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IKKε regulates the breast cancer stem cell phenotype

Zhanna Orlova[a,c], Franz Pruefer[b], Rosario Castro-Oropeza[a], Alejandro Ordaz-Ramos[a], Cecilia Zampedri[b], Vilma Maldonado[a], Karla Vazquez-Santillan[a,**], Jorge Melendez-Zajgla[b,*]

[a] Epigenetics, Instituto Nacional de Medicina Genomica, Periferico Sur No.4809, Col Arenal Tepepan, Tlalpan, Mexico City 14610, Mexico
[b] Functional Genomics Laboratories, Instituto Nacional de Medicina Genomica, Periferico Sur No.4809, Col Arenal Tepepan, Tlalpan, Mexico City 14610, Mexico
[c] Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot 7610001, Israel

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ABSTRACT

The Inhibitor of Nuclear Factor Kappa B Kinase Subunit Epsilon (IKKε) is an oncogenic protein that is up-regulated in various types of human cancers, including breast tumors. This kinase regulates diverse processes associated with malignant progression including proliferation, invasion, and metastasis. To delve into the molecular mechanisms regulated by this kinase we performed RNA-seq and network analysis of breast cancer cells overexpressing IKKε. We found that the TNF/NF-κB cascade was clearly enriched, and in accordance, NF-κB pathway inhibition in these cells resulted in a decreased expression of IKKε target genes. Interestingly, we also found an enrichment of a mammary stemness functional pathway. Upregulation of IKKε led to an increase of a stem CD44+/CD24$^{-/low}$ population accompanied by a high expression of stem markers such as ALDH1A3, NANOG, and KLF4 and with an increased clonogenic ability and mammosphere formation capacity. These results were corroborated with *in vivo* dilution assays in zebrafish embryos which showed a significant increase in the number of Cancer Stem Cells (CSCs). Finally, we found that Triple-Negative breast tumors, which are enriched in CSCs, display higher levels of IKKε than other breast tumors, supporting the association of this kinase with the stem phenotype. In conclusion, our results highlight the role of IKKε kinase in the regulation of the stem cell phenotype in breast cancer cells, as assessed by expression, functional and *in vivo* assays. These results add to the potential use of this kinase as a therapeutic target in this neoplasia.

1. Introduction

Breast cancer constitutes a group of complex and highly heterogeneous diseases. At least five different subtypes have been identified based on biological markers, including Luminal A, Luminal B, Her2+, Basal, and Claudin-low [1–3]. Breast Cancer Stem Cells (CSCs) comprise a small but critical cell subpopulation in breast tumors. CSCs are thought to initiate and support tumor self-renewal as well as potentially lead to therapy resistance and cancer relapse [4]. Each subtype of breast cancer is thought to originate from a CSC at a particular state of differentiation [5,6]. Therefore, gaining insight into the mechanisms that promote the CSC phenotype in the different breast cancer subtypes is of uttermost relevance.

Protein kinases are frequently overexpressed in human neoplasms. Deregulation of kinase activity has a critical role in tumor initiation and progression as they control key regulatory pathways involved in cell proliferation and growth [7,8]. The IKK-related kinase IKKε, encoded by the *IKBKE* gene, has been reported as an oncogenic protein up-regulated in various types of human cancers including breast, prostate, ovarian, glioma, stomach and pancreatic cancer [8–13]. Moreover, *IKBKE* overexpression promotes cancer initiation, tumor progression and strongly correlates with poor prognosis in ovarian cancer [14,15].

IKKε was first discovered as a cytokine-inducible kinase that shared 30% similarity to the canonical members of the IKK family of protein kinases, which includes IKKα and IKKβ [16]. Similar to its canonical counterparts, IKKε regulates NF-κB activity by phosphorylating the Inhibitor of kappa B kinase IκB [17]. Interestingly, it also modulates this cascade at multiple points, providing alternative transcriptional outcomes [18]. Upon IκB phosphorylation, NF-κB transcription factors are released and translocate into the cell nucleus where they regulate the transcription of genes related to a plethora of biological processes including innate and adaptive immunity, stress responses, survival, tumorigenesis, inflammation, and development [19]. Furthermore, we and others have previously shown that NF-κB regulates breast CSC phenotype [20–24].

IKBKE is a breast cancer oncogene that is overexpressed in 30% of

* Correspondence to: J. M. Zajgla, Functional genomics laboratory, INMEGEN, Mexico City 14610, Mexico.
** Correspondence to: K. V. Santillan, Epigenetics laboratory, INMEGEN, Mexico City 14610, Mexico.
E-mail addresses: kivazquez@inmegen.gob.mx (K. Vazquez-Santillan), jmelendez@inmegen.gob.mx (J. Melendez-Zajgla).

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breast cancer cell lines and primary tumors [25]. Amplification of *IKBKE* is generally present in luminal breast cancers, whereas its overexpression in other types of breast cancer may be dependent upon cytokine signaling. For instance, a recent study by Barbie et al. concluded that IKKε overexpression defines a subset of Triple Negative Breast Cancer (TNBC) characterized by the expression of cytokines IL-6 and CLL-5. Additionally, inhibition of the TBK1/IKKε/JAK cytokine network impaired growth and proliferation of TNBC cells, which further supports the oncogenic potential of IKKε in triple negative breast cancer subtypes [26]. This kinase promotes tumorigenesis in breast tissues by activating NF-κB at multiple levels of the pathway [27,28]. In addition to NF-κB, additional oncogenic pathways such as Wnt and Foxo3a are regulated by this kinase [29]. Accumulating evidence indicates that IKKε induces cell growth and invasion; however, there is a paucity of information about the potential role of this kinase in other cancer-related cellular processes, such as the stem cell phenotype.

In this study, we overexpressed IKKε in the luminal breast cancer cell line MCF-7 and performed a genome-wide expression analysis by RNA-sequencing. Notably, most of the deregulated genes were involved in a cancer stem cell phenotype. Accordingly, gene set enrichment analysis showed that forced expression of IKKε results in an overexpression of genes commonly up-regulated in mammary stem cells. Supporting this, we found that MCF-7 cells that overexpress IKKε harbor a higher number of cancer stem cells, which correlated with an increased ability to form tumors *in vivo*. These results provide insight into the function of *IKBKE* in stem cell behavior.

2. Material and methods

2.1. Cell culture and reagents

MCF-7 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in EMEM medium (ATCC, Manassas, VA, USA) supplemented with 5% Fetal Bovine Serum (FBS). Cells were maintained in a humidified atmosphere at 37 °C with 5% CO_2.

2.2. Plasmids and cell transfection

Loss and gain of function *IKBKE* experiments were carried out in the MCF-7 cell line. To overexpress IKKε a pcDNA-Flag plasmid containing the wild-type sequence was employed (Addgene #26201). The *IKBKE* mutant sequence with an inactive kinase domain (K38) was also present in a pcDNA-Flag plasmid. As a transfection control, a plasmid containing the LacZ gene (pT-REz/GW30/Lacz, Thermo Fisher Scientific) was used. The IKKε overexpressing plasmids were kindly provided by Dr. Tom Maniatis (Columbia University). MCF-7 cells were transfected with 5 μg of plasmid and 1.5 μl of Xfect (Clontech, WI, USA). Stable cell lines overexpressing wild-type or mutant IKKε were generated by Geneticin selection (600 μg/ml). For NF-κB pathway inhibition, MCF7 cells were transfected with a PLXSN plasmid harboring a dominant-negative IκB (mutated at IκB S32/S36) or a PLXSN empty vector (Mock). The PLXSN-IκB plasmid was kindly provided by Dr. P. Bauerle (University of Munich).

To decrease IKKε expression, a shRNA (GGAGCATTGGAGTGAC CTT) was designed using the RNAi Target Sequence Selector tool (Clontech). A double-stranded oligonucleotide containing the sequence for the shRNA was cloned into the pSIREN RQ vector (Clontech). As a control, we used a shRNA against luciferase. BT20 and MCF7 cells were transfected with 5 μg of plasmid using Xfect and stable cell lines were generated by Puromycin selection (500 ng/ml and 400 ng/ml respectively).

2.3. RT-PCR and quantitative real-time PCR

Total RNA was isolated with Trizol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA integrity was analyzed by spectrophotometry (NanoDrop, Thermo Fisher Scientific, Rockford, IL, USA) and capillary electrophoresis (Agilent RNA 6000, Agilent Technologies, Waldbronn, Germany). Two μg of RNA was reverse-transcribed with the Maxima First Strand cDNA Synthesis Kit with DNAse according to the manufacturer's instructions (Thermo Fisher Scientific, Rockford, IL, USA). qRT-PCR was performed with the SYBR-select Master Kit and amplified using the 7900HT (Applied Biosystems, Rockford, IL, USA) real-time quantitative PCR system. For each reaction, 12.5 ng of cDNA was mixed with 0.3 μM of each forward and reverse primers and 10 μl of the master mix kit. Primers used for qRT-PCR are listed in Table 1.

Table 1
Primers used for q-RT-PCR analysis.

Gene name	Primers	Sequence	TM
ALDH1A3	Forward	GCATACCGTGGAAGGGCG	57
	Reverse	GCTCTCTGGGCTATTGATTCTGTC	
ALDH8A1	Forward	CAGGCTACTTTATGCTTCCCAC	55
	Reverse	GCTCTTTCAATCACCTCCTCTT	
OCT4	Forward	ACATCAAAGCTCTGCAGAAAGAAC	56
	Reverse	CTGAATACCTTCCCAAATAGAACCC	
NANOG	Forward	AGGCAAACAACCCACTTCTG	57
	Reverse	TCTGCTGGAGGCTGAGGTAT	
IKKε	Forward	CAACGAGGAGCAGATTCACA	56
	Reverse	GTCCTGAAGGAGCTGGTGAG	
TBP	Forward	TGCCACACCCTGCAACTCAACA	60
	Reverse	CCCCTTCATTGACCTCAACT	
KLF4	Forward	GACTTCTTTCAGTGCTTC	56
	Reverse	CGTTGAACTCCTCGGTCTC	
S100A7	Forward	CCAACTTCCTTAGTGCCTGTG	58
	Reverse	AATCTTCTTATCCTCATTCTTGTCCTT	
CCL5	Forward	ACCGCCAAGTGTGTGCCAA	60
	Reverse	GTTCAGGTTCAAGGACTCTCCATCC	
CXCR4	Forward	TCCACGCCACCAACAGTCAGAG	60
	Reverse	TCCAGACGCCAACATAGACCACC	
CXCL17	Forward	AGCACACCTACCAGACACTC	60
	Reverse	GGCTAAGACTGACGAGAGAAGA	
LIF	Forward	ACGAGGATGTGGCTGTTGAGA	60
	Reverse	TGAAGCAGGAAGGAGAAGGCA	
MDC1	Forward	ATGAAGGAAGAGGTGAAG	55
	Reverse	AAAATCGTAGGTGAAGGA	
CENPF	Forward	ACTACCTACTCATCTCTTCA	55
	Reverse	CTATTCTCTCCTCTTTGG	
CBX5	Forward	CTACCAAGAACCACCACT	55
	Reverse	TCCTAAAATCCAGAAAGCAAG	
KIF4	Forward	ATGAAGGAAGAGGTGAAG	55
	Reverse	AAAATCGTAGGTGAAGGA	
IFNL1	Forward	ACATCATCTTGGATTGCCCATTTT	55
	Reverse	AGCCCTTCCCAGTTGTGGTG	

2.4. Droplet digital PCR

The ddPCR assays were done in accordance with the manufacturer's recommendations (Bio-Rad Laboratories). Each ddPCR reaction was performed using EvaGreen SMX (1864034), each reaction was emulsified with Droplet Generation Oil (1864006). The reactions were fractioned in up to 20,000 drops in the QX200 Droplet Generator (Bio-Rad), drops were transferred in 96-well plates (10023379) to carry out a ddPCR reaction in a conventional PCR System thermocycler (Applied Biosystems). At the end of the amplification, the individual fluorescence of drops in each reaction was measured with the QX200 Droplet Reader (Bio-Rad). Data analysis was made with the QuantaSoft droplet reader (Bio-Rad). The absolute expression values in the copy number/μl were calculated using statistics for a Poisson type distribution. The results of these measurements are represented in copy number/μl of amplifiable cDNA.

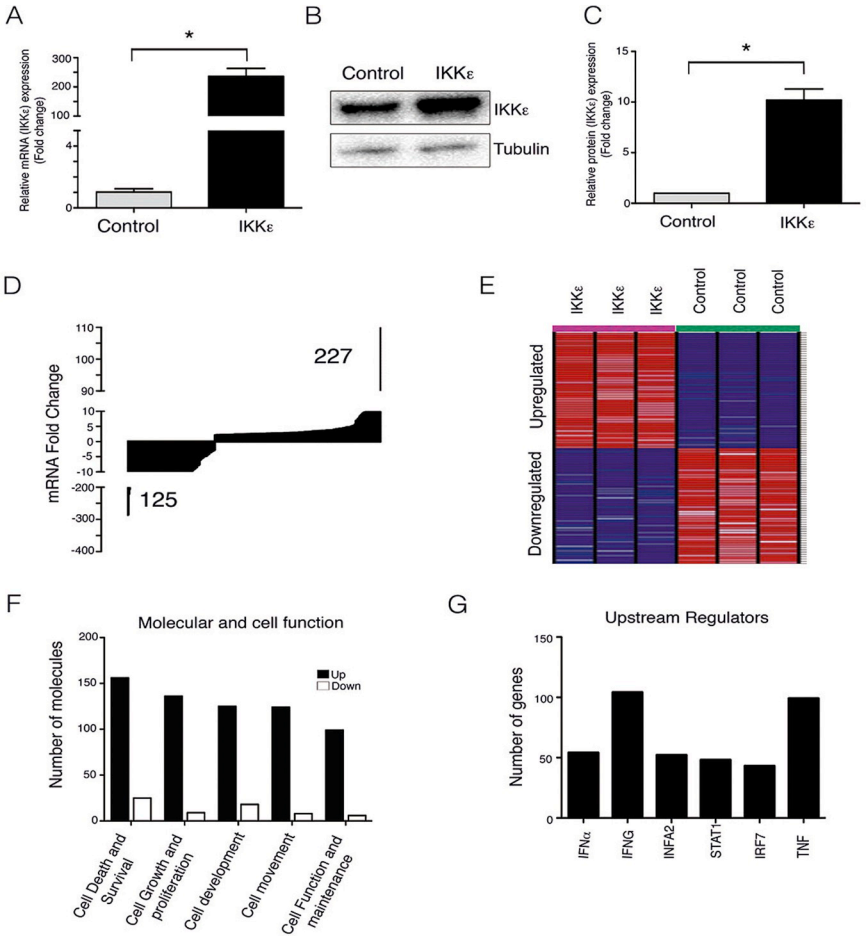


Fig. 1. Transcriptome analysis of MCF7 cells overexpressing IKKε. (A) qRT-PCR analysis showing increased *IKBKE* expression in MCF7 IKKε-overexpressing cells when compared to MCF7 control cells. ($n = 3$, error bars are s.e.m., $*p < 0.05$). (B) Western blot of IKKε in IKKε-overexpressing cells and in MCF7 control cells. Tubulin was used as a loading control. (C) Densitometry scans from three independent assays were quantified, normalized to Tubulin and calculated as fold change. (D) Fold change of modulated mRNAs after IKKε overexpression. (E) Heatmap of the top 50 upregulated genes and the top 50 downregulated genes in MCF7 cells overexpressing IKKε. (F) Top five molecular and cell functions enriched due to IKKε overexpression. (G) Top six upstream regulators predicted to be activated in MCF7 overexpressing IKKε cells.

2.5. Cell fractions

Cell fractioning was carried out with the PARIS ™ Kit (Ambion by Life technologies AM1921). The protein extracts of the nuclear and cytoplasmic fractions of MCF7 cells were always treated with protease inhibitors (Roche 11,852,700) and phosphatases (Sigma P0044). Subsequently, the fractions were separated by electrophoresis and then transferred to poly (vinylidene difluoride) (PVDF) membranes (Millipore Corp). The purity of the fractions was checked by using the Tubulin antibody (Santa Cruz SC53646) for cytoplasm and the Lamin A/C antibody (Cell signaling 4777) for the nucleus.

2.6. Western blotting

Total proteins were isolated with RIPA Buffer (Upstate Biotechnologies, Inc., New York, USA) supplied with protease and phosphatase inhibitors. Proteins were quantified by the Bradford method and then resolved in 10% SDS-PAGE. After electrophoresis, proteins were transferred onto poly-vinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline supplied with 0.1% Tween for two hours at 4 °C. Membranes were then incubated overnight at 4 °C with the antibodies IKKε (1:500, sc-52,931, 72B583), Tubulin (1:2000, sc_53646, clone I0D8), p65 (1:500, sc-372, clone C-20), p52(1:1000, 05–361) and Relb (1:500, sc-28,689, clone H-200). Antibodies were obtained from Santa Cruz (Santa Cruz Biotechnologies Inc., California, USA) with the exception of p52 antibody, which was obtained from Millipore (Billerica, MA, USA). Membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Promega, Madison, WI, USA) for 1 h at room temperature, then, antibody binding was detected with the Immobilon Western Kit (Millipore) using the ChemiDoc XRS + (Bio-Rad Laboratories Inc., Hercules, California, U.S.A).

2.7. RNA sequencing

Only samples with an RNA-integrity number higher than 9.0 were considered for transcriptome sequencing. Sequencing of total RNA was performed using a NextSeq500 (Illumina) system. Three biological replicates were used for the analysis. Clean reads (range 15183341–29348265 reads) were aligned to the GRCh 38 version of the human genome using the STAR algorithm [30] and quantified with the RSEM package [31]. Differential expression was assessed with EBSeq [32]. Only genes with expression fold change higher than 2 or less than −2 and a PPEE (Posterior Probability of Equal Expression) < 0.05 were further considered for subsequent analysis. A similar approach was used for the Sequence Read Archive's raw data downloaded from NCBI.

2.8. Pathway analyses

The differential expressed gene list was used to identify essential cellular pathways and upstream regulators with the Ingenuity Pathway Analysis Software (IPA, QIAGEN). Gene set enrichment analysis (http://software.broadinstitute.org/gsea/index.jsp) was performed using default settings; we used a permutation of 1000 with a weighted enrichment statistics and a signal-to-noise metric for gene ranking. Gene sets were downloaded from the MSigDB database (Cell Cycle, Apoptosis, DNA Repair, Development, Differentiation). Normalized enrichment scores (NES) were calculated to measure differences in gene set size as well as correlation with other gene signatures. The false discovery rates (FDR) threshold used was < 0.25.

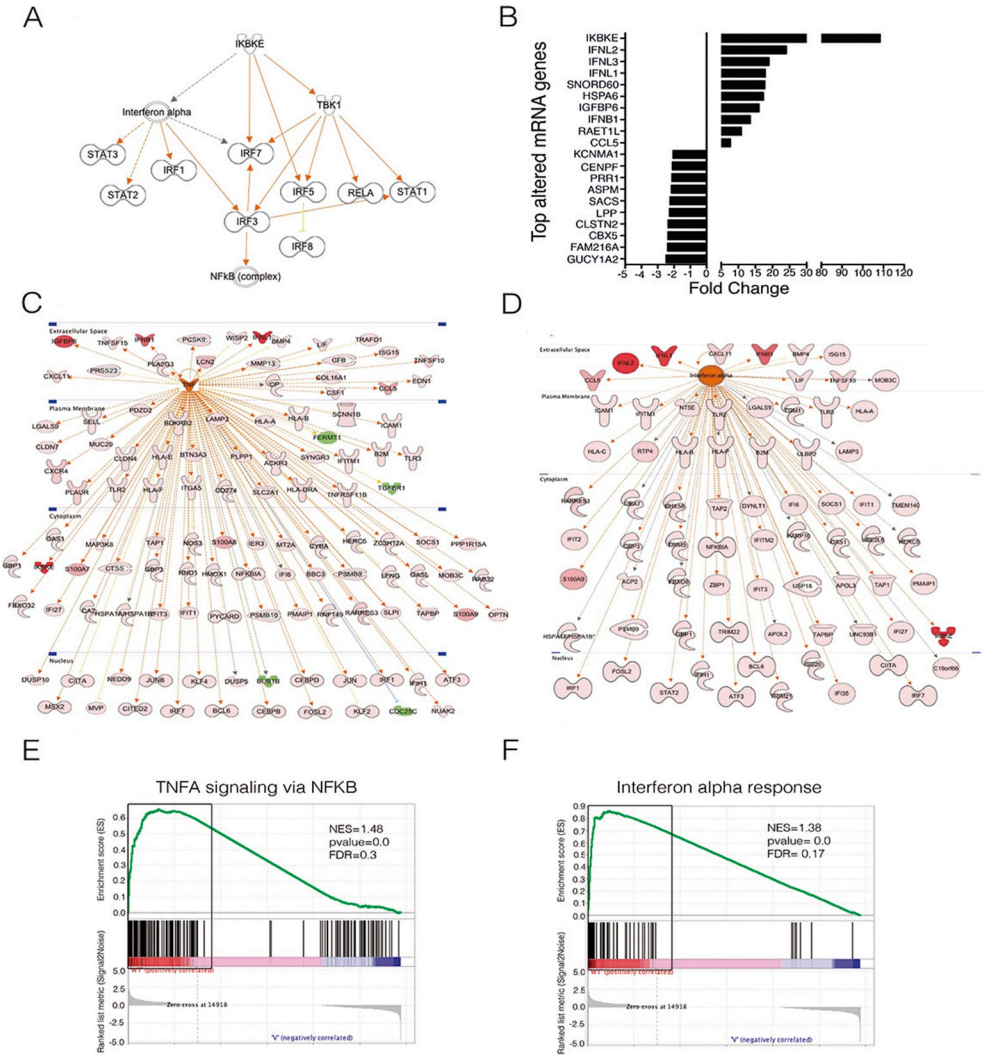


Fig. 2. IKKε upregulates developmental genes. (A) Upstream regulator analysis of differentially expressed genes after IKKε overexpression. *IKBKE*, Interferon alpha, and NF-κB pathways were predicted to be activated upon IKKε overexpression. (B) Top ten deregulated mRNA genes. (C) Pathway analysis showing that TNF/NF-κB signaling is activated in IKKε-overexpressing cells and regulates developmental gene expression. (D) IPA analysis predicts that Interferon alpha pathway is activated in IKKε-overexpressing MCF7 cells and regulates developmental genes expression. The pathways show differential expressed genes regulated by each signaling pathway. Red color indicates overexpression and highlighted genes are involved in stem cells regulation. (E) Gene set enrichment analysis (GSEA) showing a significant enrichment of the TNF-NF-κB signaling pathway in IKKε-overexpressing cells. (F) Gene set enrichment analysis (GSEA) showing a significant enrichment of the Interferon alpha signaling pathway in the IKKε overexpressing cells.

2.9. Flow cytometry analyses

Cells were detached with Accutase (Life Technologies, Carlsbad, CA, USA) and washed with phosphate buffer saline (PBS) 1%. A total of 1×10^6 cells were resuspended in the staining buffer (PBS with FBS 1%) and incubated for 30 min at 4 °C with CD44/FITC (BD Biosciences, 555478, 4230724), CD24/PE (BD Biosciences, 555428, 5049759). FITC-IgG1 (Miltenyi Biotec) and PE-IgG1 (Miltenyi Biotec) were used as isotypes controls. Cells were analyzed using the FACSaria (Beckton Dickinson, Franklin Lakes, NJ, USA) and data were analyzed with the FlowJo software package (Treestar, Ashland, OR, USA). Cells were sorted on a FACSAria (Becton Dickinson, Franklin Lakes, NJ, USA). A total of 1×10^8 MCF7 cells were sorted into $CD44^+/CD24^{-/low}$ and $CD44^-/CD24^+$ subsets and then were harvested and grown under standard culture conditions for 24 h prior to any further procedures.

2.10. Colony formation assays

Briefly, 6000 MCF-7 single cells were plated in 6-well plates coated with 0.5% low-melting-point agarose and 5% FBS. Cells were seeded in DMEM medium containing 0.3% low-melting-point agarose and 5% FBS. After 30 days in culture, colonies were stained with 0.1% crystal violet and were visualized under an inverted microscope. Plates were photographed and colonies were counted using the ImageJ software [33].

2.11. Sphere formation assays

The potential of self-renewal was assessed by plating 2×10^4/ml single cells over ultra-low attachment surface flasks. Cells were cultured in Mammocult (Stem Cell Technologies) supplemented with 0.48 μg/ml hydrocortisone and 4 μg/ml heparin. After 7 days, the spheres were counted, dissociated and sub-culture under the same conditions. This process was repeated twice to obtain first, second and third sphere generations. Experiments were carried out in triplicate.

2.12. In vivo tumorigenic assays

Wild-type zebrafish (*Danio rerio*) embryos were obtained from natural crosses of TAB WIK genetic background fishes kindly provided to us by Professor Ernesto Maldonado from ICMyL-UNAM. Adult zebrafishes were maintained in a constant pH, temperature and dark-light cycle (pH 7.2–7.4; 26–28 °C; 14 h on and 10 h off light cycle). Zebrafish embryos obtained by natural crosses were placed in Petri dishes with embryo medium (EM) [34,35] (60 embryos per plate) and maintained at 28.5 °C for 2 days. At 48 h post fertilization, the embryos were dechorionated and anesthetized with tricaine (MS-222; Sigma, Mexico). Five cell dilutions (10, 50, 75, 125 or 250 cells) of MCF-7-Ctl, MCF-7-IKKε/wild-type, and MCF-7-IKKε/mutant cell lines were suspended in 10 nl of DMEM medium and injected into the embryonic yolk sacs of 48 hpf embryos by using a microinjector PLI-100A (Warner Instruments, Harvard Apparatus, Holliston, Massachusetts) equipped with a

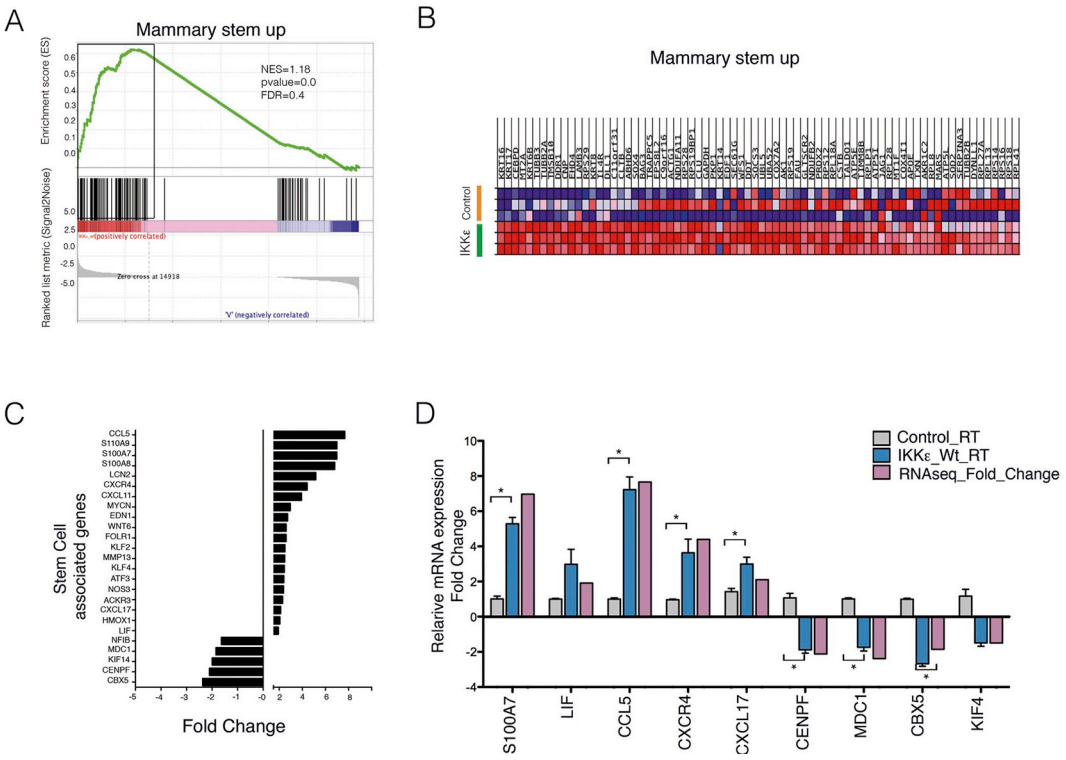


Fig. 3. IKKε expression and a mammary stem signature. (A) Gene set enrichment analysis (GSEA) showing a significant enrichment of a mammary stem cell signature in IKKε-overexpressing cells. (B) Top twenty-five stem cell-associated genes disrupted in MCF7 cells overexpressing IKKε. (C) Expression profiles of 5 upregulated and 4 downregulated genes were analyzed by RT-qPCR to validate RNA-seq results. ($n = 3$, error bars are s.e.m., $^*p < 0.05$). D) Fold-change in stem phenotype associated genes in control MCF7 cells (grey) and IKKε-overexpressing cells (blue) compared to the fold-change obtained from the RNA-Seq experiment (pink).

borosilicate glass needle. After injection, the embryos were maintained at 34 °C until the end of the experiment. *In vivo* limiting dilution assays were analyzed using the Extreme Limiting Dilution Assays (ELDA) software [36] that employs a generalized linear model used to compare active cell frequencies in cell populations. Animal husbandry followed international ethics standards. Zebrafish xenotransplants assays were approved by INMEGEN's ethic Committee (09/2015/I).

2.13. Proliferation assays

A total of 3000 cells were cultured in 96-well plates in RPMI medium enriched with 5% FBS. The MTT assay was performed according to the recommendations of the suppliers with the CellTiter 96® Non-Radioactive Cell Proliferation Assay (MTT) kit (Cat. # G4000, Promega). Cells were incubated with the reagent for 1 h at 30 min at 37 °C in the dark and 5% CO_2, then, the dye was allowed to solubilize for 24 h. The results were analyzed at times 0, 24, 48, 72, 96 and 120 h in the Beckman Coulter DTX 880 Multimode Detector spectrophotometer at a wavelength of 596 nm.

2.14. Statistical analyses

All statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA), and considered a 95% confidence interval. An ANOVA test was used to calculate statistical significance between groups.

3. Results

3.1. IKKε overexpression affects the expression of both TNF and IFN related genes

To delve into the oncogenic mechanisms of IKKε, we first performed a genome-wide expression analysis of MCF-7 cells overexpressing the kinase. As shown in Fig. 1A–C, transient transfection of a pcDNA-based IKKε expression plasmid resulted in a significant overexpression of the kinase. A genome-wide RNA-Seq differential expression (DE) analysis identified 352 deregulated genes (227 up and 125 downregulated genes) (Fig. 1D and E and Supplementary Table 1 and Supplementary Fig. 1A–B). We then sought to integrate these results in a common framework using the Ingenuity Pathway Analysis (IPA) suite. Fig. 1F shows that five main cellular and molecular pathways were enriched in our DE genes (cell death, cell growth, cell movement, development, and maintenance). Interestingly, an upstream regulator analysis showed that interferon-related pathways and effectors, along with the Tumor Necrosis Factor (TNF) cascade were the top regulators of the found networks (Fig. 1G). Supporting this, we not only found that one of the top regulated networks was the STAT/IRF/NF-κB module (Fig. 2A) but that several IFN-related molecules were present among the top 10 DE RNAs (Fig. 2B and Table 1). To further support these results we performed an enrichment analysis using the GSEA (Gene Set Enrichment Analysis) algorithm [37]. As expected, we found a significant enrichment in both Interferon and TNF-NF-κB pathways (Fig. 2C–F).

3.2. IKKε induces the expression of mammary stem cell-related genes

Interestingly, one of the top processes regulated by IKKε was a Mammary Stem Cell signature (Fig. 3A–B). This result was based on a large number of stem-associated genes that included important molecules such as CCL5, LIF, CXCR4, CXCL17 and a family of S100 calcium binding proteins (S100A7, S100A8, S100A9 and S100A16), which have been recently involved in the CSC maintenance [38] (Fig. 3C). We were able to validate the DE of 9 of these genes by qRT-PCR (a 100% validation rate) (Fig. 3D). These results prompted us to explore if IKKε could be involved in maintaining a stem phenotype in breast cancer cells. For this, we first analyzed the expression of stem-associated genes

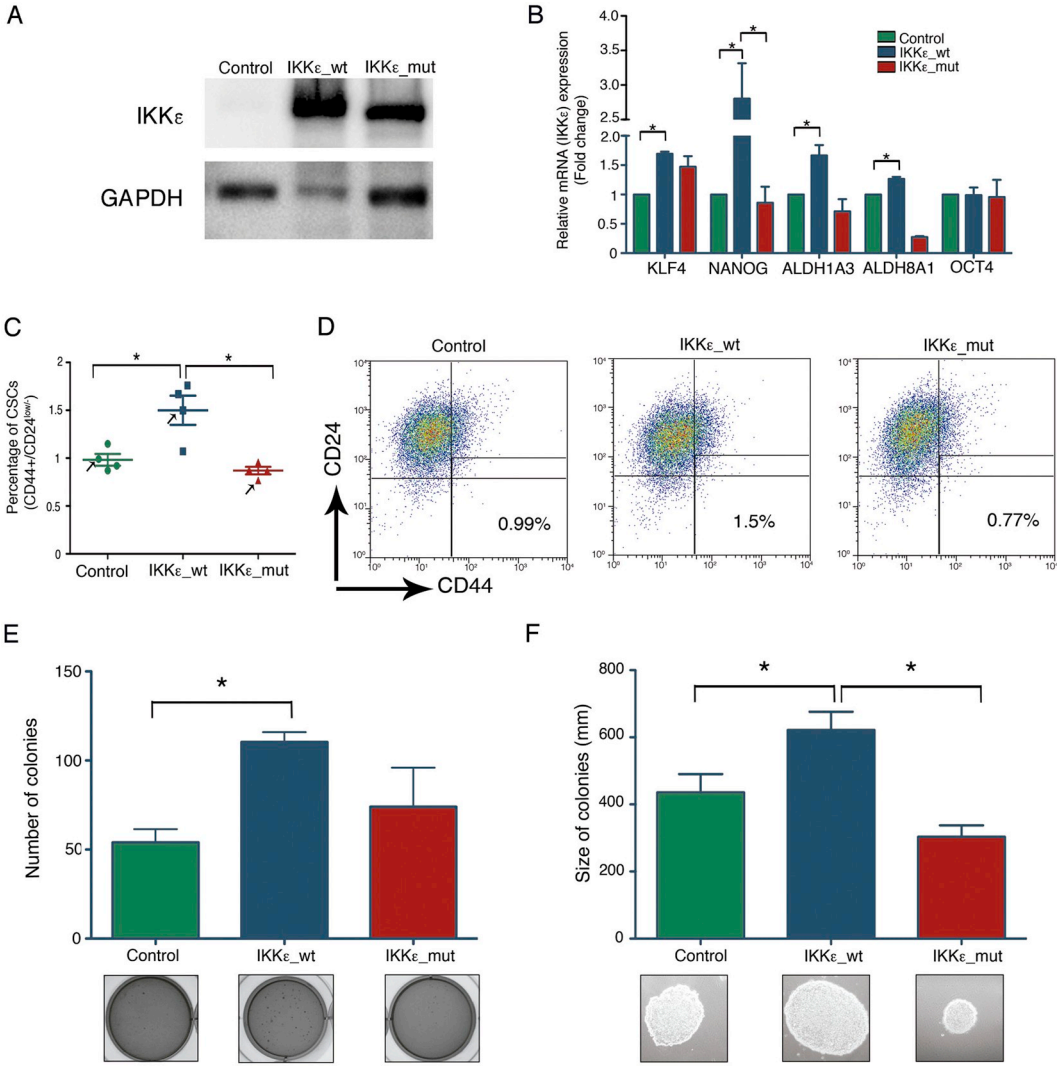
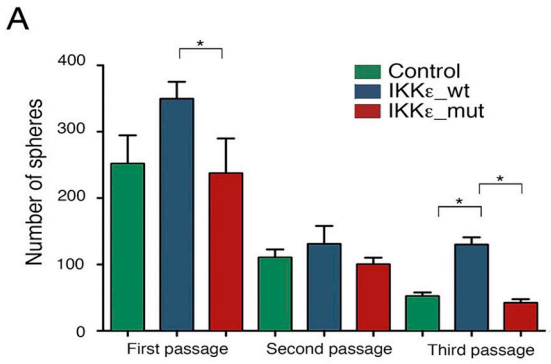


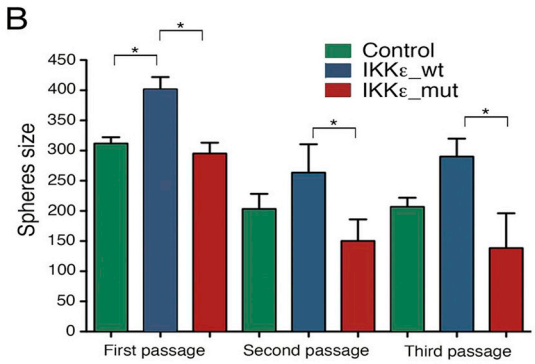
Fig. 4. The impact of IKKε overexpression in the stem phenotype. (A) Expression levels of IKKε in IKKε_wt or IKKε-mut overexpressing MCF7 cells. (B) CSC markers were assessed by real-time RT-PCR in IKKε wild-type (wt) or IKKε kinase-dead mutant (mut) -overexpressing cells. All qPCRs were normalized to 18 s rRNA. ($n = 3$, error bars are s.e.m., $^*p < 0.05$). (C) Percentage of CSCs (CD44 + /CD24-) in MCF7 cells overexpressing wild-type (wt) IKKε, or IKKε kinase-dead mutant (mut) and MCF7 control cells, as assessed by flow cytometry ($n = 4$, error bars are s.e.m., $^*p < 0.05$). Black arrows indicate the replicates shown in Fig. D. (D) Flow cytometry analysis of CD24 and CD44 cell surface showing proportions of CSCs in the MCF7 cells. (E) Clonogenic assays showing that forced expression of IKKε wt, but not its kinase-dead mutant increased the clonogenic ability of MCF7 cells ($n = 3$, error bars are s.e.m., $^*p < 0.05$). (F) Graph showing that forced expression of IKKε-wt produced bigger cell colonies. Mutant IKKε overexpression results in decreased colony sizes.

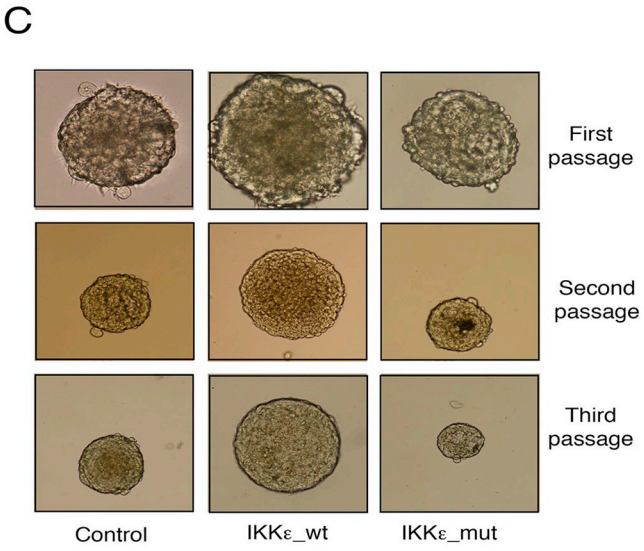
in MCF-7 cells overexpressing IKKε. Fig. 4A shows the level of IKKε in MCF7 cells stably overexpressing an IKKε wild-type or an IKKε Kinase-inactive mutant (IKKε_mut). This protein has a defective mutant [$Lys^{38} \rightarrow Ala^{38}$ (K38A)] affecting its kinase domain. It has been reported that IKKε(K38A) fails to translocate to the nuclei of mouse embryonic fibroblasts [39], therefore, in order to prove the identity and function of the plasmid harboring IKKε (K38A), we performed cell fractioning experiments and measured the IKKε expression in both the nuclear and cytosolic fraction of IKKε-wt or IKKε-mut-overexpressing MCF7 cells. Supplementary Fig. 2 shows that IKKε-mut-overexpressing cells exhibit less nuclear IKKε than IKKε-wt cells, thus confirming the identity and function of IKKε(K38A). As shown in Fig. 4A–B, we found that overexpression of this kinase was accompanied by an increase in the steady-state levels of the stem-associated markers ALDH1A3, NANOG, KLF4, and to a lesser extent ALDH8A1. Interestingly, forced expression of an IKKε kinase-inactive mutant (K38A) resulted in diminished levels of NANOG, ALDH8A1, and ALDH1A3, suggesting that their expression is regulated by IKKε's kinase activity.

3.3. Forced IKKε overexpression increases clonogenic and tumorigenic abilities of MCF7 breast cancer cell lines

Next, we sought to analyze the number of Breast Cancer Stem Cells (CSCs), as defined by a $CD44^+/CD24^{-/low}$ phenotype [40]. Fig. 4C–D shows an increased percentage of CSCs in IKKε-overexpressing cells. This phenotype was dependent on IKKε's kinase activity since overexpression of an IKKε inactive-kinase (K38A) mutant failed to increase the number of $CD44+/CD24^{low/-}$ cells (Fig. 4C–D). The higher number of CSCs was accompanied by a concomitant increase in clonogenic capacity of IKKε-overexpressing cells, as assessed by soft-agar assays (Fig. 4E). Interestingly, not only the number of colonies was increased, but also their size (Fig. 4F). As one of the most important cancer stem hallmarks is the ability to both initiate and maintain tumors in the absence of cellular interactions, we next performed serial tumorsphere passages. As shown in Fig. 5A–C, we found that cells overexpressing wild-type IKKε but not its kinase-inactive mutant generated an increased number and size of spheres after serial passages. We then performed *in vivo* xenotransplant assays in a zebrafish model. Fig. 5D





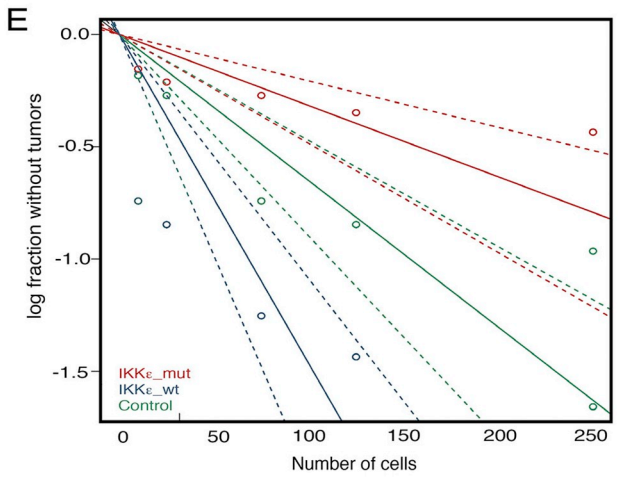


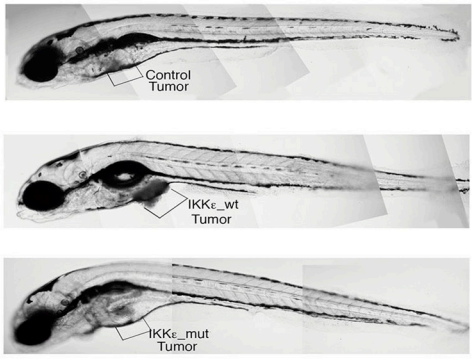
D

Phenotype	Cell number	Tumors
Control	10	3/18(16.6%)
	25	5/21(23.8%)
	75	11/21(52.3%)
	125	12/21(57.1%)
	250	13/21(61.9%)
IKKε_wt	10	11/21(52.3%)
	25	12/21(57.1%)
	75	15/21(71.4%)
	125	16/21(76.1%)
	250	17/21(80.9%)
IKKε_mut	10	3/21(14.3%)
	25	4/21(19.1%)
	50	5/21(23.8%)
	125	5/17(29.4%)
	250	6/17(35.3%)

F

Phenotype	CSC frequency	p value
Control	1/153.4	< 0.05
IKKε_wt	1/68.2	
IKKε_mut	1/315.4	





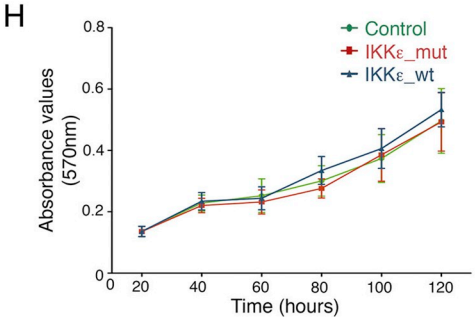


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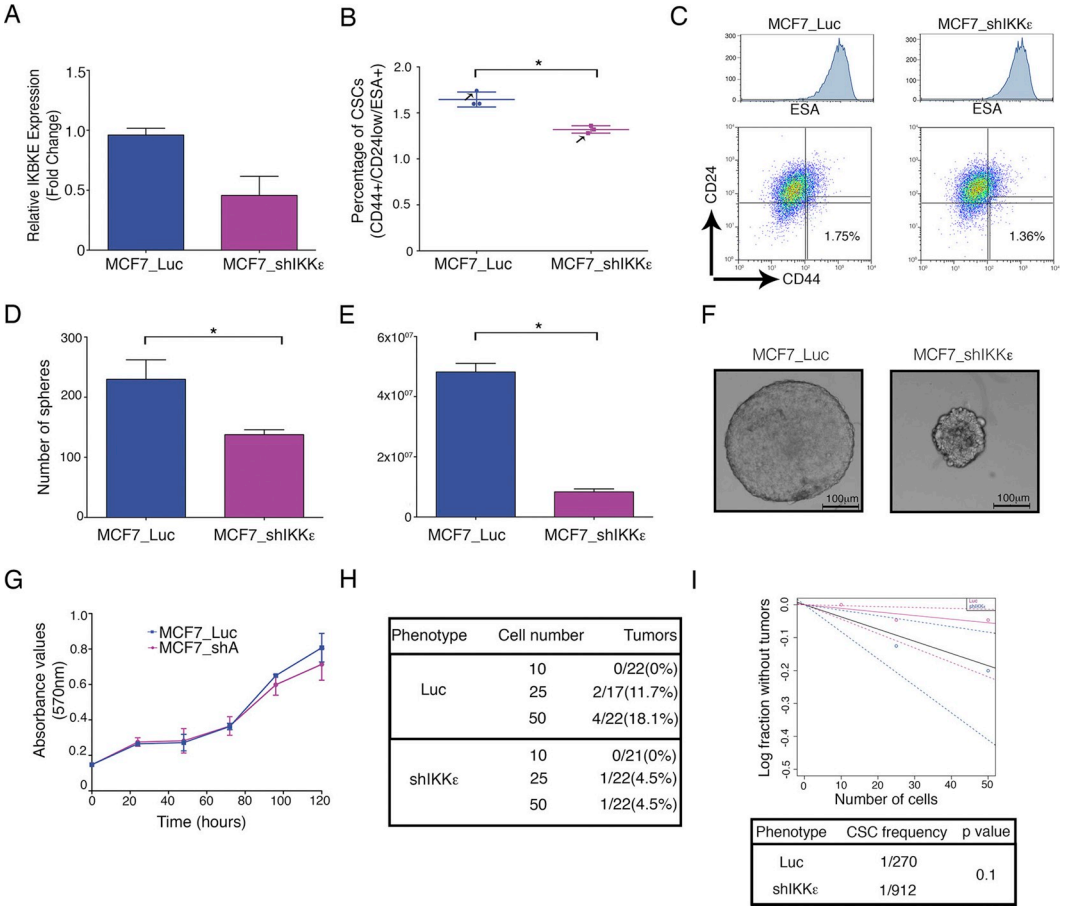
Fig. 5. Forced expression of IKKε increases clonogenic and tumorigenic abilities of MCF7 cells. (A) tumorspheres number derived from IKKε-wt expressing cells, IKKε-mut expressing cells and control cells after three consecutive passages. (B) Tumorsphere size of wt and mut IKKε-expressing cells. Sphere-forming assays showed that self-renewal capacity of CSC is increased in wt IKKε-overexpressing MCF7 cells. (C) Representative images of tumorspheres formed by wt IKKε-overexpressing MCF7 cells, mt IKKε-overexpressing MCF7 cells or control cells after consecutive cell passages. (D) Zebrafish embryos were inoculated with 10, 25, 75, 125 or 250 wt or mut IKKε-overexpressing MCF7 cells or MCF7 control cells. Animals were monitored each day for 5 days. The number of cells inoculated to zebrafish embryos and the proportion of tumors formed by each cell line is shown. (E) ELDA software was used to calculate CSC frequency. The number of cells injected *versus* the log fraction of animals without tumors is plotted. (F) Table shows the calculated CSC frequency. (G) Representative image of a tumor developed in zebrafish embryos at 5 days post-injection (dpi). (H) MTT proliferation assays were performed in IKKε-wt or IKKε-mut -overexpressing MCF7 cells during 24, 48, 72, 96, 120 h.



Phenotype	Cell number	Tumors
Luc	10	0/22(0%)
	25	2/17(11.7%)
	50	4/22(18.1%)
shIKKε	10	0/21(0%)
	25	1/22(4.5%)
	50	1/22(4.5%)

Phenotype	CSC frequency	p value
Luc	1/270	0.1
shIKKε	1/912	

Fig. 6. Functional effect of IKKε inhibition in MCF7 cells. (A) IKKε inhibition using a shRNA against IKKε. As a control, we used a shRNA directed against luciferase. The graph shows the fold decrease of IKKε ($n = 2$, error bars are s.e.m). (B–C) Flow cytometry analysis of CD24 and CD44 cell surface showing proportions of CSCs in IKKε-depleted cells ($n = 3$, error bars are s.e.m., $^*p < 0.05$), black arrows indicate the replicates shown in Fig. C. (D–E) Tumorspheres derived from IKKε-depleted cells (shIKKε or Luc cells). (D) Mammosphere number of Luc and shIKKε cells, (n = 3, error bars are s.e.m., $^*p < 0.05$). (E) Volume of mamoespheres grown by Luc and shIKKε cells, (n = 3, error bars are s.e.m., $^*p < 0.05$). (F) Representative images of tumorspheres formed by Luc or IKKε-depleted cells. (G) MTT proliferation assays were performed in Luc or shIKKε MCF7 cells during 24, 48, 72, 96, 120 h, (n = 3, error bars are s.e.m., $^*p < 0.05$). (H–I) Zebrafish embryos were inoculated with 10, 25, 75, 125 or 250 Luc or shIKKε MCF7 cells or MCF7 control cells. Fish were monitored each day for 5 days. The number of cells inoculated in the embryos and the proportion of tumors formed by each cell line is shown. (I) ELDA software was used to calculate CSCs frequency. The number of cells injected *versus* the log fraction of animals without tumors is plotted. Table shows the calculated CSCs frequency.

shows that cells overexpressing wild-type IKKε but not its kinase-dead mutant presented an increased tumorigenic capacity. An ELDA (Extreme Limiting Dilution Analysis) [41] showed that IKKε-overexpressing cells presented a significant increase in CSCs frequency, from 1 in 151 cells to 1 in 92 cells (Fig. 5E–G). Finally, we performed proliferation assays to assess whether IKKε kinase effects on clonogenicity and tumorsphere assays could be due to increased proliferation. This was not the case since overexpressing IKKε did not affect proliferation (Fig. 5H).

3.4. Depletion of IKKε affects clonogenic and tumorigenic abilities of breast cancer cell lines

To investigate whether IKKε depletion affected the CSC population, we employed a shRNA against IKKε in a luminal (MCF7, Fig. 6A) and a triple negative cell line (BT20, Fig. 7A). As expected from the previous results, IKKε inhibition reduced the CSC fraction in both cell lines (Figs. 6B–C and 7B–C). In addition, IKKε depletion also affected the number and size of tumorspheres (Fig. 6D–F). Knockdown of IKKε in BT20 also dramatically affected the size of spheres formed (Fig. 7D–F). To assess whether IKKε depletion impairs proliferation, we performed MTS assays which showed that IKKε inhibition did not affect cell proliferation in either MCF7 (Fig. 6G) or BT20 (Fig. 7G) cell lines. Finally, we performed *in vivo* xenotransplant assays in a zebrafish model. Fig. 6H–I shows that inhibition of IKKε resulted in lower tumorigenic ability of cancer cells. Thus, IKKε inhibition reduced the CSC fraction and impaired the clonogenic and tumorigenic abilities of both MCF7 and BT20 cell lines.

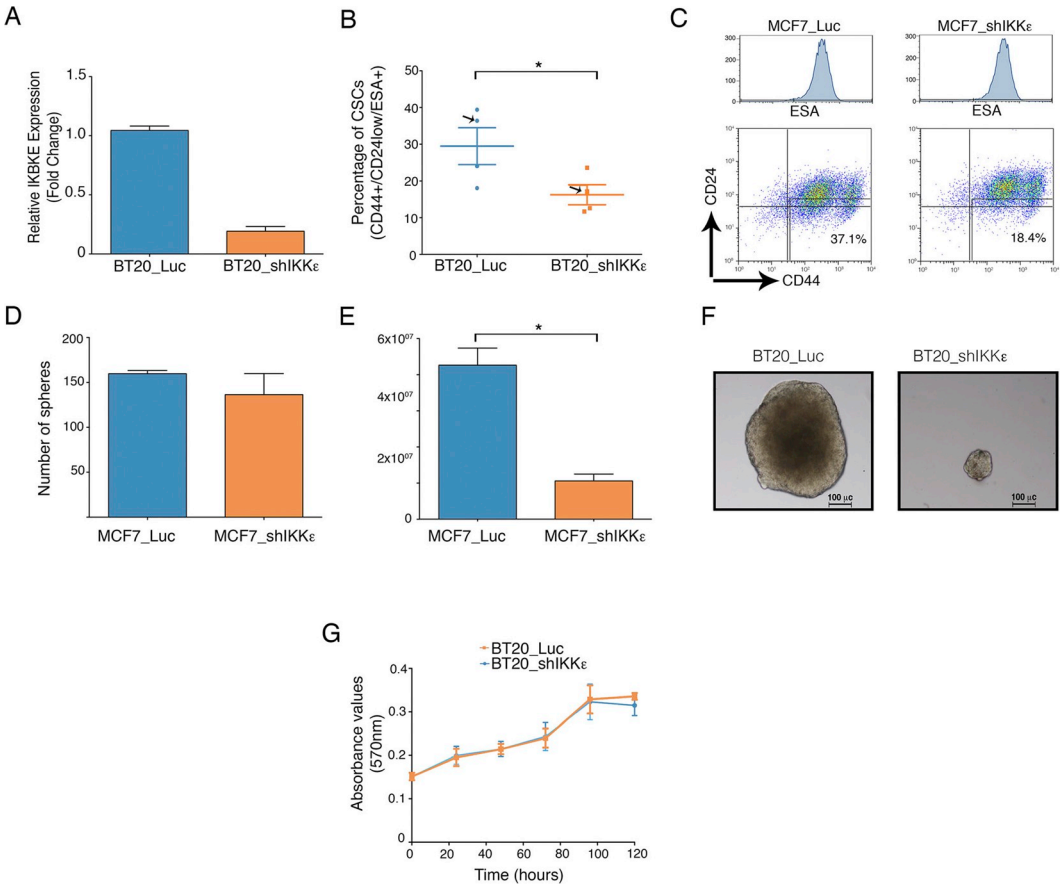


Fig. 7. Functional effect of the inhibition of IKKε in BT20. Functional effect of the inhibition of IKKε in BT20 (A) Inhibition of IKKε using a shRNA against IKKε. As a control, we used a shRNA against luciferase. The graph shows the fold decrease of IKKε (n = 2, error bars are s.e.m). (B–C) Flow cytometry analysis of CD24 and CD44 cell surface showing proportions of CSCs in IKKε-depleted cells (n = 4, error bars are s.e.m., $*p < 0.05$, black arrows indicate the replicates shown in Fig. C). (D–E) Tumorspheres derived from IKKε-depleted BT20 cells (shIKKε or Luc cells). (D) Tumorsphere number of Luc and shIKKε cells, (n = 3, error bars are s.e.m., $*p < 0.05$). (E) Tumorsphere volumes in Luc and shIKKε cells, (n = 3, error bars are s.e.m., $*p < 0.05$). (F) Representative images of tumorspheres formed by Luc or IKKε-depleted cells. (G) MTT proliferation assays were performed in Luc or shIKKε MCF7 cells after 24, 48, 72, 96 or 120 h in culture (n = 3, error bars are s.e.m., $*p < 0.05$).

3.5. IKKε is preferentially expressed in cancer stem cells

We next sought to investigate whether IKKε expression was associated with the stem cell fraction. For this, we isolated CSCs (CD44+/CD24$^{-/low}$) and a non-CSCs population (CD44-/CD24+) derived from MCF7 cells and measure the kinase expression by digital PCR. We found that CSCs express higher levels of IKKε since the non-CSCs population had < 20 copies of IKKε/µl, whereas CSCs contained approximately a 3-fold higher number of IKKε mRNA copies per µl (Fig. 8A–B). As sphere-growing cells are enriched in CSCs, we re-analyzed previously published gene expression data in primary bulk tumors grown as monolayers and mammospheres derived from them. An analysis of 15 mammospheres and 11 breast tumors cultured as monolayers (GSE7515) [42] showed a clear increase of IKKε expression in mammospheres (Fig. 8D). To verify this association, we then evaluated IKKε expression in MCF-7 cells grown as monolayers and mammospheres. As shown in Fig. 8E, mammospheres exhibited higher levels of IKKε, supporting its association with the stem cell phenotype. To investigate a possible association between IKKε and clinical parameters in breast cancer patients, we re-analyzed a previously published cohort of 140 breast cancer patients [43]. In this set, we found that IKKε was overexpressed in cancer samples when compared to normal breast tissues (Fig. 8F). Interestingly, triple-negative samples, which are enriched in CSCs, had significantly higher IKKε levels (Fig. 8G), supporting the association of this kinase with the stem phenotype.

3.6. IKKε regulates stem-cell related genes through the NF-κB pathway

Finally, we hypothesized that IKKε regulates stem cell-related genes through the activation of NF-κB signaling. To test this, we overexpressed a dominant-negative form of IκB (IκBαDN) in MCF7 cells (Fig. 9A). This mutant is resistant to phosphorylation and degradation, so its overexpression leads to the inhibition of the pathway. We expressed transiently IκBαDN or a control plasmid (PLXSN) in IKKε-wt or IKKε-mut-overexpressing MCF7 cells (Fig. 9B–C). We then analyzed the expression of several stem cell markers (S100A7, CCL5, CXCR4, CXCL17, and IFNA1). As expected, NF-κB pathway inhibition through the expression of IκBαDN mutant abrogated the upregulation of the IKKε target genes (Fig. 9D). Therefore, to further evaluate whether IKKε regulates the CSC subpopulation through this pathway, we measured the CSC fraction in IKKε overexpressing cells transiently expressing the IκBαDN. Fig. 9E–F shows that IKKε overexpression failed to increase the CSCs fraction after inhibition of NF-κB by the expression of the IκBαDN protein. Similarly, we also found that IκBαDN overexpression precluded the increase the number and size of spheres induced by IKKε (Fig. 9G–I). These findings collectively support the notion that IKKε plays an essential role in stem cell biology, possibly through regulation of the NF-κB pathway (see Fig. 10).

4. Discussion

IKBKE amplification/overexpression is a highly recurrent event in

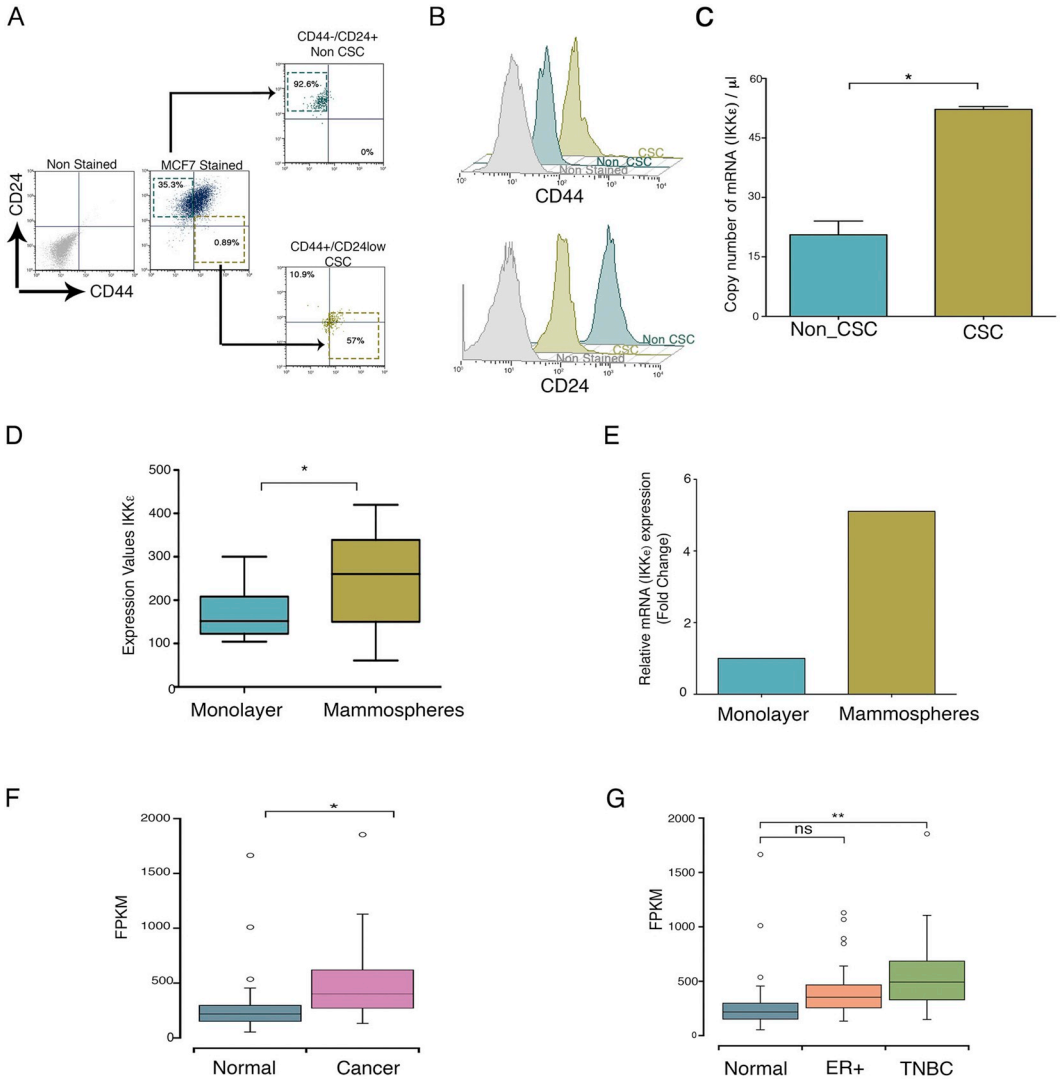


Fig. 8. (A) Fluorescence-activated cell sorting (FACS) analysis of cell surface proteins showing the proportion of CSCs. (B) The efficiency of cell sorting was determined through the expression analysis of CD44 and CD24 in sorted CSC and non-stem cancer cells (non-CSC). (C) IKKε expression in CSC and non-CSC analyzed by droplet digital PCR (n = 3, error bars are s.e.m., *$p < 0.05$). (D) IKKε expression levels in 15 tumorspheres and 11 breast tumors cultured as monolayer. Data derived from GEO dataset GSE7515. *$p = 0.04$. (E) IKKε expression levels were assayed by qRT-PCR in mammosphere and monolayer MCF7 cells (n = 2, error bars are s.e.m). (F) Boxplots of IKKε expression levels in breast cancer and normal tissues of 140 patients derived from GEO dataset GSE58135. * Posterior Probability of equivalent expression (PPEE) = 1×10^{-6}. (G) Boxplots of IKKε expression levels in Estrogen Receptor-positive, Triple Negative and Normal breast tissues of 140 patients derived from GEO dataset GSE58135. * ns **PPEE = 3.5×10^{-7}.

breast cancer cell lines and patient-derived tumors [25,26]. Forced expression of *IKBKE* transforms human immortalized mammary cells, whereas in breast cancer its upregulation induces tumor growth [44]. In a subset of breast cancers, *IKBKE* amplification occurs as an early event in tumorigenesis [25]. IKKε is required for the proliferation and survival of breast cancer cell lines harboring *IKBKE* amplification; however, the mechanism by which IKKε promotes cell growth and survival remains elusive. Here, we performed whole transcriptome analysis of IKKε-overexpressing MCF7 cells and found > 300 regulated genes. Most of the dysregulated genes were implicated in tumorigenic processes such as cell growth and proliferation, angiogenesis, motility, inflammation, invasion, and metastasis. Moreover, we observed an increased level of transcripts of genes related to developmental pathways such as LIF, S100A7, CCL5, CXCR4, and CXCL17. IKKε also induced the expression of S100 calcium binding proteins, namely S100A7, S100A8, S100A9 and S100A16. Alteration of S100 protein expression levels has been shown to correlate with breast tumor progression and metastasis [45]. Interestingly, the S100 protein family has been recently recognized as a promoter of stemness, where S100A7/8/9 genes drive breast tumorsphere growth [38]. These genes and additional developmental genes regulate the stem cell phenotype by activating signaling pathways that control self-renewal and multipotency in normal stem cells from different tissues. In normal conditions, the self-renewal and multipotent capacity of stem cells facilitate tissue regeneration. However, in cancer, expression of developmental genes in a set of cells is associated with the cancer stem cell phenotype, which in turn is linked to tumor progression.

IKKε's role in tumorigenesis has been well documented, however, its function in the CSC phenotype has not been elucidated yet. Cancer stem cells are an important source of tumor heterogeneity and tumor relapse after treatment [46]. Given that IKKε expression in the MCF7 cell line correlated with an increase in the expression of developmental genes, it is possible that *IKBKE* could be a key regulator of the cancer stem cell phenotype. To support this, we performed further analysis to understand IKBKE role in stem cell phenotype maintenance and found that forced expression of IKKε induced the expression of pluripotency stem-cell markers such as NANOG and KLF4 which are known to prevent stem cells differentiation [47]. This is consistent with the increase of the

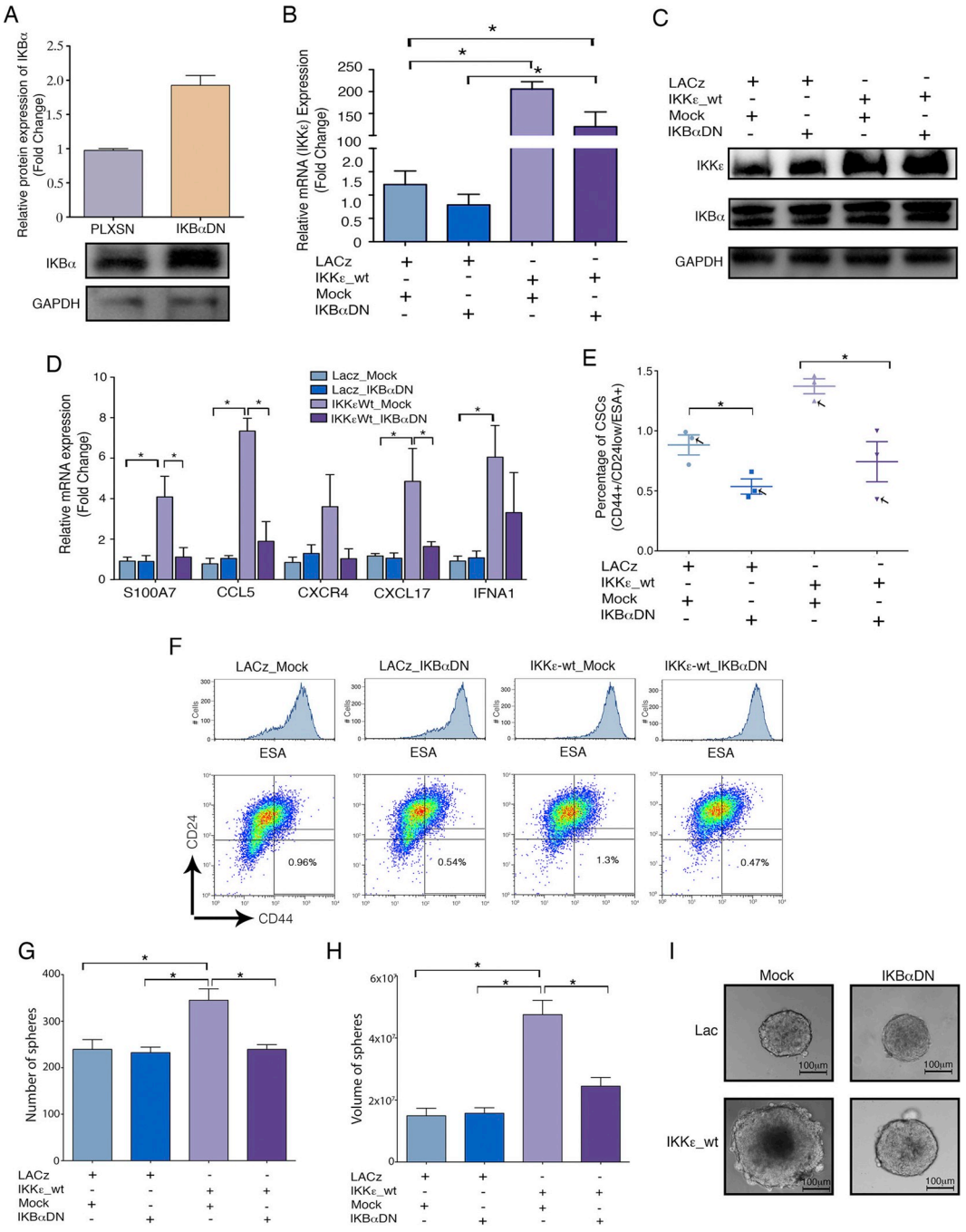


Fig. 9. NF-κB inhibition disrupts the expression of stem cell-related genes induced by IKKε. (A–C) MCF7 cells were co-transfected with IKKε-wt and an IκBα dominant-negative or Mock vector. (A) Graph shows IKBα expression in MCF7-IKBαDN overexpressing cells (n = 2, error bars are s.e.m). (B) qRT-PCR analysis shows that both Mock and IκBα cells exhibit high IKKε overexpression (n = 3, error bars are s.e.m., *$p < 0.05$). (C) Western Blot analysis of IKKε and IκBα in MCF7 cells co-transfected with IKKε-wt or Mock vector. (D) qRT-PCR analysis of S100A7, CCL5, CXCR4, CXCL17 and IFNA1 in IKKε-overexpressing MCF7 cells with NF-κB cascade inhibited through the IκBα mutant overexpression. (*E*–F) Flow cytometry analysis of CD24, CD44 and EpCAM cell surface showing proportions of CSCs in IKKε-overexpressing cells cotransfected with IκBαDN or PLXSN. (E) The graph shows the proportion of CSC in each condition (n = 3, error bars are s.e.m., *$p < 0.05$, black arrows indicate the replicates shown in Fig. F). (F)Dot blot showing the combined expression of CD44 and CD24 in each condition. (G-I) Tumorspheres derived from IKKε-overexpressing cells co-transfected with IκBαDN or PLXSN. (G) Tumorsphere number in each condition, (n = 3, error bars are s.e.m., *$p < 0.05$). (H) Volume of tumorspheres in IKKε-overexpressing cells co-transfected with IκBαDN or PLXSN, (n = 3, error bars are s.e.m., *$p < 0.05$). (F) Representative images of tumorspheres in each condition.

CSC population, mammosphere formation and clonogenic ability found after IKKε overexpression. Notably, IKKε overexpression reduced CD24 expression levels. Interestingly while wild-type IKKε overexpression allowed cancer stem cell expansion, a kinase mutant version of IKKε (K38A) failed to promote CSC self-renewal and enhanced tumorigenicity. The K38A mutation not only leads to the inhibition of its kinase phosphotransferase activity but it also prevents its nuclear import [39]. In addition, Wang et al. showed that IKKε's kinase activity is essential for tumorigenesis, since the K38A mutation abolished tumor formation [48], giving further support to our results.

IKKε has been implicated in breast cancer development by inducing NF-κB activation. As expected, we found that NF-κB target genes were

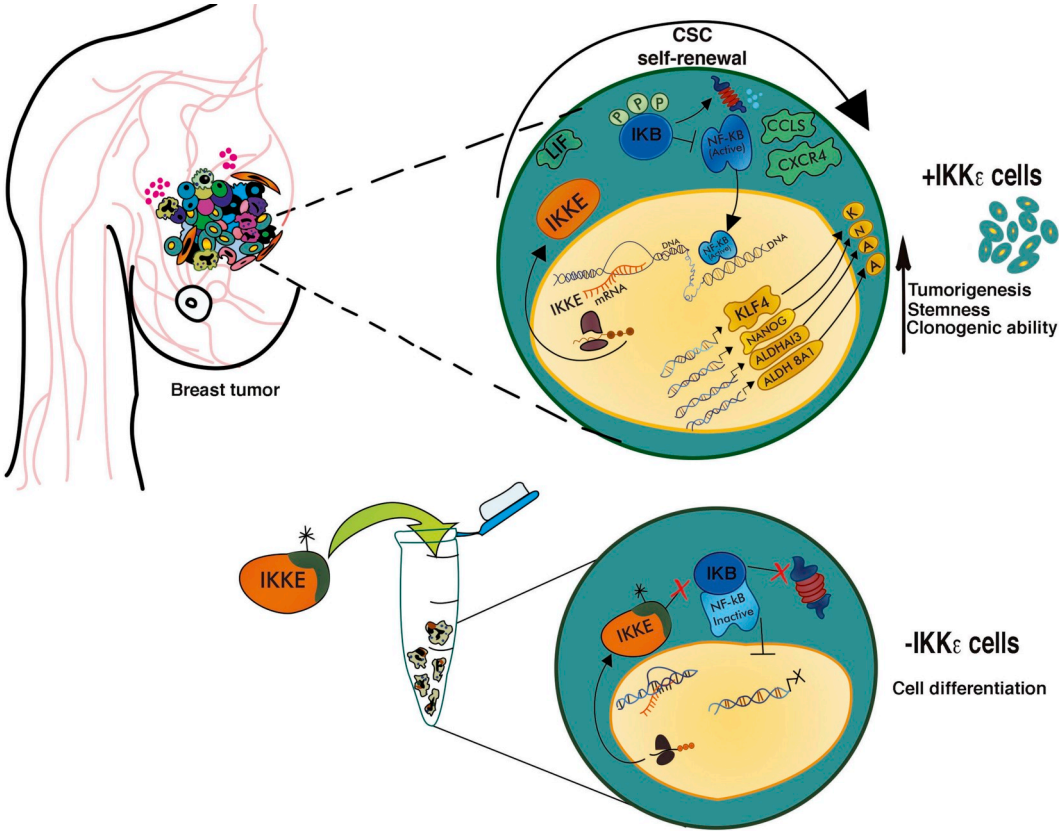


Fig. 10. Graphic representation of IKKε's role in the stemness phenotype of breast cancer cells. CSCs express higher levels of IKKε than non-CSC. Overexpression of IKKε resulted in a higher stemness, tumorigenic and clonogenic ability. The kinase domain of IKKε is required for the stemness role of the kinase, since transfection of an inactive kinase mutant (K38A) was unable to increase the tumorigenic and clonogenic ability of breast cancer cells. IKKε may regulate stemness through the NF-κB pathway.

enriched in IKKε overexpressing cells, suggesting that IKKε triggers NF-κB activation. This signaling pathway plays a fundamental role in the CSC phenotype [24]. In fact, it has been shown that the kinase-defective mutant of IKKε (K38A) effectively reduces NF-κB signaling, probably by retaining RelA in the cytoplasm cell fraction [48].

We found that NF-κB inhibition through the overexpression of IκBα dominant-negative mutant resulted in a slight reduction of the CSC population and a decreased expression of the upregulated IKKε target genes, many of which are involved in stem cell maintenance. Notably, overexpression of IκBαDN resulted in the blockage of tumorsphere formation induced by IKKε. Strikingly, IκBαDN itself slightly reduced the percentage of CSCs, however it did not affect the tumorsphere formation of the MCF7 cell line. This could be due to the low level of basal NF-κB activity in this cell line. It is well known that triple negative breast cancer cell lines exhibit high NF-κB activation levels as opposed to and luminal cell lines such as MCF7 which have very low NF-κB levels [24,49,50]. This is supported by the previous observation that overexpression of IκBαDN is enough to impact the tumorsphere formation ability in triple negative cancer cell lines such as SUM149 and MDA-MB-231 cell lines [21,51]. A direct comparison between these cells lines would be needed in order to solve this discrepancy.

In addition to NF-κB, IKKε is able to activate proteins whose function is also involved in promoting a cancer stem cell phenotype. It was recently shown that, in gliomas, IKKε overexpression directly activates the Hippo pathway proteins YAP1 and TEAD2 [52,53]. These Hippo proteins are known to induce a stem cell phenotype [54]. Interestingly, in these experiments, YAP1 and TEAD lead to Epithelial-Mesenchymal Transition (EMT) which is functionally linked to stemness [55]. Moreover, Gli1, a transcription factor from the Sonic Hedgehog pathway that induces cancer stemness [56,57], is known to be activated by IKKε in pancreatic cancer cells. Finally, it has been shown that Gli1 binds the IKBKE promoter and induces its transcription, allowing a positive feedback loop [58].

It is well known that IKKε mediates phosphorylation of interferon regulatory factor 3 (IRF3) to activate the IFN signaling pathway [59]. RNA-Seq transcriptome analysis allowed us to identify several target genes regulated by IKKε/IRF pathway such as IFNA2, IFNB1, Interferon alpha, STAT3, STAT1, NF-κB, CCL5 and ISG20, among others. It has been established that IKKε participates in the innate immune response triggered by viral infection, by means of modulating the induction of the Interferon pathway. Interestingly, recent reports have shown that interferon alpha promotes the expression of CSC markers and thus, enhance the enrichment of CSCs in pancreatic and oral squamous cell carcinomas [60,61]. Notably, our transcriptome analysis by IPA predicted that interferon alpha could be an upstream regulator of the IKKε target genes. In addition, STAT3, which we found to be also regulated by IKKε, is not only a downstream target of the Interferon pathway but is also required for the viability of a subpopulation of breast cancer MCF7 stem cell-like cells [62]. Further studies should be aimed to establish the relevance of these pathways in the stem-inducing IKKε activities.

There is compelling evidence from different research groups that IKKε promotes several protumorigenic phenotypes in cancer cells. Moreover, it was recently shown that suppression of IKKε in Tumor Infiltrating Lymphocytes (TILs) increased T-cell antitumor activity [63], and thus could also be a potential immune checkpoint target. Consequently, small molecules that inhibit IKKε could halt cancer by both targeting cancer cells and improving immune reactions against the tumor.

5. Conclusions

In conclusion, our results highlight the role of the IKKε kinase in the regulation of the stem cell phenotype in breast cancer cells, as assessed by expression, functional and *in vivo* assays. We also present evidence showing that this new function requires its kinase activity since a mutant IKKε fails to regulate the CSC population. The clinical correlation between the expression of this kinase and patient prognosis could be due, at least in part, by its role in promoting stemness in breast cancer cells.

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Transparency document

The Transparency document associated with this article can be found, in online version.

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Declarations

Ethics approval and consent to participate. Not applicable.

Consent for publication. Not applicable.

Availability of data and material. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests. The authors declare that they have no competing interests.

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Authors' contributions

ZO: Performed experiments, analyzed and interpreted data.

AOR: Performed experiments.

RCO: Performed blots assays and evaluated mRNA expression by ddPCR.

CZ: Performed the *in vivo* assays.

FP: Analyzed, interpreted data and contributed to the manuscript writing.

VML: Analyzed and interpreted data, was a major contributor in writing the manuscript.

KVS: Performed experiments, analyzed and interpreted data, was a major contributor in writing the manuscript.

JMZ: Analyzed and interpreted data, was a major contributor in writing the manuscript.

All authors read and approved the final manuscript.

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