Oestrogen causes degradation of KLF5 by inducing the E3 ubiquitin ligase EFP in ER-positive breast cancer cells

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INTRODUCTION

Krüppel is a segmentation gene in Drosophila melanogaster which encodes a protein composed of several zinc-finger motifs [1]. KLFs (Krüppel-like factors) are mammalian proteins homologous with the zinc-finger part of Krüppel. KLF5 (also named IKLF or BTEB2) is a transcription factor with a proline-rich N-terminal region and a C-terminus that contains three consecutive zinc-finger motifs [2]. Preceding the zinc-finger motifs is a short basic region that may contribute to the binding motifs is a short basic region that may contribute to the ability of KLF5 to bind to a GC-rich promoter. In our previous study demonstrating a negative regulation of ER (oestrogen receptor α) function by KLF5 in breast cancer cells [Guo, Dong, Zhao, Sun, Li and Dong (2010) Int. J. Cancer 126, 81–89], we noticed that oestrogen reduced the protein level of KLF5. In the present study, we have tested whether and how oestrogen/ER signalling regulates KLF5 protein. We found that oestrogen caused the degradation of KLF5 protein, and the degradation was sensitive to proteasome inhibitors, but not other inhibitors. The oestrogen-inducible E3 ligase EFP (oestrogen-responsive finger protein) was identified as a key player in oestrogen-mediated degradation of KLF5, as knockdown and overexpression of EFP increased and decreased KLF5 protein levels respectively, and the decrease continued even when protein synthesis was blocked. EFP-mediated degradation impaired the function of KLF5 in gene transcription. Although only unubiquitinated EFP interacted with KLF5, overexpression of EFP appeared to prevent the ubiquitination of KLF5, while resulting in heavy ubiquitination of the E3 itself. Furthermore, ubiquitination of EFP interrupted its interaction with KLF5. Although the mechanism for how EFP degrades KLF5 remains to be determined, the results of the present study suggest that oestrogen causes the degradation of KLF5 protein by inducing the expression of EFP in ER-positive breast cancer cells.

Key words: breast cancer, Krüppel-like factor 5 (KLF5), oestrogen, oestrogen receptor (ER), oestrogen-responsive finger protein (EFP).

KLF5 (Krüppel-like factor 5) is a multifunctional transcription factor involved in cell proliferation, differentiation and carcinogenesis. In addition to frequent inactivation in different types of human cancers, including breast cancer, KLF5 has been identified as an essential co-factor for the TGF-β (transforming growth factor β) tumour suppressor. In our previous study [Guo, Dong, Zhao, Sun, Li and Dong (2010) Int. J. Cancer 126, 81–89], we noticed that oestrogen reduced the protein level of KLF5. In the present study, we have tested whether and how oestrogen/ER signalling regulates KLF5 protein. We found that oestrogen caused the degradation of KLF5 protein, and the degradation was sensitive to proteasome inhibitors, but not other inhibitors. The oestrogen-inducible E3 ligase EFP (oestrogen-responsive finger protein) was identified as a key player in oestrogen-mediated degradation of KLF5, as knockdown and overexpression of EFP increased and decreased KLF5 protein levels respectively, and the decrease continued even when protein synthesis was blocked. EFP-mediated degradation impaired the function of KLF5 in gene transcription. Although only unubiquitinated EFP interacted with KLF5, overexpression of EFP appeared to prevent the ubiquitination of KLF5, while resulting in heavy ubiquitination of the E3 itself. Furthermore, ubiquitination of EFP interrupted its interaction with KLF5. Although the mechanism for how EFP degrades KLF5 remains to be determined, the results of the present study suggest that oestrogen causes the degradation of KLF5 protein by inducing the expression of EFP in ER-positive breast cancer cells.

Abbreviations used: CHX, cycloheximide; CMV, cytomegalovirus; EFP, oestrogen-responsive finger protein; ER, oestrogen receptor α; FBS, fetal bovine serum; FGF-BP, fibroblast growth factor-binding protein; GFP, green fluorescent protein; HA, haemagglutinin; IP, immunoprecipitation; KLF, Krüppel-like factor; mAb, monoclonal antibody; NTA, Ni; NTA; Ni²⁺-nitrilotriacetate; RNAi, RNA interference; RT, reverse transcription; siRNA, small interfering RNA; TGF-β, transforming growth factor β; WWP1, WW-domain-containing E3 ubiquitin protein ligase 1.

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patient survival [28], and functional studies have demonstrated that EFP plays an oncogenic role in breast cancer, at least by targeting proteolysis of 14-3-3ε, a tumour suppressor that suppresses cell-cycle progression [29]. In the antiviral response of human cells, interferon up-regulates EFP and induces its conjugation with the ubiquitin-like protein ISG15 [30], and EFP is indeed an ISG15 E3 ligase for 14-3-3ε and its ISGylation enzyme activity is RING-domain-dependent [27]. Interestingly, EFP is also autoISGylated, and the autoISGylation negatively regulates its ISG15 E3 ligase activity [31].

In the present study we characterized whether and how ER signalling regulates KLF5. We found that ER signalling regulated KLF5 at both the RNA and protein levels, mediating a dual regulation. However, the regulation of the RNA transcript was observed to be a late event, whereas KLF5 protein regulation was observed to be a prominent and early event. We found that ER signalling down-regulated KLF5 protein through the proteasome machinery in ER-positive breast cancer cells, and the oestrogen-induced EFP E3 ubiquitin ligase played a crucial role in ERE-induced proteolysis of KLF5. EFP interacted with KLF5, but did not cause obvious ubiquitination of KLF5. Rather, EFP itself was ubiquitinated, and its ubiquitination appeared to prevent the ubiquitination of KLF5. These results suggest the dual regulation of KLF5 at both the RNA and protein levels by ER signalling and that the protein regulation of KLF5 precedes the RNA level regulation of KLF5. KLF5 protein is down-regulated by ER signalling through the EFP ubiquitin E3 ligase.

EXPERIMENTAL

Plasmid constructions and transient transfection

Expression plasmids pcDNA3-KLF5, pcDNA3-FLAG-KLF5 (FLAG tag added to the N-terminus), pcDNA3-KLF5-FLAG (FLAG tag added to the C-terminus), HA (haemagglutinin)-ubiquitin (HA-tagged ubiquitin) and Myc–WWP1 (Myc-tagged WWP1) have been described in our previous study [32]. The ubiquitin (HA-tagged ubiquitin) and Myc–WWP1 (Myc-tagged WWP1) were treated with 1 μM oestrogen (E2) and were harvested at the indicated times to isolate total RNA and protein for analysis. In a similar way, MCF-7 cells cultured in hormone-free medium were treated with 1 μM tamoxifen for 4 h, and subsequently total RNA and protein were isolated for analysis.

Construction of EFP mutants

Deletion mutants of EFP were created by PCR-based approaches with FLAG–EFP as the template and FLAG–pcDNA3 as the vector. Primer sequences for creating deletion mutants FLAG–EFP-M1 and FLAG–EFP-M2 are summarized in Supplementary Table S1 (at http://www.BiochemJ.org/bj/437/bj4370323add.htm). To construct FLAG–EFP-cs and FLAG–EFP-kR substitution mutants, a PCR-directed mutagenesis method was used using FLAG–EFP as the template. PCR primers used for these mutants have been summarized in Supplementary Table S2 (at http://www.BiochemJ.org/bj/437/bj4370323add.htm).

Cell culture and treatment

An ER-positive MCF-7 breast cancer cell line was purchased from the A.T.C.C. and maintained in Eagle’s minimum essential medium supplemented with 10% FBS (fetal bovine serum), 0.01 M Hepes, 1 mM sodium pyruvate, 0.15% sodium bicarbonate, 0.45% glucose, and 1% penicillin/streptomycin solution (10000 units of penicillin and 10000 μg/ml streptomycin; Invitrogen). ER-positive T-47D cells were also purchased from the A.T.C.C. and maintained in RPMI 1640 medium supplemented with 10% FBS, 0.01 M Hepes, 1 mM sodium pyruvate, 0.15% sodium bicarbonate, 0.45% glucose and 1% penicillin/streptomycin solution. The COS-1 African green monkey kidney cell line, which is ER-negative, was also purchased from the A.T.C.C. and propagated following the procedures described by the A.T.C.C. Oestrogen (E2), tamoxifen, the proteasome inhibitors MG132, MG115 and PS341, and the lysosome inhibitor ammonium chloride were purchased from Sigma. These reagents were dissolved and used following the manufacturer’s recommendations and our previously published papers [22,33].

To investigate the direct role of oestrogen responsiveness of EFP and KLF5 degradation, MCF-7 and T-47D cells were maintained in Phenol Red-free medium supplemented with 10% charcoal-dextran-stripped FBS for at least 3 days. Subsequently, the cells were treated with 1 μM oestrogen (E2) and were harvested at the indicated times to isolate total RNA and protein for analysis. In a similar way, MCF-7 cells cultured in hormone-free medium were treated with 1 μM tamoxifen for 4 h, and subsequently total RNA and protein were isolated for analysis.

Western blot analysis and IP (immunoprecipitation)

A total of 5 × 10⁵ cells were seeded on to each well of a six-well plate, to which plasmids were transfected using the Lipofectamine™ 2000 reagent (Invitrogen). At 48 h after transfection, cells were treated with 20 μM MG132 for 4 h, and collected with RIPA buffer [50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS and 1% proteinase inhibitor I cocktail (Sigma)] for Western blot analysis. Anti-FLAG, anti-β-actin and anti-HA rabbit polyclonal antibodies were purchased from Sigma. Anti-EFP mouse mAb (monoclonal antibody) was purchased from BD Biosciences. Anti-ubiquitin mAb and all secondary antibodies were purchased from Cell Signaling Technology. For IP, cell lysates were prepared from 60-mm dishes with 0.5 ml of RIPA buffer, and incubated with 30 μl of FLAG-M2 beads with rotation at 4°C overnight. Beads were then washed three times with washing buffer [20 mM Tris/HCl (pH 8.0), 100 mM KCl, 5 mM MgCl₂, 0.2 mM EDTA, 10% glycerol and 0.1% Tween 20, with 10 mM 2-mercaptoethanol and 1% proteinase inhibitor cocktail added just prior to use]. Protein precipitates were boiled with 2× SDS loading buffer (Bio-Rad) and subjected to Western blot analysis.
siRNA (small interfering RNA) transfection

An siRNA with the sequence 5'-GCACUGGAUGUGAGAA-3' was used to target EFP. An siRNA targeting the luciferase gene with the sequence 5'-CUUACGCU-GAGUACUUGC-3' was used as a negative control. Both of the siRNAs were chemically synthesized (Dharmacon). MCF-7 cells were transfected with 100 nM of each siRNA and transfection was carried out using the siPORT Amine Reagent (Ambion) in six-well plates. At 48 h after transfection, total protein was collected for analysis using Western blotting.

CHX (cycloheximide) chase assay

COS-1 cells were seeded on to six-well plates at a density of 5 × 10^5 cells per well. After incubating at 37°C overnight, cells were transfected with untagged KLF5 construct in the presence or absence of EFP plasmid with Lipofectamine™ 2000 (Invitrogen). At 24 h after transfection, cells were treated with 50 μg/ml CHX for different time periods. Total proteins were collected and analysed by Western blotting for KLF5, EFP and β-actin. The intensity of protein bands on a film was measured using the ImageJ program (http://rsb.info.nih.gov/ij), and the degradation curves were plotted as described in the legend to Figure 2.

RNA extraction and real-time quantitative RT (reverse transcription)–PCR

An RNeasy kit (Qiagen) was used to isolate total RNA, and the iScript cDNA synthesis kit (Bio-Rad) was used to perform RT. SYBR-based real-time PCR was performed to examine the expression of KLF5, EFP and β-actin. The primers used for the study are summarized in Supplementary Table S1 at http://www.BiochemJ.org/bj/437/bj4370323add.htm. The ABI PRISM 7000 sequence detection system (AB Applied Biosystems) was used to perform SYBR real-time PCR in triplicate. The ΔCt method, in which β-actin was co-amplified to normalize the amount of RNA added to a reaction, was used to determine threshold values according to previously published procedures [34].

Ni-NTA (Ni²⁺-nitritotriacetate)–agarose purification

COS-1 cells were co-transfected with HA- and histidine-tagged KLF5 (pcDNA3-HA-KLF5-His), HA-tagged ubiquitin, and FLAG-tagged EFP or Myc-tagged WWP1 for 48 h. After treatment with MG132 at 20 μM for 4 h, cells were washed with PBS and lysed in RIPA buffer containing 10 mM imidazole. A 20 μl aliquot of Ni-NTA–agarose beads (Qiagen) was added to the cell lysate and rotated at 4°C for 6 h. Precipitates were washed three times with washing buffer containing 20 mM imidazole, boiled with 2× SDS loading buffer and subjected to Western blot analysis.

Promoter-luciferase reporter assay

The FGF-BP (fibroblast growth factor-binding protein) proximal promoter (−128 to +61) cloned into the pGL3-BASIC plasmid was provided by Dr Ceshi Chen (Albany Medical College, Albany, NY, U.S.A.) [35]. MCF-7 or T-47D cells were seeded on to a 12-well plate at a density of 1 × 10^5 cells per well. The following day these cells were transfected with the FGF-BP promoter-reporter construct (0.5 μg per well) and siEFP (75 nM final concentration) using Lipofectamine™ 2000 reagent following the manufacturer’s protocol. The siRNA for KLF5, siKLF5, has been described previously [9], and the siRNA for luciferase (siLuc) was used as a control. At 48 h after transfection, cells were lysed and a luciferase assay was carried out using the luciferase assay kit from Promega as described previously [36]. Experiments were carried out in triplicate.

RESULTS

Oestrogen immediately down-regulates the protein, but not the RNA, of KLF5 in ER-positive MCF-7 breast cancer cells

In our previous study describing the role of KLF5 in regulating ER function, we noticed that oestrogen treatment decreased the protein level of transfected KLF5 in the MCF-7 ER-positive breast cancer cell line, whereas depletion of hormone in culture medium slightly increased the protein level of KLF5 [25]. This result led us to hypothesize that ER signalling might cause the degradation of KLF5 protein. To test this possibility, we cultured MCF-7 cells in hormone-free medium, and then added oestrogen (E2) into the medium for different time periods. As shown in Figure 1(A), oestrogen (E2) treatment mediated a time-dependent decrease in KLF5 protein level. Oestrogen (E2) decreased the amount of KLF5 protein as early as 2 h after treatment, which corresponded to the induction of EFP protein by oestrogen (E2) (Figure 1A). Similarly, in another ER-positive cell line T-47D, oestrogen (E2) treatment also mediated the time-dependent decrease in KLF5 protein with a corresponding increase in EFP protein (Figure 1B). KLF5 protein was observed to decrease as early as 6 h after treatment with oestrogen (E2) with an increase in the EFP level. In another ER-positive cell line, ZR-75-1, the same treatment also decreased KLF5 protein, but the effect was not detectable until 24 h after treatment (Supplementary Figure S1A at http://www.BiochemJ.org/bj/437/bj4370323add.htm). Consistently, induction of EFP protein was not noticeable until 24 h after oestrogen (E2) treatment in ZR-75-1 cells (Supplementary Figure S1A). Induction of EFP correlated with a decrease in KLF5 at different time points, suggesting a role for EFP in the down-regulation of KLF5 protein.

We also examined the effect of oestrogen (E2) treatment on the RNA levels of KLF5 and EFP at different time points. In MCF-7 cells, oestrogen (E2)-induced EFP transcription was detectable as early as 2 h after treatment (Figure 1C), which is consistent with the increase in its protein levels; in T-47D cells, oestrogen (E2) induced EFP expression as early as 4 h (Figure 1D), with the increase in its protein level at 6 h (Figure 1B); but in ZR-75-1 cells the induction was not detectable until 8 h after treatment (Supplementary Figure S1B). In all of the cell lines, the transcription of KLF5 showed no changes within 24 h, but decreased at 48 or 72 h after oestrogen (E2) treatment (Figures 1C and 1D, and Supplementary Figure S1B), indicating that oestrogen (E2) also down-regulates KLF5 transcription, but the decrease in RNA is not as early as the decrease in protein.

We also treated MCF-7 cells maintained in hormone-free medium with the non-steroidal anti-oestrogen tamoxifen and oestrogen (E2) individually and in combination, and measured the protein levels of KLF5. Treatment of cells with oestrogen (E2) for 4 h resulted in a decreased level of KLF5 protein (Figure 1E). The decrease in the KLF5 protein level was not seen when the cells were treated with either tamoxifen alone or a combination of tamoxifen with oestrogen (E2) (Figure 1E). The RNA expression of KLF5 was not affected by either tamoxifen or oestrogen (E2) alone, or in combination (Figure 1F). We also treated MCF-7 cells maintained in regular medium with the non-steroidal anti-oestrogen tamoxifen, and measured the KLF5 protein level.
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Figure 1 Oestrogen treatment causes the degradation of KLF5 in ER-positive breast cancer cells, and the ER-induced EFP E3 ligase is involved

Although weak, treatment of cells with tamoxifen for 4 h or longer resulted in a detectable level of KLF5 protein (Supplementary Figure S1C), and the RNA expression of KLF5 was not affected by tamoxifen treatment (Supplementary Figure S1D). These results suggest that inhibition of oestrogen/ER signalling leads to an increased protein level of KLF5, whereas the RNA level is not affected.

The oestrogen-responsive protein EFP, an ubiquitin E3 ligase, plays a role in oestrogen-mediated down-regulation of KLF5 protein

Previous studies have indicated that KLF5 is regulated by the UPP (ubiquitin–proteasome pathway). WWP1 [22,33] and Fbw7 [37,38] have been identified as E3 ubiquitin ligases mediating the ubiquitination and degradation of KLF5. However, neither WWP1 nor Fbw7 are oestrogen- or ER-responsive (Supplementary Figure S1E). It is thus possible that one or more other E3 ligases mediate oestrogen-induced degradation of KLF5. There are at least seven ubiquitin E3 ligases that are ER-responsive, including SKP2 (S-phase kinase-associated protein 2), Cul-4A, E6-AP, EFP, BCA2 (breast-cancer-associated gene 2), MDM2 (murine double minute 2) and RNF11 (RING finger protein 11). In our search for ER-responsive E3 ligases that are inducible by oestrogen and ER in ER-negative cells after transfection with ER and treatment with oestrogen (E2), we found that EFP was the only ER-responsive E3 ligase among these seven in ER-negative cells (X. Y. Dong, X. Fu, S. Fan, P. Guo, D. Su, X. Sun and J. T. Dong, unpublished work). We therefore tested whether EFP could be the E3 ligase involved in the degradation of KLF5. As expected, the expression of EFP was induced by oestrogen (E2) treatment at both the RNA and protein levels in MCF-7, T-47D and ZR-75-1 cells, although the induction in ZR-75-1 cells was slower than that in MCF-7 and T-47D cells (Figures 1A–1D, and Supplementary Figures S1A and S1B). The increase in EFP protein correlated with the decrease in KLF5 protein at different time points in...
EFP mediates oestrogen-caused KLF5 degradation

Figure 2  Oestrogen-induced expression of EFP degrades KLF5 protein

(A and B) Transfection of siRNA against EFP (siEFP) into MCF-7 cells cultured in regular medium (A) or in hormone-free medium but treated with 1 μM oestrogen (E2) for 18 h (B) increases protein levels of KLF5, as determined by Western blot analysis. (C) MCF-7 cells grown in regular medium were treated with the proteasome inhibitors MG132, MG115 and PS341, or the lysosome inhibitor ammonium chloride (NH4Cl), and protein expression of KLF5, EFP and β-actin was evaluated by Western blotting. (D) Different amounts of EFP plasmid (from 0 to 0.8 μg) were co-transfected with 0.8 μg of KLF5 plasmid into COS-1 cells, and Western blotting was performed to detect protein levels of KLF5 and EFP in the presence (+) or absence (−) of MG132. (E) MCF-7 cells in regular medium (left-hand panel) and COS-1 cells (right-hand panel) were transfected with expression plasmids for FLAG-tagged EFP and untagged KLF5, KLF5 with a FLAG tag at the N-terminus (F-KLF5), or KLF5 with a FLAG tag at C-terminus (KLF5-F), and cell lysates were analysed by Western blot analysis for protein expression of KLF5 and EFP. The presence or absence of EFP is indicated by ‘+’ or ‘−’. (F and G) CHX chase assay of COS-1 cells transfected with KLF5 and vector control or EFP. Western blotting was performed with cells treated with CHX for the indicated times to detect protein levels of KLF5 and EFP (F). Signal intensities for bands of KLF5 and β-actin were quantified using the ImageJ program, and relative levels of KLF5 were plotted against times of CHX treatment (G).

these cell lines (Figures 1A and 1B, and Supplementary Figure S1A), suggesting a role for EFP in the degradation of KLF5. Consistently, treatment for 4 h with tamoxifen, an anti-oestrogen agent, interfered with the induction of EFP by oestrogen (E2) at both the RNA and protein levels and the decrease in KLF5 protein level (Figures 1E and 1F). Again, the RNA level of KLF5 was not affected by tamoxifen with 4 h of treatment (Figure 1F).

To further test whether EFP functions in oestrogen-mediated degradation of KLF5, we knocked down the expression of EFP by RNAi (RNA interference) in MCF-7 cells grown in either regular medium (Figure 2A) or hormone-free medium supplemented with oestrogen (E2) (Figure 2B), and analysed the expression of KLF5 protein. Although it is more difficult to detect KLF5 protein due to degradation in cells in normal medium, we could still detect an increase in the protein level of KLF5 upon the knockdown of EFP (Figure 2A). In MCF-7 cells cultured in hormone-free medium and treated with oestrogen (E2), knockdown of EFP also increased the protein level of KLF5 in cells treated with or without oestrogen (E2) (Figure 2B). Similar experiments were done in MCF-7 cells using different siRNAs against EFP, and it was observed that the knockdown of EFP did increase the KLF5 protein level (Supplementary Figure S2 at http://www.BiochemJ.org/bj/437/bj4370323add.htm). These results indicate that the ER-responsive EFP E3 ubiquitin ligase plays a role in oestrogen-mediated down-regulation of KLF5 protein.
Protein degradation is responsible for EFP-mediated down-regulation of KLF5

To determine whether the proteasome pathway is responsible for the oestrogen-mediated decrease in KLF5 protein, we treated MCF-7 cells grown in regular medium with the proteasome inhibitors MG132, MG115 and PS-341. The lysosome inhibitor ammonium chloride was also used. Although KLF5 protein was hardly detectable in MCF-7 cells grown in regular medium, each of the three proteasome inhibitors enriched KLF5 protein to a detectable level (Figure 2C). Cells treated with ammonium chloride still had no detectable KLF5 (Figure 2C).

To further test whether protein degradation is responsible for EFP-mediated down-regulation of KLF5, we transfected KLF5 plasmid and different amounts of EFP plasmid into COS-1 cells in the presence or absence of the MG132 proteasome inhibitor. Ectopic expression of EFP caused a dose-dependent decrease in the KLF5 protein level, and treatment with MG132 enriched protein levels of KLF5, but did not completely inhibit the effect of EFP (Figure 2D). We also transfected expression plasmids for EFP and different forms of KLF5 (wild-type, N-terminally tagged and C-terminally tagged) into MCF-7 cells in regular medium and found that ectopic expression of GFP dramatically decreased protein expression for each form of KLF5 (Figure 2E, left-hand panel). In a similar way, we transfected the same sets of plasmids into the COS-1 monkey kidney cells, and again found a dramatic decrease in the KLF5 protein level in EFP-expressing cells (Figure 2E, right-hand panel). Because KLF5 expression was driven by the CMV (cytomegalovirus) promoter in these experiments, and the CMV promoter is a viral promoter not usually affected by the regulatory mechanisms in mammalian cells, these results suggest that protein degradation rather than transcriptional regulation is responsible for the EFP-mediated reduction in the KLF5 protein level. To examine whether KLF5 degradation caused by EFP is a specific event, we transfected expression plasmids for GFP (green fluorescent protein) and KLF5 into COS-1 cells along with EFP, and found that ectopic expression of GFP dramatically decreased the protein level of KLF5, but had no effect on GFP protein (Supplementary Figure S3A at http://www.BiochemJ.org/bj/437/bj4370323add.htm). We also transfected a fusion expression plasmid in which GFP is cloned at the N-terminus of KLF5, and observed that ectopic expression of GFP could no longer degrade the GFP–KLF5 fusion protein (Supplementary Figure S3B).

To more definitely determine whether EFP decreases the KLF5 protein level by protein degradation, we performed a CHX chase assay by transfecting EFP and KLF5 into COS-1 cells, treating cells with the protein synthesis inhibitor CHX, and measuring the protein expression of KLF5 by Western blot analysis (Figure 2F) and signal quantification (Figure 2G). Consistent with previous findings showing protein degradation of KLF5 in different cells [22,32,33], KLF5 protein expression decreased in EFP-expressing cells treated with CHX for 2, 4 and 6 h (Figures 2F and 2G), whereas the level of KLF5 protein did not change in cells without EFP expression (Figures 2F and 2G). The EFP-induced decrease in KLF5 protein levels was 20%, 38% and 84% of control levels for CHX treatments of 2, 4 and 6 h respectively (Figure 2G). We also performed a CHX chase assay in MCF-7 cells where we depleted hormone for 3 days and then treated cells with oestrogen (E2) and CHX for the indicated times and performed Western blotting to examine protein expression. We found that hormone depletion increased the level of KLF5 protein, and treatment with CHX did not decrease the level of KLF5 in the absence of oestrogen (Supplementary Figure S4 at http://www.BiochemJ.org/bj/437/bj4370323add.htm). Similarly, CHX treatment did not alter the EFP level in the absence of oestrogen (E2) (Supplementary Figure S4). In the presence of oestrogen (E2), whereas the KLF5 level decreased considerably and the EFP level increased as expected, CHX treatment unexpectedly increased the KLF5 level and decreased the EFP level (Supplementary Figure S4), and the increase in KLF5 levels with different CHX concentrations was significantly correlated with the decrease in EFP levels (P < 0.005, linear regression and correlation test). These results suggest that EFP itself could undergo protein degradation, and EFP degradation could then lead to increased KLF5 protein levels. These results further suggest that EFP down-regulates KLF5 through protein degradation.

EFP interacts with KLF5 at the protein level

It is well established that, in the proteasome pathway, E3 ligases interact with substrates to transfer ubiquitin and cause their degradation by the proteasome. To test whether EFP interacts with KLF5, we transfected plasmids for KLF5 and FLAG-tagged EFP into COS-1 cells, and performed an IP with FLAG-M2 beads. Expression of transfected genes were confirmed by Western blotting (Figure 3A, Input). In the protein complex pulled down by FLAG-M2 beads against FLAG–EFP, KLF5 protein was detected (Figure 3A). We used WWP1 as a positive control, which was previously identified as an E3 ligase that interacts with KLF5 to ubiquitinate and degrade KLF5 [22]. As expected, KLF5 was detected in the protein complex precipitated by an antibody against WWP1 (Figure 3B).

To determine whether the KLF5–EFP interaction occurs between endogenous KLF5 and EFP, we performed an IP with...
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Figure 4 Characterization of KLF5 ubiquitination, EFP ubiquitination and protein interaction between EFP and KLF5 in COS-1 cells co-transfected with ubiquitin and treated with MG132

COS-1 cells were transfected with different combinations of plasmids, as indicated above the images, for 48 h. MG132 treatment of 20 μM was applied to cells during the last 15 h of transfection. Cell lysates were subjected to Western blotting (Input, all panels), IP combined with Western blotting (IP, A–C), or Ni-NTA purification combined with Western blotting (KLF5 pull down, D) to detect different proteins indicated at the left-hand side of each panel. HA-tagged ubiquitin plasmid and anti-HA antibody were used for ubiquitin (Ub), Myc-tagged WWP1 plasmid and anti-Myc antibody for WWP1, and FLAG-tagged EFP plasmid and FLAG-M2 beads for EFP. For KLF5, untagged pcDNA3-KLF5 plasmid was used in (A–C), whereas pcDNA3-HA-KLF5-His plasmid was used in (D). Anti-KLF5 serum was used to detect KLF5 protein in all panels. Arrows indicate the two EFP bands predominantly detected in cells transfected with EFP but not ubiquitin.

Although EFP itself is ubiquitinated, it does not appear to ubiquitinate KLF5

To determine whether EFP ubiquitinates KLF5 as an E3 ligase, we further characterized its interaction with KLF5 in the presence of ectopically expressed ubiquitin and the MG132 proteasome inhibitor. We transfected COS-1 cells with KLF5, FLAG-tagged EFP and HA-tagged ubiquitin, treated cells with MG132, and performed IP and Western blotting. Expression of KLF5, EFP and ubiquitin was confirmed by Western blotting (Figure 4, Input). We first precipitated ubiquitinated proteins using the anti-HA antibody against ubiquitin. As expected, a smear of proteins was detected with the anti-HA antibody in each group regardless of EFP and KLF5, and the smear was much darker in precipitated samples than in input samples (Figure 4A, Ub panel). For KLF5, its expression was strong, EFP did not make an obvious difference, and no ladder of ubiquitinated KLF5 was visible in the input samples (Figure 4A, KLF5 panel, lanes 1 and 3). In the precipitated ubiquitin complexes, on the other hand, a ladder of ubiquitinated KLF5 was strong in cells without EFP, whereas no KLF5 band was visible in the unubiquitinated form (Figure 4A, KLF5 panel, lane 4). Unexpectedly, expression of EFP dramatically decreased the level of ubiquitinated KLF5, leaving only a weak band of KLF5 that appeared to have one unit of ubiquitin (Figure 4A, KLF5 panel, lane 6). For EFP, strong smears were detected in cells transfected with EFP (Figure 4A, EFP panel, lanes 2 and 3), and the smears were darker in ubiquitin precipitates (Figure 4A, EFP panel, lanes 5 and 6), indicating that EFP is heavily ubiquitinated with the co-transfection of ubiquitin.

As a positive control for the ubiquitination of KLF5, we also transfected WWP1 into COS-1 cells and performed an IP with an anti-HA antibody, and Western blotting with antibodies against WWP1, KLF5 and ubiquitin. Again, a smear of proteins was detected with the anti-HA antibody for ubiquitin in each group regardless of WWP1 and KLF5, and the smear was much darker in precipitated samples (Figure 4B, Ub panel, both Input and IP). For KLF5, it was again strongly expressed in the unubiquitinated form in cells not subjected to IP, and there were no obvious bands of ubiquitinated KLF5 (Figure 4B, KLF5 panel, lanes 1 and 3). A band of WWP1 was detected in WWP1-transfected cells not subjected to IP (Figure 4B, WWP1 panel, lane 3), but no WWP1 was detected in ubiquitin precipitate (Figure 4B, WWP1 panel, lane 6). In ubiquitin complexes precipitated with an anti-HA antibody against ubiquitin, a ladder of ubiquitinated KLF5 was detected in cells with and without WWP1 transfection (Figure 4B, KLF5 panel, lanes 4 and 6), and expression of WWP1 did not obviously decrease larger bands of ubiquitinated KLF5 while...
dramatically decreasing the smallest band of ubiquitinated KLF5 (close to the size of KLF5 with one unit of ubiquitin) (Figure 4B, KLF5 panel, lane 6).

We then precipitated EFP complexes with FLAG-M2 beads in cells transfected with ubiquitin and different combinations of KLF5 and EFP, and performed Western blot analysis to detect each of the molecules. Consistent with the results in Figure 4(A), a smear of EFP was detected in cells transfected with EFP, regardless of IP or KLF5 expression (Figure 4C, EFP panel, both Input and IP lanes), and similar signals were also detected using the anti-HA antibody for ubiquitin in the precipitate (Figure 4C, Ub panel). For KLF5, although strong KLF5 expression was detected in cells not subjected to IP (Figure 4C, KLF5 panel, lanes 1 and 3), which is consistent with the results in Figures 4(A) and 4(B), there was no detectable KLF5 signal in the EFP precipitates (Figure 4C, KLF5 panel, lanes 4 and 6). This result suggests that ubiquitinated EFP no longer interacts with KLF5.

We also examined the presence of EFP and ubiquitin in KLF5 protein complexes in cells transfected with histidine-tagged KLF5, HA-tagged ubiquitin and FLAG-tagged EFP or Myc-tagged WWP1. Cell lysates were incubated with Ni-NTA–agarose beads to pull down KLF5 protein complexes, which were then subjected to Western blotting. WWP1 was used as a positive control that interacts with KLF5. Although expression of either WWP1 or EFP decreased the protein level of KLF5 in cells not subjected to pull down, abundant KLF5 was detected in each of the pull-down groups (Figure 4D, KLF5 panel). In the KLF5 protein complex, both WWP1 and EFP were clearly detected (Figure 4D, pull down). It is worth noting that EFP bands detected in the KLF5 protein complex were primarily in an unubiquitinated form (Figure 4D, EFP panel, lane 6), which is different from those detected in EFP or ubiquitin precipitates.

Role of the RING finger domain of EFP in the degradation of KLF5

EFP comprises three essential domains: a RING finger domain, a B-box coiled-coil domain and a C-terminal SPRY domain [39]. The essential E3 ligase activity of EFP is mostly conserved to its RING finger domain and the cysteine residues in the RING finger. We examined the role of the RING finger domain in degrading KLF5. We generated some deletion mutants of EFP (Figure 5A), and analysed their effect on the degradation of KLF5 by Western blotting. We also analysed the interaction between KLF5 and EFP by IP. As shown in Figure 5(B), the EFP-M1 mutant, comprising only the RING finger, could not interact with and degrade KLF5, suggesting that the RING finger domain alone is not sufficient for the EFP–KLF5 interaction and the degradation of KLF5. The EFP-M2 mutant, in which the RING finger domain was deleted, also could not degrade KLF5, although it still interacted with KLF5 (Figure 5B). When the two conserved cysteine residues in the RING finger of EFP were replaced with serine residues, EFP could no longer interact with KLF5, nor could it degrade KLF5 (Figure 5C). The EFP-kR mutant, in which Lys117 was changed to arginine, and thus the ISGylation of EFP was interrupted as demonstrated in a previous study [31], had no obvious effect on the EFP–KLF5 interaction and the degradation of KLF5 (Figure 5C). Therefore the degradation of KLF5 by EFP is dependent on both its interaction with KLF5 and its RING finger domain, including the conserved cysteine residues.

EFP negatively regulates the function of KLF5

Among the KLF5-regulated genes, FGF-BP has been shown as a direct target of KLF5 that involves the binding of KLF5 to the GC-rich/Sp1 site of the FGF-BP promoter [35]. Furthermore, up-regulation of FGF-BP partially mediates the pro-proliferation function of KLF5 in breast cells [35]. Therefore we examined the effect of EFP on the function of KLF5 in the regulation of FGF-BP1. Knockdown of EFP in MCF-7 and T-47D cells grown in regular medium, which did not change the RNA level of KLF5 but increased the protein level of KLF5, significantly increased the RNA level of FGF-BP1 (Figure 6A). Consistently, oestrogen (E2) treatment in MCF-7 and T-47D cells, which significantly decreased KLF5 protein levels (Figures 1A and 1B), also decreased the RNA levels of FGF-BP in these cells (Figure 6B). With the FGF-BP1 promoter-luciferase activity as a readout, knockdown of EFP increased the promoter activity of FGF-BP1 in both MCF-7 and T-47D cells (Figure 6C). These results suggest that EFP can negatively regulate the transcriptional function of KLF5.
DISCUSSION

Oestrogen causes KLF5 degradation primarily through the oestrogen-inducible E3 ubiquitin ligase EFP

In eukaryotic cells, the proteasome is the major intracellular proteolytic machinery for protein destruction [40,41]. Previous studies have established that the KLF5 transcription factor is degraded by the proteasome, and the degradation could be ubiquitin-dependent or -independent [32,33]. The WWP1 E3 ubiquitin ligase has been demonstrated to interact with KLF5, causing its ubiquitination and degradation [22]. In the present study, we found that in the ER-positive MCF-7, T-47D and ZR-75-1 breast cancer cell lines, the activation of oestrogen/ER signalling down-regulated KLF5 protein while inducing the EFP ubiquitin E3 ligase (Figures 1A and 1B, and Supplementary Figure S1A), and inhibitors for proteasome, but not for lysosome, could interrupt ER-mediated down-regulation of KLF5 (Figure 2C). In MCF-7 cells grown in both regular medium and hormone-depleted medium supplemented with oestrogen (E2), knockdown of the oestrogen-inducible E3 ubiquitin ligase EFP increased endogenous KLF5 protein (Figures 2A and 2B, and Supplementary Figures S2A–S2E), whereas ectopic expression of EFP decreased ectopically expressed KLF5 protein (Figure 2E). Furthermore, transfection of EFP into ER-negative COS-1 cells also decreased the protein level of transfected KLF5 (Figure 2E), and the decrease was sensitive to the inhibition of protein synthesis (Figures 2F and 2G). Functionally, the oestrogen-induced EFP impaired the function of KLF5 in the regulation of its target genes such as FGF-BP1, whereas knockdown of EFP enhanced the function of KLF5 in gene regulation (Figure 6). These results not only indicate that the oestrogen/ER signalling causes the degradation of KLF5, they also establish EFP as another E3 ligase that mediates the degradation and modulates the function of KLF5.

Although EFP interacts with KLF5, it does not appear to ubiquitinate KLF5, and the mechanism for EFP-mediated KLF5 degradation remains to be determined

Proteasomal degradation often involves ubiquitination, in which the substrate is attached with a chain of polyubiquitin through its interaction with an E3 ligase. Ubiquitinated substrates are then recognized by the 19S regulatory complex of the 26S proteasome for degradation. For example, the WWP1 HECT-domain-containing E3 ligase mediates the ubiquitination and degradation of KLF5 through its interaction with KLF5 [22,42]. Similarly, the RING-finger-containing EFP E3 ligase has been shown to ubiquitinate 14-3-3σ to mediate its degradation [29]. In the degradation of KLF5 by EFP, EFP interacted with KLF5 at the protein level (Figures 3 and 4D), and such an interaction appeared to be necessary for KLF5 degradation, as deletion of the B-box coiled-coil domain of EFP interrupted its interaction with and the degradation of KLF5 (Figures 5A and 5B). As expected for a typical E3 ligase in protein degradation, the RING domain and its conserved cysteine residues of EFP are essential for KLF5 degradation (Figure 5). Unexpectedly, EFP did not cause detectable ubiquitination of KLF5 (Figure 4A, KLF5 panel, lane 6). In fact, expression of EFP appeared to abolish the ubiquitination of KLF5 (Figure 4A, KLF5 panel, lane 6), which is different from the WWP1 positive control (Figure 4B, KLF5 panel, lane 6). It is likely that in the presence of ubiquitin, EFP has more preference of being ubiquitinated over KLF5.

We further noticed that EFP itself was ubiquitinated (Figure 4), and co-expression of exogenous ubiquitin enhanced the ubiquitination of EFP (Figures 4A and 4C), which is consistent with a previous report in which auto-ubiquitination of the EFP E3 ligase was described [43]. Unexpectedly, ubiquitination of EFP prevented the interaction of EFP with KLF5, as no KLF5 was detected in the protein complexes pulled down by an anti-EFP antibody (Figure 4C, KLF5 panel, lane 6) or by an anti-ubiquitin antibody (Figure 4A, KLF5 panel, lane 6), whereas strong smears were detected for both ubiquitin and EFP. It is unlikely that the lack of KLF5 ubiquitination is due to excessive degradation caused by EFP, because the MG132 proteasome...
inhibitor was applied to cells and abundant unubiquitinated KLF5 was detected in the input (Figures 4A–4C, Input for KLF5). Furthermore, in precipitates pulled down by an anti-KLF5 antibody (Figure 4D), the predominant form of EFP was unubiquitinated. Therefore, in the complex between EFP and KLF5, it appears that neither EFP nor KLF5 is ubiquitinated.

In addition to its ubiquitin E3 ligase activity, EFP is also an ISG15 E3 ligase that mediates the ISGylation of 14-3-3 proteins including DDX58/RIGI [47], ER [43] and also other proteins [48–50]. Although the ISGylation activity of EFP appears to be dispensable for its function in KLF5 degradation in the present study where no interferon treatment or viral infection was applied (the kr mutant in Figure 5), we speculate that ubiquitination of EFP could impair its function as an E3 ligase in the degradation of KLF5 similar to the effect of its autoISGylation and, when too much EFP is available, more EFP becomes ubiquitinated to decrease its activity in KLF5 degradation. It is even possible that ubiquitination of EFP leads to its protein degradation because, in the CHX chase assay, the EFP protein level decreased in CHX-treated MCF-7 cells in the presence of oestrogen (Supplementary Figure S4). Whether ubiquitination of EFP causes its degradation and alters its function in the degradation of KLF5 remains to be determined, as does how EFP mediates the degradation of KLF5.

Mutation of the two cysteine residues in the RING finger domain of EFP, which dramatically impairs the catalytic activity of EFP in its autoISGylation [27], almost abolished the interaction between KLF5 and EFP (Figure 5C), although deletion of the entire RING finger did not have an obvious effect on the interaction (Figure 5B). One possibility is that a third protein, protein X, could be ubiquitinated by EFP and interact with both EFP and KLF5, and ubiquitinated protein X could be responsible for the transfer of KLF5 to the proteasome for degradation. EFP with the cysteine mutations could still fold and bind to protein X, but could not efficiently ubiquitinate protein X. Consequently, mutated EFP could prevent other E3 ligases from binding and ubiquitinating protein X, which could otherwise occur as compensation if the RING domain is deleted.

**A negative regulatory relationship between KLF5 and oestrogen/ER signalling**

In our previous study, we found that KLF5 inhibits oestrogen-induced gene transcription and cell proliferation [25]. In the present study we found that oestrogen negatively regulates KLF5 in two ways. First, oestrogen promptly induces one of the E3 ligases, EFP, which causes protein degradation of KLF5, although the mechanism of the degradation is unclear. Secondly, oestrogen gradually down-regulates the transcription of KLF5, which takes a longer time to occur. Taken together, from our previous and present studies it appears that KLF5 and oestrogen signalling regulate each other through a feedback mechanism (Figure 7). As the presence of more KLF5 helps inhibit the function of oestrogen by interacting with the ER, control in the level of KLF5 is achieved by the up-regulation of EFP, which immediately down-regulates the protein level of KLF5 by protein degradation. Oestrogen/ER signalling also gradually down-regulates the RNA level of KLF5 for a more extensive control of KLF5 (Figure 7). EFP is predominantly expressed in oestrogen-responsive tissues including mammary glands, uteri and osteoblasts [44–46]. As an E3 ligase, EFP has been shown to ubiquitinate and degrade a number of proteins including DDX58/RIGI [47], ER [43] and 14-3-3 proteins [29], thus playing a critical role in the regulation of various pathways. Functional and expression studies suggest an oncogenic role of EFP in human cancer [26,29]. KLF5, on the other hand, has been suggested as a tumour suppressor because it has frequent genomic deletion in breast cancer, its re-expression inhibits the proliferation of breast cancer cells [19,48] and it participates in the inhibitory function of the TGF-β tumour suppressor in cell proliferation [8,9,14]. It also appears to interact with oestrogen receptor β to suppress tumour growth [49]. Although the KLF5–EFP interaction could play a role in the structure and function of normal mammary tissue, interruption of this interaction could contribute to mammary tumorigenesis.

In summary, we found that the oestrogen/ER signal regulates KLF5 at both the RNA and protein level. This dual regulation is accomplished at an early stage by increasing the EFP E3 ligase level, which mediates the degradation of KLF5 protein, and at a late stage by decreasing the RNA transcript level of KLF5. We found that the oestrogen/ER signal caused the degradation of KLF5 in ER-positive MCF-7 breast cancer cells through the proteasome machinery. These results suggest that the oestrogen/ER signal negatively regulates the KLF5 transcription factor, and EFP-mediated proteasome degradation is one of the mechanisms for the negative regulation of KLF5 in breast epithelial cells.

**AUTHOR CONTRIBUTION**

Ke-Wen Zhao, Deepa Sikriwal, Xueyuan Dong, Peng Guo and Xiaodong Sun performed the experiments, and analysed and interpreted the data. Ke-Wen Zhao, Deepa Sikriwal and Jin-tang Dong designed the research project, analysed the data, and drafted and finalized the paper and Figures.

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EFP mediates oestrogen-caused KLF5 degradation
SUPPLEMENTARY ONLINE DATA

Oestrogen causes degradation of KLF5 by inducing the E3 ubiquitin ligase EFP in ER-positive breast cancer cells

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EXPERIMENTAL

Cell culture and treatment

The ZR-75-1 ER-positive breast cancer cell line was purchased from the A.T.C.C.) and maintained in RPMI 1640 medium supplemented with 10 % FBS, 0.01 M Hepes, 1 mM sodium pyruvate, 0.15 % sodium bicarbonate, 0.45 % glucose and 1 % penicillin/streptomycin solution. To investigate the direct role of oestrogen responsiveness of EFP and KLF5 degradation, ZR-75-1 cells were maintained in Phenol Red-free medium supplemented with 10 % charcoal-dextran-stripped FBS for at least 3 days. Subsequently, the cells were treated with 1 μM 17β-oestradiol (E2) and were harvested at the times indicated to isolate total RNA and protein for analysis.

To investigate the direct role for oestrogen responsiveness of EFP and KLF5 degradation, MCF-7 cells cultured in regular medium were treated with 1 μM tamoxifen (Tam) for the times indicated, 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 treatment the cells were lysed with RIPA buffer [50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 1 % Nonidet P40, 0.5 % sodium deoxycholate, 0.1 % SDS and 1 % proteinase inhibitor I cocktail (Sigma)]. The lysates were then processed for Western blotting using standard protocols and later probed with appropriate antibodies.

siRNA transfection

siRNAs were obtained from Santa Cruz Biotechnology (catalogue numbers sc-37825 and sc-37825b) and were used to target EFP. Another siRNA against EFP was chemically synthesized (Dharmacon) with the sequence 5′-GGUGGAGCAGCUACAAACA-3′, and was also used to target EFP. MCF-7 cells were transfected with 100 nM of each siRNA, and transfection was carried out using the siPORT Amine Reagent (Ambion) in six-well plates. At 48 h after transfection, total protein was collected for analysis using Western blot analysis.

CHX chase assay of MCF-7 cells

MCF-7 cells were maintained in Phenol Red-free medium supplemented with 10 % charcoal-dextran-stripped FBS for 3 days. Subsequently, the cells were treated with 1 μM 17β-oestradiol (E2) for 4 h. Following oestrogen treatment, cells were treated with 50 μg/ml CHX for different time periods. Total proteins were collected and analysed by Western blotting for KLF5, EFP and β-actin.

1 These two authors contributed equally to this work
2 To whom correspondence should be addressed (email j.dong@emory.edu).
Figure S1  Oestrogen 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 oestrogen (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 (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 signalling inhibitor tamoxifen (Tam) for the 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 tamoxifen for the indicated times were analysed by real-time PCR for RNA expression of EFP and KLF5. (E) RNA samples of MCF-7 cells from (C) were subjected to real-time PCR to detect RNA expression of WWP1 and FBW7.
EFP mediates oestrogen-caused KLF5 degradation

Figure S2 Knockdown of EFP increases the protein level of KLF5

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

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Figure S3  EFP down-regulates 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 (B) for 48 h, and cell lysates were analysed by Western blotting for protein expression of KLF5, EFP, GFP and β-actin. The presence or absence of a construct is indicated by ‘+’ or ‘−’. The molecular mass in kDa is indicated on the right-hand side.

Figure S4  CHX chase assay of MCF-7 cells in the presence and absence of oestrogen (E2)

(A) MCF-7 cells cultured in hormone-free medium for 3 days were incubated with 1 μM oestrogen (E2) or solvent control for 4 h, followed by CHX treatment for the 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 ImageJ 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 oestrogen treatment, was defined as 1 and the ratios for other lanes were normalized accordingly and plotted against CHX treatments.
Table S1  Primer sequences (5′→3′) for creating EFP-deletion mutants
The underlined sequences represent the restriction sites

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<th>Reverse primer</th>
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<td>GGGGAATTCTGATGGCAGAGCTGTGCCCCTG</td>
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<td>EFP-M2</td>
<td>GGCGGTACCCGGAATGCCCAGCCCATGGGCCCCTG</td>
<td>CCGGAGAATTCTTTGGGAGGAGGGAGATGGAGTG</td>
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Table S2  Primer sequences (5′→3′) for creating EFP-substitution mutants
The underlined sequences represent the amino acid mutated

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<td>EFP-kR</td>
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Table S3  Primer sequences (5′→3′) for real-time PCR analysis

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<th>Name</th>
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<td>EFP</td>
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<td>TAAGCCACCCCTGAAGTCATACAG</td>
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