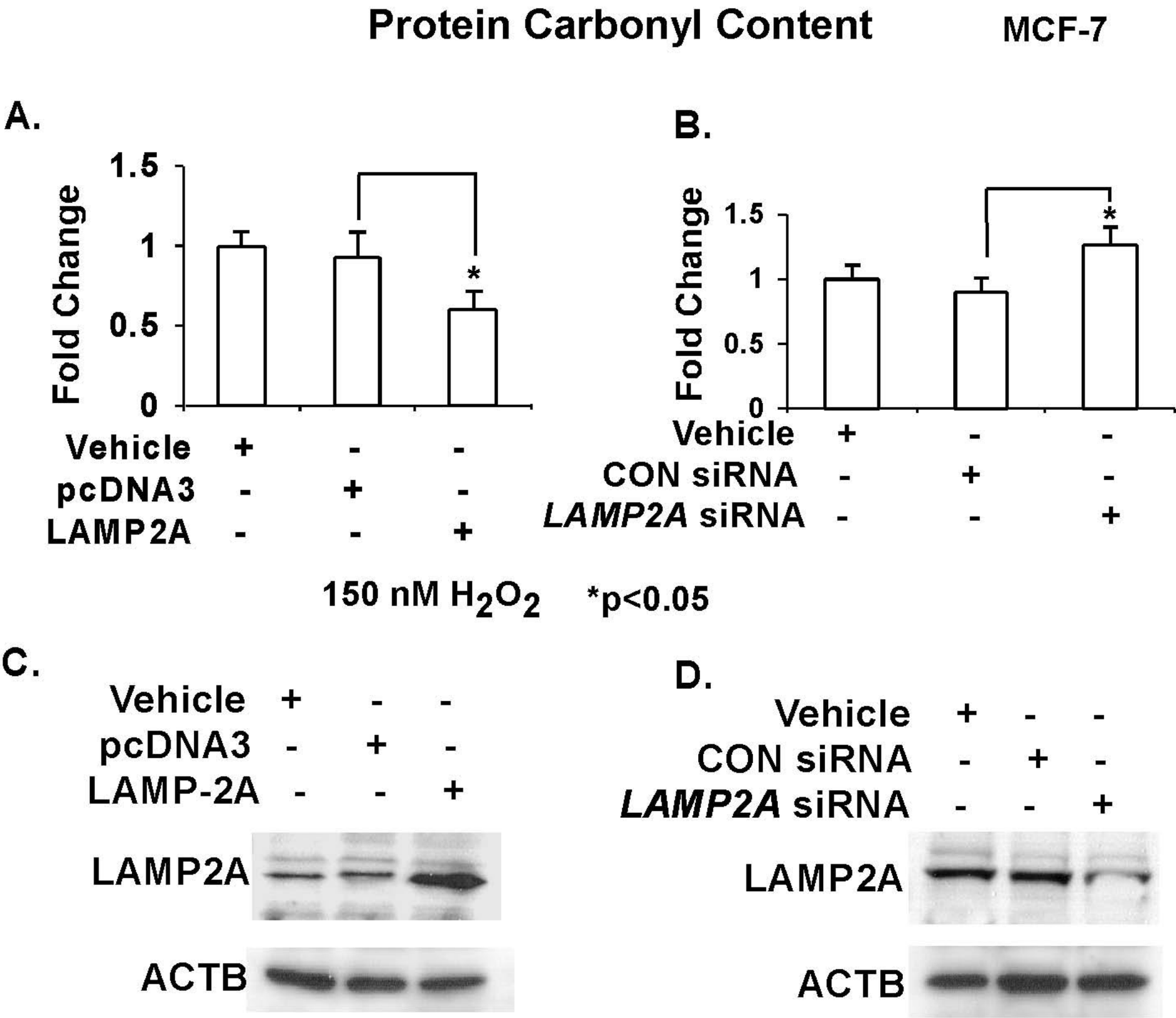

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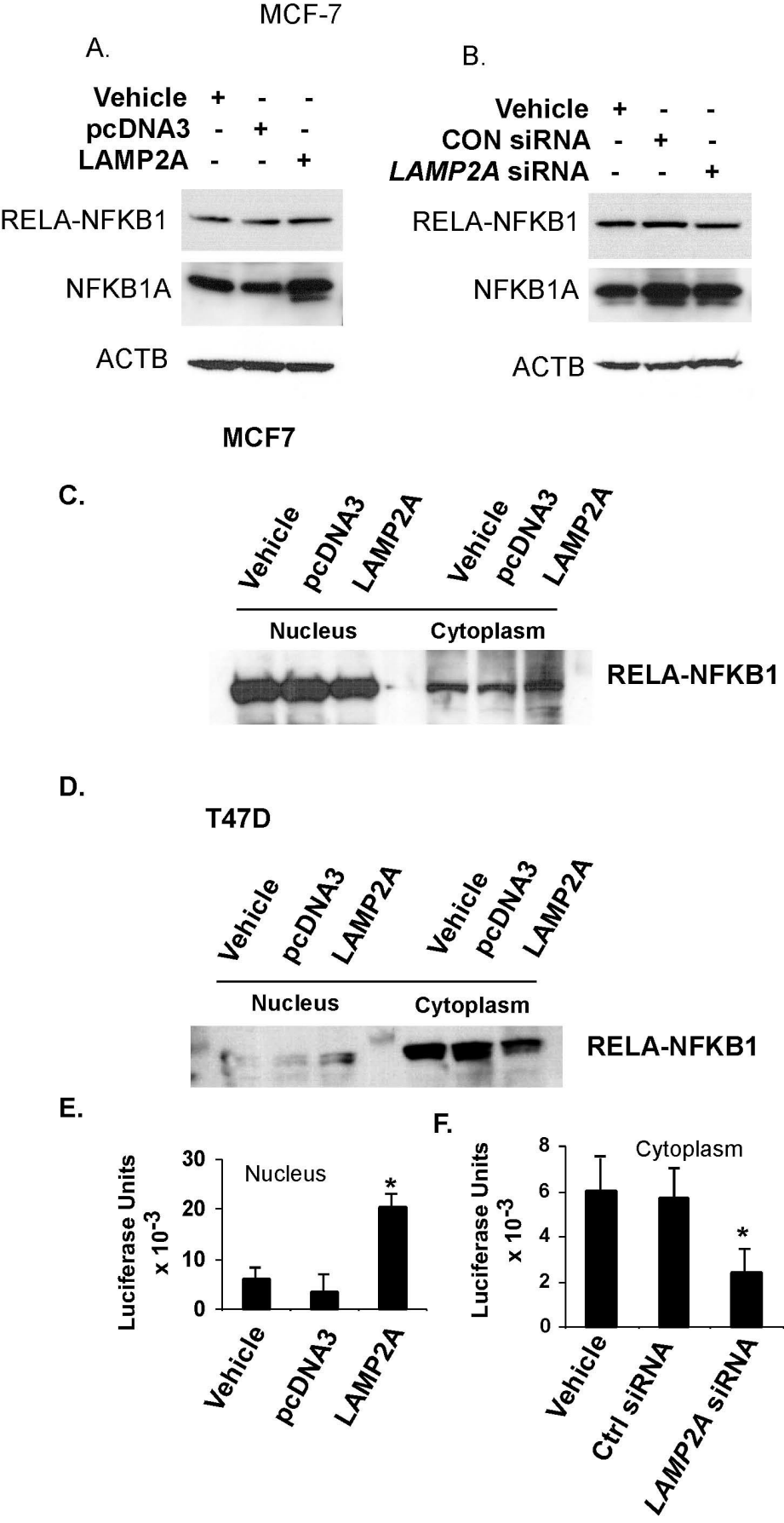
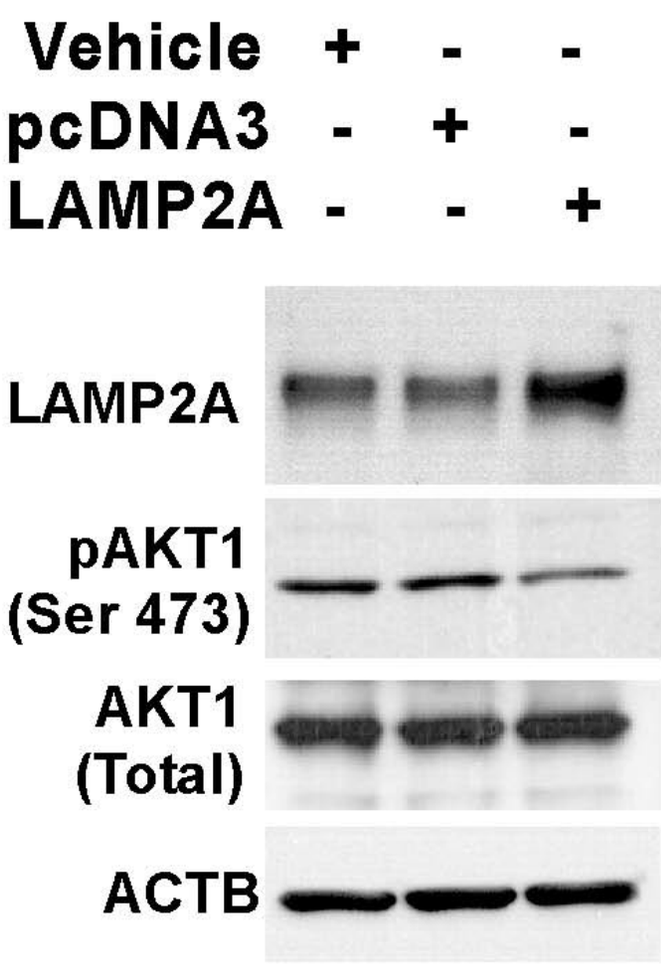


Figure S7. Saha 2012

MCF7

A.



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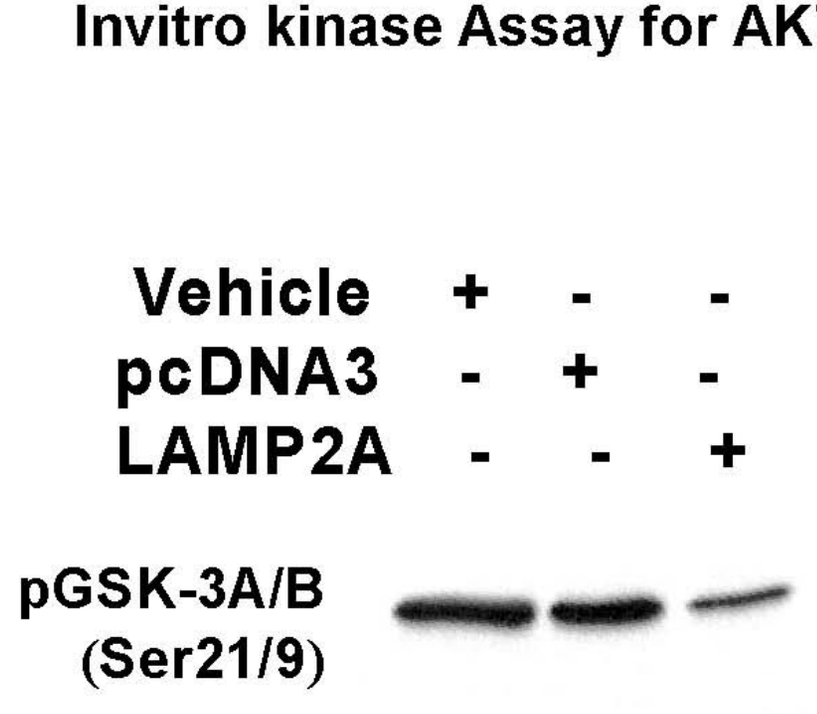

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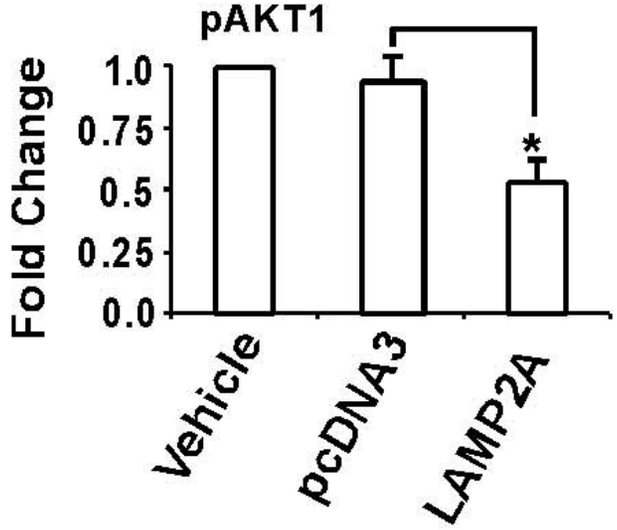

D.

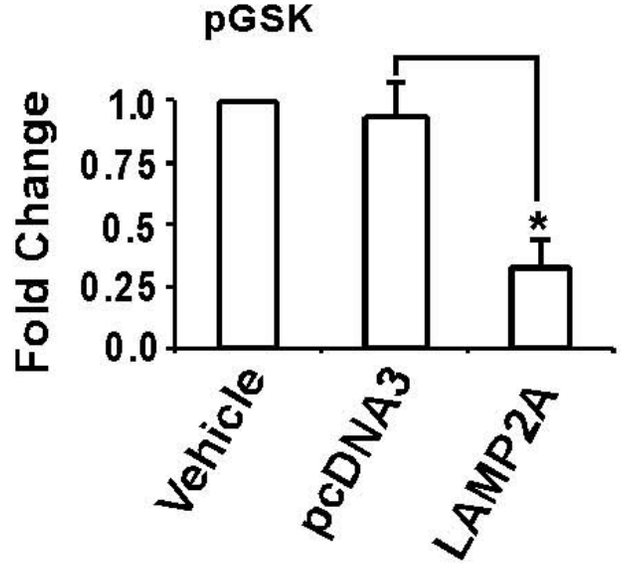

Figure S8. Saha 2012

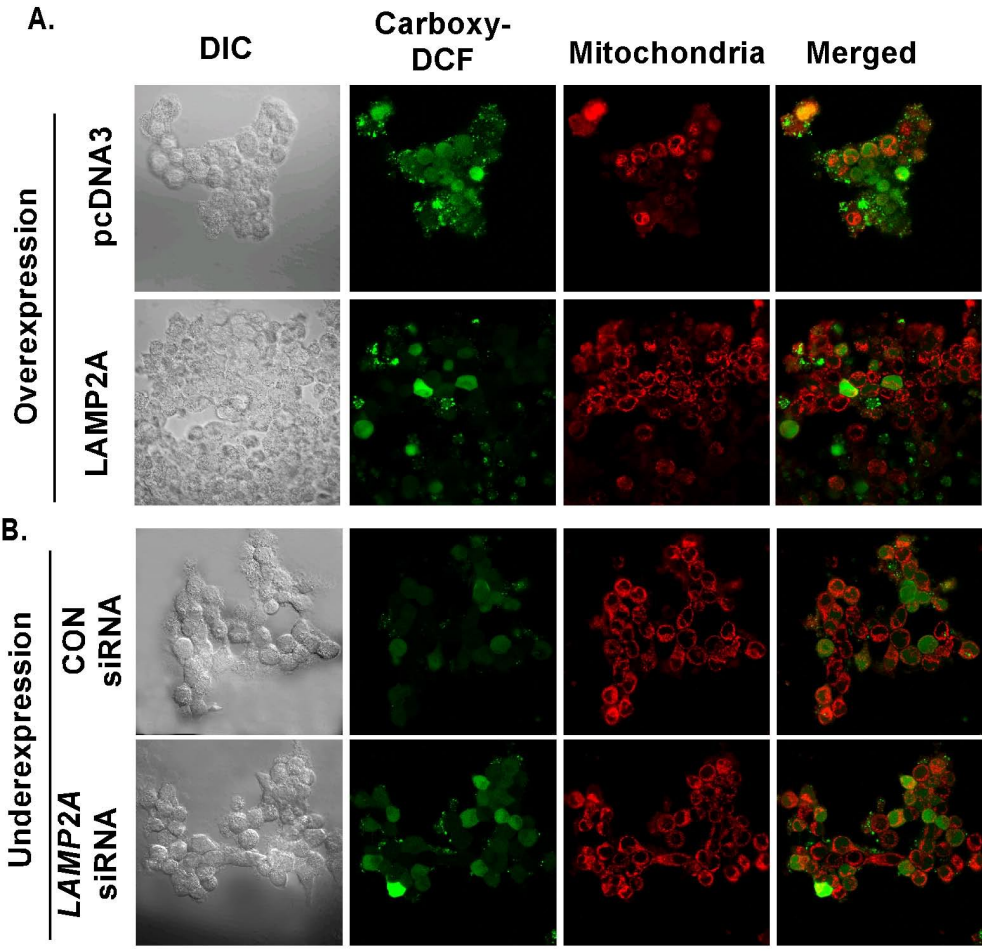

Figure S9. Saha 2012