SUPPLEMENTAL DATA

FIGURE LEGENDS

Fig. 1. CaMKK2 expression in clinical specimens of PCa. Shown are representative images of CaMKK2 IHC staining in patient samples with various histological Gleason scores[1] representing disease advancement and predicting aggressiveness of prostate tumors. (*A*) CaMKK2 shows stronger epithelial staining in malignant glands (black arrows) compared to adjacent benign glands (red arrows) in a patient specimen of GS 3+4. (*B*) Image A (boxed area) at higher magnification. (*C*) CaMKK2 expression differences in a specimen of GS 3+4 from a second patient. (*D*) Image C (malignant area, boxed) at higher magnification. Note the perinuclear CaMKK2 staining (arrows). (*E*) CaMKK2 expression is higher in a GS 4+4 tumor area (right) compared to an adjacent HGPIN area (left). Arrows indicate perinuclear CaMKK2 staining. (*F*) CaMKK2 staining is uniformly intense in a GS 4+5 tumor specimen. Note the appearance of nuclear CaMKK2 staining (blue arrows) apart from the perinuclear staining (black arrows). Magnifications: (*A, C*) 10X, (*B, E*) 20X, (*D, F*) 40X.

Fig. S2. Specificities of the CaMKK2 antibodies employed in this study. Left, CaMKK2 antibody (Abnova) used for western blotting. Right, CaMKK2 antibody (Prestige, Sigma-Aldrich) used for immunohistochemistry (IHC). Western blots of lysates from LNCaP cells treated with non-specific, NS- or CaMKK2- siRNA (#1) were probed with the two antibodies as indicated to confirm their specificities. GAPDH was used as loading control. Note that

CaMKK2 typically electrophoreses as a doublet. The basis for generation of the doublet has not been established.

Fig. S3. Expression of CaMKK2 during tumor progression in the transgenic adenocarcinoma of mouse prostate (TRAMP) model. Non-transgenic wild type (C57BL/6 x FVB) prostate tissue and TRAMP prostate tissue and tumors were isolated from mice at various stages of disease progression. Wt, non-transgenic prostate tissue; 8 wk, prostate tissue of an 8 wk TRAMP mouse; 15 wk, prostate tissue of a 15 wk TRAMP mouse; tumor, prostate tumor of a 27 wk TRAMP mouse; castrated, tumor of a 24 wk castrated TRAMP mouse. Samples were harvested and assessed by semi-quantitative RT-PCR for CaMKK2 mRNA expression. GAPDH was used as loading control. A representative agarose gel of three independent experiments is shown.

Fig. S4. The AR antagonist, Casodex (bicalutamide) blocks the DHT-induced increase in CaMKK2 expression. LNCaP cells were cultured in steroid-depleted media for 24 h then treated with DHT (10 nM) or vehicle (EtOH) in the presence or absence of Casodex (CDX, 10 μM) or its vehicle (DMSO) for 72 h. CaMKK2 and PSA protein levels were assessed by western blotting. Casodex is an AR antagonist able to block the effect of androgen on both PSA and CaMKK2 expression (compare -/+ DHT in the presence of CDX; lanes 3, 4). However, Casodex also shows partial agonist activity (compare -/+ CDX in the absence of DHT; lanes 1 and 3). This property of Casodex has been previously reported (Hara T et al. Cancer Res 2003 63:149–53.)

Fig. S5. (A) CaMKK2 knockdown has no effect on proliferation in PC3 cells. PC3 cells were transfected with either non-specific NS- or CaMKK2- siRNAs and grown for the indicated time periods. Numbers of viable cells were determined by cell counting with Trypan-blue exclusion. Representative blots are shown beneath. Results represent means ± S.E. (n=2 independent experiments) for each siRNA used. GAPDH is used as loading control. (B) PI-Flow cytometry results table. LNCaP cells were transfected with either NS- or CaMKK2- siRNAs for 4 days and analyzed by PI-flow cytometry. Results represent means ± S.E. (n=2 independent experiments) for each siRNA used. CaMKK2 siRNA #1, $P < 0.05$, $P < 0.01$ for G1 and S phase differences respectively between NS- and CaMKK2- siRNA treated cells. CaMKK2 siRNA #2, $P < 0.05$ for both G1 and S phase differences between NS- and CaMKK2- siRNA treated cells. (C) Knockdown of CaMKK2 does not change cyclin D1 mRNA levels. LNCaP cells were transfected with NS- and CaMKK2- siRNAs and grown for 72 h. Cyclin D1 mRNA levels were assessed by qRT-PCR and normalized to GAPDH. Results represent means ± S.E. (n=3 independent experiments). $P = 0.18$

Fig. S6. (A) CaMKK2 knockdown reduces PSA protein levels in LNCaP cells. LNCaP cells were transfected with NS- or CaMKK2- siRNAs and grown for 72 h. Levels of PSA protein quantified by western blotting are shown with representative blots beneath for each siRNA used. Results represent means ± S.E. (n=3 independent experiments). *$P < 0.05$, ***$P < 0.001$, relative to NS- treated control cells. GAPDH is used as loading control. (B) DHT is ineffective at raising CaMKK2 levels in LNCaP cells treated with CaMKK2 siRNA. Left panel, LNCaP cells were transfected with NS siRNA- or CaMKK2- siRNA (#2) in normal media for 48 h, switched to steroid-depleted media for 24 h and then treated with 10 nM DHT or vehicle (EtOH) for an

additional 24 h. Levels of CaMKK2 protein were quantified by western blotting and normalized to GAPDH. Right panel, LNCaP cells were transfected with NS- or CaMKK2- siRNA (# 2) in normal media for 24 h, switched to steroid-depleted media for 72 h and then treated with 10 nM DHT or vehicle (EtOH) for an additional 16 h. Levels of CaMKK2 mRNA were quantified by qRT-PCR and normalized to GAPDH. Results represent means ± S.E. in arbitrary units (n=3 independent experiments). *P <0.05, **P <0.01 either of DHT treated cells relative to EtOH treated cells (over bars) or CaMKK2- relative to NS- siRNA-treated cells (over brackets). (C) CaMKK2 knockdown does not reduce AR protein levels. AR protein levels were quantified by western blotting under the conditions described in (B). GAPDH is used as loading control.

Fig. S7. Androgen depletion potentiates the inhibition of LNCaP cell proliferation by the CaMKK inhibitor STO-609. The inhibitory effects of various concentrations of STO-609 on proliferation were assessed in LNCaP cells grown in either normal FBS-containing media or in steroid-depleted media (CSS). Cells were plated at a density of 2000 cells per well in 96-well plates and grown for 24 h prior to treatment. They were then treated with various concentrations of STO-609 or vehicle (NaOH) and grown in media with FBS or CSS for 6 d. Cell proliferation was evaluated by the MTS proliferation assay kit, according to manufacturer's instructions (Promega). Absorbance was measured directly from 96-well plates at 490 nm. Left, bars represent numbers of viable cells (proportional to the measured optical density) grown in FBS-containing media at each drug concentration as a percentage of respective vehicle control cells. Right, bars represent the numbers of viable cells grown in CSS-containing media at each drug concentration as a percentage of respective control cells (vehicle treated cells grown in CSS-media). Results represent means ± S.E. (n=3 independent experiments under each condition).

Figure S1

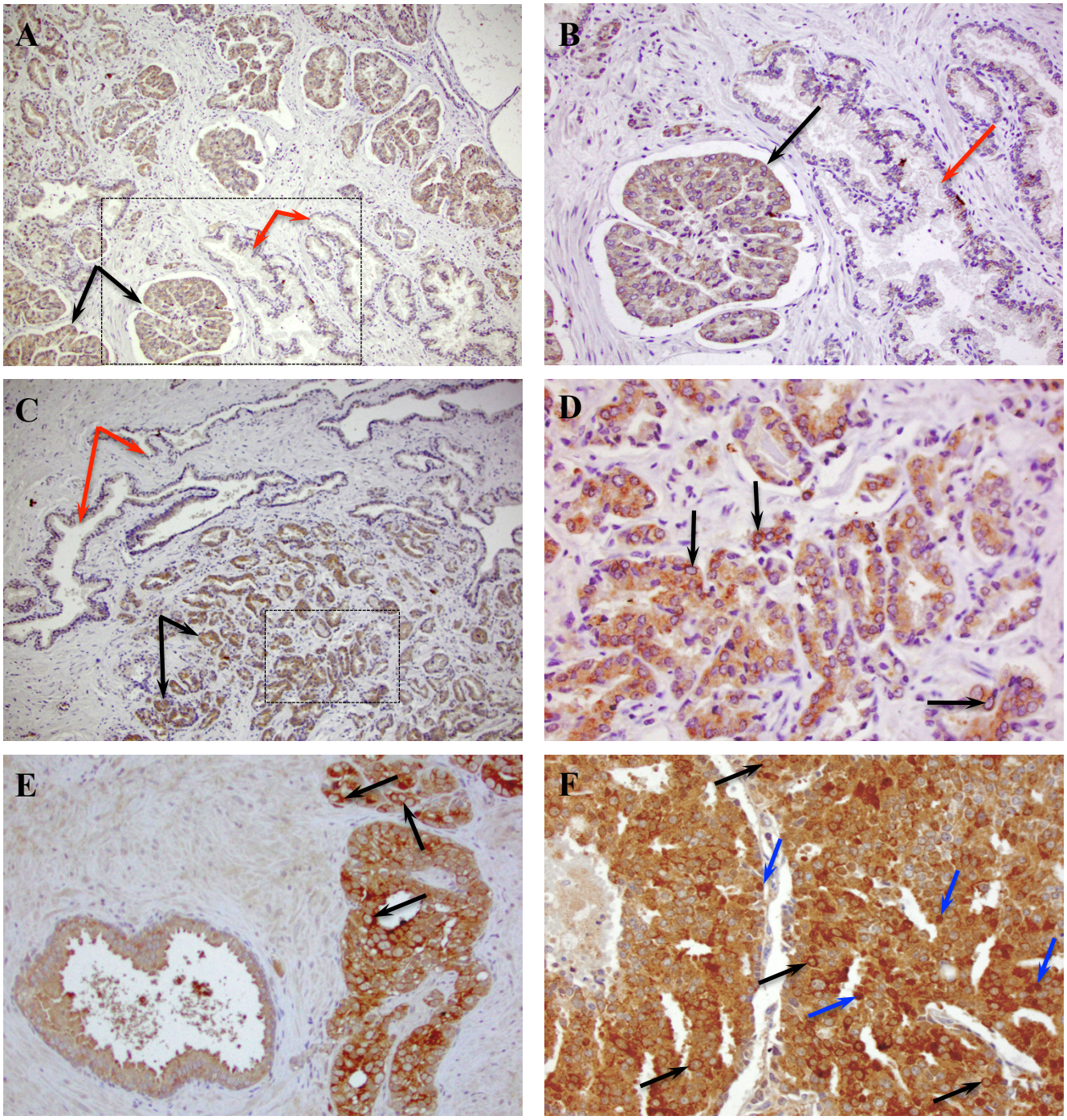


Figure S2

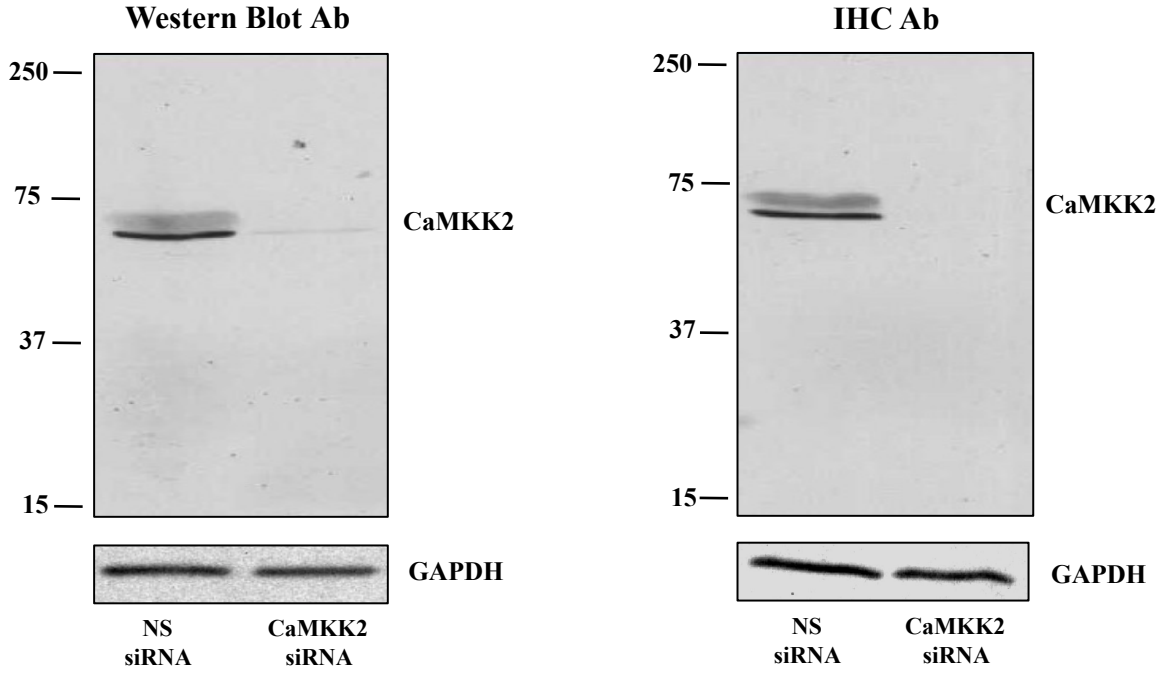

Western Blot Ab
250
75
37
15
CaMKK2
GAPDH
NS siRNA
CaMKK2 siRNA
IHC Ab
250
75
37
15
CaMKK2
GAPDH
NS siRNA
CaMKK2 siRNA

Figure S3

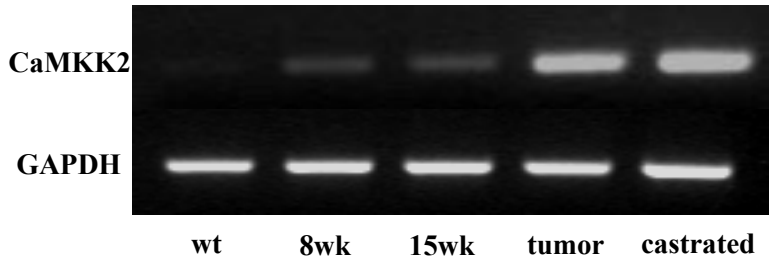


Figure S4

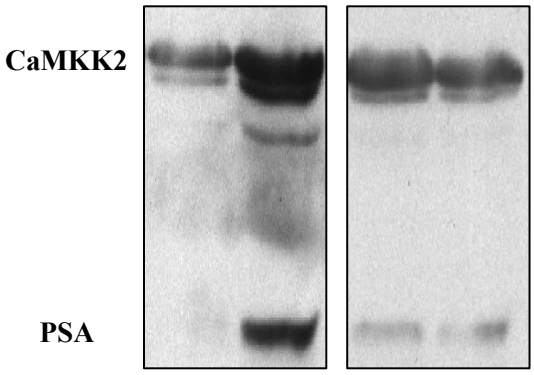
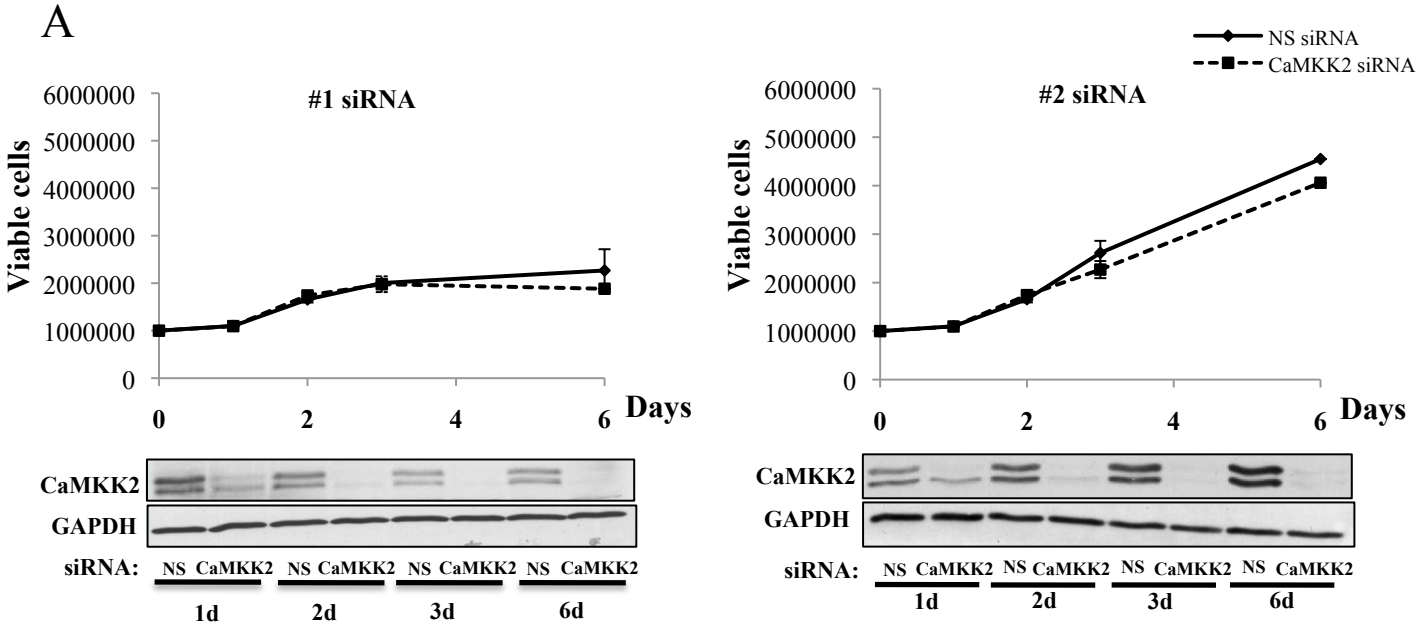


Figure S5

A



B

	#1 siRNA		#2 siRNA	
	NS	CaMKK2	NS	CaMKK2
G1	70.35±0.78	80.92±2.48	64.11±0.34	71.81±0.63
S	19.56±0.49	7.34±3.37	23.85±1.17	17.86±0.66
G2	10.08±0.29	11.75±0.99	12.04±0.84	10.33±0.04

C

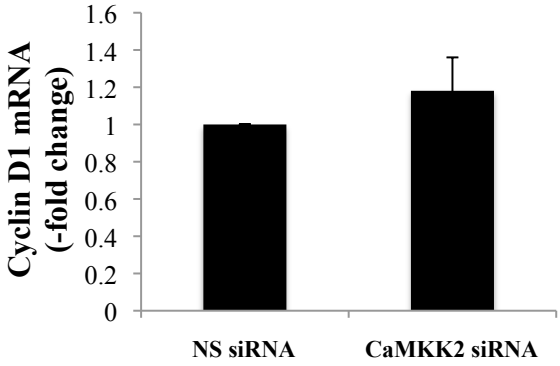
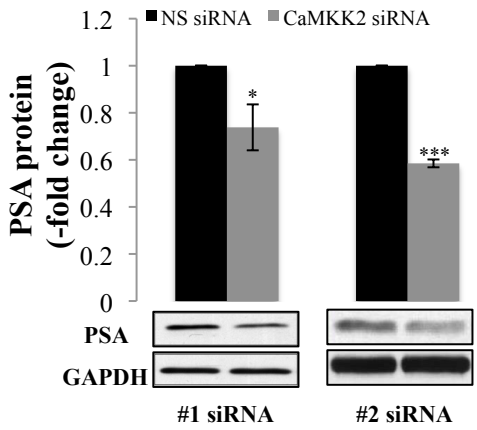
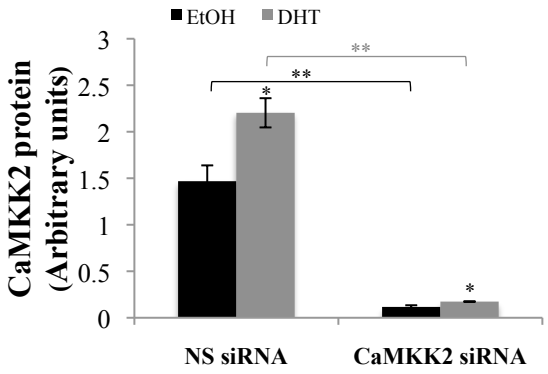


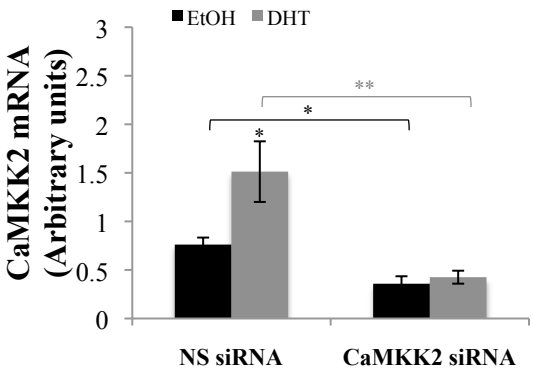
Figure S6

A



B





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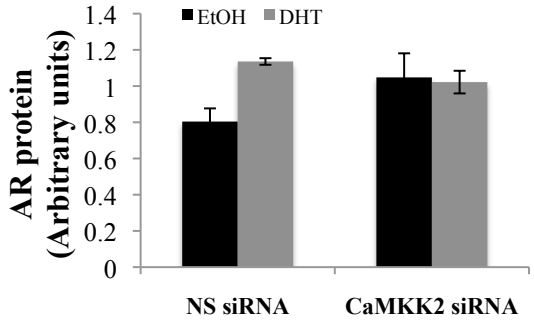
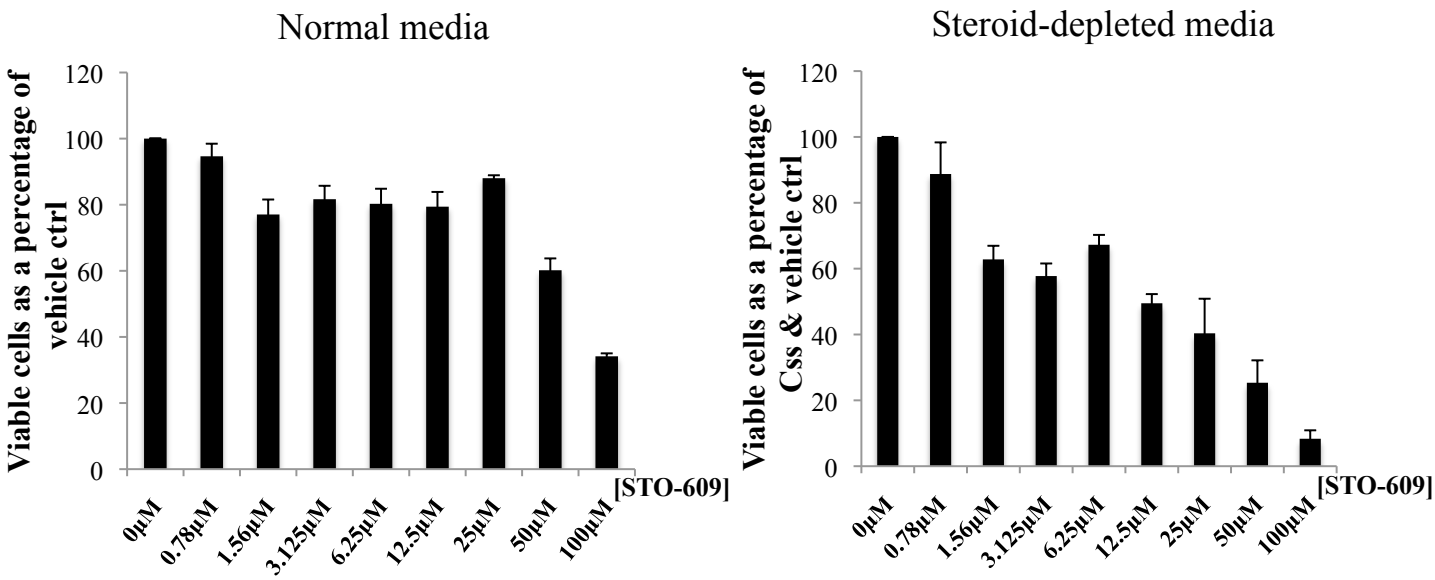


Figure S7


Normal media
Viable cells as a percentage of vehicle ctrl
120
100
80
60
40
20
0
0μM
0.78μM
1.56μM
3.125μM
6.25μM
12.5μM
25μM
50μM
100μM
[STO-609]
Steroid-depleted media
Viable cells as a percentage of Css & vehicle ctrl
120
100
80
60
40
20
0
0μM
0.78μM
1.56μM
3.125μM
6.25μM
12.5μM
25μM
50μM
100μM
[STO-609]