EXPERIMENTAL METHODS FOR SUPPLEMENTARY FIGURES

Cell culture and treatment

The ZR-75-1 ER positive breast cancer cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 0.01 M HEPES, 1 mM sodium pyruvate, 0.15% sodium bicarbonate, 0.45% glucose, and penicillin and 1% streptomycin. To investigate the direct role of estrogen responsiveness of EFP and KLF5 degradation, ZR-75-1 cells were maintained in phenol red-free medium supplemented with 10% charcoal-dextran-stripped fetal bovine serum for at least 3 days. Subsequently, the cells were treated with 1 μM of 17β-estradiol (E2) and were harvested at indicated times to isolate total RNA and protein for analysis.

To investigate the direct role of estrogen responsiveness of EFP and KLF5 degradation, MCF-7 cells cultured in regular medium were treated with 1 μM of tamoxifen (Tam) for the indicated times and subsequently total RNA and protein were isolated for analysis.

Western blot analysis

Cell lysates for western blotting were prepared as described in the Experimental section of the main text. Briefly, following the treatment the cells were lysed with RIPA buffer containing 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS and 1% Sigma cocktail proteinase inhibitors I. The lysates were then processed for western blotting using standard protocol and later probed with appropriate antibodies.

SiRNA transfection

Small interfering RNAs (siRNA) were procured from Santa Cruz Biotechnology (Catalog # sc-37825 and sc-37825b, Santa Cruz, CA) and were used to target EFP. Another siRNA against EFP was chemically synthesized (Dharmacon, Chicago, IL) with the sequence of 5'-GGUGGAGCAGCUACAACAA-3' and was also used to target EFP. MCF-7 cells were transfected with 100 nM of each siRNA and transfection was carried out by using the siPORT Amine Reagent (Ambion, Austin, TX) in 6-well plates. Forty-eight hours after transfection, total protein was collected for analysis using western blot.

Cycloheximide (CHX) chase assay of MCF-7 cells

MCF-7 cells were maintained in phenol red-free medium supplemented with 10% charcoal-dextran-stripped fetal bovine serum for 3 days. Subsequently, the cells were treated with 1 μM of 17β-estradiol (E2) for 4 hours. Following the estrogen treatment cells were treated with 50 μg/ml CHX for different time periods. Total proteins were collected and analyzed by western blot for KLF5, EFP and β-actin.

Table S1: Primer sequences (5' to 3') for creating EFP deletion mutants

Mutants	Forward primer	Reverse primer
EFP-M1	GGGGAATTCTGATGGCAGAGCTGTGCCCCCTG	CCCTCTAGATGCTGGG TGCAGAGGCGCGG
EFP-M2	GGGGGTACCCCGAATGCCCAGGTGGCCTGC	CCCGAATTCCTACTTGGGGGAGCAGATGGAGAGTG

Table S2: Primer sequences (5' to 3') for creating EFP substitution mutants

Mutants	Primer-1	Primer-2
EFP-cS	CCATACCTGAGCCCGCAGAGCCGCGCCGTC	GACGGCGCGGCTCTGCGGGCTCAGGTATGG
EFP-kR	GAGGCCGCCGTGAGGACGTGCTTGGTG	CACCAAGCACGTCGTCACGGCGGCCTC

The underlined sequences represent the amino acid mutated

Table S3: Primer sequences (5' to 3') for real-time PCR analysis

	Forward primer	Reverse primer
KLF5	CAGAGGACCTGGTCCA GACAAGATG	GAGGCCAGTTCTCAGGTGAGTGATG
EFP	GTGACCACGGCTTTGTCATCT	TAAAGTCCACCC TGAACTTATACATCAG
β-actin	GAAACTACCTTCAACTCCATC	CTAGAAGCATTTGCGGTGGACGATGGAGGGGCC
FGF-BP	GCAGATGGGCTGCTACTGAG	CGGGCAACTTGTTTCCAATA
WWP1	GTATGGATCCTGTACGGCAGCA	GTTGTGGTCTCTCCCATGTGGT
Fbw-7	CCCGGAGCTGTGCAGCAA	CAGATGTAATTCGGCGTCGTT

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Estrogen treatment causes the degradation of KLF5 in ER-positive breast cancer cells. (A) ZR-75-1 cells grown in hormone-free medium and treated with 1 μM estrogen (E2) for the indicated times were subjected to western blotting to detect protein expression of KLF5, EFP and β-actin. (B) ZR-75-1 cells in panels A were subjected to real-time PCR for RNA expression of EFP and KLF5. (C) MCF-7 cells grown in regular medium were treated with the ER signaling inhibitor tamoxifen (Tam) for indicated times, and protein expression of KLF5, EFP and β-actin was evaluated by western blotting. (D) MCF-7 cells grown in regular medium and treated with 1 μM Tam for indicated times were analyzed by real-time PCR for RNA expression of EFP and KLF5. (E) RNA samples of MCF-7 cells from Figure 1C were subjected to real-time PCR to detect RNA expression of WWP1 and Fbw7.

Figure S2. Knock-down of EFP increases protein level of KLF5. (A, C) Transfection of a mixture of EFP siRNA (siEFP) purchased from Santa-Cruz (A) or a chemically synthesized siRNA (C) or a single siEFP from Santa-Cruz (E) into MCF-7 cells cultured in regular medium increases protein levels of KLF5. (B, D) MCF-7 cells cultured in hormone-free medium were transfected with either the Santa-Cruz siRNA (B) or synthesized siRNA (D) or control siRNA for 24 hours, treated with 1 μM estrogen (E2) for 18 hours, and subjected to western blotting for protein expression of KLF5, EFP and β-actin.

Figure S3. EFP downregulates KLF5 by protein degradation. COS-1 cells were transfected with expression plasmids for EFP, KLF5 and GFP (A) or EFP and GFP-KLF5 fusion protein for 48 hours and cell lysates were analyzed by western blotting for protein expression of KLF5, EFP, GFP and β-actin. Presence or absence of a construct is indicated by "+" or "-".

Figure S4. Cycloheximide (CHX) chase assay of MCF-7 cells in the presence and absence of estrogen (E2). (A) MCF-7 cells cultured in hormone-free medium for 3 days

were incubated with 1 μM estrogen (E2) or solvent control for 4 hours, followed by CHX treatment for indicated times. Western blotting was performed to detect the protein expression of KLF5, EFP, and β-actin. (B) Band intensities for all proteins were determined using the Image J program, and the intensity for KLF5 or EFP in each lane was divided by that for β-actin to indicate the relative level of KLF5 or EFP. The ratio for either KLF5 or EFP without CHX treatment, with or without estrogen treatment, was defined as 1 and the ratios for other lanes were normalized accordingly and plotted against CHX treatments.

Figure S1 (Zhao et al)

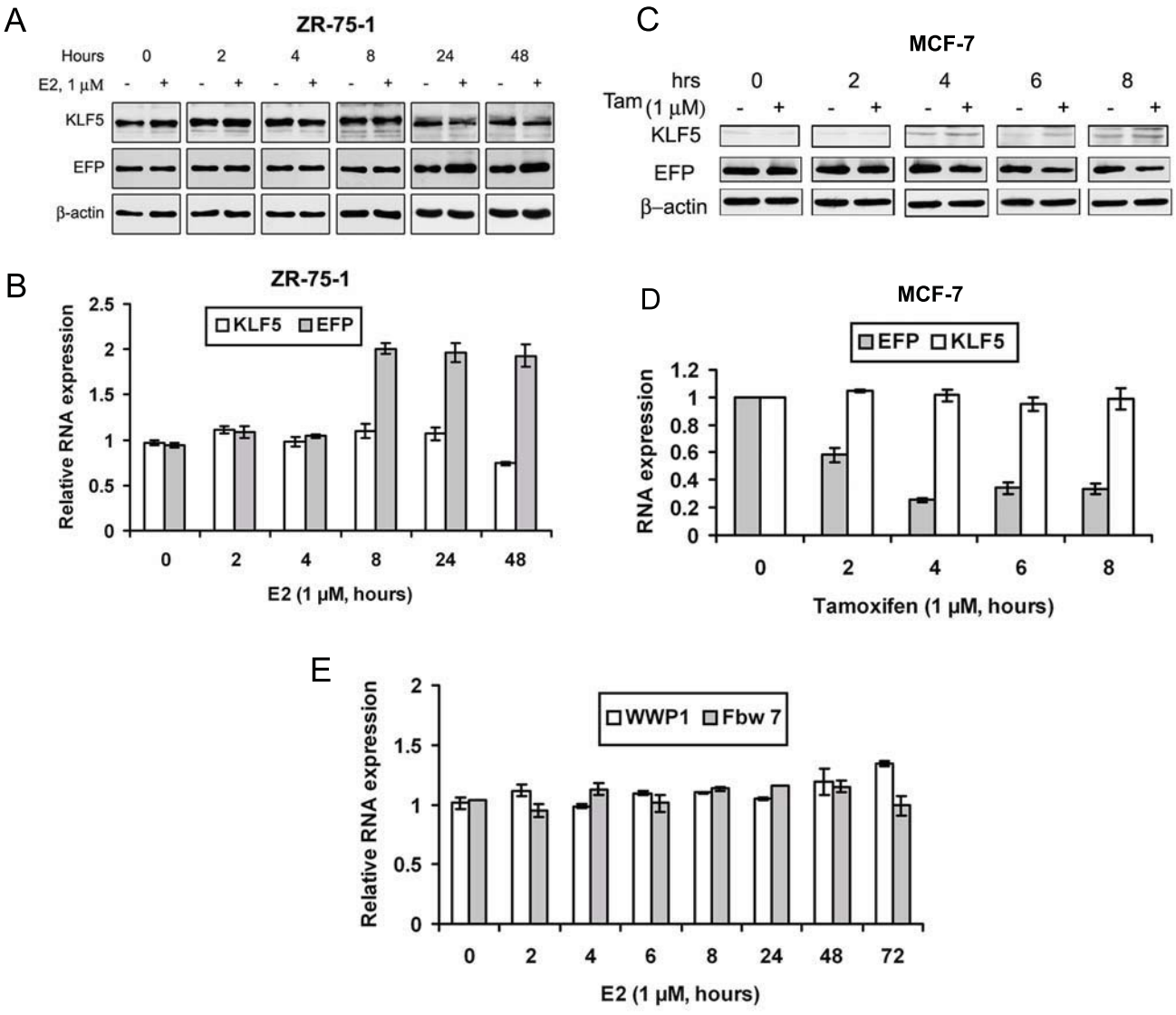


Figure S2 (Zhao et al)

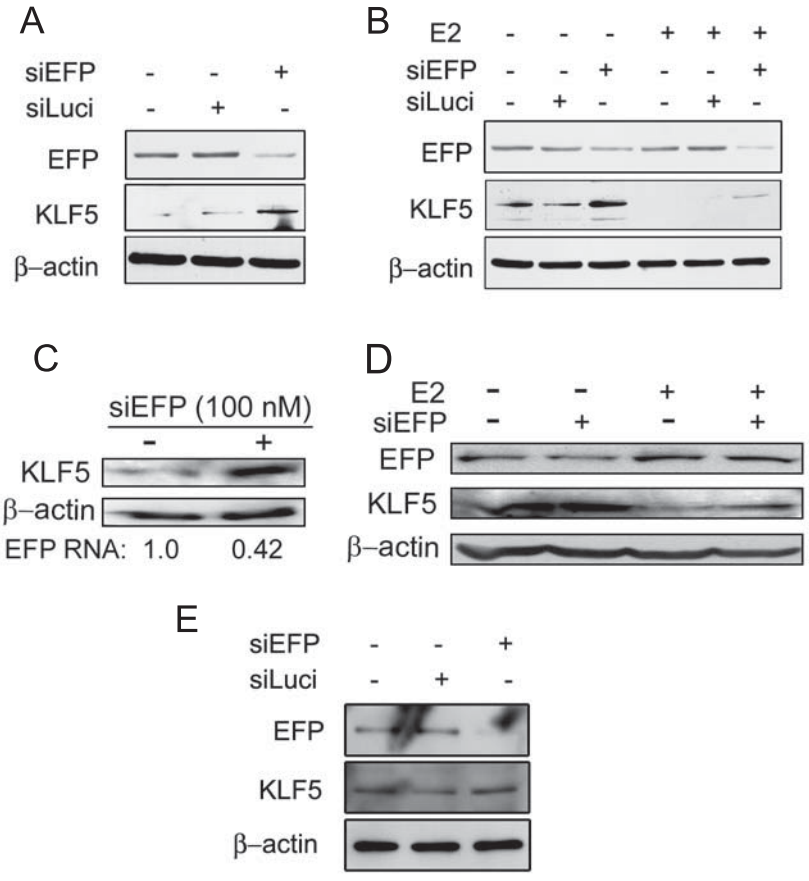


Figure S3 (Zhao et al)

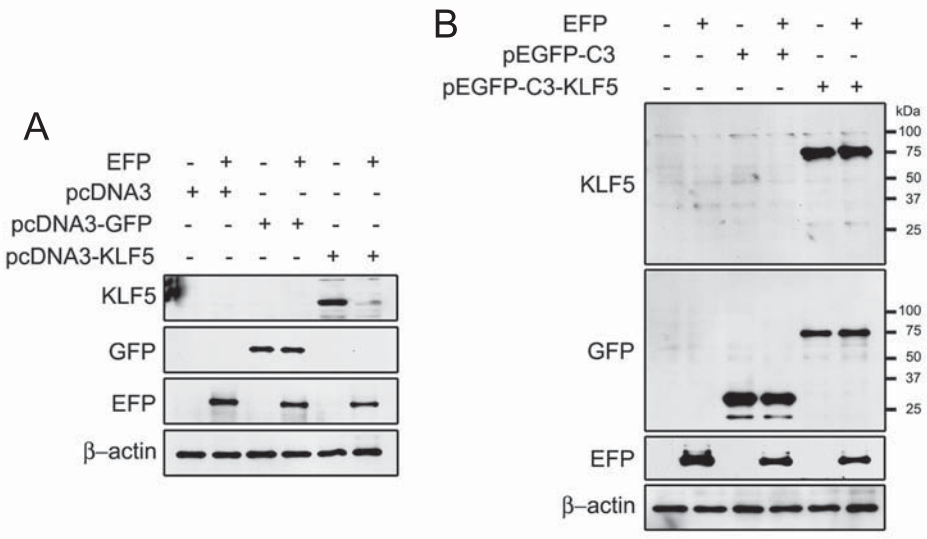
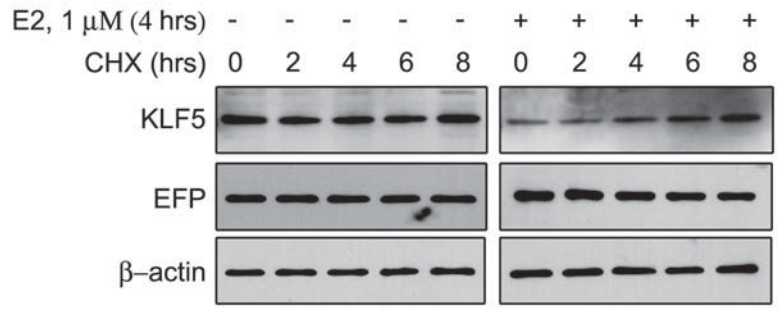


Figure S4 (Zhao et al)

A



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