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IκB kinase-ε-mediated phosphorylation triggers IRF-1 degradation in breast cancer cells

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Abstract

Interferon Regulatory Factors (IRFs) are key regulators of immunity, cell survival and apoptosis. IRF transcriptional activity and subcellular localization are tightly regulated by posttranscriptional modifications including phosphorylation. The IκB kinase family member IKK-ε is essential in regulating antiviral innate immunity mediated by IRFs but is now also recognized as an oncoprotein amplified and overexpressed in breast cancer cell lines and patient-derived tumors. In the present study, we report that the tumor suppressor IRF-1 is a specific target of IKK-ε in breast cancer cells. IKK-ε-mediated phosphorylation of IRF-1 dramatically decreases IRF-1 protein stability, accelerating IRF-1 degradation and quenching IRF-1 transcriptional activity. Chemical inhibition of IKK-ε activity, fully restores IRF-1 levels and function and positively correlates with inhibition of cell growth and proliferation of breast cancer cells. By using a breast cancer cell line stably expressing a dominant negative version of IRF-1 we were able to demonstrate that IKK-ε preferentially exerts its oncogenic potential in breast cancer through the regulation of IRF-1 and point to the IKK-ε-mediated phosphorylation of IRF-1 as a therapeutic target to overcome IKK-ε-mediated tumorigenesis.

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Introduction

The nuclear factor kappa-B kinase (IκB kinase)-ε is a non-canonical member of the IκB kinase family [33,45] initially recognized for its role in the activation of specific NF-κB pathways [28]. Together with the IκB-related kinase TANK-binding kinase 1 (TBK-1), IKK-ε has been identified as the kinase activating IRF-3/7 and signal transducer and activator of transcription (STAT)-1 in response to viral infection or cellular stimulation [33,45,14,42,48]. In addition to its role in innate immunity, IKK-ε is recognized as an important element in cell proliferation and oncogenesis [8]. In particular, IKK-ε role as a proto-oncogene was emphasized by its overexpression in breast cancer cell lines and patient-derived tumors [4]. Ectopic expression of IKK-ε led to malignant transformation while its suppression by shRNA, or by a dominant negative form, induced inhibition of cellular anchorage and invasiveness of breast cancer cells [4,12,36]. IKK-ε-mediated oncogenic activity is determined by phosphorylation of multiple substrates including tumor suppressor CYLD, estrogen receptor α (ERα), tumor necrosis factor receptor-associated factor 2 (TRAF2), Forkhead box O 3a (FOXO3a), and Akt [19,20,22,44].

IRF-1 is the most multifunctional member of the interferon (IFN) regulatory factor (IRF) family of transcription factors being deeply implicated in the regulation of a broad spectrum of biological functions. These include hematopoietic differentiation, development and activation of immune cells, antiviral and antibacterial responses, cell growth control, susceptibility to transformation by oncogenes and induction of apoptosis in response to a variety of stimuli [2,39,47]. IRF-1 is recognized as a

Abbreviations: CHX, cycloheximide, CTR, control, CYLD, cylindromatosis, hEGF, human Epidermal Growth Factor, ERα, estrogen receptor α, FCS, fetal calf serum, FOXO3a, forkhead box O 3a, GST, glutathione S trasferase, HDM2, human double minute 2 protein, HS, horse serum, IFN, interferon, IKK-ε, inhibitor of nuclear factor kappa-B kinase-ε, IRFs, interferon regulatory factors, NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells, Ni-NTA, nickel-nitrilotriacetic acid, NRU, neutral red uptake, PBS, phosphate buffered saline, PCNA, Proliferating Cell Nuclear Antigen, SDS, sodium dodecyl sulphate, Stat-1, signal transducer and activator of transcription-1, TBK-1, TANK Binding Kinase 1, TRAF2, tumor necrosis factor receptor-associated factor 2, Ub, ubiquitin, WB, western blot, WCE, whole cell extract

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tumor suppressor gene and its antitumor activities are exerted either directly on tumor cells, through cyclin-dependent kinase inhibition, DNA-damage-induced cell-cycle arrest, stimulation of DNA repair proteins and induction of genes promoting apoptosis, or indirectly, by modulation of immune responses including development of natural killer cells, and differentiation of $CD4^+/CD8^+$ cells and Dendritic cells as reviewed [2,7,10]. IRF-1 is predominantly regulated at the transcriptional level [15,46] but posttranslational modifications including phosphorylation, sumoylation, acetylation and ubiquitination also play a significant, non-redundant role in the regulation of its activity [13,26,27,30,32,37,41,49].

We have previously reported that IRF-1, -3, and -7 are specific IKK-ε substrates. IKK-ε-mediated phosphorylation of IRF-1 negatively affects IRF-1 transcriptional activity in $CD4^+$T cell activation [41]. In the present work we show that IRF-1 phosphorylation by IKK-ε strongly influences the stability of the IRF-1 protein in breast cancer cells by accelerating proteasome mediated degradation. Importantly, recovering IRF-1 expression using an IKK-ε chemical inhibitor, both restores IRF-1 transcriptional activity and induces cell growth arrest signals. The antiproliferative effect exerted by IKK-ε chemical inhibition is almost completely abolished in MCF7 breast cancer cells stably expressing an IRF-1 dominant negative version (dnIRF-1) that inhibits IRF-1 regulation of its target genes. Our results indicate that the IRF-1 degradation that occurs following IKK-ε-mediated phosphorylation is the critical step in IKK-ε-induced cellular transformation leading to breast cancer.

Experimental procedures

Cell culture and reagents

HEK293 and MCF7 cells were purchased from the American Tissue Culture Collection (ATCC) and cultured in Dulbecco modified Eagle medium (DMEM) (Bio-Whittaker, Cambrex Bio Science, Verviers, Belgium), supplemented with 10% fetal calf serum (FCS), glutamine and antibiotics. MCF10A cells, a generous gift of P.G. Natali (Institute Regina Elena, Rome, Italy), were cultured in DMEM-F12 containing 5% horse serum (HS), antibiotics, hydrocortisone (0.5 μg/ml), hEGF (20 ng/mL). Insulin (10 μg/ml) was also added to MCF7 and MCF10A growth medium. MCF7/dnIRF-1 cells and MCF7/control cells were previously described [6]. Small molecule CAY10576 (Cayman chemical Company) was used at 0.5, 1 and 2 μM as indicated; CHX (Sigma) and MG132 (Sigma) were used at 25 μg/ml and 50 μM, respectively.

Plasmids, transient transfection and reporter gene assay

CMVBL, CMVBL IRF-1, mutants CMVBL IRF-1 3A, CMVBL IRF-1 3D and pCDNA3.1 IKK-ε and its mutated form (IKKε 1–361) expression vectors have been described previously [41,42]. pRL-βactin was a generous gift of T. Matsuyama (Nagasaki University School of Medicine, Nagasaki, Japan). pCDNA3.1 Ub-His(6x) was a kind gift of T. Haas (Department of Experimental Oncology, IFO, Rome, Italy). The glutathione S-transferase (GST)-IRF-1 NH_2 terminus (amino acids [aa] 1–180) and the GST-IRF-1 COOH terminus (amino acids [aa] 181–240) have been described previously [41]. IRF-1-responding luciferase reporter constructs pISRE-TA-LUC was purchased from Clontech. Transient-transfections were performed using JetPei reagent (Polyplus Transfection SA, Illkirch, France) according to the manufacturer's protocol. Reagents from Promega Corp. (Madison, WI) were used to assay extracts for dual-luciferase activity in a Lumat LB9501 luminometer (E&G Berthold, Bad Wildbad, Germany).

Whole cell extracts (WCE) 0.5 μg were added to kinase buffer (10 mM HEPES pH 7.4, 5 mM $MgCl_2$, 50 μM orthovanadate, 10 mM β-glycerophosphate, 1 mM DTT, 50 mM NaCl, 10 mM p-nitrophenylphosphate [PNPP]). An *in vitro* kinase assay was performed by adding the GST-purified constructs, GST-IRF-1 (1–180) or GST-IRF-1 (181–240) (1 μg), to 10 μM cold ATP, and 10 μCi of [γ-^{32}P] ATP in kinase buffer. The kinase reaction was performed at 30 °C for 30 min and stopped by the addition of sodium dodecyl sulphate (SDS) sample buffer. Samples were analyzed by 10% SDS-PAGE followed by Coomassie staining. The dried gels were exposed to film at −70 °C for 5 h.

CIP

WCE (50 μg) from MCF7 confluent cells were incubated with 5 U of Calf Intestine alkaline Phosphatase (CIP) (New England Bioscience) in CIP buffer for 1 h at 37 °C and then SDS PAGE loading buffer was added followed by heat (90 °C for 5 min) mediated protein denaturation and samples were assessed using SDS-PAGE and Western blot analysis.

In vitro ubiquitination assay

HEK293 cells were seeded (2×10^6 in 10 cm diameter plates) and were co-transfected with expression plasmids encoding Ubiquitin-His (6x), pCDNA3.1, IKK-ε and/or IRF-1 wild type (IRF-1 wt) or IRF-1 mutant (IRF-1 3A). Cells were lysed 24 h after transfection in 6 ml of buffer A (6 M guanidium-HCl, 10 mM Tris/HCl pH 8.0, 100 mM Na_2HPO_4/NaH_2PO_4 pH 8.0, 5 mM imidazole and 10 mM β-mercaptoethanol) and sonicated. Extracts were incubated with 70 μl of Nickel-NTA-agarose resin (Ni-NTA) (Qiagen) overnight at 4 °C. Resin was then washed once in buffer B (8 M Urea, 100 mM Na_2HPO_4/NaH_2PO_4 pH8, 10 mM Tris/HCl pH 8.0 and 10 mM β-mercaptoethanol), twice in buffer C (8 M Urea, 100 mM Na_2HPO_4/NaH_2PO_4 pH 6.3, 10 mM Tris/HCl pH6.3, 10 mM β-mercaptoethanol and 0.2% Triton X-100) and once in buffer C plus 0.1% Triton. Resin was then eluted with 50 μl of buffer D (0.15 M Tris-HCl pH 6.7, 30% glycerol, 0.72 M β-mercaptoethanol, 5% SDS supplemented with 200 mM imidazole) and put under stirring for 20 min at room temperature. Sample buffer was added and the supernatants were subjected to SDS-PAGE and Western blot analysis. Ectopically expressed IRF-1 wt, IRF-1 mutant, and IKK-ε were detected with specific antibodies. Expression of the β-actin protein was used as loading control.

Immunoprecipitation, Western blot analysis, and protein quantifications

WCE from MCF7, MCF7/dnIRF-1, MCF7/control, or MCF10A cells were prepared and subjected to Western Blot analysis or immunoprecipitation as previously described [37]. Briefly, 300 μg of WCE were incubated with 1 μg of polyclonal anti-IRF-1 antibody (sc-13041 Santa Cruz Biotechnology Inc., Santa Cruz, CA.) overnight at 4 °C and then Ultralink immobilized protein A/G-Sepharose (Pierce Biotechnology, Rockford, IL) was added for 2 h at room temperature. After extensive washing, immunoprecipitates were eluted by boiling the beads for 3 min in SDS sample buffer and then subjected to Western Blot analysis. IRF-1 and IRF-1 mutated form (IRF-1 3A) were detected by anti-IRF-1 (sc-497 Santa Cruz Biotechnology) antibody; anti-UbK48 Apu2, anti-E2F1 and anti-CyclinA were from Millipore; anti-IKK-ε was from Active Motive; anti-phospho-IKK epsilon (Ser172) antibody were from Cell Signaling Technology; anti-p21, anti-PCNA were from Santa Cruz Biotechnology. Levels of IRF-1, p21, E2F1, Cyclin A and PCNA proteins, relative to levels of endogenous actin protein were quantified using UVP Vision Works LS Image Acquisition software. Anti-actin antibody (Santa Cruz Biotechnology) was used in each experiment as protein loading control; the secondary antibody was from Calbiochem.

Neutral red uptake assay

Neutral red uptake (NRU) assay was performed as described [38]. In brief, 1×10^4 MCF7/well were seeded in 96-well plates and exposed to different concentrations of CAY10576 (0–2 μM) for 24, 48 and 72 h. At the end of the exposure time, cells were washed with phosphate-buffered saline (PBS) before being incubated for 3 h in medium supplemented with neutral red (50 μg/mL). The medium was washed off rapidly with PBS and the cells incubated for a further 15 min at R.T. in a mixture of acetic acid (1%) and ethanol (50%) to extract the dye. Absorbance was then measured at 540 nm using a micro-plate reader (Biorad). Neutral red powder was purchased from Sigma-Aldrich.

Wound healing assay

For the wound healing assay (WHA), 1×10^4 MCF7, MCF7/dnIRF1, or MCF7/control cells were seeded on a six-well plate. After achieving confluence, the cellular layer in each well was scratched using a 10 μl plastic pipette tip to create wounds and treated with different concentrations of CAY10576 inhibitor (0–2 μM), then incubated at 37 °C, 5% CO_2. The rate of wound repair was monitored using an inverted microscope (EVOS Floid Cell Imaging) and wound recovery was compared at 24, 48, and 72 h after CAY10576 treatment with untreated control cells. The percentage of wound closure was measured with the formula: percentage (%) of wound closure = $(At_0 - At_x)/At_0 \times 100$ where At_0 is the area of the wound measured immediately after the scratch, At_x the area measured hours after the scratch is performed [17]. Statistical analysis was performed between the control groups and the groups treated with CAY10576 (0.5, 1 and 2 μM) at different times (24, 48 and 72 h).

Statistical analysis

All experiments in the present study were performed at least three times and similar results were obtained. The statistical analysis was made in part through the ``two tailed paired Student's *T* test'' and in part, in the case of multiple comparisons, using the ``one-way analysis of variance'' (ANOVA), followed by a appropriate post-hoc test. The program GraphPad Prism software was used. A value of at least $P < 0.05$ was considered to be statistically significant.

Results

Activated IKK-ε-kinase induces IRF-1 phosphorylation in MCF7 breast cancer cells

IKK-ε is aberrantly expressed in a high percentage of human breast cancers [4] that are also characterized by loss of IRF-1 [9]. Since we have previously shown that IRF-1 is a target of IKK-ε-mediated phosphorylation in T cells, we sought to investigate whether a functional correlation between IKK-ε overexpression and undetectable IRF-1 levels is present in breast cancer cells. Therefore, IRF-1 and IKK-ε protein expression and activation were analyzed in MCF7 cells as compared with non-tumorigenic MCF10A breast cells. IRF-1 and IKK-ε proteins were measured in cells at different growth density since it has been reported that, in non-immune cells, IRF-1 levels are dependent on cell growth, being undetectable in growing cells and maximally expressed when cell cultures reach confluence [21,23]. Western blot analysis indicated that IKK-ε was overexpressed in MCF7 cells regardless of cell density when compared with MCF10A control cells where IKK-ε was only detectable in fully confluent cultures (compare Fig. 1A: lanes 3 and 4 with lanes 1 and 2). Analysis of IKK-ε activation using a specific antibody recognizing the phosphorylated form of the protein (IKK-ε S172) showed that the active form of IKK-ε was constantly expressed during cell growth of MCF7 cells at comparable levels, while it was totally absent in MCF10A non-tumorigenic cells, regardless of cell density.

Comparative analysis of IRF-1 expression indicated that in control cells, levels of the protein increased as cells approached confluence (Fig. 1A: lanes 1 and 2), as expected. In tumorigenic cells, IRF-1 protein was absent in growing cells (Fig. 1A: lane 3), while in confluent cells IRF-1 was detected. A protein band with a slower mobility was also present (IRF-1 II) (Fig. 1A: lane 4, versus lane 2). Based on our previous data from T cells showing that IKK-ε can phosphorylate IRF-1, we hypothesized that the IRF-1 II band could represent the phosphorylated form of the protein (Fig. 1A: lane 4). To test this hypothesis, we performed a CIP (Calf intestine alkaline phosphatase) treatment on WCEs from MCF7 confluent cells determining the disappearance of the upper band and the corresponding increase in the lower band (IRF-1 I) as shown in Fig. 1B: lane 2, demonstrating the presence of phosphorylated IRF1 in MCF7 confluent cells. Thus we performed an in vitro kinase assays (KA), by using cell extracts from MCF7 and MCF10A and, as substrates, the NH_2-terminal GST-IRF-1 1–180 and the COOH-terminal GST-IRF-1 181–240, the latter carrying the IKK-ε phosphorylation cluster that we already identified on IRF-1 [41]. As shown in Fig. 1C, a constitutive IRF-1 phosphorylation was detected within the NH_2-terminal fragment in cell extracts from both cell lines. Conversely, a clear phosphorylation of the GST-IRF-1 (181–240) COOH fragment, containing the IKK-ε cluster, was observed only in MCF7 tumorigenic cells. The specificity of the IKK-ε-mediated IRF-1 phosphorylation in transformed cells was further confirmed by the IRF-1 shift from form II to form I in confluent MCF7 cells upon treatment with an IKK-ε chemical inhibitor CAY10576 (Fig. 1D) [1].

Dose-response experiments with CAY10576 in MCF7 cells that do not express IRF-1 protein showed an increase in IRF-1 starting from 24 hours with a maximum increase at 48 hours. (Fig. 1E). Similarly, transfection with an IKK-ε dominant negative construct (IKK-ε 1–361) increased IRF-1 levels in MCF7 cells (Fig. 1F). These results support a role for IKK-ε kinase-mediated phosphorylation of IRF-1 as a driver of IRF-1 loss in breast cancer cells.

IKK-ε-mediated phosphorylation of IRF-1 in its COOH-terminal affects IRF-1 protein stability

To determine how IKK-ε regulated IRF-1 protein levels in cancer cells, we asked whether IKK-ε could modulate IRF-1 protein stability. We measured the decay rate of IRF-1 in the absence and in the presence of IKK-ε. Initially, we used the cell model system of HEK 293. Cells were transfected with IRF-1 wt or IRF-1 3D, a phosphomimetic mutant of IRF-1 in which Ser215, Ser219 and Ser221 were substituted with aspartic acid [D] [41], with or without IKK-ε expression vector, in the presence of the protein synthesis inhibitor cycloheximide (CHX) for different times. IKK-ε expression induced a significant acceleration in IRF-1 decay (compare Fig. 2A: lanes 5 and 6 with lanes 2 and 3). The accelerated IRF-1 turnover was abrogated by pretreatment with the IKK-ε inhibitor CAY10576, which appeared to stabilize the IRF-1 protein (Fig. 2A: lanes 7–9). Conversely, even in the absence of IKK-ε, the phosphomimetic IRF-1 mutant (IRF-1 3D) showed a rate of decay comparable to that of IRF-1 wt in the presence of IKK-ε (lanes 11,12 versus 5,6 and values in the graph). Densitometric quantification of IRF-1 protein levels (graph at the bottom) indicated that in the absence of IKK-ε expression, the half-life of IRF-1 was 50 min in HEK 293 cells. In the presence of IKK-ε this half-life was reduced to ∼37 min. The IRF-1 3D mutant half-life was comparable to that of IRF-1 wt in presence of IKK-ε ($T_{1/2}$: ∼33 min). Finally, CAY10576 treatment induced a stabilization of the protein ($T_{1/2}$: >120 min).

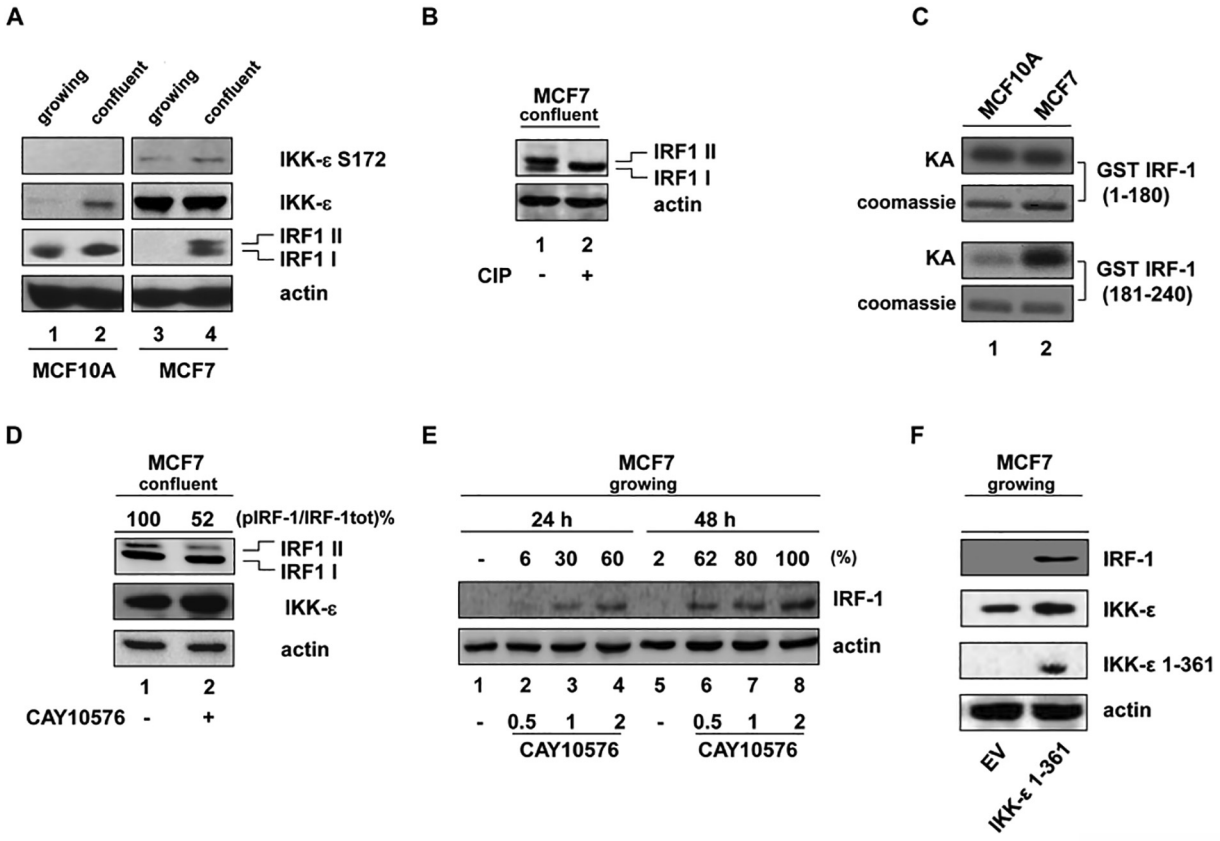


Fig. 1. IKK-ε- kinase activation induces IRF-1 phosphorylation in MCF7 breast cancer cells. (A) Whole cell extracts (WCE) (50 μg) of growing and confluent MCF10A and MCF7 cells were analyzed by Western blotting (WB) using anti-IKK-ε, anti-phosphoIKK-ε (IKK-ε S172) and anti-IRF-1 specific antibodies. (B) (WCE) (50 μg) from MCF7 confluent cells were incubated with 5U of Calf Intestine alkaline Phosphatase (CIP) in a CIP assay then analyzed by WB using anti-IRF-1 and anti-actin specific antibodies (C) WCE (0.5 μg) of MCF10A and MCF7 were used in *in vitro* kinase assay (KA) using, as substrates, recombinant GST-IRF-1 fusion proteins containing fragments of the NH2-terminal (1–180) and COOH-terminal (181–240) of IRF-1. Coomassie staining of the recombinant proteins is also shown. (D) WCE of confluent MCF7 cells were treated with the IKK-ε inhibitor CAY10576 (2 μM) for 24h and analyzed by WB using anti-IKK-ε and anti-IRF-1 specific antibodies, the % of pIRF1/IRF1tot is indicated. (E) Growing MCF7 cells were treated with increasing doses of CAY10576 or (F) transfected with a dominant negative form of IKK-ε (IKK-ε 1–361). After 24 h in (F) or as indicated in (E) WCE (50 μg) were analyzed by WB, using anti-IRF-1, anti-IKK-ε and anti-actin antibodies. In (E) the intensity of the IRF-1-specific bands was measured by densitometry and was reported as the percentage of IRF-1 expression after normalization with actin levels.

Since IRF-1 is degraded through the ubiquitin/proteasome pathway, we wondered whether IKK-ε could stimulate IRF-1 polyubiquitination and proteasomal degradation. IRF-1 and six-histidine-tagged ubiquitin (His6-Ub) were co-expressed in the presence or absence of IKK-ε. IRF-1 ubiquitination was then determined by capturing His6-Ub in cell extracts with nickel beads (Ni-NTA), followed by Western blot analysis of the purified ubiquitin conjugates with IRF-1-specific antibodies. Basal IRF-1 ubiquitination was detected in the His-Ub- and IRF-1-expressing cells. Maximum IRF-1 polyubiquitination was detected in IKK-ε-containing extracts (Fig. 2B: lane 2 versus lane 1). Treatment with CAY10576 reversed the rate of IRF-1 ubiquitination induced by IKK-ε (Fig. 2B: lane 3 versus lane 2).

To confirm further the specificity of IKK-ε-mediated IRF-1 phosphorylation on the rate of IRF-1 degradation, we also examined the mutant IRF-1 3A, where Ser215, Ser219 and Ser221 were modified to the inert residue alanine [A]. IRF1 3A is not phosphorylated by IKK-ε [41]. As shown in Fig. 3A and graph, IRF-1 3A half-life increased significantly when compared with IRF-1 wt (lanes 8,9 versus 2,3) and IRF-1 3A was not significantly affected by the expression of IKK-ε (lanes 11,12 versus 8,9 and graph). Data from the nickel capture assay confirmed that the IRF-1 3A mutant was resistant to ubiquitination induced by IKK-ε (Fig. 3B lane 4 versus 2).

IKK-ε induces K48 polyubiquitination and proteasomal-mediated IRF-1 degradation in MCF7 breast cancer cells

To demonstrate that the increased turnover and ubiquitination of IRF-1 observed in the cell model system of HEK293 cells in the presence of IKK-ε was responsible for the lack of IRF-1 expression in IKK-ε highly expressing MCF7 breast cancer cells, we examined the IRF-1 degradation rate in MCF7 as compared with MCF10A cells. Breast cells were treated with CHX in the absence and presence of CAY10576 and IRF-1 expression levels were measured by Western blotting. As shown in Fig. 4A, the levels of IRF-1 detected in confluent MCF7 cells fell rapidly along with the phosphorylated form of IRF-1. A slower turnover of IRF-1 was observed in MCF10A (Fig. 4A: lanes 5 and 6 versus lanes 2 and 3). Consistent with the hypothesis that the rapid turnover of IRF-1 in MCF7 cells was due to IKK-ε-mediated IRF-1 phosphorylation, treatment with CAY10576 restored the rate of IRF-1 decay to levels comparable to those

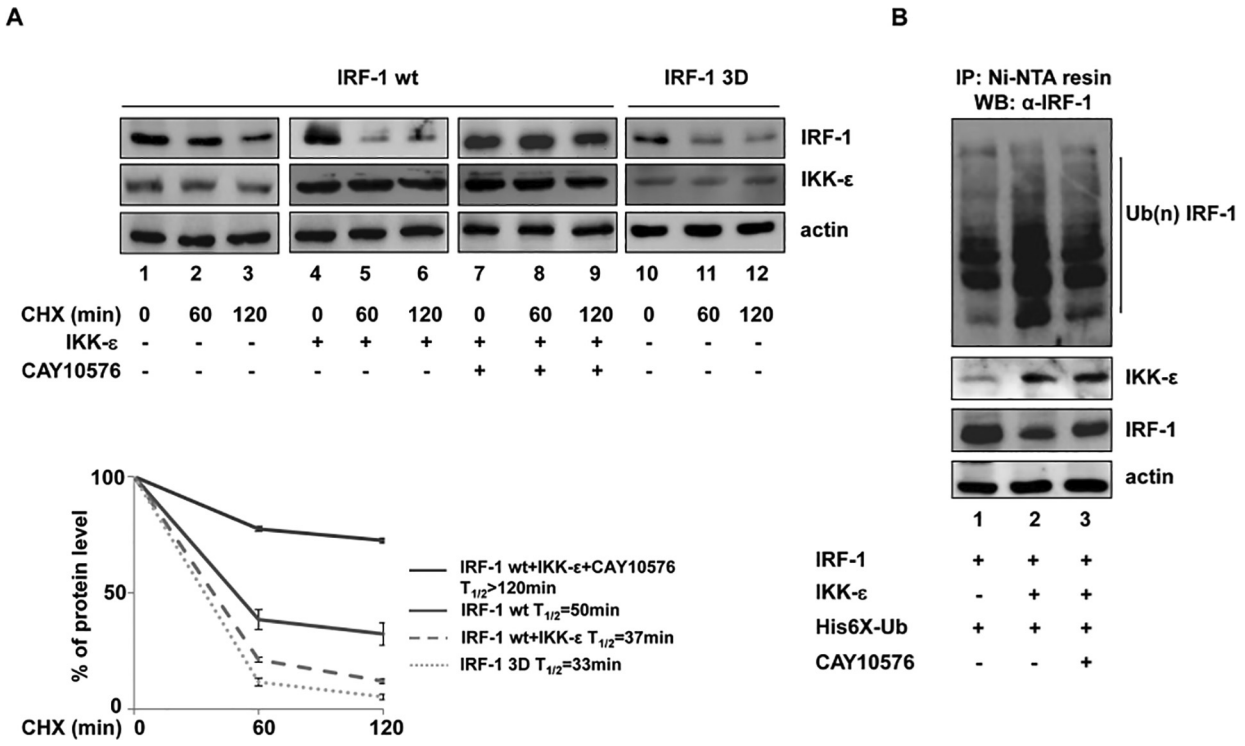


Fig. 2. IKK-ε-mediated phosphorylation of IRF-1 in its COOH-terminal affects IRF-1 protein stability. (A) HEK293 cells were transfected with IRF-1 wt or IRF-1 3D in the presence or in the absence of IKK-ε expression vector and where indicated were treated with CAY10576 (2 μM) inhibitor for 24 h and then with CHX for the indicated time. WCE (30 μg) were analyzed by WB and IRF-1, IRF-1 3D and IKK-ε proteins were detected with anti-IRF-1 and anti-IKK-ε specific antibodies, respectively. Data plotted in the graph at the bottom represent the means þ SEM, from three different assays, of IRF-1 protein bands quantified from WB, normalized to actin protein levels and presented as percentage values relative to those without CHX treatment set at 100%. (B) HEK293 cells were cotransfected with expression vectors for His6X-Ub and IRF-1 in the presence or in the absence of IKK-ε expression vector and, where indicated, treated with CAY10576 (2 μM) for 24h. His-Ub-conjugated proteins were captured by nickel-agarose beads (Ni-NTA resin), eluted, and analyzed by WB with anti-IRF-1 antibodies. WB of cell lysates shows the expression of ectopically expressed proteins.

observed in MCF10A control cells (Fig. 4A: lanes 8 and 9 versus lanes 2 and 3, and the data in the graph on the right). Furthermore, when we treated MCF7 cells with the proteasome inhibitor MG132, IRF-1 decay was inhibited and both forms of the protein were stabilized (Fig. 4A: lanes 10–12).

Since proteasomal degradation is triggered by the formation of K48-linked polyubiquitination chains, we measured the levels of IRF-1-K48 poly-ubiquitination in MCF7 and MCF10A by immunoprecipitation assay of endogenous IRF-1 followed by detection of IRF-1-linked polyubiquitination chains with antibodies specific for K48-linked ubiquitin. As shown in Fig. 4B a substantial increase in K48-polyubiquination of IRF-1 was observed in MCF7 as compared with MCF10A control cells (compare lane 2 with lane 1). Treatment with the CAY10576 inhibitor (lane 3) reduced the levels of IRF-1K48-ubiquitination to those observed in MCF10A cells. To further confirm that the K-48 ubiquitination and degradation of IRF-1 was triggered by IKK-ε-mediated IRF-1 phosphorylation, we compared the polyubiquitination of the 3A IRF-1 mutant with IRF-1 wt following their respective transfection in MCF7 cells. The results in Fig. 4C clearly show that the levels of IRF-1 3A K-48 polyubiquitination were substantially lower as compared with those of IRF-1 wt (Fig. 4C: compare lane 2 with lane 1).

To address the functional consequences of the accelerated IRF-1 turnover in MCF7 cells, we examined how IKK-ε affected IRF-1-dependent gene expression. Transcriptional activity of the IRF-1-responsive reporter construct pISRE-TA-LUC was measured in MCF7 cells co-transfected with IRF-1 wt or IRF-1 3A mutant in the absence or presence of CAY10576 (IKK-ε inhibitor). As shown in Fig. 4D, the low transcriptional activity of IRF-1 was significantly increased by treatment with the IKK-ε inhibitor (lane 4 versus lane 3). Interestingly, treatment with the inhibitor significantly increased the transcriptional activity of endogenous IRF-1 (lane 2 versus lane 1). The IRF-1 3A mutant maximally induced pISRE-TA promoter activity (lane 5) and was not affected by the CAY10576 treatment (lane 6).

The CAY10576-mediated increase in IRF-1 levels and activity decreases proliferation and migration potential of MCF-7 breast cancer cells

To examine the impact of IKK-ε inhibition on the phenotype of breast cancer cells we determined the effects of CAY10576 on the proliferation and migration potential of MCF7 cells by Neutral Red uptake assay (NRU) and wound healing assay (WHA). A marked decrease in cell proliferation was evident in cells treated with the inhibitor in a time and dose-dependent manner (Fig. 5A). This result correlated with the expression levels of IRF-1 and IRF-1-regulated genes affecting cell proliferation as p21, E2F1, CyclinA and PCNA (Fig. 5B). As shown in Fig. 5C (upper panel), MCF-7 cell migration was profoundly decreased in the presence of the IKK-ε inhibitor as assessed by the changes in wound size in the treated groups when compared with control group at the designated time points. Comparison of quantified results showed that the effect of CAY10576 on wound closure in MCF7 cells was dose- and time-dependent (Fig. 5C lower panel).

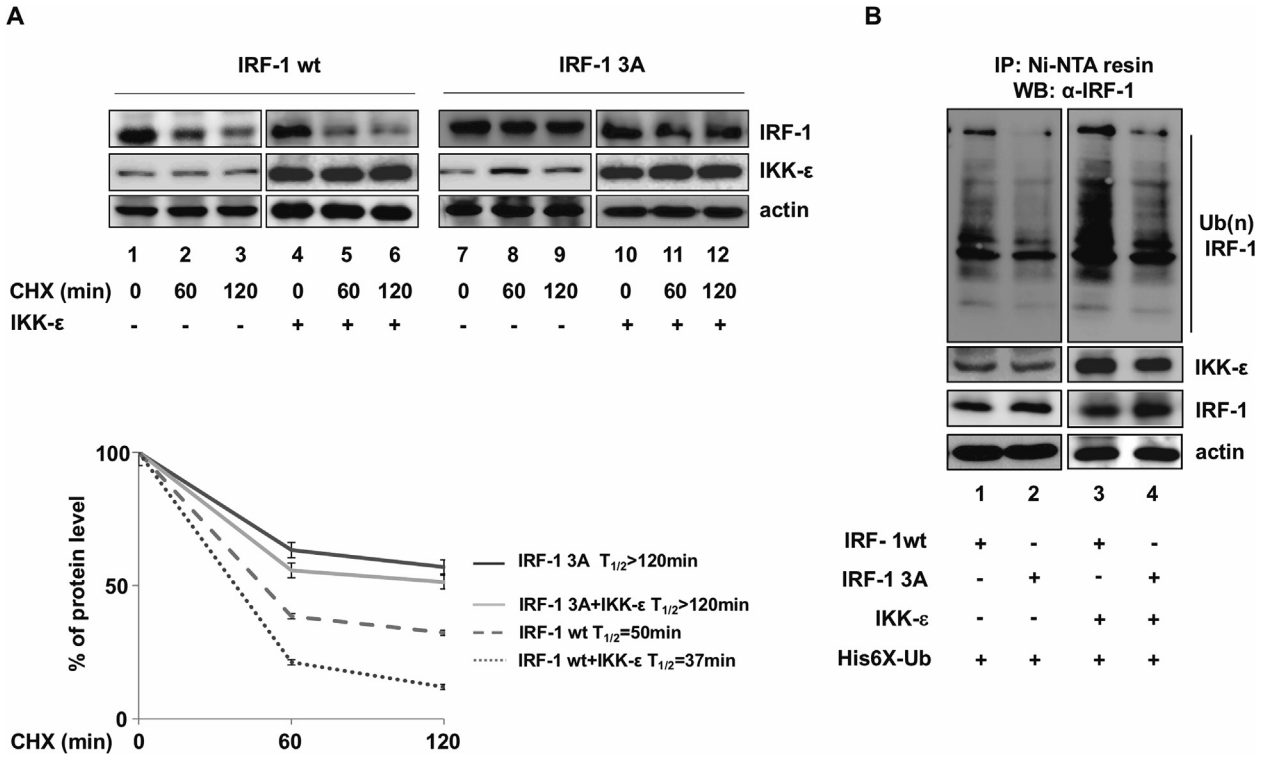


Fig. 3. IRF-1 mutated in IKK-ε phosphoacceptor target site shows an increased stability as compared with IRF-1 wt. (A) HEK293 cells were transfected with IRF-1 wt or the IRF-1 mutant IRF-1 3A, in the presence or in the absence of IKK-ε expression vector, as indicated. Twenty four hours after transfection, cells were treated with CHX for the indicated time and the expression of IRF-1, IRF-1 3A and IKK-ε was detected by WB using specific antibodies. Data plotted in the graph represent the means þ SEM from three different assays quantified as indicated in the legend of Fig. 2A. (B) HEK293 cells were cotransfected with His6X-Ub and IRF-1 wt or IRF-1 3A and ,where indicated, with IKK-ε expression vector. His-Ub-conjugated proteins were captured by Ni-NTA resin, eluted and analyzed by WB with anti-IRF-1 antibodies. WB of cell lysates shows the ectopically expressed proteins.

A dominant negative IRF1 (dnIRF-1) inhibits IRF-1-mediated attenuation of cell growth after CAY10576 treatment

We established that the IKK-ε-induced proliferation and migration of breast cancer cells was mediated by the inhibition of IRF-1 using the MCF7/dnIRF-1 cells that stably express a dominant negative IRF1 and their MCF7 empty vector controls [6]. Cells were treated with CAY10576 in a time and dose-dependent manner and cell migration was measured using the WHA. Fig. 6A (right panel) shows that wound closure in MCF7/control cells was reduced in the presence of increasing doses of the IKK-ε inhibitor. In contrast, MCF7/dnIRF1 cells recover cellular wounds at a rate comparable to control groups (CTR) and independent of IKK-ε inhibition (Fig. 6A left panel). Analysis of the quantified results in the graph at the bottom of Fig. 6A showed that the effect of CAY10576 on wound closure was dose- and time-dependent in MCF7/-controls, whereas there was no effect on MCF7/dnIRF1 cells. These results correlated with the expression levels of IRF-1 regulated genes that influence cell proliferation including p21, Cyclin A, and PCNA (Fig. 6B). In Fig. 6, panel C, the expression of IRF-1 and IRF-1 dominant negative in MCF7/dnIRF1 and MCF7/control is shows.

Discussion

The development of a cancer is a multistep and multifactorial process in which proto-oncogene mutations and deregulated function of tumor suppressor genes allow cells to escape from both cell and tissue homeostasis. Several kinases play essential roles in most human cancers and kinase inhibitors are already in clinical use [3,18]. The non-canonical member of the IκB kinase family, IKK-ε, already known for its role in innate immunity, it is now referred also as an oncogene due to the fact that it has been found over-expressed in more than 30% of human breast cancers [4]. However, the exact molecular mechanism is unclear. While studies of IKK-ε involvement in oncogenesis have implicated NF-κB as a major effector of its transformation activity [43], several other IKK-ε substrates are known to regulate key cellular processes involved in cell transformation [19,20,22,44]. Thus, other pathways and substrates may play a non-redundant role in IKK-ε-induced transformation. Previously, we identified the tumor suppressor IRF-1 as an IKK-ε-specific substrate [41]. Here, we now establish that persistently activated endogenous IKK-ε mediates IRF-1 phosphorylation and induces IRF-1K48 ubiquitination and proteasome-mediated degradation in breast cancer cells (Fig. 4). Restoration of IRF-1 levels by blocking IKK-ε activity resulted in the inhibition of tumor cell growth and migration (Fig. 5). Conversely, the presence of the dnIRF-1 did not block the increase in IRF-1 protein levels produced by CAY10576 treatment (data not shown) and prevented the inhibition of tumor cell growth and migration (Fig. 6A and B). Taken together, these findings indicate that IKK-ε-mediated IRF-1 inactivation in breast cancer cells is critical for the loss of growth control.

IRF-1 regulates a wide range of biological processes including inhibition of cell proliferation and modulation of immune responses [39,47]. Inactivation of IRF-1 is evident in several cancers [13] including breast cancer [9]. Consistent with our results, a negative correlation of IRF-1 expression with tumor grade is reported in invasive breast cancers [50].

The biological activity of IRF-1 is regulated primarily at the transcriptional level, but post-translational modifications also play an important

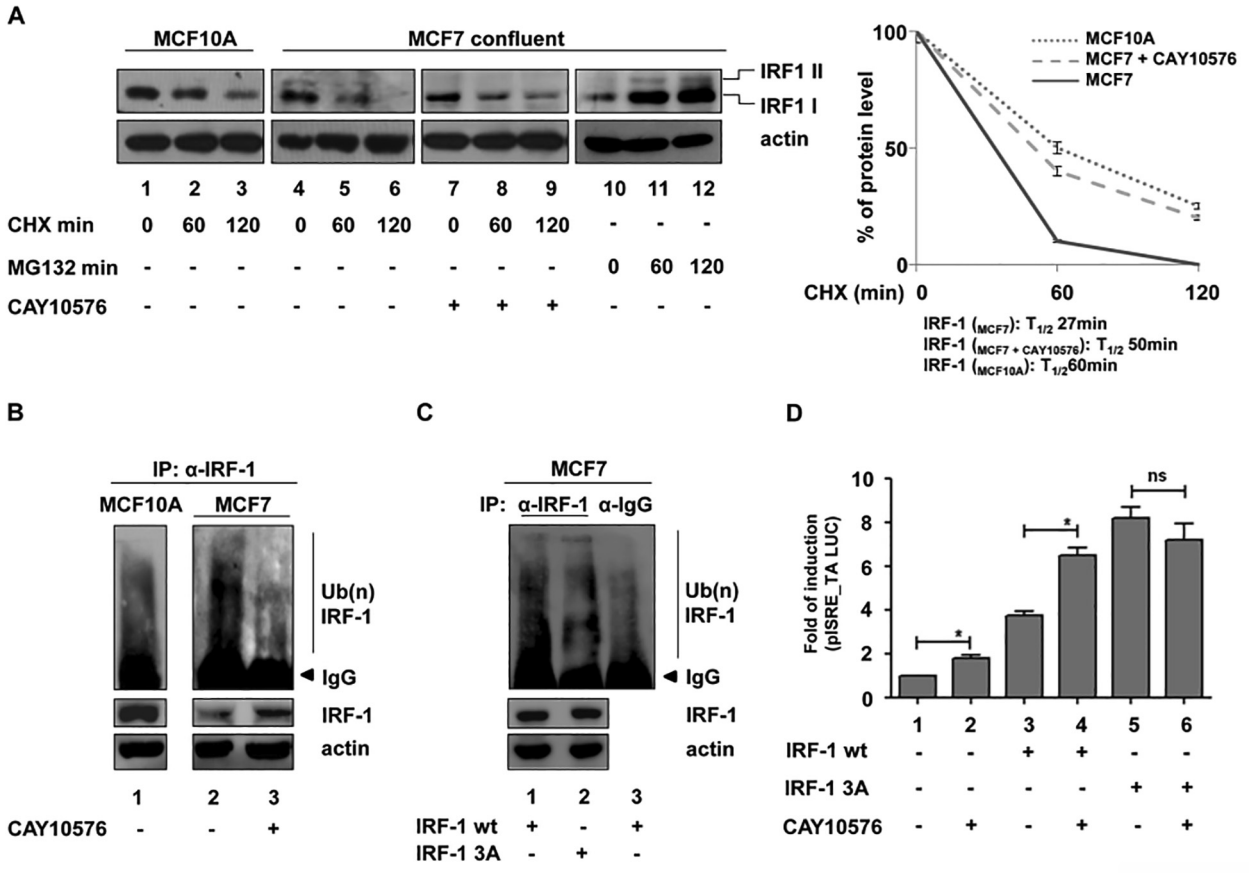


Fig. 4. IKK-ε induces accelerated IRF-1 turnover, K48 polyubiquitination and proteosomal-mediated degradation in MCF7 breast cancer cells. (A) MCF10A and MCF7 cells were treated with CHX for the indicated time and MG132 or CAY10576 inhibitor (2 μM), as indicated. Anti-IRF-1 antibodies were used to detect IRF-1 protein expression. Data plotted in the graph on the right represent the means þ SEM from three different assays of IRF-1 protein bands quantified as indicated in the legend of Fig. 2A. (B) WCE (300 μg) were prepared from MCF10A and MCF7 cells treated or not with CAY10576 (2 μM) for 24 h and immunoprecipitated with anti-IRF-1 specific antibodies. IRF-1 ubiquitinated forms ($Ub_{(n)}$ IRF-1) were then detected using anti-K48 ubiquitin antibodies (anti-UbK48). WB of cell lysates (30 μg) shows the expression of endogenous IRF-1. (C) MCF7 cells were transfected with IRF-1 wt or IRF-1 3A and WCE were immunoprecipitated using IRF-1 specific antibodies. IRF-1 ubiquitinated forms were detected as in (B). (D) MCF7 cells were co-transfected with a synthetic IRF-1-responding construct p*TA-ISRE*-Luc and expressing vectors for IRF-1 wt or IRF-1 3A. Where indicated cells were treated with CAY10576 (2 μM) for 24 h. The day after transfection, dual-luciferase assay was performed and means plus standard deviations (SD) from three separate experiments were calculated after normalization with the Renilla activity. Statistical analysis was performed using ANOVA test. Bars and asterisks indicate values significantly different (*$P < 0.05$; ns: not significative).

and non-redundant role. Specifically, sumoylation, ubiquitination, and acetylation are generally inhibitory on IRF-1 transcriptional activity and can prevent IRF-1 from binding to DNA, or cause IRF-1 degradation via the ubiquitin–proteasome pathway [24,27,29–30,32,35,37]. The modulatory role of phosphorylation on IRF-1 transcriptional activity has been similarly reported and several kinase target sequences have been identified. IRF-1 phosphorylation may result in activation or repression of IRF-1 transcriptional activity, depending on the kinase involved and the protein residues affected. However, the molecular mechanisms involved are not fully elucidated [26,31,41,49]. We have previously reported that IRF-1 is phosphorylated by IKK-ε at a consensus motif present in the C-terminal half of the protein, resembling the IKK-ε target sequence present on the tumor suppressor CYLD [22,41]. In T cells, IKK-ε-mediated IRF-1 phosphorylation repressed IRF-1 transcriptional activity by impairing the physical interaction between IRF-1 and the NF-κB RelA subunit and interfering with PCAF-mediated acetylation of NF-κB RelA [41]. Here, we have identified another mechanism of IRF-1 inactivation mediated by IKK-ε phosphorylation that regulates the degradation of IRF-1. In the presence of IKK-ε, IRF-1 degradation is accelerated (Fig. 2A, 2B, and Fig. 3A, 3B) through K48-linked polyubiquitination and proteasome activation (Fig. 4A-C).

Our studies are consistent with data showing that the IKK-ε target cluster in the C-terminal portion of IRF-1 is a regulatory domain that modulates transcriptional activity and the rate of protein degradation [34].

Recently Garvin et al. showed that phosphorylation of IRF-1 at T181 by GSK3β (glycogen synthase kinase 3β) was a signal for ubiquitin-dependent degradation of IRF-1. Fbxw7α (F-box/WD40 7) was shown to be the ubiquitin E3-ligase protein involved in this degradation process [16]. Whether the HDM2 E3 ubiquitin ligase that accelerates IRF-1 proteasome-mediated degradation in HIV-1-infected cells [37] is also involved in IKK-ε-mediated IRF-1 inactivation in breast cancer cells is under investigation.

We showed that the IKK-ε effect was specific. A mutant of IRF-1 where the IKK-ε phosphorylation target residues were replaced with alanine was more stable than the wild-type protein and lacked subsequent ubiquitination and degradation (Fig. 3). Moreover, the specific IKK-ε inhibitor CAY10576 reversed IRF-1 inactivation in breast cancer cells with a significant increase in IRF-1 expression and a full recovery of

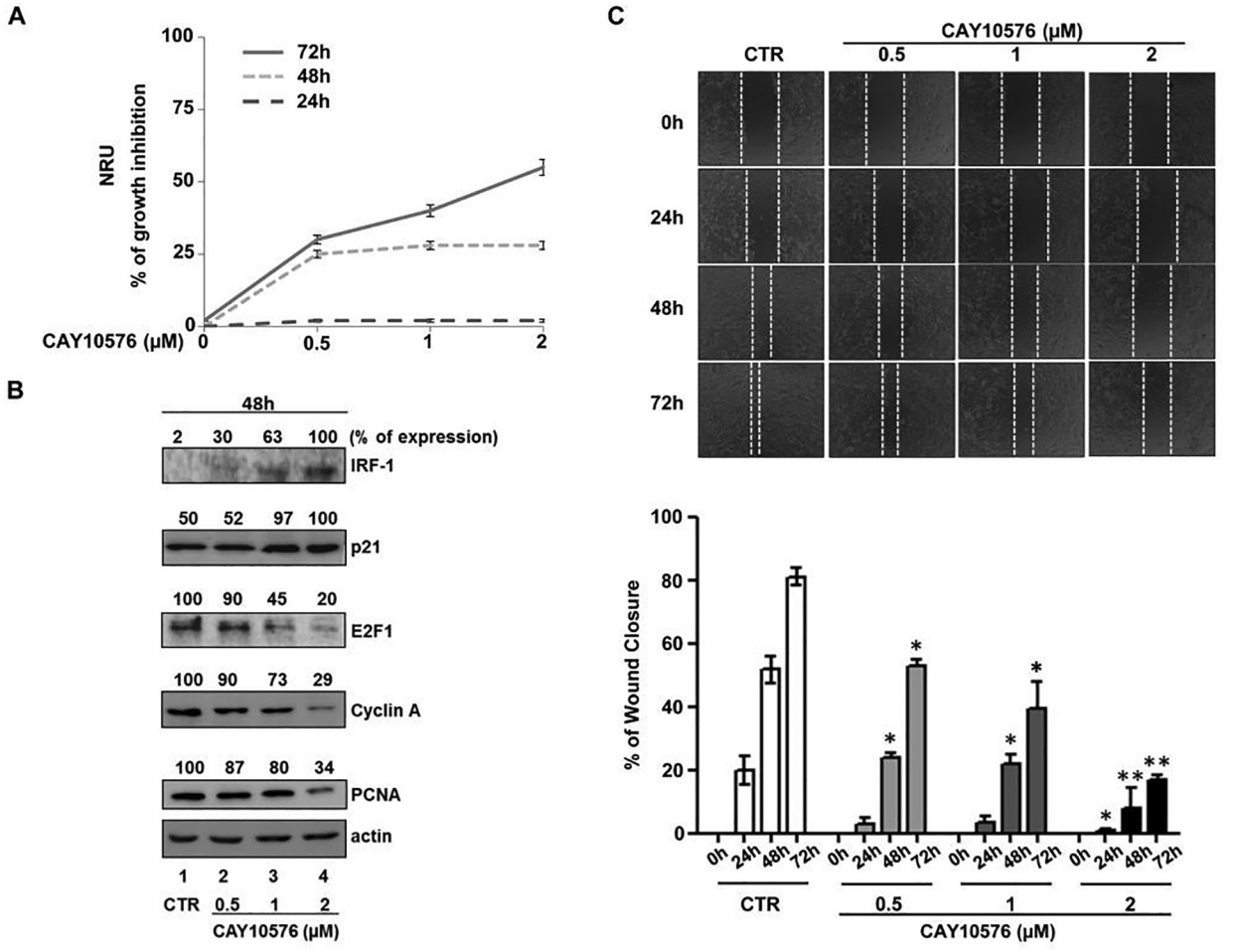
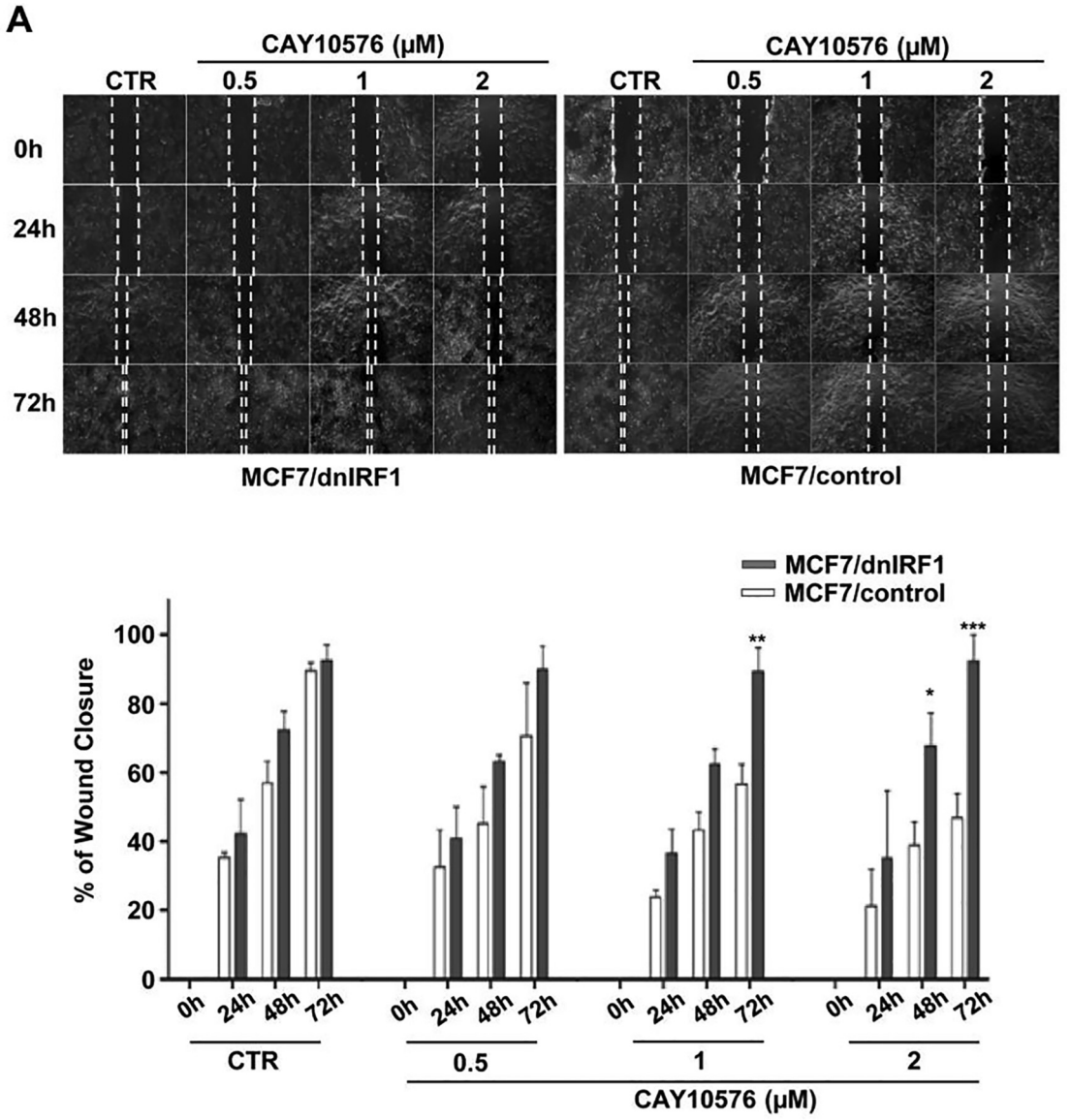


Fig. 5. The CAY10576-mediated increase in IRF-1 levels decreases proliferation and migration potential of MCF-7 breast cancer cells. (A) MCF7 cells were treated with CAY10756 at different doses (0.5–2 μM) for 24, 48, 72 h and the percentage of growth inhibition was detected using NRU assay. (B) MCF7 cells were treated with CAY10576 as in (A). WCE (50 μg) were used for WB and IRF-1, p21, E2F1, CyclinA, PCNA and actin were detected using specific antibodies. WB is representative of at least three independent experiments with similar results. (C) MCF7 cells were wounded and treated with CAY10756 as in (A). The closure of the wound was monitored with a bright field microscopy every 24 h. The picture is representative of one out of three experiments performed. The migration was quantified by the rate of the scratched area filled as indicated in ``Experimental procedures'' and graphed as percentage of wound closure. Statistical analysis was performed using pair to tales Student's *T* test comparing the treated groups at different time with the correspondent control group (*$P < 0.05$; **$P < 0.01$).

IRF-1 activity on target genes (Fig. 5B). The same recovery was not observed in breast cancer cell lines that overexpress dnIRF-1 [5] (Fig. 6B). These observations establish IRF-1 as a critical mediator of IKK-ε induced transformation. Our findings on growth regulation mediated by the IKK-ε inhibitor are consistent with the evidence that different IKK-ε chemical inhibitors are highly effective antiproliferative agents in several cancers [11,25].

The relative contribution of the different IKK-ε targets to growth inhibition following CAY10576 treatment remains to be determined. Nonetheless, our data suggest that the recovery of discrete levels of IRF-1 expression and activity is key. Consistently, in the presence of CAY10576, E2F1, CyclinA, PCNA and p21 expression levels correlated with IRF-1 expression in MCF7 cells (Fig. 5B). Hence, the regulated expression of these genes is directly related to the onco-suppressor properties of IRF-1. Several data are in agreement with the hypothesis that the loss of IRF-1 expression contributes to the dysregulation of growth in some breast cancers [40]. Consistently, increased IRF-1 levels correlate with a change in the cell phenotype, with a decrease in proliferation and

Fig. 6. MCF-7/dnIRF-1 breast cancer cells are unable to undergo the CAY10576-mediated inhibition of proliferation activity and migration potential. (A) MCF7/dnIRF-1 and MCF7/control cells were wounded and treated with CAY10756 at different doses (0.5–2 μM). The closure of the wound was monitored with a bright field microscopy every 24 h (24, 48, 72 h). The picture is representative of one out of three experiments performed. The migration was quantified by the rate of the scratched area filled as indicated in ``Experimental procedures'' and graphed as percentage of wound closure. Statistical analysis was performed using pair to tales Student's *T* test comparing the treated groups at different time with the correspondent control group (CTR) (*$P < 0.05$; **$P < 0.01$). (B) MCF7/dnIRF-1 and MCF7/control cells were treated with CAY10756 at different doses (0.5–2 μM) for 48 h. WCE (50 μg) were used for WB and IRF-1, IKK-ε, p21, CyclinA, PCNA and actin were detected using specific antibodies. (C) WCE (50 μg) by MCF7/dnIRF-1 and MCF7/control cells were used for WB IRF-1 and actin were detected using specific antibodies. WB is representative of at least three independent experiments with similar results.


A
CAY10576 (μM)
CTR
0.5
1
2
0h
24h
48h
72h
MCF7/dnIRF1
MCF7/control
MCF7/dnIRF1
MCF7/control
% of Wound Closure
100
80
60
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B
MCF7/dnIRF1
MCF7/control
100 90 90 95
38 62 69 100
(%)
p21
97 94 91 100
100 100 53 51
PCNA
100 93 90 100
100 72 29 31
CyclinA
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5 6 7 8
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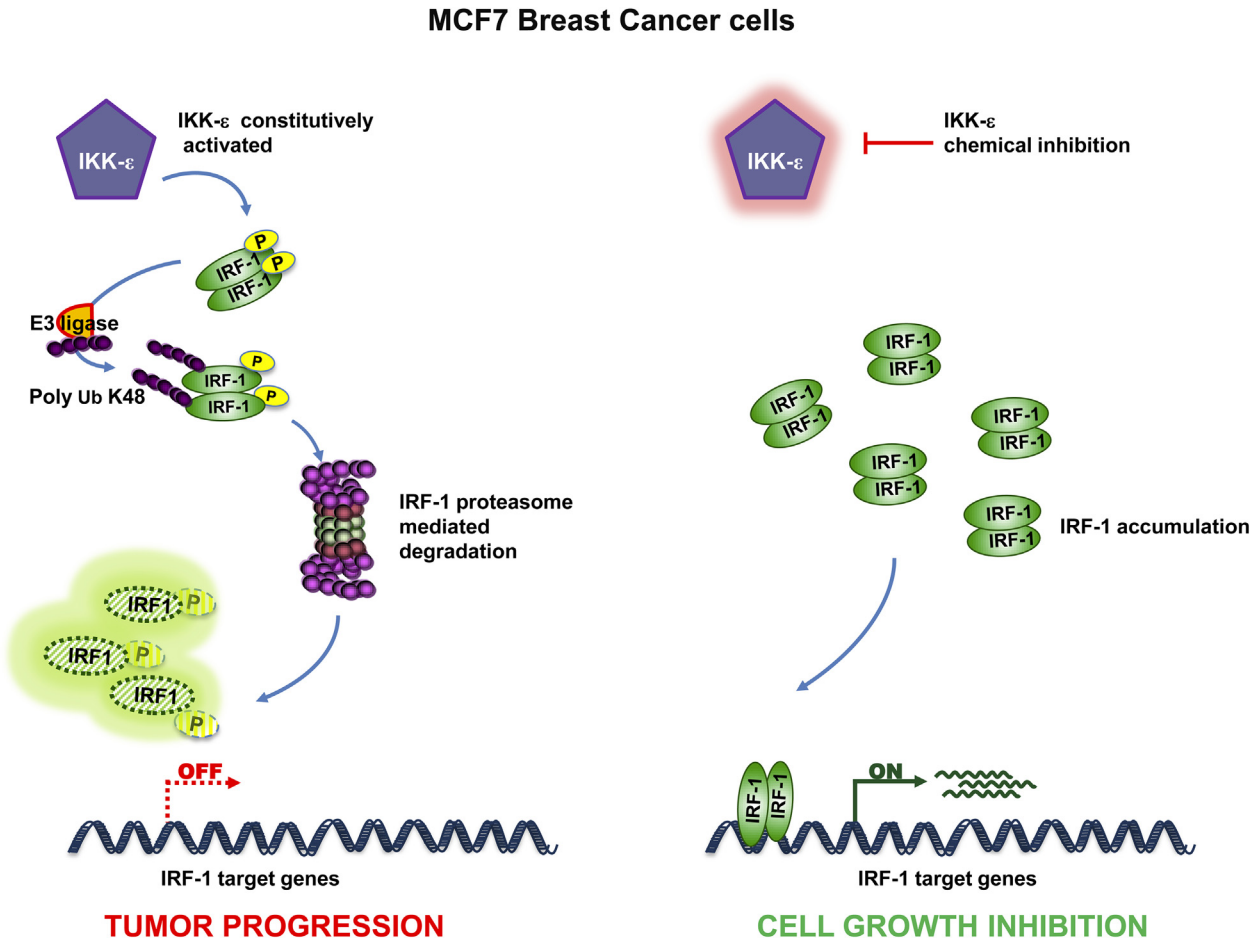

Fig. 7. Schematic representation of the IRF-1 inactivation in breast cancer cells by IKK-ε protooncogene. IKK-ε in MCF7 breast cancer cells, where is overexpressed and persistently activated, mediates IRF-1 phosphorylation, this posttranslational modification induces IRF-1 ubiquitination and proteasome-mediated degradation. IRF-1-regulated genes are then suppressed and tumor progression can now occur. Chemical inhibition of IKK-ε activity restores IRF-1 levels with full recovery of IRF-1 transcriptional potential resulting in cell growth inhibition.

migration of MCF-7 cells, and with the modulation of the expression of the related genes (Fig. 5).

Conclusions

Here we have established a new and mechanistically important regulatory mechanism of the IKK-ε/IRF-1 axis in breast cancer. Our data provide the mechanism involving the mode of IKK-ε action in inducing breast cancer cell transformation and point to the negative regulation of the onco-suppressor gene IRF-1 by IKK-ε as a key mechanism in promoting cell survival and transformation (Fig. 7). Therefore, IKK-ε-mediated degradation of IRF-1 is a potential therapeutic target that can be exploited using either IKK-ε inhibitors or molecules able to disrupt IKK-ε-IRF-1 interactions.

Authors' contributions

G.M. conceived the study and participated in its design and coordination. G.M., A.L.R. and M.S. conceived and carried out experiments, analyzed data and wrote the paper. A.B. analyzed data and wrote the paper; M.A. and R.O. performed experiments, E.P. and C.A. carried out experiments and helped to draft the manuscript. R.C. provided key engineered cell lines and edited the manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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