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Original Article

Activation of Keap1/Nrf2 signaling pathway by nuclear epidermal growth factor receptor in cancer cells

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Abstract: Nuclear translocation of EGFR has been shown to be important for tumor cell growth, survival, and therapeutic resistance. Previously, we detected the association of EGFR with Keap1 in the nucleus. Keap1 is a Kelch-like ECH-associated protein, which plays an important role in cellular response to chemical and oxidative stress by regulating Nrf2 protein stability and nuclear translocation. In this study, we investigate the role of EGFR in regulating Keap1/Nrf2 cascade in the nucleus and provide evidence to show that nuclear EGFR interacts with and phosphorylates nuclear Keap1 to reduce its nuclear protein level. The reduction of nuclear Keap1 consequently stabilizes nuclear Nrf2 and increases its transcriptional activity in cancer cells, which contributes to tumor cell resistance to chemotherapy.

Keywords: EGFR, Keap1, Nrf2, cancer, ubiquitination

Introduction

Kelch-like ECH-associated protein 1 (Keap1), a chemical and oxidative stress sensor protein in mammalian cells, is an E3 ligase that targets Nrf2 and IKKβ for degradation [1-3]. Keap1 contains three major functional domains, a broad-complex, tramtrack, and bric-a-brac (BTB) dimerization domain, a cysteine-rich intervening region (IVR) domain, and a kelch domain with 6 kelch repeats [4, 5]. Keap1 localizes to both the cytosol and the nucleus [6, 7]. Within the cytosol, Keap1 interacts with its target proteins, such as Nrf2, a bZip transcription factor that plays a role in regulating oxidative stress. In the basal state, Keap1 associates with Nrf2 and promotes the ubiquitination and proteasomal degradation of Nrf2. In response to stress, Keap1 is modified at its cysteine residues to release Nrf2 which then enters the nucleus to bind to the antioxidant response element (ARE) motif-containing promoter to turn on target gene transcription [4, 5, 8]. Post stress, nuclear translocation of Keap1 mediates the dissociation of Nrf2 from the ARE motif and subsequent nuclear export of Nrf2 for degradation in the cytosol [6].

The epidermal growth factor receptor (EGFR) tyrosine kinase is frequently overexpressed or highly activated in many human cancers, including breast, ovarian, lung, and colon cancers and glioblastoma [9, 10]. Upon ligand binding, EGFR is activated through dimerization and autophosphorylation, which then turns on its downstream signaling pathways to promote cell proliferation, survival, and drug-resistance [11-14]. Ligand binding also induces EGFR internalization and translocation to the nucleus [15], where EGFR functions in gene transcription, DNA repair, and DNA replication [10, 16, 17]. Importantly, EGFR nuclear expression has been shown to induce cancer cell resistance to therapeutic treatments [18, 19] and correlate with poor survival rate of cancer patients [10, 16].

EGFR downstream MAPK/Erk signaling pathway has been recently reported to activate Nrf2-mediated cell proliferation in lung cancer cells [20]. In our previous study, Keap1 was identified among a list of proteins pulled down by nuclear EGFR in the nucleus by an unbiased proteomic approach [21]. However, it is not clear whether EGFR itself directly regulates the Keap1/Nrf2 pathway. Here, we investigate the role of nuclear EGFR in activating Keap1/Nrf2 pathway in cancer cells.

Materials and methods

Materials

Antibodies and chemicals were used in this study including Anti-EGFR (Ab13, Lab Vision; SC-03, Santa Cruz), anti-EGFR pY1068 (ab32430, Abcam), anti-Keap1 (Proteintech), anti-lamin B1 (Santa Cruz), anti-α-tubulin (Sigma), anti-Flag (F3165, Sigma), anti-HA (Roche), anti-Myc (Roche), anti-Nrf2 (H300, Santa Cruz), anti-IKKβ (2648, Cell signaling Technology), anti-Ub (P4D1, Santa Cruz), anti-phosphotyrosine (4G10, Millipore); recombinant human EGF (Sigma), AG1478 (LC Laboratories), Erlotinib (Tarceva, Selleck), and U0126 (Sigma). Plasmids of Myc.EGFR, Myc.EGFR-ΔNLS, Myc.EGFRkd, Myc.EGFR1-644, Myc.EGFR645-1186, HA.Keap1 and HA.Keap1 deletion constructs, HA.Ub, and Myc/Flag.Nrf2 were previously described [2, 21, 22]. Flag.Keap1 was constructed by subcloning of Keap1 cDNA from HA.Keap1 into p3 × Flag.CMV10 (Sigma) expression vector using Hind*III* and Kpn*I* restriction enzyme sites. pGL2B-NQO1-ARE. Luc reporter construct was kindly provided by Dr. Anil K. Jaiswal (University of Maryland, School of Medicine) [23]. pβ-actin-Renilla promoter [21] was co-transfected with pGL2B-NQO1-ARE.Luc as an internal control to correct transfection efficiency for luciferase assay.

Cell culture, transfection, immunoprecipitation, and Western blot

Human cell lines (HEK293T, HeLa, MDA-MB-468, A431) were maintained in DMEM/F12 media containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified incubator with 5% CO_2 at 37°C. Transfection was performed with lipofectamine 2000 (Invitrogen), and cells lysis, cellular fractionation, immunoprecipitation, Western blot, and MTT assay were previously described [21]. shRNAs used in this study were non-silencing shRNA control [21], pLKO.1-EGFR: 5'-GCTGCTCTGAAATCTCCTTTA-3' (3'-UTR), and pGIPZ-Nrf2: 5'-TAATTGTCAACTTCTGTCA-3' (CDS, shRNA core facility, MD Anderson Cancer Center).

Luciferase reporter and MTT assays

Luciferase reporter and MTT (3- (4, 5-dimethylthiazol-2-yl) -2, 5-diphenyl tetrazolium bromide) assays were performed as previously described [21, 24]. For luciferase reporter assay, 2×10^5 HeLa cells were seeded in 6-well culture plates and were transfected with pGL2B-NQO1-ARE. Luc, plasmids expressing Nrf2, Keap1, or EGFR, and pβ-actin-Renilla (internal control). Cells were serum starved overnight followed by 5 hr of EGF (50 ng/ml) stimulation before cell lysis and luciferase reporter assay.

For MTT assay, 4×10^3 MDA-MB-468 cells were seeded in 96-well plate. After overnight culture, cells were treated with erlotinib (2.0 μM), cisplatin (0.75 μM), or both as indicated. Cells were continued to culture for different times. 20 μl of MTT was added to each well and incubated at 37°C, 5% CO_2 for 4 hours before cell lysis. Cell growth rate was determined by measuring optical density at 570 nm.

Mass spectrometry

To identify phosphorylation residues of Keap1, we performed mass spectrum analysis using the method previously described [24]. Briefly, HEK293T cells were cotransfected with Flag.Keap1 and Myc.EGFR. After 30 min of EGF (50 ng/ml) stimulation, cells were lysed, and Keap1 was immunoprecipitated with anti-Flag antibody followed by SDS-PAGE separation. The protein band corresponding to Keap1 was excised and subjected to in-gel digestion with trypsin. After purification of phosphopeptides with Phos-trap™ Phosphopeptide Enrichment Kit (PerkinElmer, Massachusetts, USA), MS/MS was performed to identify phosphorylation residues of Keap1.

Results

EGFR interacts with Keap1

Previously, Keap1 was identified as one of many proteins pulled down by nuclear EGFR through a non-biased mass spectrum analysis [21], which suggests that Keap1 may associate

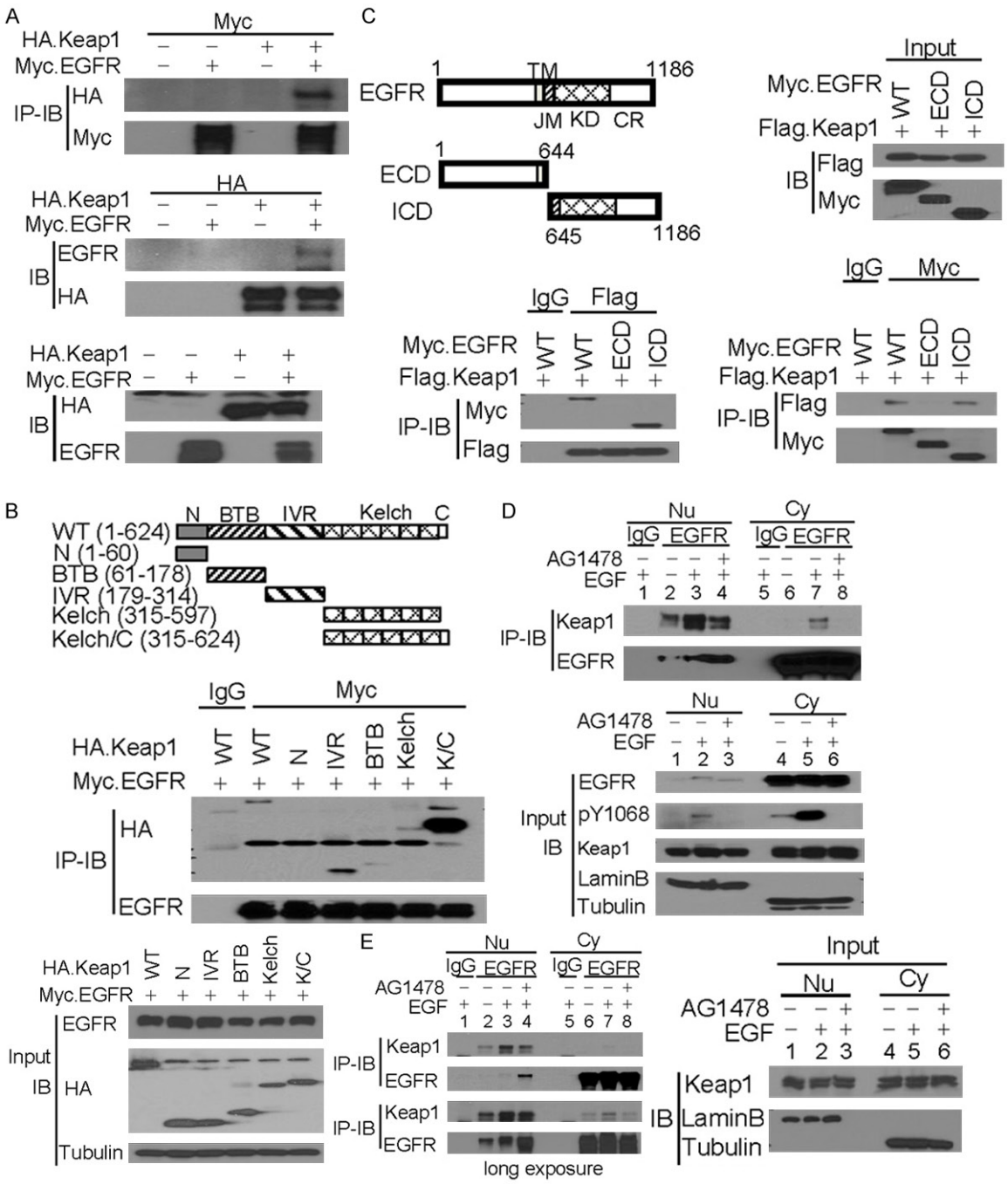

Figure 1. EGFR interacts with Keap1. A. Cell lysates from HEK293T cells transfected with indicated plasmids were immunoprecipitated by either anti-Myc (upper panel) or anti-HA (middle panel) antibodies followed by WB to detect EGFR and Keap1. Total cell lysates were used to detect the expression levels of indicated proteins (lower panel). B. HEK293T cells were transfected with EGFR and different constructs of Keap1 as indicated and cell lysates were immunoprecipitated with anti-Myc antibody followed by WB to detect the associated Keap1 domains (middle panel) in the immunoprecipitates. Schematic in upper panel shows the different domains of Keap1. The numbers shown in parentheses indicate amino acid residues. Lower panel shows the expression of these different constructs in HEK293T cells. C. HEK293T cells were transfected with Keap1 and different constructs of EGFR. Cell lysates were immunoprecipitated with anti-Flag antibody (lower left panel) or anti-Myc antibody (lower right panel) followed by WB to detect the associated EGFR domains or Keap1 in the immunoprecipitates. The numbers shown indicate amino acid residues. ECD: extracellular domain; ICD: intracellular domain; TM: transmembrane domain; JM: juxtamembrane domain; KD: kinase domain; CR: C-terminal regulatory domain. D. Interaction between endogenous EGFR and Keap1 in MDA-MB-468 cells. MDA-MB-468 cells, after overnight serum starvation, were treated with or without EGF

(50 ng/ml) for 30 min. Tyrosine kinase inhibitor AG1478 (10 μM) was used to pretreated the cells for 30 min before addition of EGF. Cells were then collected for cellular fractionation followed by IP-WB as indicated (upper panel). Input lysate was used to detect the expression levels of the indicated proteins (lower panel). Lamin B and tubulin were used to indicate the cellular fractionation efficiency. E. The same experiment as described in (D) was repeated in A431 cells. WB, Western blot; IP, immunoprecipitation.

with the nuclear EGFR. To validate the interaction between EGFR and Keap1, we coexpressed HA.Keap1 and Myc.EGFR in HEK293T cells and performed immunoprecipitation followed by Western blot (IP-WB) analysis. As shown in **Figure 1A**, Keap1 associated with EGFR as Keap1 was pulled down by anti-EGFR antibody and vice versa. Although the three domains of Keap1 (IVR, Kelch, and Kelch/C-terminus) interacted with EGFR (**Figure 1B**) through EGFR's intracellular domain (ICD) (**Figure 1C**), the strongest association appeared to be between the Kelch/C-terminus of Keap1 and EGFR (**Figure 1B**, middle), suggesting that the short C-terminal region of Keap1 plays an important role for its interaction with EGFR. To validate endogenous interaction, we analyzed cell lysates from two different cell lines, MDA-MB-468 (**Figure 1D**) and A431 (**Figure 1E**) cancer cells by IP-WB. Indeed, endogenous EGFR interacted with Keap1 in both nucleus and cytosol. Interestingly, Keap1 signals were stronger in the nucleus than in the cytosol (**Figure 1D**, top, and **1E**, left, lane 2 vs. lane 6). In addition, the association between Keap1 and EGFR was enhanced by EGF and decreased by AG1478, an EGFR tyrosine kinase inhibitor (TKI) (**Figure 1D**, top, and **1E**, left, lane 3 vs. lane 4 and lane 7 vs. lane 8). Taken together, these data indicate that nuclear Keap1 prefers to interact with nuclear EGFR, and EGF stimulation can increase their nuclear association in cancer cells.

EGFR phosphorylates Keap1

Since EGFR interacts with Keap1, we asked whether it also phosphorylates Keap1. Indeed, we found that wild type but not kinase-dead EGFR (EGFRkd) induced tyrosine phosphorylation of Keap1 (**Figure 2A**, lane 3 vs. lane 6), which was blocked by the addition of AG1478 (**Figure 2A**, lane 3 vs. lane 4). Using an *in vitro* kinase assay, we further showed that purified EGFR phosphorylated Keap1, and inhibition of EGFR kinase activity by AG1478 blocked this phosphorylation (**Figure 2B**, lane 4 vs. lane 5). Consistently, the endogenous Keap1 was phosphorylated in both MDA-MB-468 and A431 cells upon EGF stimulation (**Figure 2C** and **2D**, lane 2 vs. lane 3). Interestingly, EGF-induced Keap1 phosphorylation occurred mainly in the nucleus but not in the cytosol (**Figure 2C** and **2D**, lane 3 vs. lane 7). As expected, EGFR TKI treatment blocked the tyrosine phosphorylation of Keap1 in the nucleus (**Figure 2C** and **2D**, lane 3 vs. lane 4). Moreover, an EGFR-ΔNLS mutant that lacks the nuclear localization signal could not phosphorylate Keap1 upon EGF treatment (**Figure 2E**, lane 3 vs. lane 5) even though the EGFR-ΔNLS mutant could still activate the Erk pathway to a level similar to wild type EGFR (**Figure 2E**, right panel). This suggests that EGFR-induced keap1 tyrosine phosphorylation requires EGFR nuclear translocation and the tyrosine phosphorylation of nuclear Keap1 is EGFR-kinase dependent.

To identify which tyrosine residues of Keap1 are phosphorylated by EGFR, we performed mass spectrum analysis using the immunoprecipitate of Flag-Keap1 from HEK293T cells coexpressing Myc.EGFR and Flag. Keap1. We identified three tyrosine-phosphorylated residues of Keap1, Y206, Y263, and Y334. To validate that these sites are responsible for EGFR-induced phosphorylation, we generated Keap1 mutant by substituting these three tyrosine residues with phenylalanines (triple mutant 3F: Y206F/Y263F/Y334F). Since Y141 has been previously identified as a tyrosine phosphorylation site of Keap1 upon oxidative stress [25], we also included the Y141F mutant in our study as a control. In transiently transfected HEK293T cells, we found that the Keap1 triple mutant (3F) greatly reduced the tyrosine phosphorylation signal (**Figure 2F**, lane 3 vs. lane 7) while the Y141F mutant had little effect on Keap1 phosphorylation (**Figure 2F**, lane 3 vs. lane 5). Taken together, the data suggest that these three residues, Y206, Y263, and Y334, are the major sites of Keap1 phosphorylated by EGFR.

EGFR enhances Nrf2 protein stability

To investigate the biological effects of the EGFR/Keap1 interaction, we transfected HEK-293T cells with EGFR, Keap1, and Nrf2 and

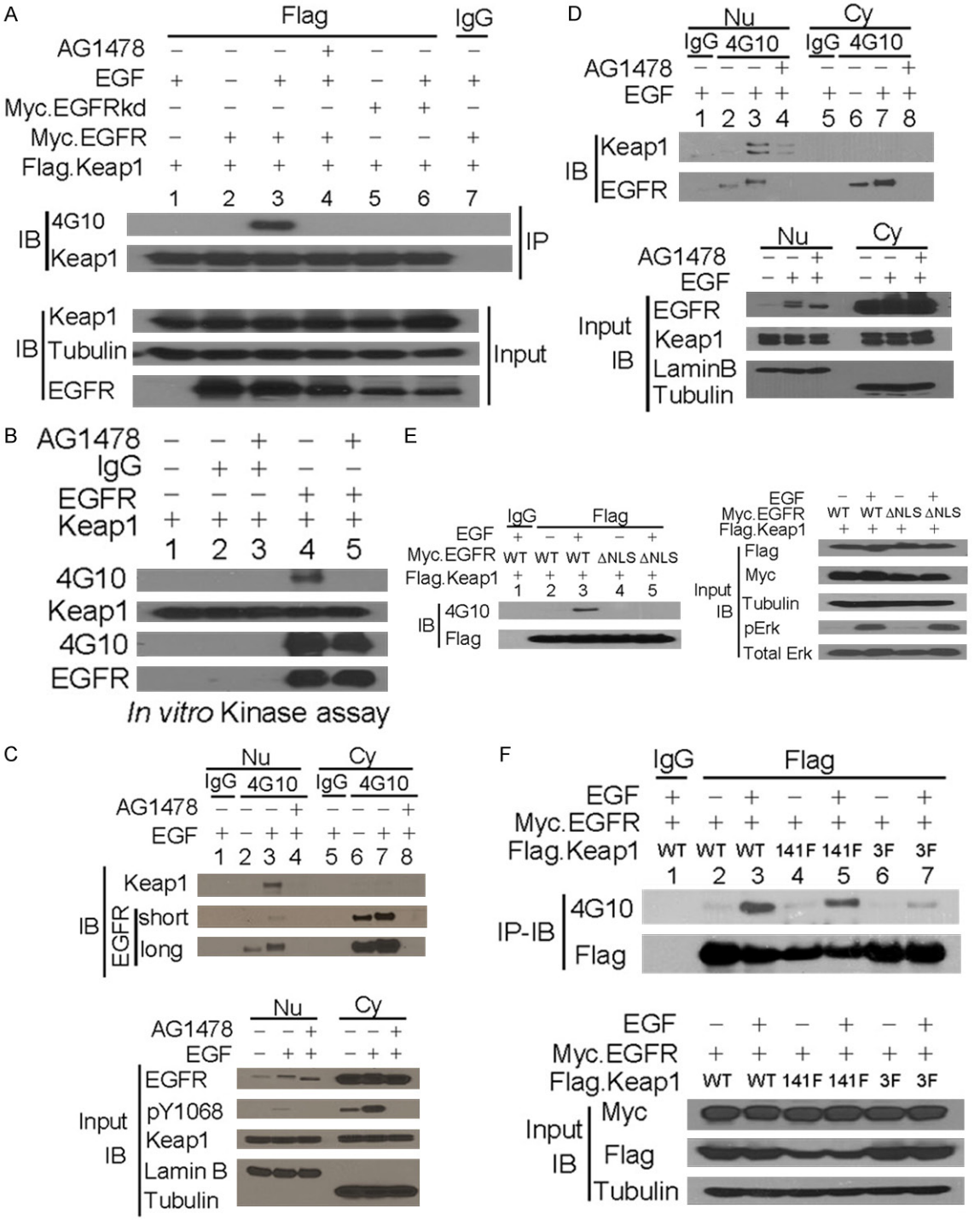

Figure 2. EGFR Tyrosine phosphorylates Keap1. (A) Cell lysates from transfected HEK293T cells treated with EGF (50 ng/ml) and AG1478 (10 μM) as indicated were immunoprecipitated by anti-Flag antibody followed by immunoblotting with anti-tyrosine phosphorylation antibody (4G10). (B) Immunoprecipited EGFR and purified Flag.Keap1 were used for in vitro kinase assay. AG1478 (10 μM) was added to block EGFR kinase activity. The same membrane was first blotted with 4G10 antibody and then with antibodies against either Keap1 and EGFR to detect the protein levels in these samples. IgG: immunoprecipitate with normal IgG. (C) MDA-MB-468 cells were treated as indicated and then lysed followed by cellular fractionation and IP-WB to detect the phosphorylation levels of Keap1 and EGFR (upper panel). Lower panel shows the WB results from input. Nu: nuclear fraction; Cy: cytosolic fraction. (D) The same experiment as described in (C) was repeated in A431 cells. Lower panel shows expression of indicated proteins in both nuclear and cytosol fractions. (E) Cell lysates from HEK293T cells transfected by Flag.Keap1 and Myc.

EGFR (either wild type or EGFR-deltaNLS mutant) and treated with EGF (50 ng/ml, 30 min) were immunoprecipitated by anti-Flag antibody, followed by WB to detect phosphorylated Keap1 and total Keap1 as indicated (left panel). Right panel shows the expression level of the indicated proteins in HEK293T cells. (F) HEK293T cells cotransfected by indicated plasmids were treated as indicated. Equal amounts of cell lysate were used for immunoprecipitation with anti-Flag antibody followed by blotting with 4G10 (upper panel). Lower panel shows the expression levels of both EGFR and Keap1 in the cells.

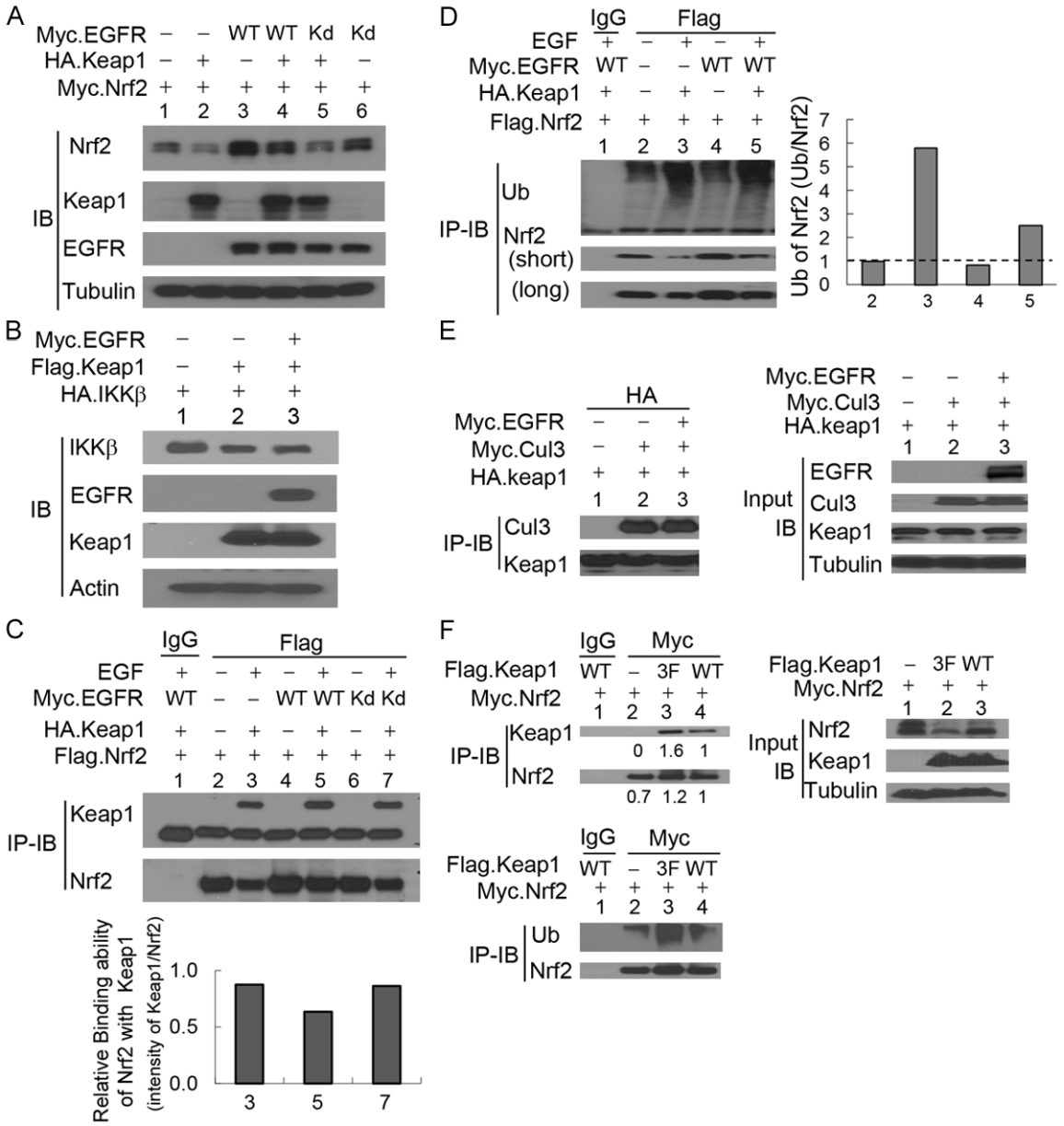

Figure 3. EGFR activation increases Nrf2 protein stability. A. Cell lysates from HEK293T cells transfected with indicated plasmids were subjected to WB analysis to detect the indicated proteins. B. HEK293T cells were transfected with plasmids as indicated, and equal amounts of cell lysate were subjected to Western blot analysis to detect the protein levels of HA.IKKβ, Flag.Keap1, Myc.EGFR and actin. C. HEK293T cells transfected with plasmids as indicated and equal amounts of lysates were used for IP-WB. The relative association of Keap1 with Nrf2 was calculated by normalizing the band intensity of Keap1 to that of Nrf2 from the same membrane and is shown on the lower pannel. D. HEK293T cells transfected with plasmids as indicated were treated with proteasome inhibitor MG132 for 5 hr before lysis. Equal amounts of cell lysates were used for immunoprecipitation of Nrf2 by anti-Flag antibody, followed by immunoblotting with indicated antibodies (Ub first, then Nrf2). The relative Ub level of Nrf2 is shown on the right by normalizing the band intensity of ubiquitination to that of corresponding Nrf2. E. HEK293T cells were transfected

with the indicated plasmids, and equal amounts of lysate were immunoprecipitated with anti-HA antibody, followed by Western blot analysis to detect the associated Cul3 with Keap1. Total cell lysates were used for WB to show the expression levels of these proteins as indicated (right panel). F. HEK293T cells were transfected with indicated plasmids and treated with MG132 for 5 hr before lysis. Equal amounts of cell lysates were immunoprecipitated with anti-Myc antibody followed by immunoblotting with either antibodies against Keap1 and Nrf2 (left upper panel) or antibodies against ubiquitin and Nrf2 (left lower panel). Equal amounts of lysates from cells transfected with Nrf2 and Keap1 but without MG132 treatment were subjected to WB analysis to detect the expression levels of indicated proteins (right panel).

asked whether EGFR affects Keap1-mediated protein degradation of Nrf2. As shown in **Figure 3A**, co-transfection of Nrf2 with Keap1 reduced the Nrf2 protein level (lane 1 vs. lane 2), which is consistent with the previous reports that Keap1 is a major regulator of Nrf2 stability [26, 27]. However, cotransfection of EGFR in these cells not only increased the basal level of Nrf2 (**Figure 3A**, lane 1 vs. lane 3) but also inhibited Keap1-mediated Nrf2 degradation (**Figure 3A**, lane 2 vs. lane 4). In contrast, EGFRkd mutant did not block the Keap1-mediated Nrf2 degradation (**Figure 3A**, lanes 2 and 4 vs. lane 5). Interestingly, under our experimental conditions, co-expression of EGFR had no effect on Keap1-mediated degradation of IKKβ (**Figure 3B**, lanes 1 and 2 vs. lane 3) even though IKKβ has been reported as a Keap1 target [2], suggesting that Keap1-mediated protein degradation is likely dependent on its target protein location in the cells and the status of its post-translational modification.

Next, we asked how EGFR affects the stability of Nrf2. To this end, we transfected HEK293T cells with Nrf2, Keap1, and EGFR to investigate the ubiquitination of Nrf2 and its interaction with Keap1. The results showed that EGFR reduced the interaction of Nrf2 with Keap1 (**Figure 3C**, lane 3 vs. lane 5), which in turn decreased Keap1-mediated ubiquitination of Nrf2 (**Figure 3D**, lane 3 vs. lane 5). In contrast, we did not observed any differences in the interaction between Keap1 and Cul3, a scaffold protein of the Keap1 E3 ubiquitin ligase complex, when co-expressed with EGFR (**Figure 3E**, upper panel, lane 2 vs. lane 3). Co-expression of EGFRkd did not affect the interaction of Keap1 with Nrf2 (**Figure 3C**, lane 3 vs. lane 7). In addition, we found that mutations of Keap1 at Y206, Y263, and Y334 residues enhanced its interaction with Nrf2 (**Figure 3F**, upper left panel, lane 3 vs. lane 4), increased the ubiquitination of Nrf2 (**Figure 3F**, lower left panel, lane 3 vs. lane 4), and consequently decreased the Nrf2 protein stability (**Figure 3F**, right panel, lane 2 vs. lane 3). Taken together, these results support that EGFR-mediated phosphorylation of Keap1 reduces its interaction with Nrf2 and decreases Nrf2 ubiquitination, leading to increased Nrf2 stability.

EGFR activation increases Nrf2 transcriptional activity

Since EGFR activation stabilizes Nrf2, we asked whether EGFR stimulates Nrf2 transcriptional activity by analyzing ARE-containing promoter-luciferase reporter gene expression. To this end, we choose HeLa cells for the luciferase reporter assay as they respond well to EGF after overnight serum starvation. Upon EGF stimulation, EGFR not only increased the basal level of luciferase expression driven by ARE-containing promoter (**Figure 4A**, lanes 5 and 6 vs. lanes 1 and 2) but also blocked the Keap1-mediated downregulation of the ARE-containing promoter activity (**Figure 4A**, lanes 7 and 8 vs. lanes 3 and 4). In addition, pretreatment of cells with EGFR TKI abolished the inhibitory effect of EGFR on Keap1-mediated downregulation of ARE-containing promoter activity (**Figure 4A**, lanes 9 and 10 vs. lanes 7 and 8). In contrast, pretreatment of cells with MEK inhibitor U0126 moderately inhibited the EGF-stimulated promoter activity (**Figure 4A**, lanes 11 and 12 vs. lanes 7 and 8), suggesting other possible regulation of Keap1/Nrf2 pathway besides the EGFR/MEK cascade upon EGF treatment.

Our data demonstrated that the interaction of EGFR with Keap1 mainly occurred in the nucleus. Thus, we asked whether nuclear EGFR stimulates Nrf2 transcriptional activity. To this end, we examined the effects of wild type EGFR and EGFR-ΔNLS mutant on Nrf2 target gene's promoter activity. As shown in **Figure 4B**, wild type EGFR but not ΔNLS mutant increased the promoter activity (lanes 5 and 6 vs. lanes 1 and 2 for EGFR; lanes 9 and 10 vs. lanes 1 and 2 for EGFR-ΔNLS) and blocked the Keap1-mediated suppression of luciferase expression (lanes 7

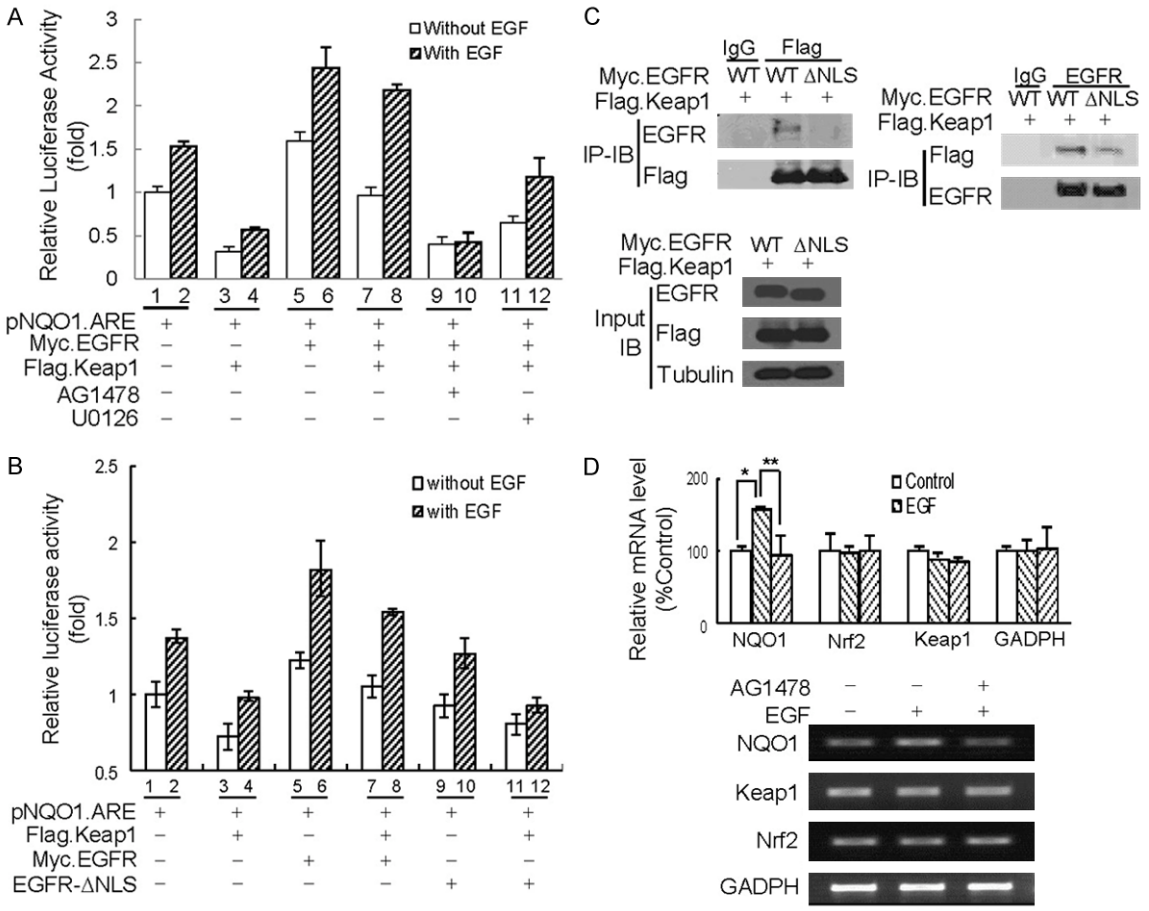

Figure 4. EGFR increases Nrf2 transcriptional activity. A. HeLa cells transfected with indicated plasmids were serum starved and treated with EGF, AG1478, or U0126 as indicated for 5 hr. Cells were then lysed for luciferase assay (n = 3). pNQO1.ARE: pGL2B-NQO1-ARE.Luc. B. HeLa cells transfected with indicated plasmids were treated with or without EGF for 5 hr after overnight serum starvation and then subjected to luciferase assay after cell lysis (n = 3). C. HEK293T cells were transfected with indicated plasmids. Equal amounts of cell lysate were immunoprecipitated with antibodies against either Flag (upper left panel) or EGFR (right panel) followed by immunoblotting to detect the indicated proteins. Equal amounts of input were subjected to WB analysis to detect the expression levels of the indicated proteins in HEK293T cells (lower left panel). wt: EGFR wild type, ΔNLS: EGFR-ΔNLS. D. Cells with different treatment as indicated were lysed with TRIZOL for total RNA extraction. Equal amounts of total RNA from each sample were used for reverse-transcription followed by PCR to detect the expression levels of NQO1, a target gene of Nrf2. Upper panel: quantitative RT-PCR (n = 3); lower panel: regular RT-PCR. Error bar: ± SD. **p* = 0.001, ***p* = 0.027.

and 8 vs. lanes 3 and 4 for EGFR; lanes 11 and 12 vs. lanes 3 and 4 for EGFR-ΔNLS). In line with the results from the promoter-reporter assay, the association of Keap1 with EGFR-ΔNLS was reduced (**Figure 4C**). Furthermore, EGF treatment increased the mRNA expression level of Nrf2 target gene NQO1, which was inhibited by EGFR TKI (**Figure 4D**). However, the mRNA levels of Nrf2 and Keap1 remained unchanged upon EGF treatment (**Figure 4D**), suggesting that a posttranslational mechanism may be responsible for the EGFR-mediated Nrf2 protein stability and activity.

EGFR regulates nuclear Keap1 protein levels

In addition to the observation of a strong nuclear interaction between EGFR and Keap1 (**Figure 1**), results from our cellular fractionation experiments indicated that the levels of nuclear Keap1 gradually decreased while nuclear Nrf2 concurrently increased in a time-dependent manner upon EGF stimulation in both A431 (**Figure 5A**, upper panel) and MDA-MB-468 (**Figure 5B**, upper panel) cells. In contrast, the cytosolic levels of Keap1 remained relatively similar during the course of EGF stimulation,

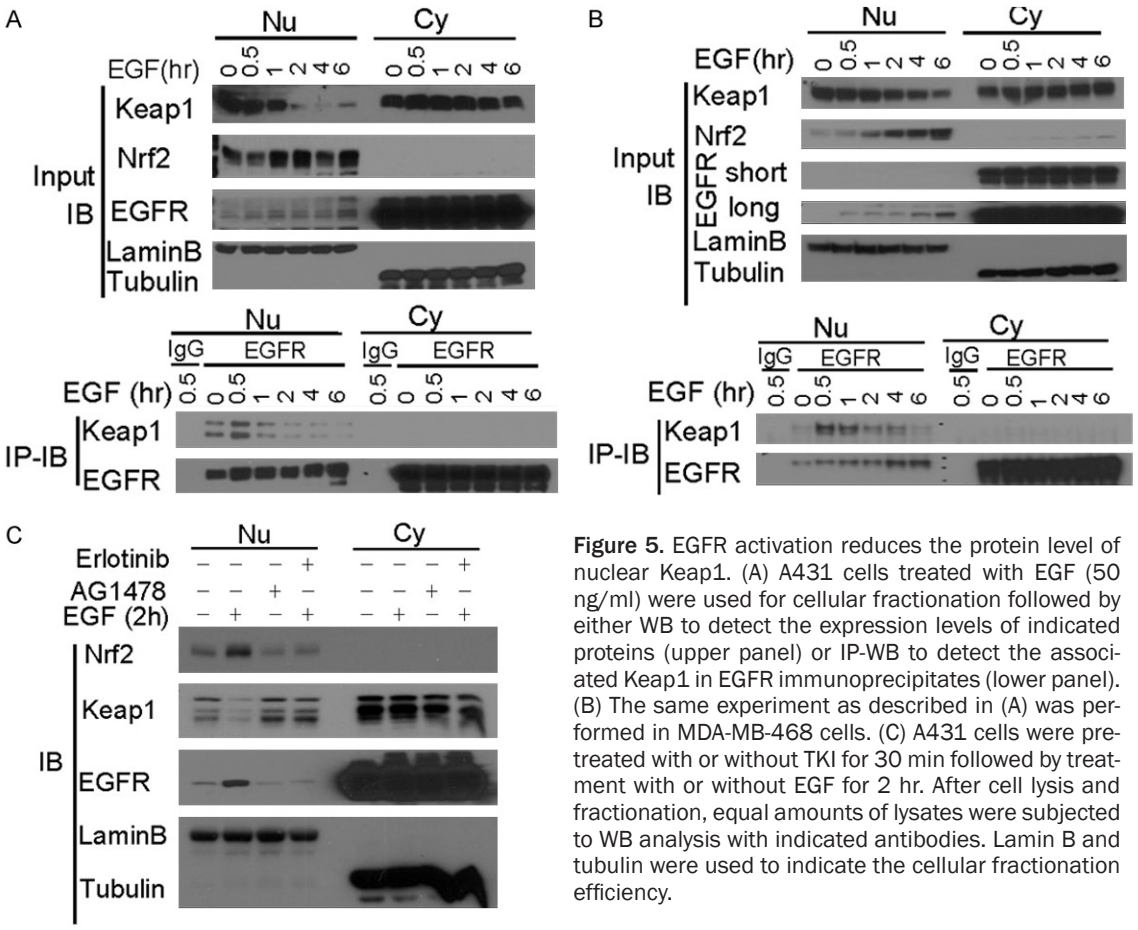

Figure 5. EGFR activation reduces the protein level of nuclear Keap1. (A) A431 cells treated with EGF (50 ng/ml) were used for cellular fractionation followed by either WB to detect the expression levels of indicated proteins (upper panel) or IP-WB to detect the associated Keap1 in EGFR immunoprecipitates (lower panel). (B) The same experiment as described in (A) was performed in MDA-MB-468 cells. (C) A431 cells were pretreated with or without TKI for 30 min followed by treatment with or without EGF for 2 hr. After cell lysis and fractionation, equal amounts of lysates were subjected to WB analysis with indicated antibodies. Lamin B and tubulin were used to indicate the cellular fractionation efficiency.

suggesting that a possible nuclear-specific regulation of Keap1 is involved. In addition, the nuclear interaction between EGFR and Keap1 increased after 30 min EGF stimulation (**Figure 5A** and **5B**, IP-WB, lower panel). Addition of TKIs (erlotinib and AG1478) blocked the EGF-induced decrease of nuclear Keap1 and increase of nuclear Nrf2 (**Figure 5C**). To determine whether EGF reduces nuclear Keap1 protein stability, we treated cells with EGF and proteasome inhibitor. Nuclear protein level of Keap1 was increased in the presence of proteasome inhibitor MG132 (**Figure 6A**). Moreover, we found that EGF treatment induced the ubiquitination of Keap1 in the nucleus but not in the cytosol, and EGFR TKI blocked the EGF-induced ubiquitination of nuclear Keap1 (**Figure 6B**). Consistently, wild type EGFR but not EGFR-ΔNLS mutant increased Keap1 ubiquitination in a transient transfection study (**Figure 6C**, lane 3 vs. lane 4). In addition, EGF treatment reduced the protein half-life of nuclear Keap1 considerably (**Figure 6D**, from 8.5 h to 4 h), which is in line with the reduction of nuclear Keap1 by EGF stimulation (**Figure 6A**). Together, these data suggest that activation of nuclear EGFR signaling reduces the protein expression levels of nuclear Keap1 through a ubiquitination pathway. We also co-expressed EGFR with Keap1 (wild type or 3F mutant) in HEK293T cells to determine whether EGFR-induced tyrosine phosphorylation is important for Keap1 stability. We found that mutation of Keap1 at tyrosine residues of Y206, Y263, and Y334 greatly increased the half-life of Keap1 (**Figure 6E**), supporting that EGFR-induced tyrosine phosphorylation contributes to Keap1 protein instability.

To further validate the role of EGFR in Keap1 protein instability, we knocked down endogenous EGFR by shRNA in HeLa cells and found that EGFR knockdown increased Keap1 protein level, which also led to a reduction in Nrf2 protein level (**Figure 7A**, lane 1 vs. lane 2). In addition, ectopic Nrf2 protein level was significantly increased by EGF but decreased by EGFR TKI in HeLa cells with endogenous EGFR expression

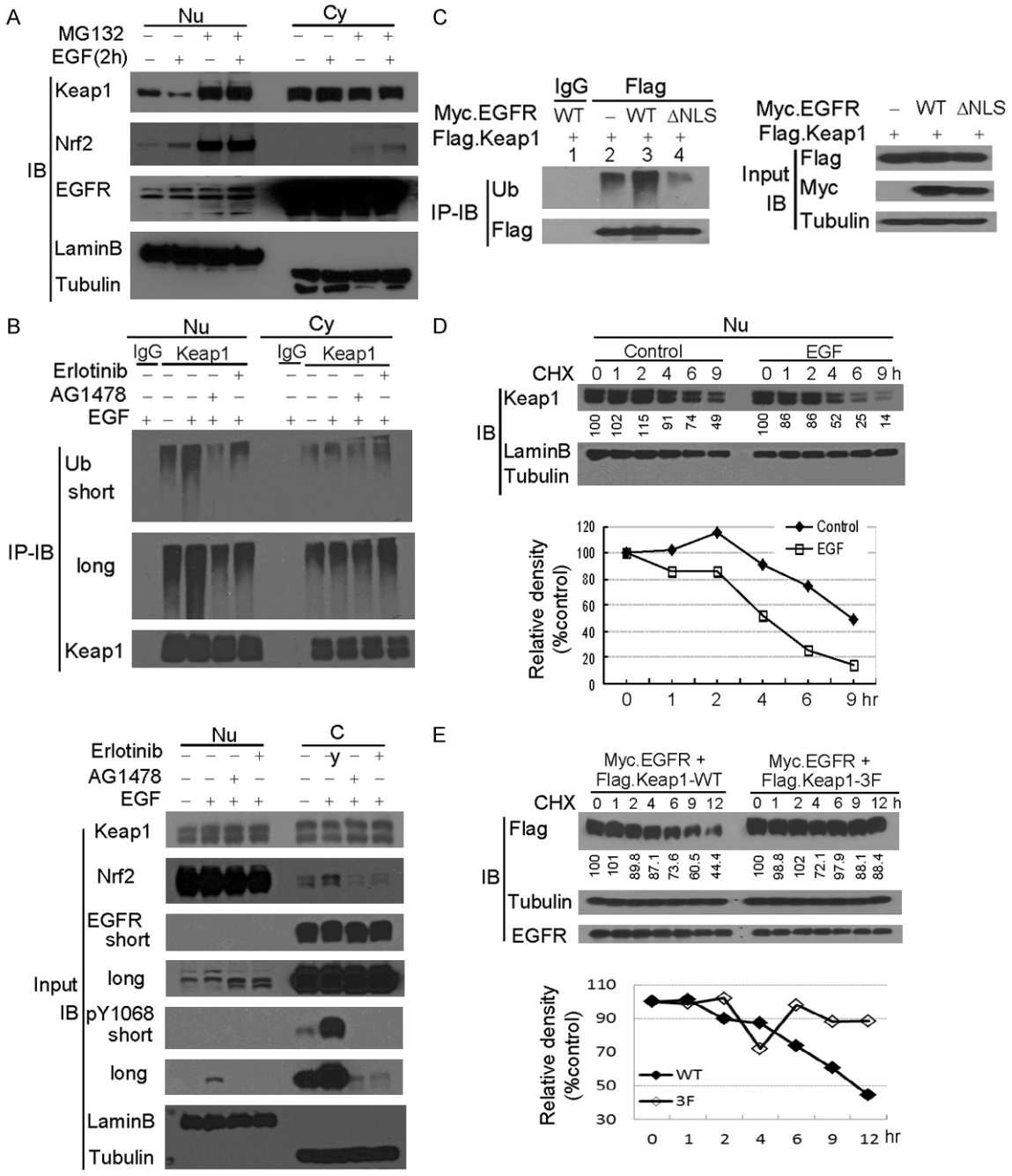

Figure 6. EGF treatment reduces nuclear Keap1 expression. (A) A431 cells were treated with EGF (50 ng/ml) with or without MG132 (10 μM) for 2 hr followed by cell lysis and fractionation. Equal amounts of cell lysates were subjected to WB analysis with the indicated antibodies. (B) A431 cells were pretreated with or without EGFR TKI for 30 min followed by treatment with or without EGF for 5 hr in the presence of MG132 (10 μM). Cells were lysed, fractionated, and immunoprecipitated by anti-Keap1 antibody followed by immunoblotting with anti-Ub antibody. The same membrane was reblotted with anti-Keap1 antibody to show Keap1 protein level. (C) HEK293T cells transfected with indicated plasmids were treated with MG132 for 5 hr. Equal amounts of cell lysates were immunoprecipitated with anti-Flag antibody followed by immunoblotting with indicated antibodies. Left panel shows the ubquitination of immunoprecipitated Keap1. Right panel shows the expression levels of proteins in HEK293T cells. ΔNLS: EGFR-ΔNLS. (D) After overnight serum starvation, A431 cells were treated with protein synthesis inhibitor cycloheximide (100 μg/ml) with or without EGF (50 ng/ml) for different times. Equal amounts of nuclear fractionation were subjected to Western blot analysis to detect the protein levels of nuclear Keap1 (upper panel). The band intensity at zero time point was set as 100%, and the intensities of other bands were normalized to the value at the zero time point. Lamin B and tubulin were used to show the cellular fractionation efficiency. Middle panel shows the quantitative analysis

of the protein half-life of nuclear Keap1. (E) HEK293T cells were transfected with indicated plasmids and treated with cycloheximide (100 μg/ml) for different times before cell lysis. Equal amounts of cell lysates were subjected to WB analysis to detect the expression levels of indicated proteins (upper panel). Lower panel shows the quantitative analysis of the protein half-life of Flag.Keap1 using the same method as described in (D).

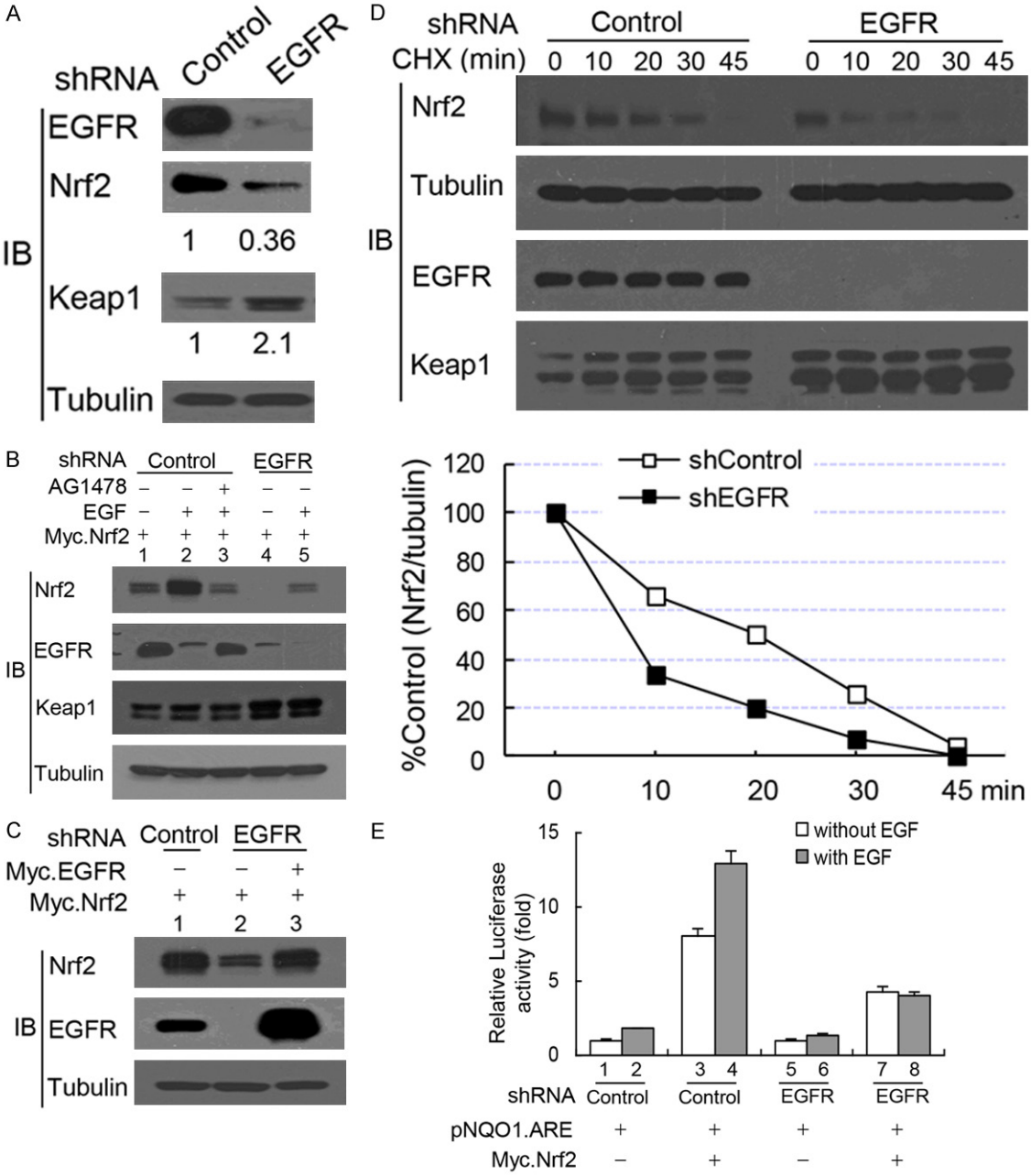

Figure 7. EGFR knockdown increases Keap1 and decreases Nrf2 protein levels. A. HeLa cells stably expressing non-targeting shRNA (control) or shRNA targeting EGFR (3'-UTR) were collected and lysed. Equal amounts of cell lysates were subjected to WB analysis to detect the protein levels of EGFR, Nrf2, Keap1, and tubulin. B. HeLa cells expressing the indicated shRNAs and Myc.Nrf2 were serum starved and treated with or without EGF and AG1478. Equal amounts of cell lysates were subjected to WB analysis to detect the expression levels of indicated proteins. C. HeLa cells with EGFR-knockdown were transiently transfected with Myc.Nrf2 and shRNA-resistant Myc.EGFR. Cells lysates were subjected to WB analysis to detect the expression levels of Myc.Nrf2, Myc.EGFR, and tubulin. D. HeLa cells with EGFR-knockdown were treated with protein synthesis inhibitor cycloheximide (100 μg/ml) for different times. Equal amounts of cell lysates were subjected to WB analysis to detect the protein levels as indicated (upper panel). Lower panel shows quantification analysis of Nrf2 half-life. The band intensity of Nrf2 at zero time point was set as

100%, and the band intensity of others was calculated by normalizing it to the zero time point. E. HeLa cells expressing the indicated shRNAs were transfected with pNQO1.ARE (pGL2B-NQO1-ARE.Luc) and Myc.Nrf2 plasmids. After overnight serum starvation, cells were treated with or without EGF for 5 hr. Cells were then lysed and subjected to luciferase assay (n = 3). Error bars: ± SD.

(**Figure 7B**, lane 1 vs. lane 2 and lane 2 vs. lane 3, respectively). However, compared with cells expressing control shRNA, ectopic Nrf2 was undetectable without EGF stimulation and barely detectable upon EGF stimulation in EGFR-knockdown cells (**Figure 7B**, lane 1 vs. lane 4 and lane 2 vs. lane 5, respectively). The reduced protein level of ectopic Nrf2 was restored by re-expression of shRNA-resistant exogenous EGFR in the EGFR-knockdown cells (**Figure 7C**, lane 2 vs. lanes 1 and 3). In contrast to the increase of Keap1 protein stability, knocking down endogenous EGFR reduced the protein half-life of Nrf2 (**Figure 7D**) and subsequently Nrf2-stimulated ARE-containing promoter activity (**Figure 7E**). Taken together, these data support that nuclear EGFR activation downregulates nuclear Keap1 and therefore increases nuclear Nrf2.

EGFR inhibitor enhances the cytotoxicity of cisplatin

One of major mechanisms of cisplatin-mediated cell apoptosis is through induction of reactive oxygen species (ROS) [28], and Nrf2 is a key transcription factor that protects cells from ROS-induced apoptosis through activating expression of cytoprotective genes. Indeed, several studies have demonstrated that Nrf2 increases cancer cell proliferation and induces cancer cell resistance to cisplatin treatment [29-31]. Nuclear translocation of EGFR is also reported to induce cisplatin resistance [22]. Therefore, we hypothesized that nuclear EGFR-induced Nrf2 stability as demonstrated above may be one of the mechanisms contributing to cisplatin resistance in cancer cells and that inhibition of EGFR by TKI may enhance the cytotoxicity of cisplatin. Indeed, we found that the combination of EGFR TKI, e.g., erlotinib (2 μM), with a low dose of cisplatin (0.75 μM) significantly increased the cytotoxicity of cisplatin and strongly inhibited the proliferation of MDA-MB-468 cells *in vitro* (**Figure 8A**). Similar to the addition of erlotinib, knockdown of Nrf2 also sensitized breast cancer cells to the low dose of cisplatin (**Figure 8B**). Taken together, these results suggest that EGFR TKI may sensitize cancer cell to cisplatin through EGFR-mediated regulation of Keap1/Nrf2 pathway in the nucleus.

Discussion

In this study, we investigated the role of nuclear EGFR in regulating Keap1/Nrf2 pathway and found that nuclear EGFR interacts with and phosphorylates nuclear Keap1 to reduce its protein level, and therefore stabilizes nuclear Nrf2. Although both EGFR and Keap1 localize predominately in the cytoplasm and only a small percentage of them are located in the nucleus [7, 32], the interaction of EGFR with Keap1 and EGFR-induced tyrosine phosphorylation of Keap1 were mainly detected in the nucleus. Indeed, nuclear interaction between EGFR and Keap1 is consistent with the observation of that EGFR only affects the Keap1 target protein Nrf2 but not IKKβ [2], as IKKβ is generally localized in the cytosol while Nrf2 is located in both the nucleus and the cytoplasm.

Keap1 has been reported to be tyrosine phosphorylated by an unknown kinase at Y141, which stabilizes Keap1 and accelerates Nrf2 degradation [25], and at Y85, which regulates nuclear Keap1 export upon antioxidant treatment [33]. In this study, we identified three new tyrosine residues of Keap1, Y206, Y263, and Y334 phosphorylated by EGFR. Substitution of all the three residues by phenylalanines greatly reduced the tyrosine phosphorylation level of Keap1 stimulated by EGFR, suggesting that these three tyrosine residues are major target sites of Keap1 phosphorylated by EGFR. Although the protein level of Keap1 Y141F mutant was lower than that of wild type Keap1, which is consistent to previous study [25], this mutant did not affect the tyrosine phosphorylation of Keap1 upon EGF stimulation, indicating that EGFR is not responsible for this phosphorylation. Thus, the phosphorylation of Keap1 at Y141 is likely functionally different from the EGFR-induced phosphorylation of Keap1. The function of Y85 phosphorylation appears to be also different from that of the Y206/Y263/Y334 phosphorylation by EGFR. For instance, antioxidative stress-mediated tyrosine phos-

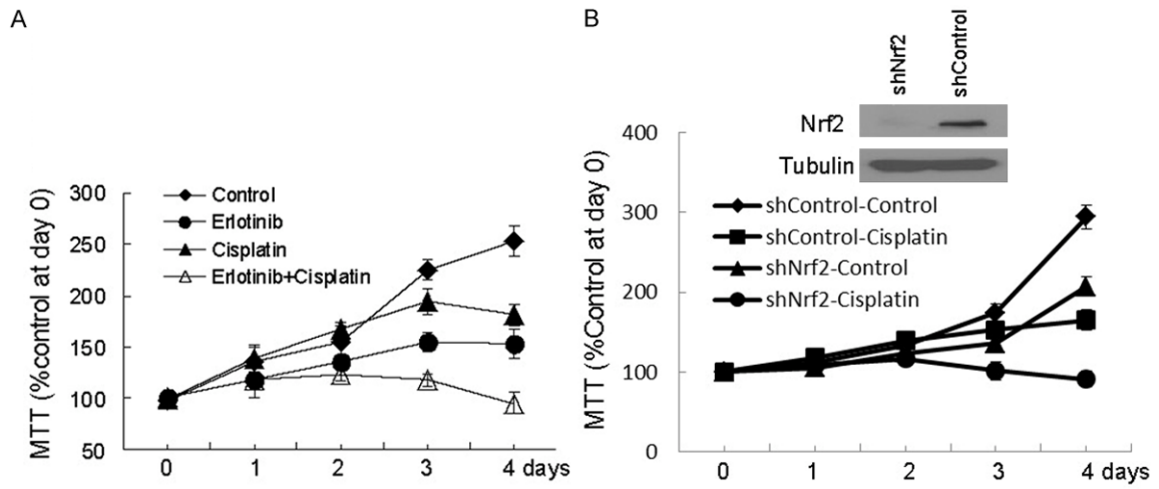

Figure 8. EGFR increases Nrf2-mediated drug insensitivity. (A) MDA-MB-468 cells in 96-well plates were incubated overnight followed by treatment with erlotinib, cisplatin, or both. Cells were then cultured for the indicated times. Cell proliferation was monitored by MTT assay (n = 6). (B) MDA-MB-468 cells stably expressing the indicated shRNAs (non-targeting shControl, and shNrf2) were used for MTT assay to monitor the cell proliferation under the indicated condition. Knockdown efficiency was examined by WB analysis shown in the upper panel of (B) (n = 6). Error bars: ± SD.

phorylation at Y85 of Keap1 decreased its nuclear protein level within 30 min and increased Keap1 ubiquitination in the cytosol [33] whereas activation of EGFR induced the reduction of nuclear Keap1 much slower (after one hour of EGF treatment) with increasing Keap1 ubiquitination in the nucleus but not in the cytosol. Therefore, Keap1 may regulate Nrf2 stability by different mechanisms in response to different stimuli.

Our finding indicate that EGFR induces phosphorylation and ubiquitination of nuclear Keap1, leading to proteasome-dependent reduction of Keap1 in the nucleus as proteasome inhibitor can stabilize nuclear Keap1. Our study is different from the previous report that ubiquitinated Keap1 is degraded via a proteasome-independent manner under oxidative stress [34]. This discrepancy may come from different experimental conditions. However, our data together with the study by Zhang *et al.* support the notion that cells can respond to different stimuli (e.g., tBHQ vs. EGF) in different ways to maintain cell homeostasis against environmental stress.

Our finding that EGF/EGFR-induced Nrf2 stability in the nucleus is in line with a recent report that EGFR activation regulates the function of Nrf2 in cell proliferation in non-small-cell lung cancer cells (NSCLCs) [20]. In NSCLC cells expressing both wild type EGFR and wild type Keap1 (e.g., NCI-H292), activation of EGFR increases Nrf2 protein stability and enhances cell proliferation under non-oxidative stress condition [20]. Although EGFR downstream MAPK pathway has been demonstrated to regulate Nrf2 activity in NSCLC [20], the results from promoter-reporter assay in our study indicated that EGFR itself is also important for directly regulating Nrf2 activity in cancer cells as MEK inhibitor did not completely block EGF/EGFR-induced ARE-containing promoter activity as did EGFR inhibitor. In addition, the loss of stimulatory effect of EGFR-ΔNLS on the phosphorylation and ubiquitination of Keap1 and consequently on the promoter activity of Nrf2 target gene further supports a direct functional role of nuclear EGFR in regulating nuclear Keap1/Nrf2 signaling. In conclusion, the novel function of nuclear EGFR in regulating Keap1/Nrf2 pathway we identified may have an important role in cell growth and drug resistance in human cancers.

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Disclosure of conflict of interest

None.

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