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Supplemental Data

Control of Cyclin D1 and Breast Tumorigenesis by the EglN2 Prolyl Hydroxylase

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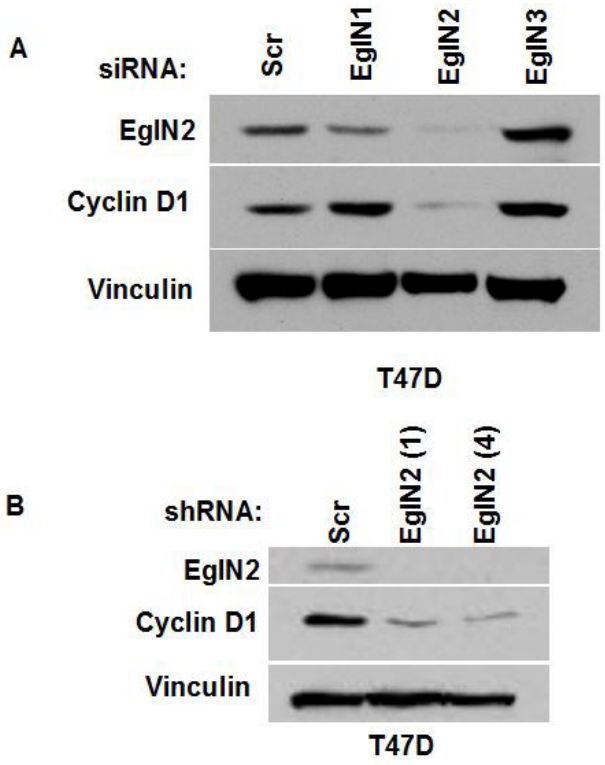


Figure S1. EglN2 Regulates Cyclin D1
(A) Immunoblot analysis of T47D cells 48 hours after transfection with siRNAs targeting EglN1, EglN2, EglN3, or a scrambled control siRNA.
(B) Immunoblot of T47D cells infected with two independent lentiviral shRNA vectors (#1 and #4) targeting EglN2.

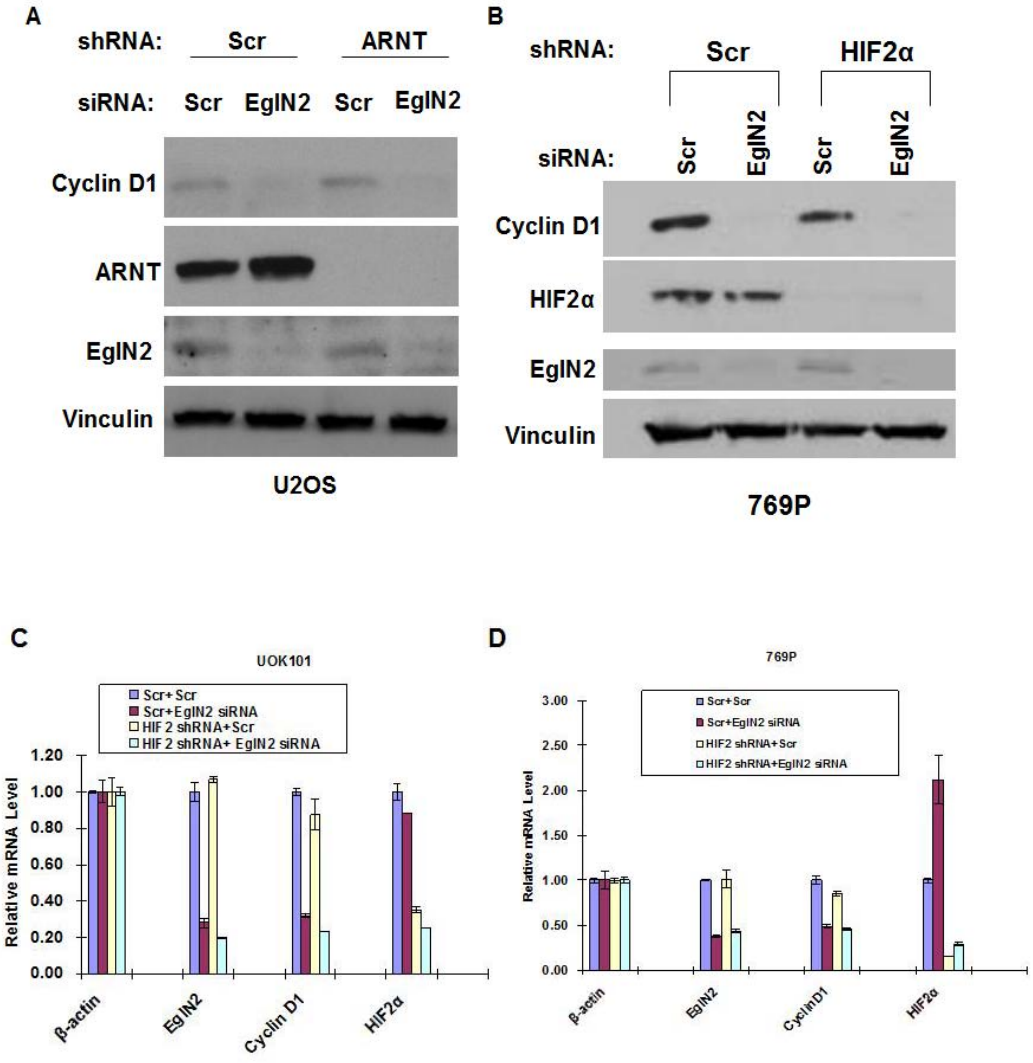


Figure S2. Loss of Cyclin D1 After EglN2 Knockdown is HIF-Independent

(A) Immunoblot analysis of U2OS cells infected with a lentivirus encoding an ARNT1 shRNA or scrambled control (Scr) followed by transfection with siRNA against EglN2 or scrambled control.
(B) Immunoblot analysis of 769P cells infected with a lentivirus encoding a HIF2α shRNA or scrambled control (Scr) followed by transfection with siRNA against EglN2 or scrambled control (Scr). (C and D) Real-time RT-PCR analysis of UOK101 (C) and 769P (D) cells infected with a lentivirus encoding a HIF2α shRNA or scrambled (Scr) shRNA followed by transfection with siRNA against EglN2 or scrambled control (Scr). mRNA values were normalized to the β-actin mRNA level in that sample and then expressed relative to the β-actin mRNA level in the scrambled control cells. Error bars equal one standard error of the mean.

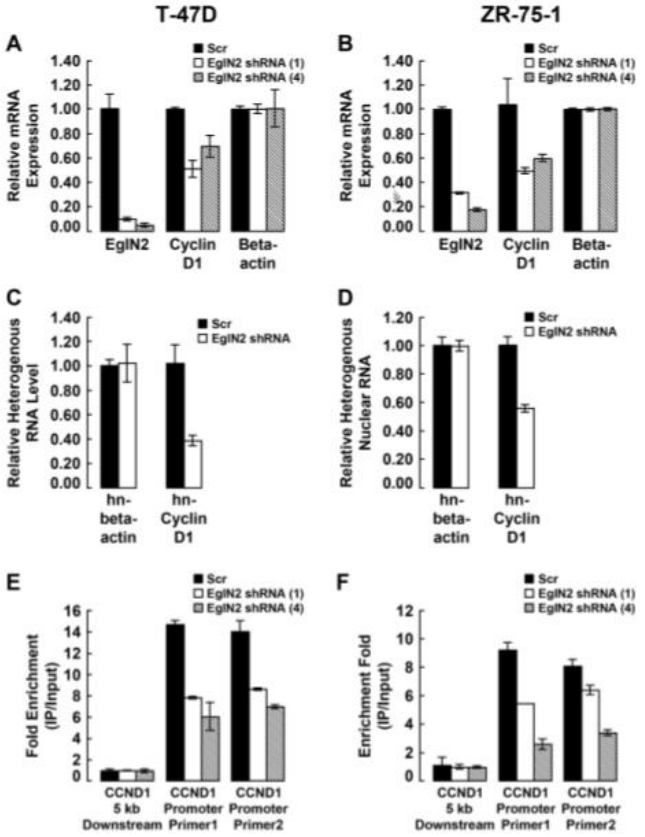


Figure S3. EglN2 Regulates Cyclin D1 Transcriptionally.

(A and B) Real-time RT-PCR analysis for the indicated mRNAs in T47D (A) and ZR-75-1 cells (B) infected with a lentivirus encoding either of two different EglN2 shRNAs (corresponding to sequences #1 or #4) or scrambled control (Scr) shRNA. mRNA values were normalized to the β-actin mRNA level in that sample and then expressed relative to the β-actin mRNA level in the scrambled control cells.

(C and D) Real-time RT- PCR analysis for heterogenous nuclear (hn) Cyclin D1 or β-actin RNA in T47-D (C) and ZR-75-1 (D) cells infected with a lentivirus encoding an EglN2 shRNA (corresponding to sequence #4) or scrambled control. Cyclin D1 values were normalized to the β-actin level in that sample and then expressed relative to the β-actin mRNA level in the scrambled control cells. Primers were: heterogenous nuclear Cyclin D1 (F: 5'-ACA GCC TCC TTC CCT CTC TC-3' R: 5'-TGA GGC GGT AGT AGG ACA GG-3'); heterogenous nuclear β-actin (hn-β-actin) (F: 5'- CCC AGC ACA ATG AAG ATC AA-3' R: 5'-GTG AGG ACC CTG GAT GTG AC-3').

(E and F) Chromatin immunoprecipitation analysis of T47D (E) and ZR-75-1 (F) cells infected as in panels (A) and (B), respectively, using anti-RNA Pol II antibody. Bound genomic DNA corresponding to the Cyclin D1 promoter (primer set 1 or primer set 2) or 5kB downstream of the promoter was measured by real-time PCR. Values are expressed relative to input DNA and normalized to the scrambled value for the 5 kB downstream site.

Error bars = 1 SEM.

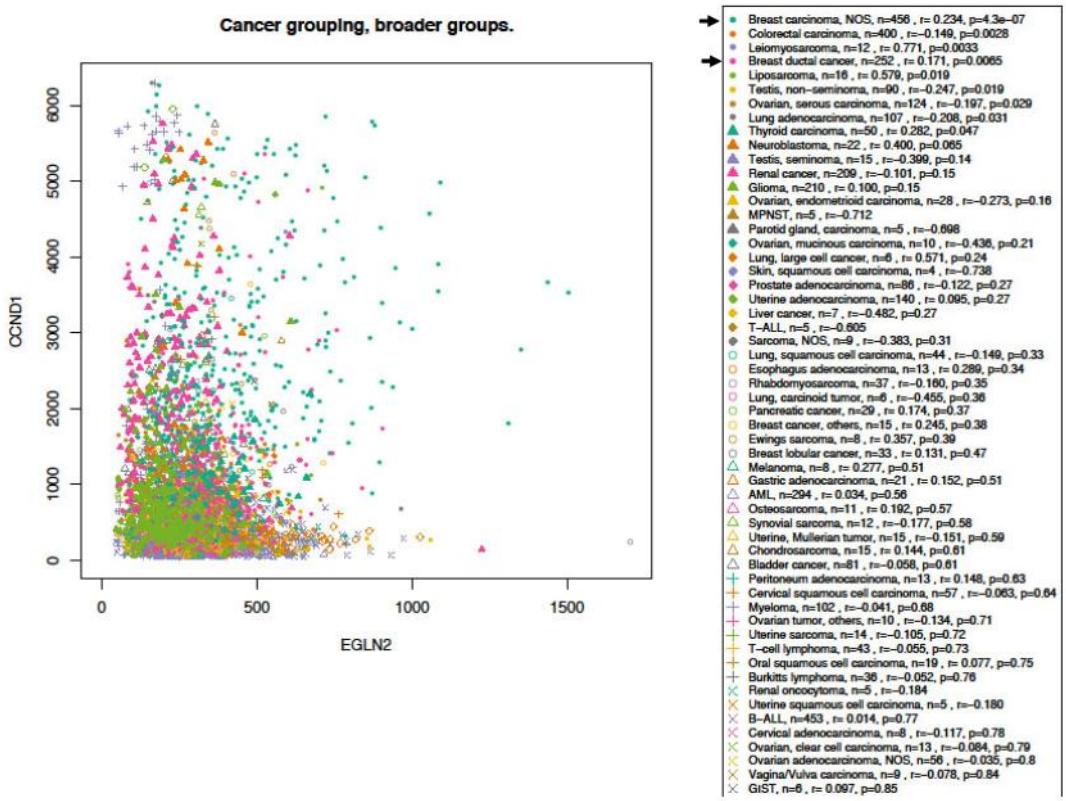


Figure S4. Correlation of CCND1 (Cyclin D1) and EglN2 (EGLN2) mRNA Levels across Multiple Cancers

In silico analysis of CCND1 and EglN2 mRNA levels across multiple tumors using www.genesapiens.org. Note p values for Breast cancer, not otherwise specified (NOS), and Breast ductal cancer.

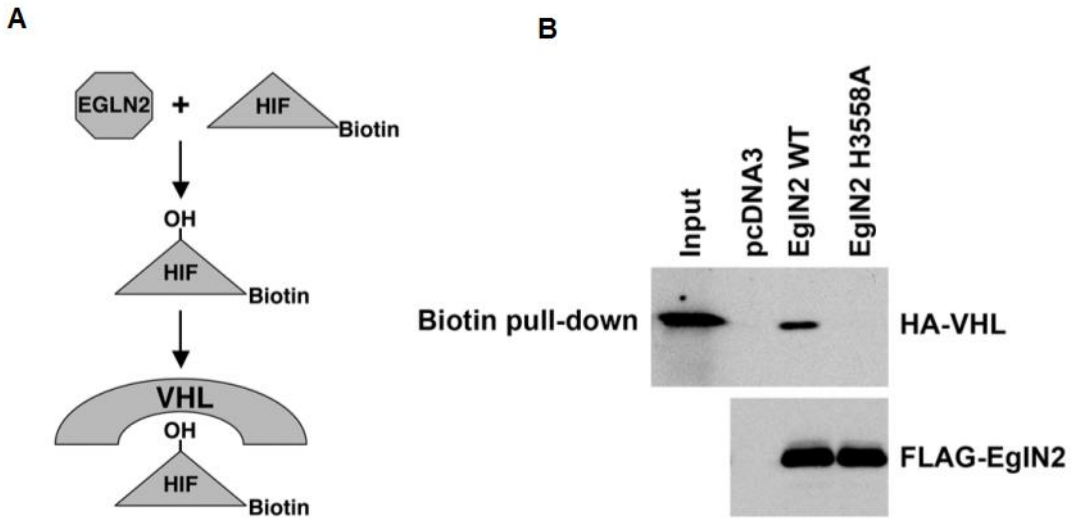


Figure S5. EglN2 H358A is Hydroxylase-defective

(A) Schematic of in vitro hydroxylation assay

(B) Recovery of ^{35}S-labelled pVHL by a biotinylated HIF1α peptide after preincubation with *in vitro* translation products of plasmids encoding EglN2 (wild-type), EglN2 (H358A), or empty vector (pcDNA3). Recovery of pVHL indicates hydroxylation of the HIF1α peptide.

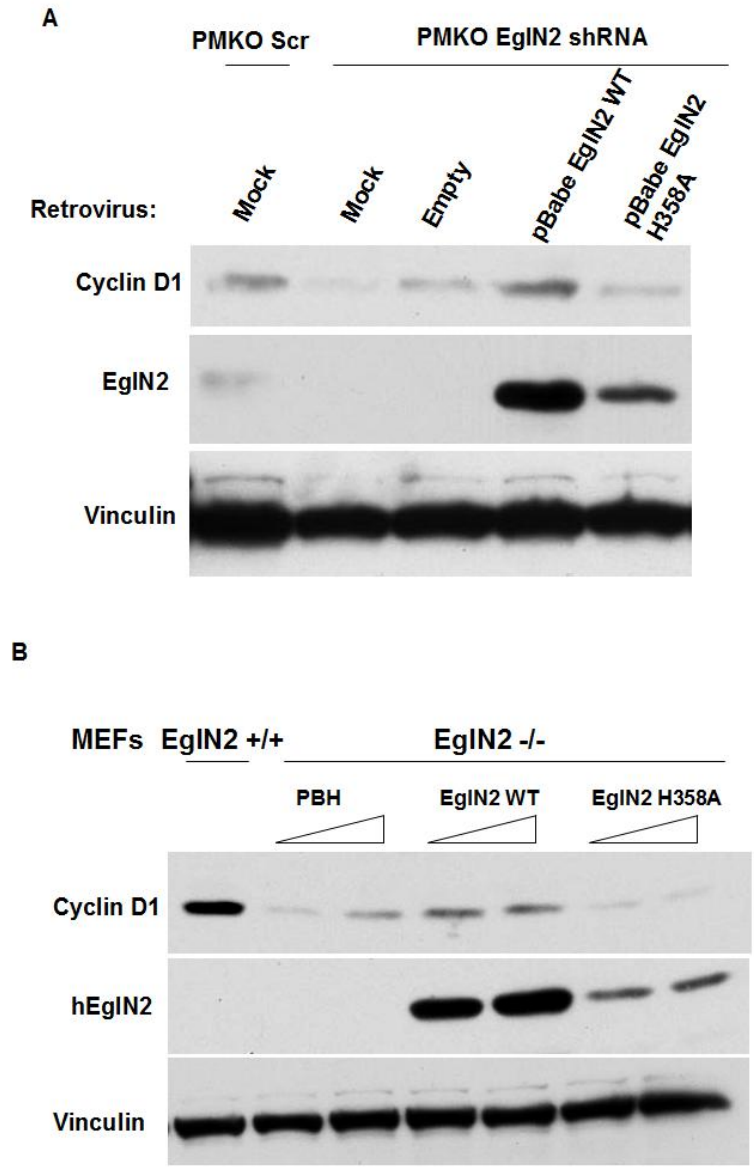


Figure S6. Exogenous Wild-type, but not Catalytic-dead, EglN2 Rescues Cyclin D1 Levels in Cells Lacking Endogenous EglN2

(A) Immunoblot analysis of T47D cells that were first infected with a retrovirus expressing an shRNA-resistant mRNA that encodes wild-type or catalytic-dead (H358) EglN2 (or with the backbone pBabe vector) and then infected with a EglN2 shRNA retrovirus (or scrambled shRNA retrovirus).

(B) Immunoblot analysis of EglN2-/- MEFs infected with increasing amounts, as indicated by the triangles, of retroviruses encoding wild-type EglN2, EglN2 H358A, or backbone virus (PBH). EglN2+/+ MEFs were analyzed in lane 1 as a control.

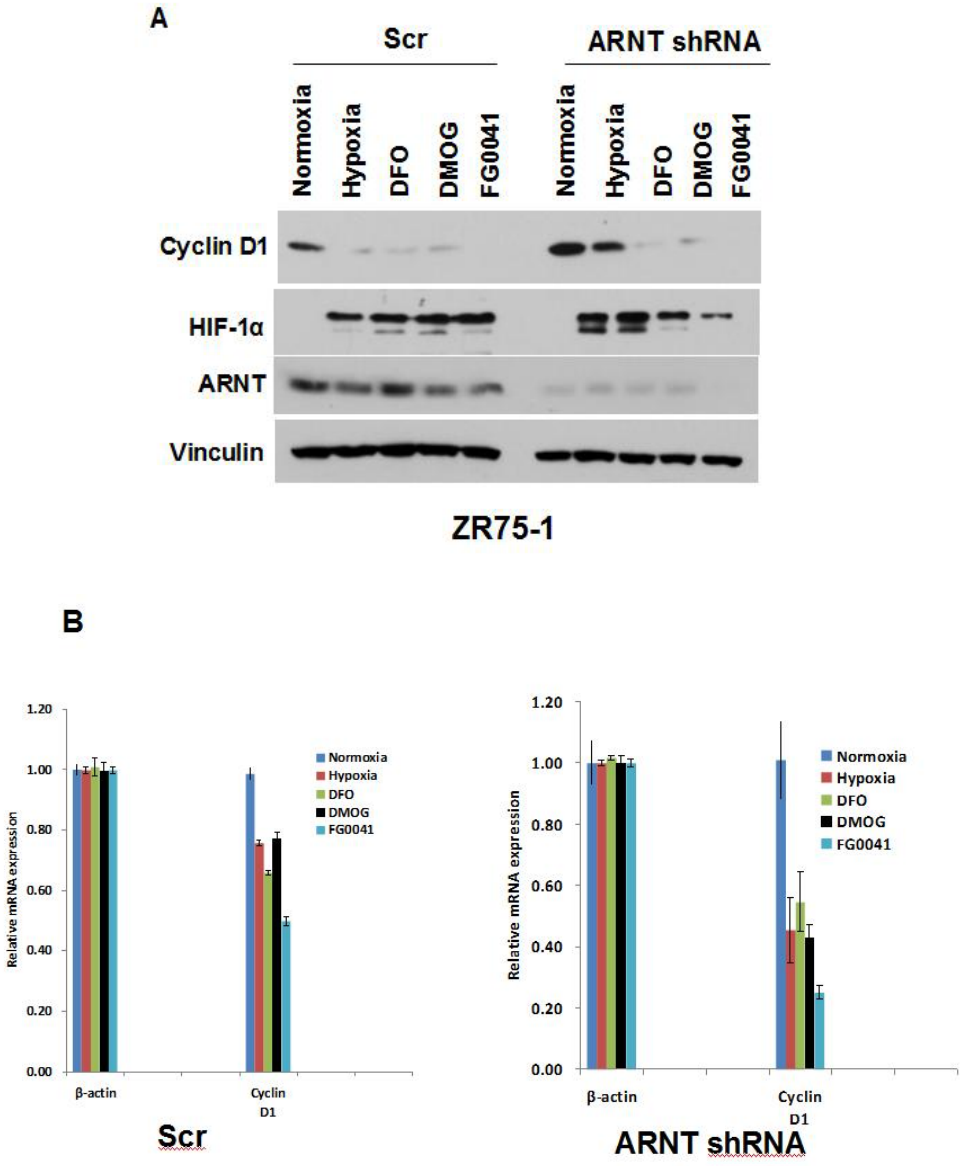


Figure S7. Cyclin D1 Regulation by Hydroxylase Inhibitors is Largely HIF-independent

(A) Immunoblot analysis of ZR75-1 cells that were first infected with a lentivirus encoding an ARNT1 shRNA (or scrambled control) and then treated overnight with hypoxia (0.2% oxygen), DFO (200 μM), DMOG (1 mM) or FG0041 (40 μM) (Ivan et al., 2002).

(B) Quantitative real-time PCR analysis of RNA isolated from cell treated as in (A). EglN2 mRNA values were first normalized to β-actin mRNA levels and then normalized to the values from cells treated under normoxic conditions. Error bars equal one standard error of the mean.

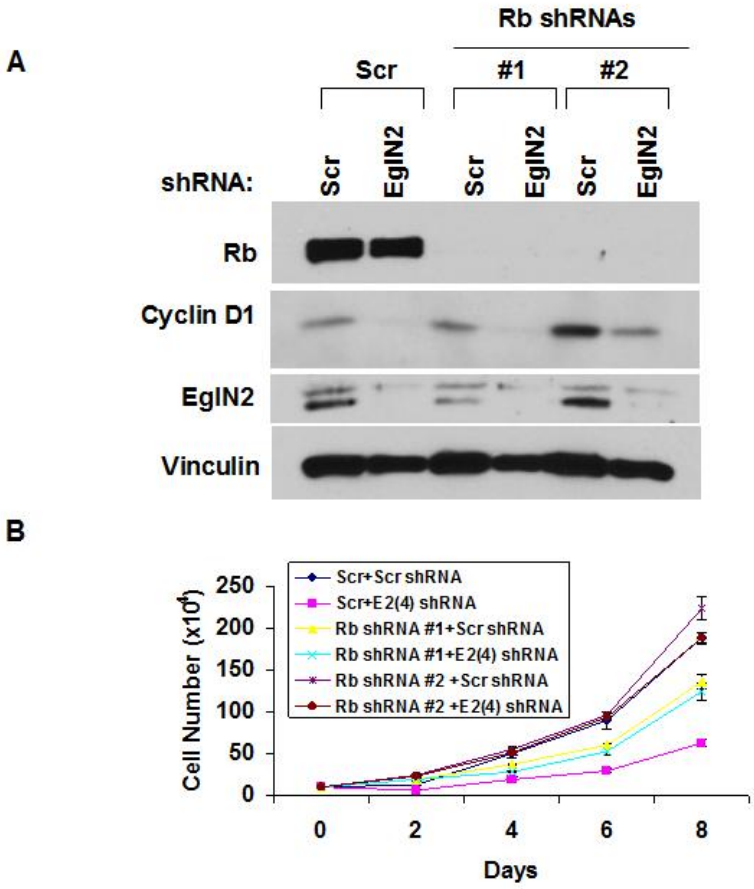
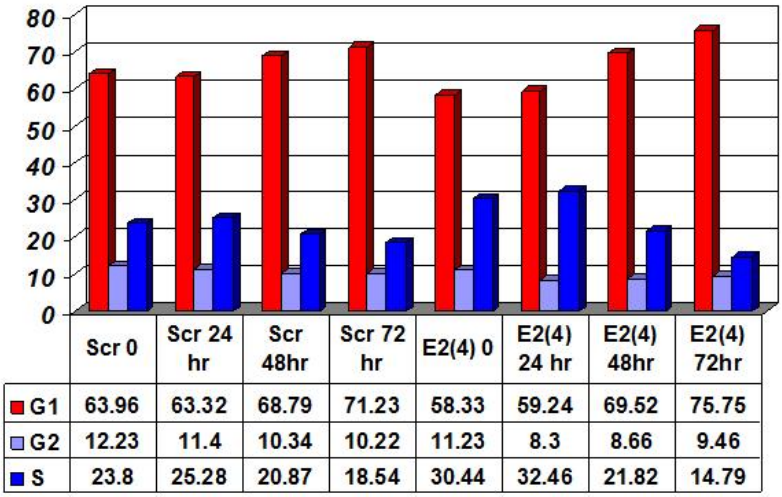


Figure S8. Impaired Proliferation after EglN2 Loss is pRB-Dependent

(A and B). Immunoblot (A) and cell proliferation (B) assay of T47D cells that were infected with lentiviruses encoding shRNAs against RB1 [sequence 1 or 2](or scrambled control) and subsequently infected with a retrovirus encoding EglN2 shRNA (corresponding to siRNA #4)(or scrambled control). Note that hyperphosphorylated and hypophosphorylated pRB are not resolved under these electrophoretic conditions. Error bars = 1 SEM.

ZR-75-1 Dox treatment



	Scr 0	Scr 24 hr	Scr 48hr	Scr 72 hr	E2(4) 0	E2(4) 24 hr	E2(4) 48hr	E2(4) 72hr
G1	63.96	63.32	68.79	71.23	58.33	59.24	69.52	75.75
G2	12.23	11.4	10.34	10.22	11.23	8.3	8.66	9.46
S	23.8	25.28	20.87	18.54	30.44	32.46	21.82	14.79

Figure S9. EglN2 Loss Impairs Cell-Cycle Progression
ZR75-1 cells were infected with a lentivirus encoding doxycycline-inducible hairpin against EglN2 (corresponding to siRNA sequence #4) or scrambled control shRNA. Cells were stained with propidium iodide at the indicated timepoints after doxycycline addition and cell-cycle distribution was determined by FACS.

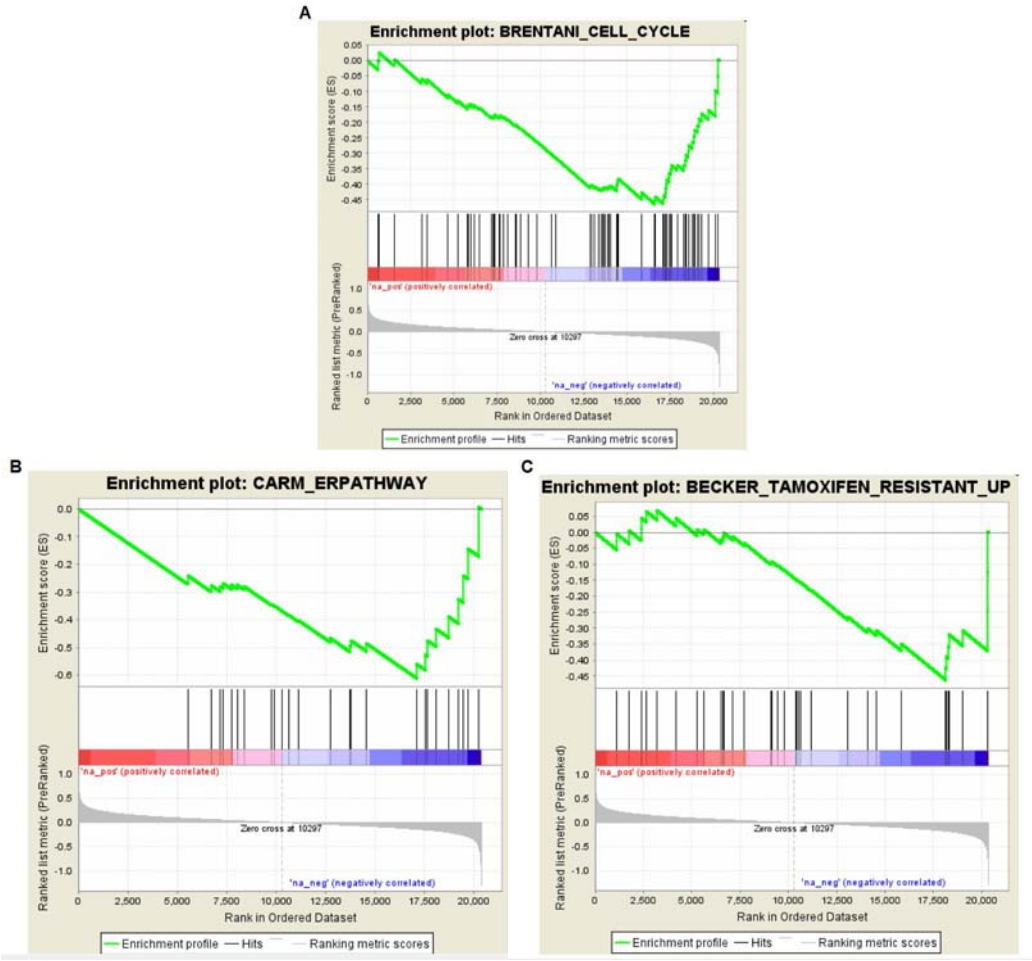


Figure S10. Gene Set Enrichment Analysis

Gene set enrichment analyses (GSEA) (Subramanian et al., 2005) was used to determine whether any a priori defined sets of genes (gene sets) differed in a statistically meaningful way between T47D cells producing EglN2 or scrambled shRNAs. 1892 curated gene sets from the Molecular Signatures Database (MSigDB, http://www.broadinstitute.org/gsea/msigdb/index.jsp) were interrogated in this study. The reference gene list *L* was generated by ranking the fold change of all of 33297 genes on *A*ffymetrix Human Gene ST1.0 array. The Q value-estimated FDR of 25% was then used to select overrepresented gene sets. Shown are data for set of genes linked to Cell-Cycle Progression (Panel A; see also Supplementary Table 1), ER Signaling (Panel B; see also Supplementary Table 2), and Tamoxifen Resistance (Panel C; see also Supplementary Table 3). Each vertical bar represents an individual gene from the predefined gene set. Genes upregulated by EglN2 loss appear toward the left and downregulated by EglN2 appear toward the right. The enrichment score is shown in green at the top.

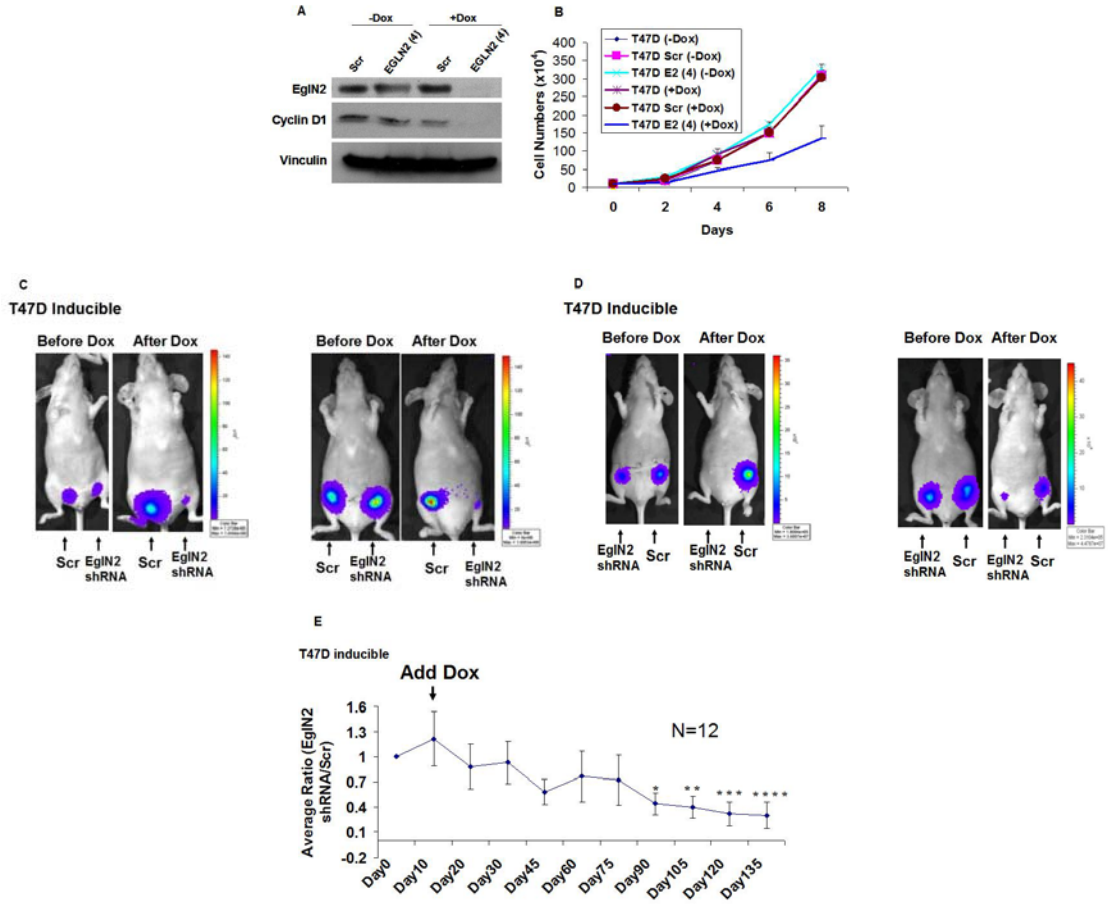


Figure S11. Downregulation of EglN2 Suppresses T47D Tumorigenesis

(A) and (B). Immunoblot (A) and cell proliferation assay (B) of T47D breast carcinoma cells infected with doxycycline (DOX)-inducible lentiviruses encoding shRNAs against EglN2 [4](or scrambled control). Cells were grown in RPMI supplemented with 10% fetal bovine serum in the presence or absence of doxycycline. Error bars equal one standard error of the mean.

(C and D). Representative bioluminescent images of orthotopic tumors formed by T47D cells as in (A) that were then superinfected with a retrovirus encoding firefly luciferase. 6 x 10^6 cells were injected into the 4rd mammary glands of nude mice implanted with estrogen pellets. Shown are images obtained prior to addition of doxycyline (3 weeks after implantation) and after 16 weeks of doxycycline treatment.

(E). Quantitation of imaging studies as in (C and D). *p<0.01 for comparison between Day 90 and Day 0, **p<0.001 for comparison between Day 105 and Day 0. **p<0.001 for comparison between Day 105 and Day 0. ***p<0.001 for comparison between Day 120 and Day0. ****p<0.001 for comparison between Day 135 and Day 0. Error bar = 1 SEM.

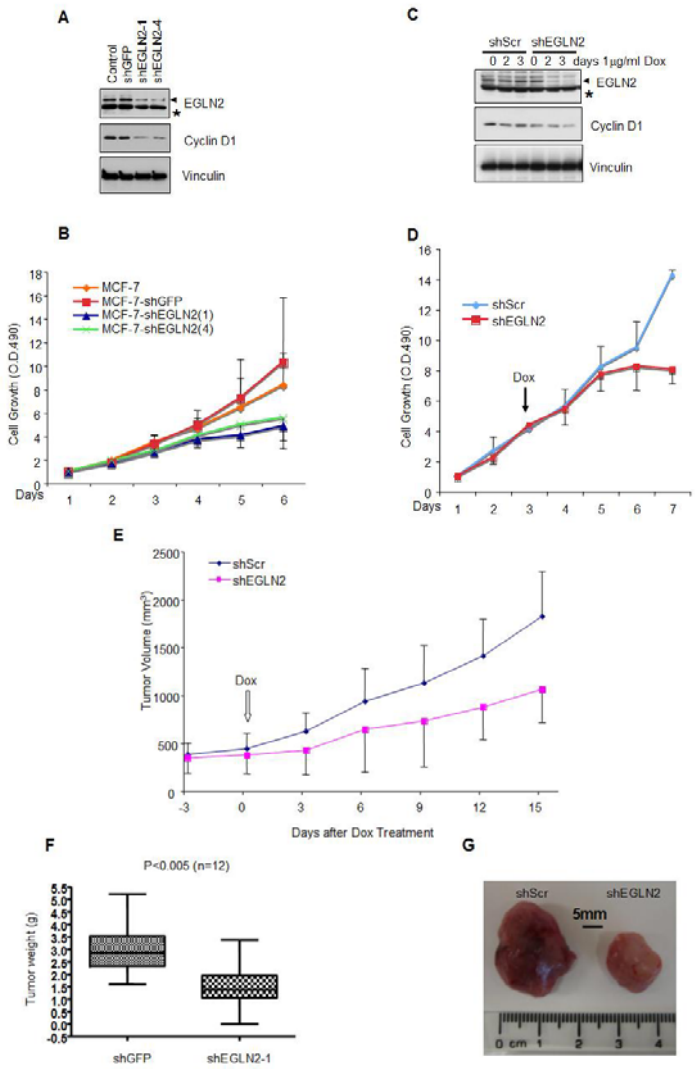


Figure S12. Downregulation of EglN2 Suppresses MCF7 Tumorigenesis

(A and B). Immunoblot (A) and XTT cell proliferation assay (B) of MCF7 cells infected with retrovirus encoding shRNA against EglN2 (sequence 1 or 4) or a scrambled control shRNA (* indicates nonspecific bands).

(C and D). Immunoblot (C) and XTT cell proliferation assay (D) of MCF7 cells infected with doxycycline (DOX)-inducible lentiviruses encoding shRNA against EglN2 [siRNA sequence #4] or scrambled control (* indicates nonspecific bands).

(E). Tumor burden, as determined by caliper measurement, in nude mice in which the cell analyzed in (C and D) were implanted in opposite mammary glands of nude mice.

(F). Tumor weights at necropsy of mice analyzed in (E). Mice were fed with doxycyline containing water 5-6 weeks after tumor implantation. Tumors were harvested around 9 weeks after initial tumor implantation.

(G). Representative tumors at necropsy.

Error bars equal one standard error of the mean.

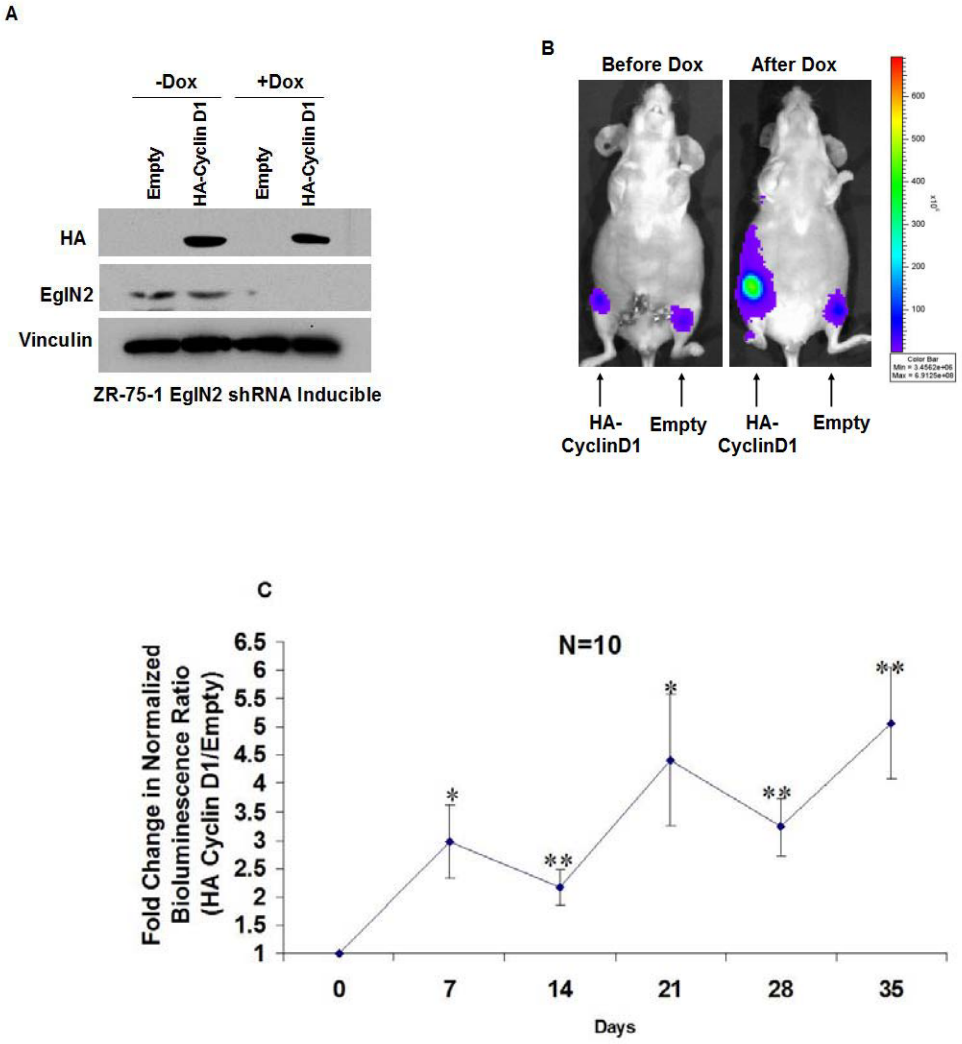


Figure S13. Tumor Suppression following EglN2 Depletion can be Rescued by Cyclin D1.

(A) Immunoblot analysis of ZR-75-1 infected with a lentivirus encoding a doxycycline-inducible hairpin against EglN2 (corresponding to siRNA sequence #4) and then infected with a retrovirus encoding HA-Cyclin D1 (or the backbone vector). Where indicated cells were treated with doxycycline (+DOX) for 48 hours.

(B) Representative bioluminescent images of orthotopic tumors formed by ZR75-1 cells as in (A) that were then superinfected with a retrovirus encoding firefly luciferase. 8 x 10^6 cells were injected into the 4th mammary glands of nude mice implanted with estrogen pellets. Bioluminescent images were obtained one week later (day 0) and serially after mice were begun on chow containing doxycycline (day 3). Shown in (B) are day 0 image (Before Dox) and day 35 (After Dox).

(C). Quantitation of imaging studies as in (B). *$p<0.05$ for comparison between Day 7, 21 and Day 0, **$p<0.005$ for comparison between Day 14, 28, 35 and Day 0. Error bars = 1 standard error of the mean. See Methods for normalization.